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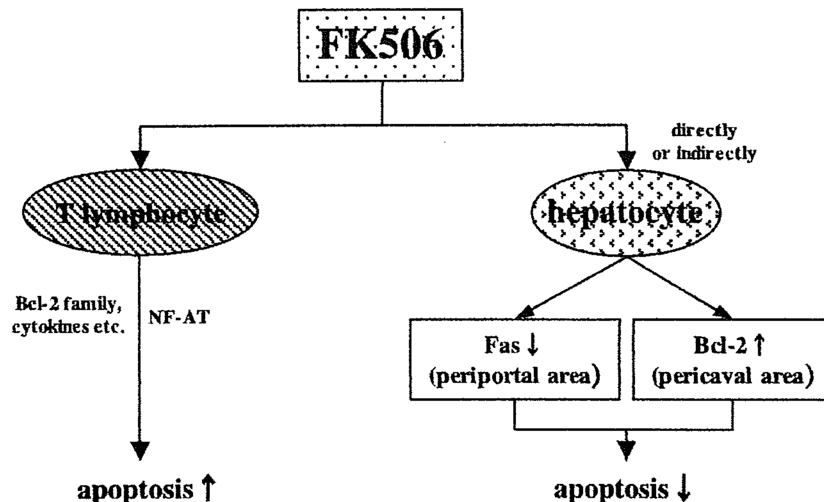

FIG. 7. Scheme of the multipotent functions of FK506 in allogeneic rat liver transplantation. It is possible that FK has hepatoprotective effects not only by the suppression of Fas-Antigen (Fas) on hepatocytes in the periportal areas, and the up-regulation of Bcl-2 on hepatocytes in the pericaval areas but by the direct promotion of the pro-apoptotic genes in infiltrating T lymphocytes, and the indirect induction of apoptosis in activated T lymphocytes through the deprivation of several cytokines.

[28, 29], FK may rescue hepatocytes from apoptosis through the induction of infiltrating T lymphocyte apoptosis, as well as directly making hepatocytes resistant to apoptosis.

The current study showed that FK reduced the expression of Fas in the grafted liver, where the expression in hepatocytes around the periportal area seemed to be closely associated with apoptosis. Apoptosis is regulated by several molecules, including Fas and FasL [30,31]. The expression of Fas is directly suppressed by FK in cultured hepatocytes, and the suppression of Fas expression leads to the inhibition of apoptosis [32]. However, considering that FK suppresses hepatocyte apoptosis in either the grafted liver or the injured liver through the reduced expression of several cytokines, such as IL-4, IFNγ, and TNFα [33–35], as well as the inhibition of the caspase cascade reaction [36], FK apparently plays multifunctional roles in the blockade of Fas-dependent hepatocyte apoptosis. In contrast, FasL expression was not affected by FK, indicating that FK treatment affected the expression of Fas in hepatocytes but not the expression of FasL in rat OLT.

Moreover, the Bcl-2 family, such as Bcl-2 and Bax, is another major group of regulators of apoptosis [37–39]. In this study, there was a significant difference in the expression of Bcl-2 between the FK treated group and the untreated group, while Bax expression showed no difference in the allografted groups. The most striking finding was the zonal expression of Bcl-2 in hepatic lobules. The immune reaction following liver transplantation is observed mostly in the periportal area (zone 1). Bcl-2 was expressed largely in the pericaval area (zone 3). In the syn group, the Bcl-2 expression level in the pericaval area was less than that of the allografts with FK. In addition, FK allowed the allografts to maintain the same level of Bcl-2 expression, even though its expression in the pericaval area was markedly reduced in the allografts. Pericaval hepatocytes (zone 3) are responsible for liver specific functions and, unfortunately, they are less tolerant of cellular stress, such as I/R injuries [40–42]. Therefore, if zone 3 is severely injured, the liver loses most of its specific functions [40]. In addition, the apoptosis of the hepatocytes was frequently detected in the centrilobular zones after ischemia-reperfusion in human liver allografts [41]. Although transplantation was completed immediately after graft harvesting in our study, the liver graft was damaged by I/R injury on day 1 after OLT [43, 44]. Accordingly, the allo-FK group showed a greater Bcl-2 expression in the pericaval hepatocytes than in the syn group on day 1 after OLT. As a result, FK was thus suggested to ameliorate I/R injury after OLT via mediating the Bcl-2 expression on hepatocytes in zone 3. The observation that the liver function was better preserved in the FK group and that the area (pericaval area) of Bcl-2 expression was different from the area with T lymphocyte accumulation, FK seemed to maintain the cellular viability of zone 3 hepatocytes *via* enhanced Bcl-2 expression [37–41, 45], independently of T lymphocyte action. This is the first report regarding to FK and Bcl-2 expression in liver transplantation.

Several approaches to avoid of hepatocyte apoptosis have been explored recently, by either the administration of FK or the genetic modification of apoptosis regulating factors [28, 29, 36, 46–50]. Moreover, several attempts have successfully achieved better protection of transplanted liver, including (1) the use of antibodies

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to neutralize Fas action [51, 52]; (2) overexpression of Bcl-2 by gene transfer [47]. As summarized in Fig. 7, however, FK plays multifunctional roles in the protection of hepatocytes at different points and also is widely accepted for clinical use. Therefore, FK could be a better candidate as a therapeutic strategy against hepatocyte apoptosis.

In conclusion, the current study suggested that FK has beneficial antiapoptotic effects on hepatocytes in the allografted rat livers through both the down-regulation of Fas expression in the periportal areas and the up-regulation of Bcl-2 expression in the pericaval areas. Although further studies are required, particularly to elucidate the molecular mechanism underlying the FK contributions, FK might be a potentially powerful agent for use during organ transplantation.

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INTERNAL MEDICINE

CASE REPORT

Multiple Immune Abnormalities in a Patient with Idiopathic CD4+ T-Lymphocytopenia

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Abstract

Idiopathic CD4+ T-lymphocytopenia (ICL) is a new disease entity characterized by CD4+ T-lymphocyte depletion without evidence of HIV infection. We report a 27-year-old ICL patient with a long history of multiple immune abnormalities. His CD4+ T-lymphocyte count started to decrease after generalized lymphadenopathy of an unknown cause at age 3. He satisfied the criteria for ICL at age 9, and the decreased CD4+ T-lymphocyte count persisted for more than 18 years. This is probably the first childhood-onset ICL case in which the trigger event for the development was known together with the patient's autoimmune background.

Key words: ICL, CD4, T lymphocytopenia

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Introduction

Idiopathic CD4+ T-lymphocytopenia (ICL) is a rare syndrome that was first reported in 1992 by the Centers for Disease Control and Prevention (CDC) of the USA (1). In the same year, a similar case was also reported in Japan (2). This disease is characterized by an absolute decrease in CD 4+ T lymphocyte count to less than 300/μL or to less than 20% of total T lymphocytes without evidence of human immunodeficiency virus-1 (HIV-1), HIV-2, human T-cell leukemia virus type-1 (HTLV-1), or HTLV-2 infection, a known immunodeficiency syndrome, or therapy-related CD4+ T-lymphocyte depression (3-8). ICL is a cluster of heterogeneous diseases with diverse clinical courses and immunologic characteristics. Although some patients present with opportunistic infections such as cryptococcosis, human papillomavirus (HPV), and nontuberculous mycobacteria, others remain in a relatively healthy condition for years (8). Since the prognosis of ICL has not been well defined, a prospective study to evaluate the history of this disease was performed. Thirty-nine cases were collected, and the details of the study were reported recently (8). Here, we report a unique ICL patient with childhood onset and a long history of multiple immune abnormalities.

Case Report

A 27-year-old man was referred to our hospital because of hypoproteinemia with monoclonal gammopathy. The patient showed no remarkable findings on physical examination except for redness of the face and conjunctival hyperemia. He was in good physical condition with a good appetite, but his serum total protein was very low due to hypoalbuminemia (Table 1). No protein was detected in his urine. His immunoglobulin levels were nearly normal, but he had a small amount of IgA-kappa monoclonal immunoglobulin; i.e., monoclonal gammopathy of undetermined significance (MGUS). There was no sign of liver dysfunction or renal dysfunction, and his serum levels of asparate-aminotransferase (AST), alanine-aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine were all within the appropriate reference range. In a peripheral blood examination, he was found to be polycythemic (Table 1). Al-

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Table 1. Routine Laboratory Data

Hematologic		Serum chemistry	
Red blood cells	6.02×10^6/μL	Total protein	5.5 g/dL
Hemoglobin	18.4 g/dL	Albumin	2.7 g/dL
Leukocytes	5,600 /μL	IgG	1,140 mg/dL
Neutrophils	81%	IgA	344 mg/dL
Lymphocytes	12% (672 /μL)	IgM	147 mg/dL
Monocytes	4%	Na	136 mEq/L
Eosinophils	3%	K	4.0 mEq/L
Platelet	17.6×10^4/μL	Cl	105 mEq/L
		BUN	12 mg/dL
Flow-Cytometric		Creatinine	0.78 mg/dL
CD3	46.8% (314 /μL)	Total bilirubin	0.4 mg/dL
CD4	7.5% (50 /μL)	AST	21 IU/L
CD8	40.2% (270 /μL)	ALT	33 IU/L
CD22	6.9% (46 /μL)	LD	151 IU/L
CD16	43.2% (290 /μL)	Anti-HIV antibody	(-)
CD25	6.4% (43/μL)	Anti-HTLV-1 antibody	(-)
CD56	43.1% (290 /μL)	Anti-nuclear antibody	(-)
		Anti-CMV antibody	
		IgG	7,600
		IgM	0.6

BUN: blood urea nitrogen, AST: asparate-aminotransferase, ALT: alanine-aminotransferase, LD: lactic dehydrogenase, CMV: cytomegalovirus

though his white blood cell count was normal at 5,600/μL, his absolute lymphocyte count was very low at 672/μL. A lymphocyte subset analysis indicated a marked decrease in his absolute CD4+ T-lymphocyte count to only 50/μL (Table 1 and Fig. 1). His absolute B-lymphocyte count was also low at 43/μL, but natural killer cell compartment was well preserved (Table 1 and Fig. 1). Serum tests for HIV-1, HTLV-1 and anti-nuclear antibody (ANA) were negative. He had a very high anti-cytomegalovirus (CMV) IgG antibody titer with negative IgM antibody indicating past CMV infection (Table 1).

A review of his medical records showed that he had a long history of immune abnormalities (Fig. 2), although his family history was not significant. There was no consanguineous marriage. First, he developed autoimmune hemolytic anemia (AIHA) and idiopathic thrombocytopenic purpura (ITP) at age 1, which was successfully treated with prednisolone. Next, he developed measles pneumonia at age 3 and then suffered from repeated episodes of generalized lymphadenopathy with polyclonal hypergammaglobulinemia (IgG: 3,000-6,000 mg/dL) from ages 3 to 9. Rheumatoid arthritis test (RA test) was positive suggesting an autoimmune background. The result of Epstein-Barr virus (EBV) antibodies (VCA-IgG positive, VCA-IgM negative and EBNA positive) indicated the past EBV infection. The size of the lymph nodes was as large as 4 cm in diameter, but biopsies of the lymph nodes revealed reactive lymphadenitis. Analysis of his peripheral blood lymphocyte subsets was performed at age 4, and an inversion of the CD4/CD8 ratio was noticed. His absolute lymphocyte count including CD4+ T-lymphocytes decreased gradually, and he satisfied the criteria for ICL at age 9. A serum test for HIV was negative. At age 12, he developed cryptococcal pneumonia, which was successfully treated with fluconazole. At age 14, he developed mucosa-associated lymphoid tissue (MALT) lymphoma of the stomach with multiple swelling of abdominal lymph nodes, which gradually regressed within several years without therapy. His serum total protein started to decrease from age 15 and never returned to the normal level. He suffered a recurrence of his ITP at age 17 and developed thrombotic thrombocytopenic purpura (TTP) at 20, which were successfully treated with a short course of prednisolone and steroid pulse therapy with plasma exchange, respectively. He became polycythemic after recovering from these episodes, and his RBC count exceeded 600×10^4/μL. His serum erythropoietin was slightly higher than the normal upper limit at 37.5 mU/mL (reference range: 8-36 mU/mL). Since then, he has not experienced any serious diseases that required hospitalization and has dropped out of follow-up.

Discussion

In a nationwide study of ICL in the USA, 57 patients were recruited between 1992 to 2006 (8). Eighteen patients were excluded from the study because they did not fulfill the criteria for the definition of ICL (1, 5). The excluded patients had a CD4 T-lymphocyte count of more than 300/μL, HIV antibody, or were complicated with hematological malignancies that could have caused lymphocytopenia (8). One patient was excluded because of an alternative diagnosis

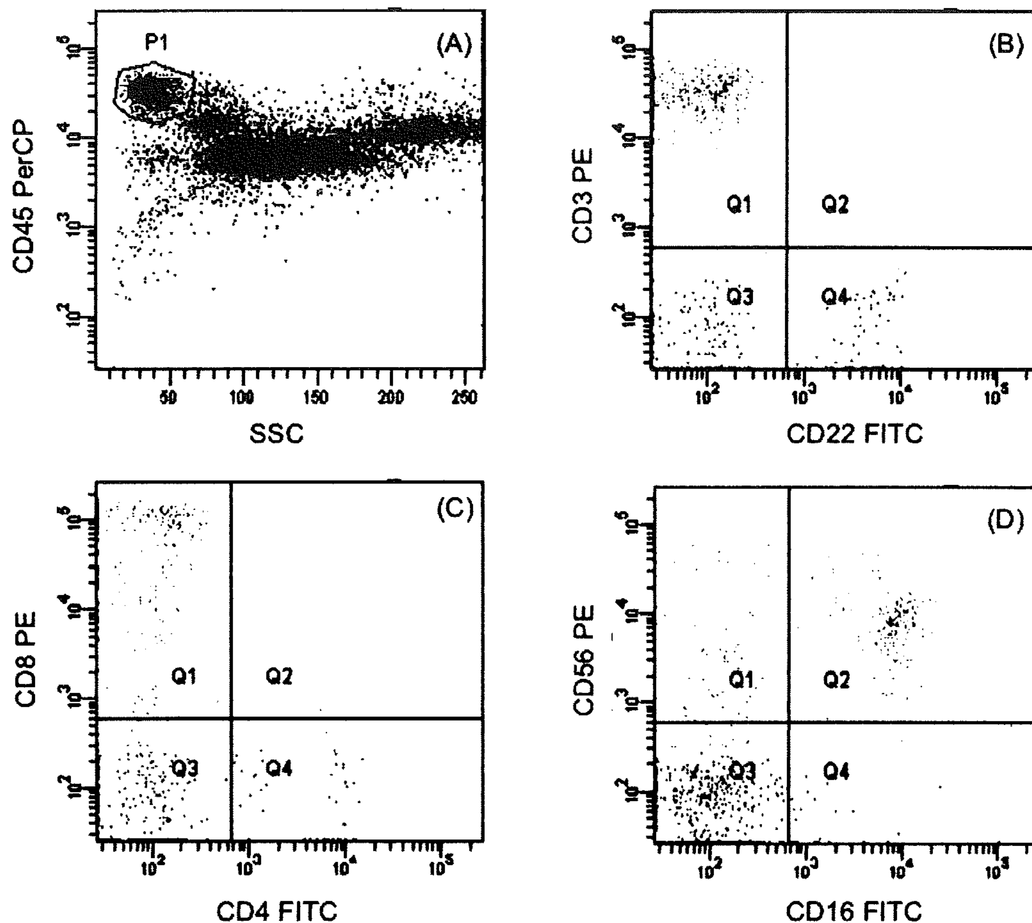

Figure 1. Lymphocyte subset analysis of the peripheral blood. Cells were stained with various combinations of fluorescein isothiocyanate (FITC), phycoerythrin (PE), or peridinin-chlorophyll-protein (PerCP)-conjugated murine monoclonal antibodies, and the results of gated cells of figure (A) (lymphocyte population: low SSC with high CD45 antigen expression) are shown in figures (B), (C) and (D). Marked decreases in CD4+ T-lymphocytes and CD22+ B-lymphocytes are shown.

(common variable immunodeficiency). The present patient does not meet the criteria for any known immunodeficiency diseases other than ICL. Severe combined immunodeficiency (SCID) was ruled out, and humoral immunodeficiencies such as common variable immunodeficiency and hyper-IgM syndrome were also ruled out because of his normal immunoglobulin class levels. Wiskott-Aldrich syndrome and ataxia telangiectasia were ruled out by the absence of their characteristic clinical features and laboratory data.

ICL usually occurs in middle-aged or elderly adults, and the number of patients aged from 20 to 30 years old in the cohort study was only 2 out of 39 cases (5.1%), and there were no patients younger than 20 years old (8). The present patient met the criteria for ICL from age 9. Childhood ICL cases are rare although there have been a few reports (9, 10). There is no common feature of childhood ICL, and there is heterogeneity even within childhood cases; two cases showed IgA deficiency with progressive pulmonary infection and another case showed a significant increase in γδTCR-bearing T cells (9, 10). In the present case, if we consider his peculiar past history including the development of autoimmune diseases at age 1 and recurrent episodes of lymphadenopathy from age 3, it is possible that he possessed genetic defects that led to immunodeficiency. Alternatively, the prolonged and intermittent use of prednisolone to control these episodes may have influenced or accelerated the development of ICL. ICL cases are divided into 2 subgroups in terms of their CD8+ T-lymphocyte levels: those with levels lower than the lowest 2.5% of the CD8+ T-lymphocyte counts in the control population (<180/μL) and those with levels higher than that. Since the absolute CD8+ T-lymphocyte count of this patient was 270/μL, he was classified into the latter group. In the cohort study, 5 patients (13.8%) developed AIDS defining clinical conditions during the first 24 months of observation (8). The present patient has not met the criteria for such conditions in spite of the very long follow-up duration. The most common infection in ICL is cryptococcal infection, and this patient also experienced cryptococcal pneumonia; however, that is not an AIDS defining clinical condition (11). Autoimmunity is also common in ICL, and 9 patients (23.1%) developed autoimmune diseases in the cohort study, either before diagnosis or in the follow-up period (8). One patient developed autoimmune hemolytic anemia, as in our patient, but no patient de-

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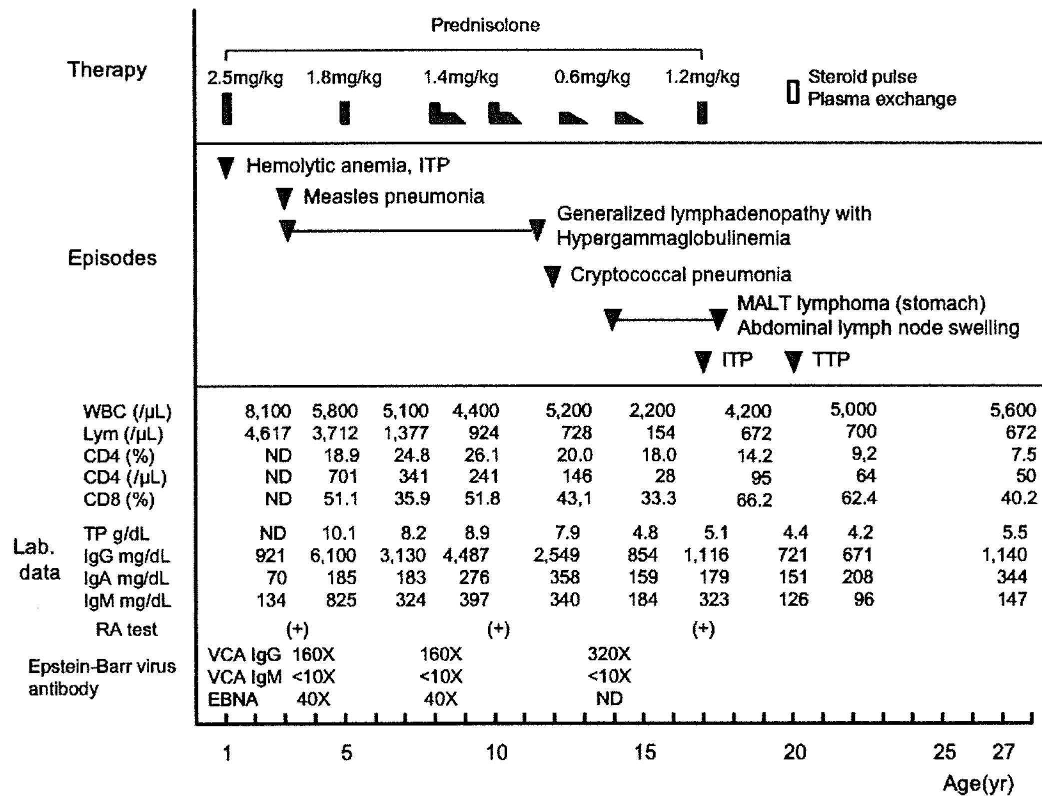

Figure 2. Clinical course and laboratory data. ITP: idiopathic thrombocytopenic purpura, TTP: thrombotic thrombocytopenic purpura, MALT: mucosa-associated lymphoid tissue, TP: total protein, RA test: rheumatoid arthritis test, VCA: viral capsid antigen, EBNA: Epstein-Barr virus nuclear antigen

veloped ITP. Hypoalbuminemia was one of the characteristic features of this patient. There may be some association between poor nutritional state and CD4+ T-lymphocytopenia, as suggested in very elderly people (12). It has also been reported that 6 of 103 chronic obstructive pulmonary disease (COPD) patients examined (5.8%) showed ICL, and there was a positive correlation between serum albumin level and CD4+ T-lymphocyte count (13). Among the unique features observed in this patient, the most characteristic feature was his long-term survival in spite of an extremely low CD4+ lymphocyte count. As for the prophylaxis to prevent opportunistic infections, the cohort study suggested that it should only be considered for the subsets of ICL patients with the worst prognoses, such as those with low CD8 counts or patients presenting with an AIDS defining condition (8). The present patient did not receive any prophylaxis for more than 7 years. Although the presence of anti-CD4+ lymphocyte antibody and overexpression of Fas/CD95 with enhanced apoptosis have been reported in some cases (14, 15), the etiology of ICL is still largely unknown. Further accumulation of ICL or ICL-like cases, especially childhood cases, is desired for its etiological classification and for the development of effective therapies against ICL.

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Screening for genetic heterogeneity in the interferon sensitivity determining region of the hepatitis C virus genome by polymerase chain reaction with melting curve analysis

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Abstract

Background: Although mutations in the interferon (IFN) sensitivity determining region (ISDR) of hepatitis C virus (HCV) have been reported to be useful as a predictive viral factor for IFN therapy in patients infected with HCV-1b, such laboratory research has not been favorably translated into the clinic. To promote such translation, we attempted the establishment of a rapid and simple polymerase chain reaction (PCR) combined with melting curve analysis (MCA) to screen for mutations in the ISDR and for the monitoring of HCV quasispecies.
Methods: A PCR-MCA protocol was established using in-house primers and hybridization probes designed according to the results of direct sequencing of 34 HCV-1b samples. Then, the performance of PCR-MCA was verified by comparing with mutation profiles obtained by direct sequencing and sequencing after cloning.
Results: The MCA assay revealed that melting temperature (Tm) was inversely correlated with the number of nucleotide (nt) and amino acid substitutions in the ISDR deduced on the basis of the results of direct sequencing. A boundary Tm of 58.0°C allowed us to discriminate HCV genomes into two groups: one with a Tm >58.0°C had no or a low number of nt substitutions, while the other genomes with a Tm <58.0°C had a high number of nt substitutions, corresponding to wild-type in the former and mutant-type in the latter in respect of a clinical setting for IFN therapy. Moreover, this MCA assay provided precise discrimination of Tm between clones, reflecting the degree of the genetic complexity of HCV genomes.
Conclusions: This study indicates that the MCA assay is useful to rapidly and simply screen the mutational status of the ISDR of HCV, as well as in using the ISDR as one of the targets for discriminating the genetic complexity of HCV genomes. The MCA assay could also be applicable as a convenient and useful screen of the genetic heterogeneity of clones relating to HCV quasispecies.
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Keywords: hepatitis C virus (HCV); interferon (IFN); interferon sensitivity determining region (ISDR); melting curve analysis (MCA); quasispecies.

Introduction

The hepatitis C virus (HCV) is an RNA virus belonging to the Flaviviridae. It has a single strand and a genome of approximately 9.6 kb with a single open reading frame encoding four structural (C, E1, E2, and p7) and six non-structural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins (1). HCV is persistently present in plasma as a mixture of heterogeneous HCV RNA genomes, quasispecies, and causes chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) in humans. Patients with HCC have been reported to be prevalent in the Nagasaki district and a majority of them are seropositive for the virus (2). Fortunately, recent advances in the treatment of chronic hepatitis C are promising, namely interferon-ribavirin combination therapy, as the mainstay treatment for chronic hepatitis C. However, such combination therapy is not always effective in all patients with HCV-related disorders and can also cause adverse events associated with the drugs (3, 4). Since recently, the HCV core antigen has been generally used to quantitatively monitor HCV viremia, but is not appropriate for monitoring the complexity of the HCV genome relating to HCV quasispecies. For these reasons, a biomarker to predict the efficacy of interferon (IFN) therapy is required. At present, the presence of the HCV genotype-2 or -3 and a low viral load prior to treatment have been proposed and applied in a practical setting as predictive markers for the effectiveness of IFN therapy (5). Although HCV genotype 1b is resistant to IFN therapy, Enomoto et al. (6) reported that mutations in the interferon sensitivity determining region (ISDR) within the *NS5A* gene are useful as predictive markers of the response to IFN therapy for chronic hepatitis patients with a Japan-specific subtype (J-type) of the HCV genotype 1b. The ISDR corresponding to codons 2209–2248 of the HCV genome (NS5A2209–

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2248) can interact with the double-strand RNA protein kinase. This interaction has been shown to be able to block the IFN signaling pathway, leading to HCV resistance to IFN therapy (7, 8). However, the precise mechanism of IFN resistance remains unresolved, because the regions other than ISDR, such as E2, NS3/4, and NS5A, have also been reported to be involved in resistance to IFN therapy in Japanese patients (9, 10).

Mutations in the ISDR that are expected to be useful as predictive markers have not yet been introduced into the clinical setting. One of the reasons is that the methodology used for routine laboratory tests is inapplicable because it is labor-, time-, and cost-consuming.

In the present study, to evaluate the implications of nucleotide substitutions within the ISDR in the resistance to IFN therapy and in quasispecies, we examined the genetic heterogeneity in the ISDR of HCV isolates from clinical samples. Then, we attempted to develop a rapid and easy methodology for screening of nucleotide mutations using polymerase chain reaction (PCR) combined with melting curve analysis (MCA).

Materials and methods

Study samples

HCV samples were collected from plasma specimens from patients admitted to our hospital. Clinical information was referred from the hospital information system, as summarized in Table 1. HCV genotyping was determined with multiplex PCR, as described by Ohno et al. (11). Quantitation of serum HCV RNA and HCV antigens was performed according to the manufacturer's instructions using a commercial kit (Ortho HCV Antigen IRMA Test, Ortho Clinical Diagnostics, Raritan, NJ, USA). HCV isolates with genotype type-1b classified according to the typing system by Ohno et al. (11) were enrolled in the study. Clinical virological assay for the qualification and quantitation was carried out using commercial assay kits (AMPLICORE HCV Monitor test, version 2.0 and AMPLICORE HCV, version 2.0, Roche Diagnostics, Basel, Switzerland).

HCV RNA extraction, amplification by nested PCR, and sequencing

HCV RNA was extracted from serum samples using a QIAamp Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions.

Table 1 Characteristics of patients enrolled in the study.

Case no.	Patient no.	Age, years	Sex	HCV genotype	Viral load, kIU/mL	HCV Ag, fmol/L	Disease	Treatment	Response
1	No. 01	69	M	1b	313	ND	CH	NT	
2	No. 02	63	M	1b	1650	ND	AC	NT	
3	No. 04	35	M	1b	2140	ND	CH	NT	
4	No. 05	44	F	1b	1880	ND	CH	NT	
5	No. 06	63	F	1b	2430	ND	CH	NT	
6	No. 08	41	F	1b	638	ND	AC	NT	
7	No. 09	68	F	1b	2810	ND	AC	NT	
8	No. 10	65	F	1b	7980	ND	CH	NT	
9	No. 11	66	F	1b	2490	ND	CH	NT	
10	No. 12	74	M	1b	514	ND	CH	NT	
11	No. 13	65	F	1b	2510	ND	HCC	NT	
12	No. 15	55	F	1b	>5000	52,000	LC	IFN+R	NR
13	No. 16	55	M	1b	4500	19,622	CH	NT	
14	No. 17	60	M	1b	2100	3010	CH	NT	
15	No. 18	72	M	1b	ND	380	CH	IFN+R	PR
16	No. 19	55	F	1b	ND	15,600	CH	IFN+R	NR
17	No. 20	55	F	1b	2800	4420	CH	IFN+R	NR
18	No. 21	59	M	1b	1200	1560	CH	NT	
19	No. 23	54	F	1b	>5000	62,200	LC	IFN+R	PR
20	No. 25	33	F	1b	ND	1280	CH	NT	
21	No. 27	73	F	1b	490	ND	CH	NT	
22	No. 28	62	F	1b	>5000	17,800	LC	IFN+R	NR
23	No. 29	65	F	1b	ND	11,900	CH	NT	
24	No. 30	52	F	1b	ND	5480	CH	IFN+R	PR
25	No. 31	70	F	1b	ND	9280	CH	NT	
26	No. 32	46	M	1b	ND	11,000	CH	NT	
27	No. 33	62	F	1b	ND	3060	HCC	IFN+R	NR
28	No. 34	57	M	1b	1200	3210	HCC	IFN+R	SVR
29	No. 36	70	F	1b	ND	7390	AC	NT	
30	No. 37	71	M	1b	ND	10,500	CH	IFN+R	SVR
31	No. 38	56	F	1b	ND	3070	CH	IFN+R	NR
32	No. 39	65	M	1b	ND	5210	CH	IFN+R	PR
33	No. 40	58	M	1b	ND	2050	LC	IFN+R	NR
34	No. 41	47	M	1b	ND	11,200	CH	IFN+R	PR

Out of 34 cases, 14 underwent IFN+ribavirin therapy, but the remaining cases did not undergo such therapy because of severe complications, such as cancers, pneumonias, etc. ND, not done; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; AC, asymptomatic carrier; IFN+R, interferon (IFN) and ribavirin (R); NT, non-treatment; NR, nonresponse; PR, partial response; SVR, sustained virological response.

cDNA was generated using SuperScript III Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA, USA) and antisense primer or random hexamer oligonucleotides. A fragment of 251 bp including the ISDR was amplified by nested PCR using primers, as described previously (12). The amplicons were subjected to single strand conformation polymorphism (SSCP) and direct sequencing analysis to identify mutations in the ISDR. PCR-SSCP was performed according to the standard method (13, 14). Direct sequencing was carried out with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) using an Automated DNA Sequence Analyzer (Model 310, Applied Biosystems). The nucleotide (nt) and deduced amino acid (aa) sequences of the ISDR were aligned and compared with that of the HCV-J prototype (15). From several samples, PCR products were cloned using a DynaExpress TA PCR Cloning Kit with Jet Competent Cells (BioDynamics Laboratory Inc., Tokyo, Japan) and then each clone was sequenced and subjected to the MCA assay.

PCR-MCA

To screen for mutations in the PCR amplified products in the ISDR, MCA analysis was applied using a LightCycler instrument equipped with the DNA MCA analysis program (LightCycler Software Version 3.0) (16). Briefly, reactions were performed in a 20-μL volume with 5 mM $MgCl_2$, 2 μL 1/10,000 diluted PCR product, 0.5 μM PCR primers, 0.4 μM anchor and sensor hybridization probes (Table 2), and 2 μL of 10×LightCycler FastStart DNA Master HybProbe (Roche Diagnostics). After 35 amplification cycles, the PCR products were denatured at 95°C for 20 s, annealed at 40°C for 20 s, and then raised from 40°C to 80°C at a transmission rate of 0.2°C during continuous fluorescence monitoring at 640 nm. The fluorescence intensity was analyzed using the manufacturer's program, generating melting temperature (Tm) and melting curve values.

The results of the MCA assay were validated by comparison with direct sequencing.

Results

Mutation in the ISDR and relevancy for probe setting

To evaluate the heterogeneity of mutations in the ISDR (nts 6954–7073), a fragment of the region was amplified and sequenced directly for 34 HCV samples. Compared with a reference sequence of the HCV-1b prototype (Gene Bank accession D90208), the sites of nt substitutions were mainly distributed in two regions at or near nt 6974 and nt 7070, as shown in Figure 1. Although the site of silent mutations (open columns) was widely distributed, the site of missense mutations (solid columns) was concentrated to the region of nts 6960–6990 corresponding to codons 2211–2221. The number of aa substitutions corresponding to missense mutations was from 0 to 6, with no substitution (wild-type) in 15 cases, one in nine cases, and two or more in 10 cases. As all cases had at least one nt substitution with concentration of missense mutations within the region of nts 6954–6990, the probe site was targeted in the region, as shown in Table 2. The G:C content of the anchor probe was approximately 53.9%, and sensor probe was 46.7%. Using this probe, our PCR-MCA method yielded reasonably sharp melting curves in all 34 cases with total

Table 2 Sequence profiles of primers, and sensor and anchor hybridization probes designed for the present study.

Forward primer	5′-ACCGACCCCTCTCATATTAC-3′
Reverse primer	5′-GATCGAAAGAGTACAGGATTAC-3′
Anchor probe (3′labeled FITC)	5′-GCCAGCTCTTCAGCTAGCCAGTTGTCTGCGCCTTCTTTGAAG-3′
Sensor probe (5′labeled LCRed640)	5′-CGACATGTACTACCCATCATGACT-3′-P

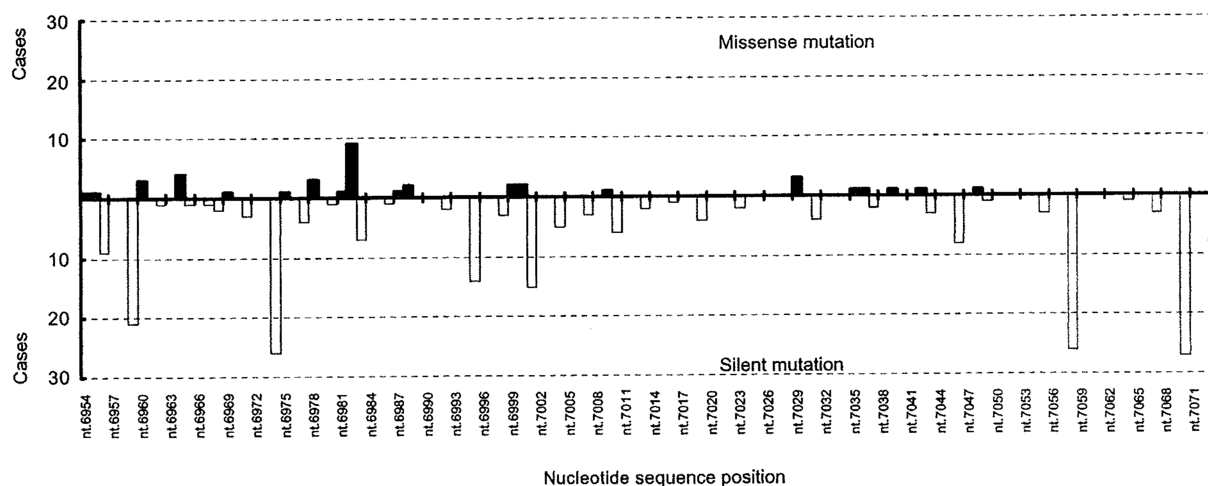

Figure 1 The frequency of nucleotide (nt) substitutions (open columns, silent mutations; solid columns, missense mutations) in the ISDR from nts 6954 to 7073.
Although silent mutations are widely distributed, missense mutations are mainly concentrated to the region of nts 6960–6990 corresponding to codons 2211–2221. At least one nt substitution was detected within the region corresponding to the probe site in all cases.

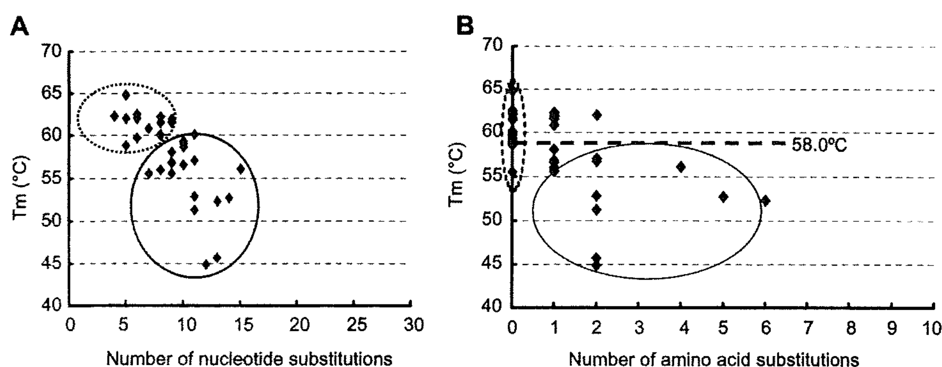

Figure 2 Correlation between melting temperature and the quantity of nt substitutions.
(A) The number of nt substitutions is inversely correlated to Tm ($r=-0.689$, $p<0.01$). There is a tendency to form two clusters: one with high Tm and low number of nt substitutions (dotted line circle), and one with low Tm and high number of nt substitutions (solid line circle). (B) The number of missense mutations (aa substitution) is more significantly correlated to Tm ($r=-0.617$, $p<0.01$). There appears to exist a boundary (dotted line) in Tm at 58.0°C, discriminating into two clusters: one cluster with high Tm and no or one aa substitution, and one with low Tm and two or more aa substitutions.

run times of approximately 3 h, as demonstrated below.

Performance of PCR with MCA discriminating heterogeneity in the ISDR

As the Tm value is essentially regulated by heteroduplex formation depending upon various genetic and epigenetic alterations, we verified whether PCR with MCA can discriminate and screen the mutation status in the ISDR. As shown in Figure 2A, Tm values of amplicons derived from blood samples varied from approximately 45°C to 65°C, and were inversely correlated with the number of nt substitutions ($r=-0.689$, $p<0.01$) and the deduced aa substitution number ($r=-0.617$, $p<0.01$). In particular, Figure 2B reveals that the boundary of a Tm of 58.0°C makes it possible to distinguish two major groups: a high Tm group of >58.0°C and a low Tm group of <58.0°C. Samples distributed in the high Tm area had mainly no or rarely one aa substitution, whereas samples distributed in the low Tm area had mainly two or more aa substitutions. However, although only samples having one aa substitution were distributed to both the high and low Tm areas, the total number of nt substitutions was different between them: approximately two times higher in samples distributed in the low Tm area rather than the high Tm area.

Table 3 Characteristics of the mutation profile of ISDR sequence and viral load in two groups classified by a Tm of 58.0°C boundary.

	Average		
	Nucleotide	Amino acid	HCV Ag
HCV with high Tm	7.7±2.0*	0.4±0.6*	15,063±18,952
HCV with low Tm	10.9±2.3*	2.1±1.7*	6291±4097

*Statistically significant ($p<0.01$).

Then, as shown in Table 3, viral and clinical parameters between the high and low Tm groups were compared. The high Tm group showed a tendency of low nt and aa mutations, high viral load, and non-response to IFN. Figure 3 shows representative cases who received IFN therapy, showing that patients 15, 19 and 33 (panel A) were non-responders with a Tm of 59.3°C, 62.5°C and 64.8°C, respectively, while

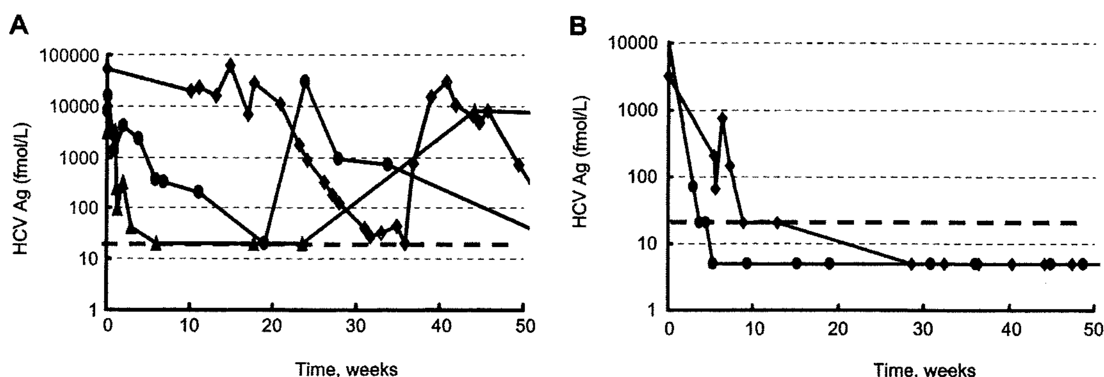

Figure 3 The difference of IFN efficacy in cases classified by Tm status (more or less than 58.0°C) by monitoring HCV viral load after IFN treatment in patients infected with HCV (solid broken line: HCV Ag detection limit of ≤20 fmol/L).
(A) Cases with Tm of 59.3°C, 62.5°C, and 64.8°C. All three cases were transiently responsive but not sustained, namely non-sustained virological responders. (B) Cases with Tm of 56.1°C and 43.7°C. These two cases with low Tm values showed sustained viral response.

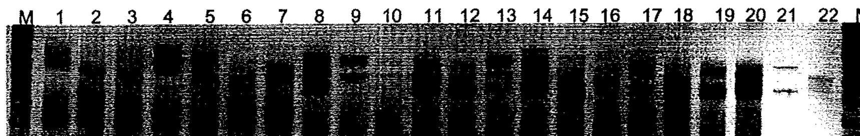

Figure 4 PCR-SSCP analysis for the NS5A 2209–2248 region, indicating the presence of the heterogeneity in the ISDR sequence in lanes 1, 3, 4, 7, 8, 11, 12, 13, 14, 15, 18, and 20. M, marker.

patients 34 and 37 (panel B) were responders with a Tm of 56.1°C and 45.7°C, respectively. A patient infected with HCV with a Tm of 62.1°C was an exceptional responder. All of these suggest that the high and low Tm values are equivalent to wild-type and mutant-type ISDR from the viewpoint of the prediction of IFN efficacy.

HCV quasispecies and MCA

HCV is present in plasma as a mixture of heterogeneous RNA genomes relating to HCV quasispecies. First of all, we examined the genetic complexity of the ISDR of HCV genomes by SSCP analysis. PCR-SSCP analysis revealed 2–7 bands (mean 5 bands, as shown in Figure 4), indicating that there were variant clones with differences in the degree of complexity even at the pre-treatment stage. Then, from four cases in lanes 3 (patient no. 17), 12 (no. 28), 20 (no. 37), and 1 (no. 15), a total of 86 clones were established and subjected to MCA assay. As shown in Figures 5 and 6B, MCA gave instructive Tm distributions forming several clusters with minor separated clones. The differences in Tm values among the clones were correlated to the nt substitution number, indicating that HCV genomes are heterogeneous in the ISDR case by case. These findings on Tm for amplicons and clones and their cluster patterns are shown in Figure 5. For example, in patient no. 17, although the Tm of the

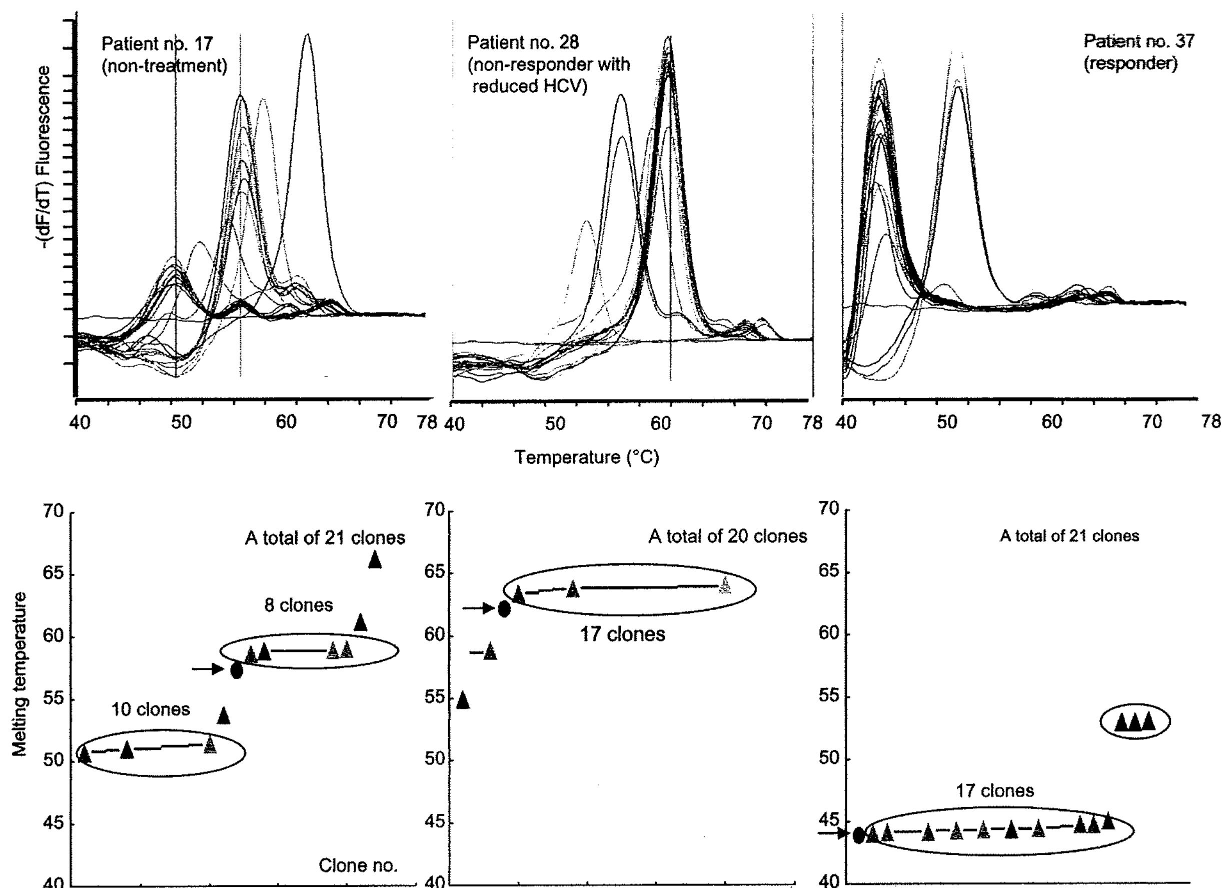

Figure 5 Melting curves (upper) and dotted plots (lower) of Tm after cloning of each isolate prior to treatment, showing that the HCV genome is a mixture of clones with subtle variation in Tm relating to HCV quasispecies.
Solid circle (arrowhead), Tm of amplicons; other triangles, Tm of clones. The MCA assay provides subtly different Tm values between clones reflecting the degree of the genetic heterogeneity of three HCV samples. In patient no. 17, they consist of two major clones with Tm values of 50.8°C and 57.8°C and several scattered clones; in patient no. 28, of one major clone with Tm of 64.0°C and rare minor clones with Tm around 55.0°C; and in patient no. 37, of one major clone with Tm of 44.0°C and other minor clones.

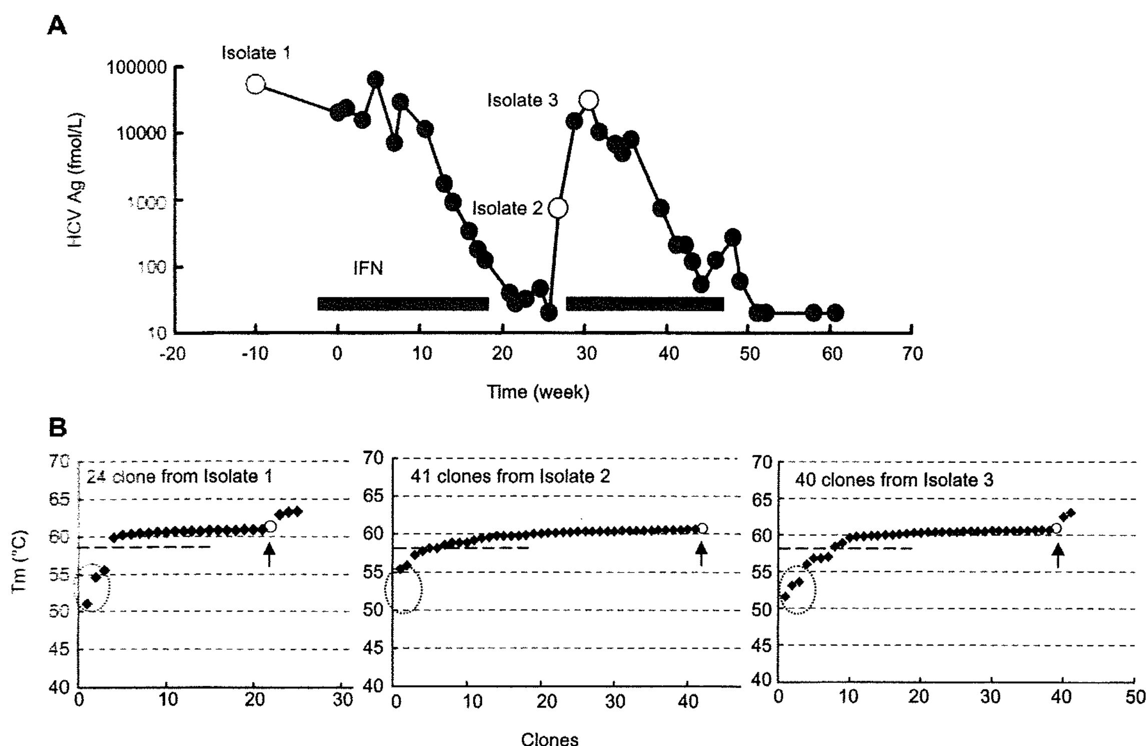

Figure 6 Observation of changes in the composition of clones by the MCA assay during IFN therapy.
(A) Changes in HCV viral load after IFN therapy. (B) Sequential observation of clones at three clinical points by MCA assay. Although the Tm value was 61.2°C in isolate 1, 60.7°C in isolate 2, and 60.9°C in isolate 3, the Tm values after cloning from each isolate showed that many different clones exist, characterized by subtly different Tm values, relating to quasispecies. In particular, the disappearance of clones with low Tm (dotted line circle in panel B) in isolate 2 is noteworthy, which reappears again in isolate 3. The major clones with high Tm remained unchanged during the entire clinical course.

amplicon was 57.2°C, the Tms of clones were variable in a range of 50.7°C–66.2°C distributing in two major clusters around 50.8°C and 57.8°C, one minor cluster, and scattered clones. The band number was 3. In patient no. 15, changes in the composition of clones defined by Tm were monitored during IFN therapy, as shown in Figure 6. The Tm of three HCV samples collected at the three clinical points of pre-treatment, discontinuation, and re-treatment was almost the same, 61.2°C, 60.7°C, and 61.0°C, respectively, whereas dot plot distribution profiles of Tm after cloning of each sample were subtly different in the range of 51.0°C–63.4°C, 55.3°C–60.7°C, and 51.6°C–62.3°C, respectively. Notably, among these clones with different Tm, clones with values less than 55.0°C disappeared after treatment with IFN, and they appeared again at the time of re-treatment. The major clones with high Tm remained unchanged.

Discussion

In this study, we developed a simple and rapid PCR-MCA method for screening the genetic heterogeneity of the ISDR of HCV genomes, indicating that this MCA assay is applicable for clinical use as a predictive marker of IFN efficacy or monitoring of the emergence of new clones. This is meaningful, because approximately 2% of the human population worldwide are infected with and suffer from HCV-related disorders. At present, IFN therapy makes it possible to relieve symptoms caused by HCV, such as chronic hepatitis, cirrhosis, and HCC (17, 18). However, the current combination therapy of IFN and ribavirin is not universally effective in patients with chronic hepatitis C, showing response rates of 90%–40% (19). The difference in responsiveness is thought to be in part explained by viral load and the genetic heterogeneity of the HCV genome (5, 6, 20). In particular, genotype 1 prevalent in Japan is known to be resistant to IFN. Enomoto et al. (6) describe a correlation between the number of mutations within a 40 aa sequence of the ISDR and the response to IFN therapy in genotype-1b infected patients. Thus, although the mechanism of IFN resistance is now being elucidated, the translation from research laboratory to clinical application is poor. The practical application of findings of ISDR mutations and IFN responsiveness is no exception. The reason why practical applications lag behind current research is due in part to technical and economic problems in translating clinical laboratory tests, with regard to time, labor, and cost. Although there are many methodologies for the detection of mutations, such as sequencing, SSCP, DHPLC, microarray technology, etc., we examined MCA combined with PCR amplification, because it is a simple and rapid method for screening of genetic alterations. MCA is based on the basis that the Tm of duplex DNA is dependent upon the length, sequence, G:C content, and Watson-Crick base pairing (21, 22).

As the Tm value precisely reflects genetic alterations in the ISDR, a sequence region of nts 6954–6990 with high nt substitutions was selected as the anchor and sensor probe sites. All 34 cases had at least one nt substitution (either silent or missense mutation) in the probe sites and especially missense mutations were concentrated in this region. In other words, sequence status of the probe sites is expected to surrogate the genetic alterations of the entire sequence in the ISDR, accounting for the Tm values reflecting mutational status of the ISDR. This is actually the confirmed by this study. Thus, our PCR-MCA method with the hybridization probe gave promising results enabling us to subcategorize HCV genomes into two major groups: one group with a Tm >58.0°C and the other with a Tm <58.0°C from the viewpoint of IFN efficacy. Compared with sequence results, the former and the latter are approximately equivalent to cases with no aa substitutions and cases with two or more aa substitutions, respectively. However, cases having the one aa substitution defined by direct sequencing were distributed between both groups. We can interpret the meaning of this finding, because the Tm value is generated as a comprehensive result based on the total nt substitution number of missense and silent mutations. Accordingly, considering the features of the MCA assay, it is reasonable to designate the high Tm group as wild-type and the low Tm group as mutant-type in a clinical setting. This stratification by Tm, despite the small number of pilot cases, suggests its usefulness in predicting the efficacy of IFN therapy: non-responsive in the high Tm group and responsive in the low Tm group. However, we need a large-scale prospective clinical trial to verify the reliability of Tm as a predictive marker, because the validity of the MCA assay in a practical setting has been partially shown in this study.

Another important point of this MCA assay is to indicate that Tm values after cloning display the composition profiles of viral variants within HCV quasispecies more clearly than SSCP. HCV genomes in plasma are generally present as a mixture of heterogeneous genomes, designated quasispecies. To better understand the resistance mechanism against IFN, HCV quasispecies are interesting and instructive, because the pressure of IFN on virus replication could allow the generation and selection of new clones with IFN resistance. Generally, although HCV quasispecies are defined by sequencing or SSCP with targets in the HCV E2 region, 5'NCR, etc. regions (23), the methodology for quasispecies remains problematic, in terms of time and cost. Our study showed that the MCA assay can simultaneously and rapidly screen the degree of the genetic complexity of HCV according to the differences in Tm of the ISDR. To understand the composition of quasispecies precisely, it requires the analysis of a large number of clones. The MCA assay could easily and efficiently pick up a newly derived clone different from other major clones by comparison of Tm.

In conclusion, this study demonstrates that the MCA assay rapidly and simply provides Tm values reflecting the genetic heterogeneity in the ISDR of the HCV genome. According to the Tm status provided by this MCA assay, HCV genomes were classified into two types with high and low Tms, corresponding to wild-type ISDR and mutant-type ISDR from the viewpoint of IFN efficacy. Moreover, the MCA assay is convenient and useful to screen newly generated clones by the difference in Tm of the clone relating to the behavior of quasispecies.

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Original Article

Hepatitis C virus kinetics during the first phase of pegylated interferon-α-2b with ribavirin therapy in patients with living donor liver transplantation

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Aim: To identify the problems of pegylated interferon (PEG IFN) with ribavirin therapy against hepatitis C virus (HCV) reinfection in living donor liver transplantation (LDLT) patients. HCV kinetics during the PEG IFN with ribavirin therapy were analyzed in LDLT patients, as well as in chronic hepatitis C (CHC) patients.

Methods: The study included 80 consecutive HCV infected patients undergoing PEG IFN with ribavirin therapy (64 CHC and 16 LDLT patients) who attended the Nagasaki University Hospital for an initial visit between January 2005 and December 2007.

Results: The sustained viral response (VR) rate of the CHC group (80%) was superior to the LDLT group (22%). The viral disappearance rate of the CHC group was also superior to the LDLT group, regardless of the HCV serotype. The HCV core antigen (cAg) titer under treatment in the LDLT group was more than that of the CHC group from day 0 to week 12. The HCV cAg decrease rate of the LDLT group on the first day of treatment was less than that of the CHC group.

Conclusion: The HCV infection of a transplanted liver is more refractory to treatment than a non-transplanted liver. The low reduction HCV cAg rate on day 1 is one of the problems of the combination therapy.

Key words: chronic hepatitis C, first phase, hepatitis C virus, interferon, living donor liver transplantation

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection is widespread throughout the world. Chronic HCV infection leads to cirrhosis and hepatocellular carcinoma. Liver transplantation for HCV-related liver disease has been an option worldwide.[1] Recently, it has been shown that the prognosis for liver transplanted (LT) patients with HCV-related disease deteriorates over time,[2] thus resulting in a poorer outcome than in the non-HCV course.[3] The transplanted liver for HCV-related disease undergoes a rapidly progressive fibrosis and acute graft failure.[3,4] Consequently, anti-HCV treatment after LT is important for the prognosis. Interferon (IFN) has been recognized as the only treatment method for HCV infection. For the transplanted liver, it is known that IFN treatment improves liver fibrosis or halts the progression.[5] Recently, the combination of pegylated IFN (PEG IFN) with ribavirin was used and produced an excellent result for non-transplanted patients with HCV.[6] However, that was not the case for the HCV re-infected transplanted liver.[7] It is important that the cause of refractory HCV infection in the transplanted liver be more fully clarified. Immunosuppressant therapy, especially with glucocorticoid, has been speculated to be the cause of the refractory nature of the transplanted liver to IFN.[8,9] The cause of this is considered to be that glucocorticoid downregulated the IFN signal transduction in the hepatocytes.[8] The authors recently found that calcineurin inhibitors also inhibited IFN induced STAT-1 phosphorylation and antiviral activity in the HCV

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replicon system.[10] Therefore, the problem of IFN signaling in the hepatocyte induced an IFN refractory condition[11] and decreased the first phase of HCV decline, which was IFN induced HCV decay during the first day of IFN treatment.[12]

In the present study, we attempted to better understand PEG IFN and ribavirin therapy by comparing patients with chronic hepatitis from HCV infection (CHC) with living donor LT (LDLT) patients. When the non-transplanted CHC patients were used as a reference against the HCV reinfected LDLT patients, we expected that the differences in the clinical data in the two groups would help to clarify the problem of IFN refractory HCV infection, and shed light on the analysis of HCV kinetics under IFN and ribavirin treatment, and to elucidate the damaged segment of the IFN induced antiviral mechanism in the LDLT condition.

PATIENTS AND METHODS

Patients

THE PRESENT RESEARCH is a prospective study. The study included 80 consecutive HCV-infected patients undergoing PEG IFN with ribavirin combination therapy (64 CHC and 16 LDLT patients) who attended the Nagasaki University Hospital for an initial visit between January 2005 and December 2007. All patients received the targeted dose of 1.5 μg/kg PEG IFN-α-2b (Pegintron; Schering-Prough K.K., Osaka, Japan) once weekly with daily ribavirin (Rebetol; Schering-Prough K.K., Osaka, Japan) for a total dose of 600 mg (bodyweight < 60 kg), 800 mg (60 kg < bodyweight < 80 kg) or 1000 mg (bodyweight > 80 kg) according to bodyweight (BW). The number of patients who were judged to have obtained a curative effect from IFN therapy was 42 in total, and 12 were LDLT patients. If the HCV-RNA had been negative in the patient serum until 12 weeks after the initiation of treatment or positive at 24 weeks, PEG IFN with ribavirin therapy was stopped at week 48. If the HCV-RNA had been negative from weeks 12 to 24, PEG IFN with ribavirin therapy was continued for 24 weeks to a predetermined 48 weeks. CHC patients were diagnosed on the basis of a persistently raised alanine aminotransferase (ALT) level and biopsy proven disease. All LDLT patients, who had undergone liver transplantation for HCV related cirrhosis at Nagasaki University Hospital from June 2002 to May 2007, had the HCV-RNA in their serum at the commencement of PEG IFN with ribavirin treatment. To prevent HCV related hepatitis after liver transplantation, pre-emptive therapy using IFN is the strategy used at the Nagasaki University Hospital. After the recovery of the general condition without ascites and icterus after transplantation, and establishment of the diagnosis using the liver biopsy, PEG IFN with ribavirin therapy was started. The interval between LDLT and IFN treatment was a mean of 281 days (range 16–989 days). Tacrolimus (Astellas, Tokyo, Japan), an immunosuppressive agent, was used together with steroids for all LDLT patients as the induction therapy. When IFN therapy was commenced, tacrolimus was switched to cyclosporin (Novartis, Tokyo, Japan) in 12/16 cases. A percutaneous liver biopsy assisted by ultrasonography was carried out in all cases. Liver histology was evaluated according to the degree of fibrosis and necroinflammatory activity.[13] The extent of fibrosis (staging) was classified as follows: F1 (periportal expansion), F2 (portoportal septa), F3 (portocentral linkage or bridging fibrosis) and F4 (cirrhosis). The necroinflammatory activity (grading) was classified as follows: A1 (mild), A2 (moderate) and A3 (severe). Liver biopsy specimens were fixed in 10% formalin, embedded in paraffin, cut to a thickness of 4 μm, and subjected to hematoxylin-eosin and Azan-Mallory staining.

Hepatitis C virus kinetics assessment

We compared the HCV viral load in both groups, determined by the HCV core antigen (cAg), at baseline (D0), day 1 (D1), week 1 (W1), week 2 (W2), week 4 (W4), week 8 (W8), week 12 (W12), week 24 (W24) and week 48 (W48). The HCV viral serotype (ST) and HCV cAg were determined using available kits. In this assay, HCV serotypes 1 and 2 correspond to genotypes 1 and 2 of Simmonds' classification,[14] respectively. The HCV cAg correlates with HCV-RNA by quantitative PCR.[15] HCV cAg was measured at the indicated times and HCV-RNA qualitative PCR, the amplicor monitor method, was used after the level was under the detection range of HCV cAg in every month. In the present study, we proposed the calculation of the decreased HCV viral load during PEG IFN with ribavirin treatment and set as follows: a negative HCV cAg was 20 fmol/L and a negative HCV-RNA qualitative PCR was 1 fmol/L.

Clinical and laboratory measurements

The body mass index (BMI) was calculated as weight (kg) divided by the square of height (m). Subjects fasted overnight before blood samples were obtained. Venous plasma glucose was measured with an automated analyzer, and basal serum insulin was measured using a standard radioimmunoassay. The index of insulin

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resistance and β-cell function was calculated using the fasting value of plasma glucose (we excluded the patients with greater than 130 mg/dL), and the serum insulin level according to the homeostasis model assessment (HOMA) method. HOMA-IR, an insulin resistance marker, is calculated as follows: fasting plasma glucose × fasting insulin/405. HOMA-β, a β-cell function marker, was calculated as follows: 360 × fasting insulin/(fasting plasma glucose-63).[16] White blood cell, red blood cell, platelet, hemoglobin A1c, ALT, aspartate aminotransferase (AST), γ-GTP, total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL), high density lipoprotein (HDL), free fatty acid (FFA), and ferritin were determined by standard hematometry and laboratory techniques.

Statistical analysis

The data were processed on a personal computer and analyzed using StatView 5.0 (SAS Institute, Cary, NC, USA). Differences between groups were analyzed by Mann–Whitney *U*-test and Pearson χ^2-test. All data in the text and tables are shown as means, unless otherwise indicated. The statistical analysis of the HCV-RNA disappearance rate was by the Kaplan–Meier method with Wilcoxon assay. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Differences of patient characteristics

FIRST, THE PRETREATMENT clinical and laboratory characteristics were compared with All-CHC and All-LDLT patients (Table 1). The BW and BMI in the All-CHC group were higher than that of the All-LDLT group. Therefore, the levels of PEG IFN dose per BW and ribavirin dose per BW were even, but the levels of PEG IFN dose and ribavirin dose in the all LDLT group were lower than in the All-CHC group. The HCV viral load in the all LDLT group was greater than that in the All-CHC group and serotype 1 was the majority in the All-LDLT group. In hematology and laboratory data, the red blood cell count and hemoglobin in the All-LDLT group was lower than that of the All-CHC group, and the FFA level was higher in the All-LDLT group. In the histological examination, fibrosis is more advanced in the All-CHC group than in the All-LDLT group. There was the tendency toward higher levels of fasting plasma glucose and lower levels of HOMA-β in the All-LDLT group than in the All-CHC group. Next, we targeted the serotype 1 and a high HCV titer (ST1H group) above 100 KIU/L by the qualitative PCR method or 300 fmol/L of the cAg assay. These were examined in the same way (Table 2). The ST1H group might have shown the same result as the All group, except the levels of fasting plasma glucose and HOMA-β did not differ with ST1H-CHC and ST1H-LDLT. The mean value of fasting plasma glucose (FPG) was higher than the normal range in the LDLT group. The discontinuance rates of treatment were almost equal, 19 cases (29.7%) and 4 cases (25%) in All-CHC and All-LDLT, respectively. The reasons for discontinuance were adverse effects in All-LDLT patients and the refractory nature of viral response in two All-CHC patients.

The HCV infection in the LDLT group is more obstinate than in the CHC group

The response rate and cure rate of PEG IFN with ribavirin therapy were compared with both groups (Table 2A, All group and B, ST1H group). The HCV response rate to treatment, viral response (VR), was determined by the disappearance of HCV-RNA or by the decline of HCV cAg to less than 1/100 before treatment. The cure rate, sustained viral response rate (SVR), was determined by a negative HCV-RNA by qualitative PCR method at 6 months post-termination of treatment. The VR rate at 8 and 12 weeks, but not at 4 weeks, and the PP-SVR in the LDLT group (Table 3A,B) was worse than that in the CHC group. Non-viral responders, who did not achieve HCV-RNA negativity during the treatment, did not show statistical significance in either SG1H group (Table 3B). As a result, we calculated the prediction of the lack of SVR by non-viral response in the LDLT group. The sensitivity, specificity, positive predictive values and negative predictive value were 1, 0, 0.917 and the acalculia for null viral responders at 24 h, 0.7, 1, 1 and 0.25 at 4 weeks, 0.6, 1, 1 and 0.2 at 8 weeks and 0.6, 1, 1 and 0.2 at 12 weeks, respectively.

The disappearance rate of HCV-RNA was evaluated by the Kaplan–Meier method (Fig. 1 ST1H group). The disappearance rate in the LDLT group was statistically lower than the CHC group. Before 14 weeks after the initiation of treatment, the HCV-RNA disappearance case was not apparent in the ST1H group (Fig. 1).

The decline of HCV load, especially early phase, is blocked in the LDLT group

For the analysis of viral kinetics, we evaluated the decline of the HCV load and the decline rate after treatment with particular emphasis of the early phase of treatment, including D1-W12. In the ST1H group (Fig. 2), the decreased rate on D1 in the LDLT group was

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Table 1 Difference of characteristics between all chronic hepatitis C cases and all living donor liver transplantation cases

Characteristics	All-CHC (n = 64)	All-LDLT (n = 16)	P-value
Age (years)	58 ± 10.8	58.8 ± 4.62	NS
Sex (male : female)	36:28	7:9	NS
Height (m)	1.60 ± 0.098	1.583 ± 0.010	NS
Bodyweight (kg)	61.0 ± 11.0	54.8 ± 8.52	0.025
Body mass index	23.6 ± 2.94	21.8 ± 2.30	0.022
PEG IFN dose (μg)	80.1 ± 18.7	71.9 ± 33.5	0.035
PEG IFN/BW	1.31 ± 0.304	1.35 ± 0.708	NS
Ribavirin dose (mg)	621.9 ± 151.7	525 ± 100	0.030
Ribavirin/BW	10.2 ± 2.23	9.72 ± 2.04	NS
Serotype (1:2)	45:17	15:1	0.081
HCV cAg (fmol/L)	5773 ± 5609	23144 ± 21059	0.001
WBC (/μL)	5006.3 ± 1335	5918.8 ± 2439	NS
RBC (10^4/μL)	445 ± 41.1	350 ± 56.7	< 0.0001
Hemoglobin (g/dL)	13.8 ± 1.06	10.9 ± 1.85	< 0.0001
Platelet (10^4/μL)	16.4 ± 4.48	18.5 ± 10.6	NS
AST (U/L)	62.9 ± 35	64.3 ± 37.2	NS
ALT (U/L)	85 ± 53.0	89.9 ± 57.1	NS
γ-GTP (U/L)	62.1 ± 56.5	138.9 ± 129.1	0.013
Ferritin (ng/dL)	218 ± 216	254 ± 259	NS
TC (mg/dL)	169.8 ± 26.6	167.3 ± 38.8	NS
TG (mg/dL)	105.3 ± 46.8	122.8 ± 44.8	0.069
HDL (mg/dL)	45.2 ± 11.9	46.6 ± 14.9	NS
LDL (mg/dL)	97.3 ± 24.3	88.8 ± 26.7	NS
FFA (mEq/L)	0.492 ± 0.261	0.686 ± 0.299	0.019
FPG (mg/dL)	91.9 ± 15.4	125.1 ± 56.9	0.090
Insulin (mIU/L)	9.16 ± 5.1	8.34 ± 5.16	NS
HOMA-IR	2.08 ± 1.22	1.75 ± 1.42	NS
HOMA-β	135.4 ± 86.2	89.7 ± 86.9	0.075
Fibrosis	1.86 ± 1.18	0.875 ± 0.806	0.004
Activity	1.03 ± 0.48	1.31 ± 0.48	0.067

Data are shown as the means ± standard deviation and values, with statistical analysis calculated by Mann–Whitney U-test for means and Pearson's χ^2-test for values.
Normal values in laboratory tests: ALT (IU/L), 5–40; AST (IU/L), 10–40; γ-GTP (IU/L), < 70 in males, < 30 in females; TC (mg/dL), 150–219; TG (mg/dL), 50–149; FFA (mEq/L), 0.14–0.85; LDL (mg/dL), 70–139; HDL (mg/dL), 40–86 in male, 40–96 in female; hemoglobin (g/dL), 13.5–17.6 in male, 11.3–15.2 in female; WBC (/μL), 3900–9800 in males, 3500–9100 in females; RBC (10^4/μL), 427–570 in males, 376–500 in females; ferritin (mg/dL), 27–320 in males, 3.4–89 in females; platelet (10^4/μL), 13.1–36.2 in males, 13–36.9 in females; insulin (IU/L), 3.06–16.9; FPG (mg/L), 70–109. HOMA-IR, HOMA-β, and BMI are described in the text.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHC, chronic hepatitis C; FFA, free fatty acid; FPG, fasting plasma glucose; HCV cAg, hepatitis C virus core antigen; HDL, high density lipoprotein; HOMA, homeostasis model assessment; LDL, low density lipoprotein; LDLT, living donor liver transplantation; PEG IFN, pegylated interferon; RBC, red blood cell count; TC, total cholesterol; TG, triglyceride; WBC, white blood cell count.

statistically lower than CHC (Fig. 2b) and the viral load of the LDLT group was larger than that in CHC from D0 to W12 (Fig. 2a). The decreased rate at the indicated time without D1 and W12 was not the difference between CHC and LDLT (Fig. 2b). We next analyzed the SG1H-group that matched the pre-treatment HCV cAg titer (Fig. 3). In a similar fashion to Figure 2, the viral load of the matched LDLT group was larger than that of the matched CHC from D1 to W12 (Fig. 3a) and the decreased rate of the matched LDLT group was lower than that of the matched CHC at D1, W2 and W4 (Fig. 3b).

DISCUSSION

IN THE PRESENT prospective study, we compared CHC and LDLT patients treated with PEG IFN and ribavirin for HCV infection. BMI, HCV cAg, red blood

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