important to investigate the host factors which prescribe responsiveness to treatment with IFN.

In the present study, gene analysis of the entire NS5A region in HCV-1b infected patients was performed before and after withdrawal of IFN therapy. There have been few reports analyzing the entire NS5A region from the same patients infected with genotype 1b of HCV before and after IFN treatment (Pawlotsky and others 1998; Cuevas and others 2008). In the ETR (n=11) and NR (n=11) groups, some amino acid substitutions were found after treatment withdrawal regardless of the therapeutic outcome in all cases. However, these substitutions were sporadic and no characteristic substitutions related to the therapeutic outcome were observed. Although further analysis is needed, these findings suggest a change of population based on a quasispecies of HCV that does not depend on the IFN treatment. Any adaptive mutations (Blight and others 2000; Krieger and others 2001; Lohmann and others 2001, 2003; Sumpter and others 2004) that strengthen replication efficiency in HCV replicon cells could not be found, and serine residues in the central and C-terminus NS5A concerning phosphorylation of NS5A (Kaneko and others 1994; Tanji and others 1995; Asabe and others 1997; Katze and others 2000) were completely conserved.

On the other hand, some features were seen in the NS5A amino acid sequence before IFN treatment. As reported by Enomoto and others (1996), mutations of ISDR in NS5A significantly correlated with SVR, suggesting that the mutations in ISDR predict the

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achievement of SVR by IFN therapy. Moreover, the proportion of the amino acid at position 2378 located in V3 region was more remarkable. A2378 was significant in the ETR and SVR group, but was never detected in the NR group, while T2378 was significantly correlated with NR. A large cohort study is needed to investigate the clinical importance of the single amino acid substitution in NS5A. However, it is thought to be clinically important at least in part that a significant change in IFN response is detected by the single amino acid substitution of HCV NS5A, although it was conducted using small sample size. It was reported that the variability of NS5A in the V3 region is correlated with the treatment outcome of IFN (Duverlie and others 1998; Nousbaum and others 2000; Puig-Basagoiti and others 2005), and was suggested that the significant amino acid found in this study located in the V3 region was concerned not only with the treatment outcome, but also for ALT normalization. Furthermore, the amino acid at 2378 was conserved in all patients before and after withdrawal of the therapy. This finding suggests that the variation in the V3 region may indicate the therapy outcome as well as normalization of the ALT level.

Examining the relationship between ALT normalization and ISDR mutations, there did not seem to be a significant difference between the ISDR mutation number and the ALT values in our study. Yoshioka and others (2005) reported that if there were more than four ISDR mutations, the ALT values were rather high. Moreover, Hiraga and others (2005) reported that the elevation of the ALT value did not show a significant correlation to the ISDR mutation

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number. Our results were basically consistent with their analysis in the aspect that the ISDR mutation number did not correlate significantly with ALT normalization.

Concerning the IFN sensitivity, the HCV replicon cells (Huh-9-13 cell) used in this study exhibit IFN sensitive phenotype in spite of having the HCV subgenome of genotype 1b (Con1 strain), which is generally considered to be IFN resistant. To clarify the role of amino acid 2378, alanine substitution for threonine at 2378 in replicon cells (T2378) was examined. T2378 replicon cells showed a reduction in IFN sensitivity and a suppression of transactivation by IFN compared with those of wild type (A2378) replicon cells. These observations suggest that amino acid at the 2378 plays an important role in the IFN response in replicon cells of this study. It is very important to clarify the underlying molecular mechanisms in regard to how T2378 in NS5A confers IFN resistance on the replicon cells. Further, the relationship between the IFN therapeutic outcomes in HCV infected patients and the IFN response in replicon cells, although both are caused by amino acid 2378 in NS5A, needs clarification.

Regarding the ALT value, A2378 was detected frequently in the ETR group with relapse but maintenance of a normal ALT value before and after withdrawal of IFN therapy, as well as in the SVR group. In contrast, A2378 was not found in the NR group, which showed abnormal ALT values in this study. Progression of the hepatitis such as development of fibrosis, cirrhosis, and HCC in HCV infected patients with persistently normal ALT showed slower

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phenotype and better prognosis than those with an abnormal ALT. It has been reported that the ETR rate in HCV infected patients with a normal ALT was higher than those with an abnormal ALT, and in addition, the SVR rate in normal ALT patients was almost the same as in abnormal ALT patients (Zeuzem and others 2004). Moreover, normalization of ALT by IFN treatment represses the development of HCC (Nishiguchi and others 1995). It is thought that not only achieving SVR by combination therapy of IFN with ribavirin, but also delay in the progression of hepatitis such as development of cirrhosis or HCC by IFN treatment with slight side effects is important, especially in Japan, because an infection with genotype 1b of HCV spreads and the age of patients is high particularly in Japan. It is still unclear whether SVR and ETR by IFN cause normalization of ALT by A2378; however, A2378 may be an important prediction factor for the normalization of ALT in this study. Although ALT varies depending on various factors other than chronic infection with HCV, establishment of a suitable evaluation to detect a relationship between a change of ALT and HCV is needed. Analyses of the molecular mechanisms such as ALT normalization and acquisition of IFN sensitivity by A2378 may contribute to development of new drug targets for HCV.

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Author Disclosure Statement

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Figure legends

FIG. 1.

A phylogenetic tree of the nonstructural protein 5A (NS5A) region in 33 patients infected with genotype 1b of hepatitis C virus (HCV). Patient number, treatment outcome, and gender (M, male; F, female) are shown for all patients. As reference strains, HCV-J and Con1 strain were included in the tree. A genetic distance was indicated in the parentheses after patient number.

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FIG. 2.

A relationship between alanine aminotransferase (ALT) value and the amino acid at position 2378 of NS5A. Bars indicated mean±SD, and open circles represented individual ALT values (IU/L). $P<0.05$ versus T2378 by the Tukey-Kramer test. ALT: alanine aminotransferase.

FIG. 3.

IFN sensitivity of T2378-harboring replicon cells. (A) Colony formations of T2378 or A2378 replicon cells in the presence or absence of Interferon α (IFNα). HCV replicon RNA possessing A2378 (wild type) or T2378 (mutant type) from Con 1 strain were transfected into naïve Huh-7 cells. These transfected cells were selected with G418 (1 mg/mL) in the

presence or absence of IFNα for 4 weeks. The colonies obtained from the selection were stained with Crystal Violet. The upper panel shows a selection without IFNα and the lower panel showed a selection with 10 IU/mL of IFNα. HCV: hepatitis C virus, wt: wild type, mt: mutant type. (B) Western blot analysis of HCV non-structural (NS) protein expression in the T2378 and the original A2378 replicon cells. The expression of β-actin was used as an internal control. Each cell was seeded on a 60-mm plate at 3×10^5 cells/well. After twenty-four hours, the cells were lysed with SDS sample buffer. Total proteins were subjected to a 2/15% SDS gradient gel, and were subsequently immunoblotted by NS3, NS5A, NS5B, and β-actin antibody, respectively. Lane 1: A2378 (wild type: wt) replicon cells. Lane 2: T2378 (mutant type: mt) replicon cells. SDS: sodium dodecyl sulfate. (C) Reactivity for IFNα in the T2378 mutant replicon cells and the original A2378 replicon cells. The cells were treated with 0, 0.03, 0.1, 0.3, 1, 3, 10, and 30 IU/mL of IFNα for 6 days, and the amount of cellular HCV RNA was measured by quantitative RT-PCR to determine the EC50 value of IFNα. Change in copy number of HCV RNA in the mutant T2378 (open circle) and the original A2378 (closed circle) replicon cells by IFNα treatment. These experiments were performed in triplicate and are shown as mean values ± SEM. In the panel, the EC50 value (IU/mL) of IFNα in each replicon and the fold reduction of the value compared to the original replicon are shown as a table. RT-PCR: reverse transcriptase polymerase chain reaction, EC50: half maximal effective concentration. (D) Transactivation of ISRE in the

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T2378 replicon cells (open circle) and the original A2378 replicon cells (closed circle) by reporter gene (pISRE/Luc) analysis. The cells were stimulated with 0.1, 1, 10, 100, and 1000 IU/mL of IFNα for 24 hours after transfection of reporter plasmid DNA. Then, the luciferase activity of the transfected cells were measured using Steady-Glo® Luciferase Assay System (Promega). These experiments were performed in triplicate and are shown as mean values ± SEM. ISRE: interferon stimulated gene responsive element.

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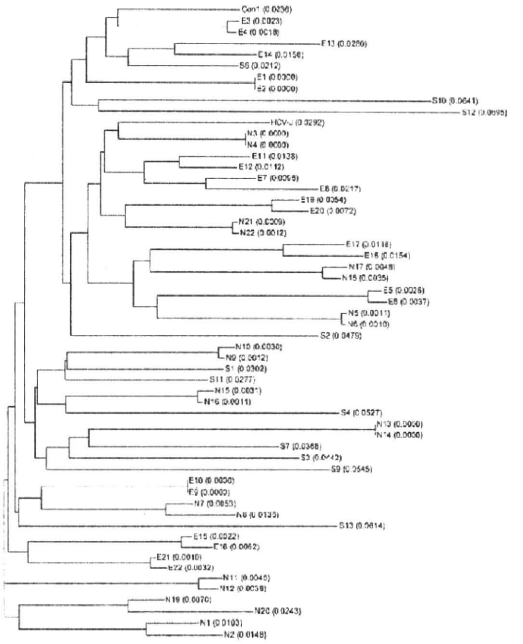
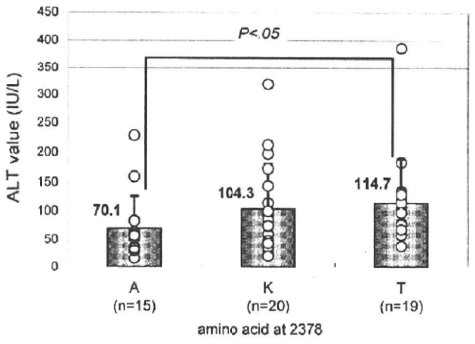
Figure 1.



A phylogenetic tree of the nonstructural protein 5A (NS5A) region in 33 patients infected with genotype 1b of hepatitis C virus (HCV). Patient number, treatment outcome, and gender (M, male; F, female) are shown for all patients. As reference strains, HCV-J and Con1 strain were included in the tree. A genetic distance was indicated in the parentheses after patient number.
210x297mm (600 x 600 DPI)

Figure 2.



A relationship between alanine aminotransferase (ALT) value and the amino acid at position 2378 of NS5A. Bars indicated mean±SD, and open circles represented individual ALT values (IU/L). $P<0.05$ versus T2378 by the Tukey-Kramer test. ALT: alanine aminotransferase.
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Figure 3.

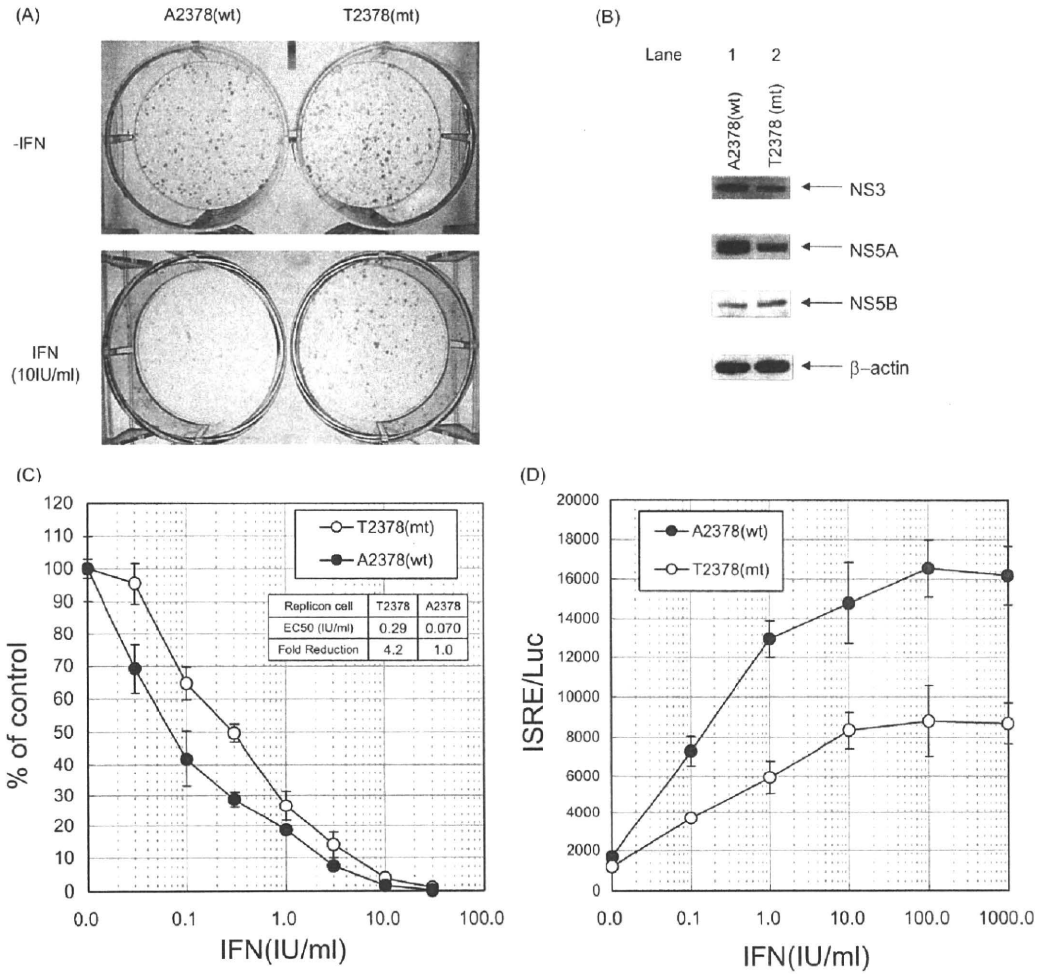
(A)
A2378(wt)
T2378(mt)
-IFN
IFN
(10IU/ml)
(B)
Lane 1 2
A2378(wt)
T2378 (mt)
NS3
NS5A
NS5B
β-actin
(C)
T2378(mt)
A2378(wt)
Replicon cell T2378 A2378
EC50 (IU/ml) 0.29 0.070
Fold Reduction 4.2 1.0
% of control
IFN(IU/ml)
(D)
A2378(wt)
T2378(mt)
ISRE/Luc
IFN(IU/ml)

Table 1. Baseline characterisics of patients with chronic hepatitis C virus subtype 1b infection

Patient No.	Sample No.	Gender	Age	Geno type	Administration of IFNα-2b		Serum collection (weeks after therapy start)	HCV RNA (copies/ml serum)	ALT value (IU/L)	Therapy outcome	Mutations in ISDR before treatment
					Period (weeks)	Total (MIU)					
1	S1	male	34	1b	24	528	0	2.4E+04	386	SVR	1
2	S2	female	54	1b	24	528	0	8.9E+04	173	SVR	8
3	S3	male	26	1b	24	528	0	6.3E+03	105	SVR	6
4	S4	male	57	1b	24	528	0	1.8E+05	170	SVR	6
6	S6	male	27	1b	24	580	0	1.0E+06	160	SVR	1
7	S7	male	63	1b	24	580	0	4.0E+05	229	SVR	3
9	S9	male	53	1b	24	600	0	1.0E+05	64	SVR	7
10	S10	male	56	1b	24	600	0	2.2E+06	144	SVR	8
11	S11	male	49	1b	24	600	0	1.6E+07	68	SVR	1
12	S12	male	50	1b	24	600	0	3.6E+06	84	SVR	5
13	S13	female	48	1b	24	600	0	7.4E+04	60	SVR	4
14	E1 E2	female	47	1b	24	528	0 48	4.1E+06 4.3E+06	55 30	ETR	1
15	E3 E4	female	56	1b	24	528	0 48	2.5E+05 5.9E+06	61 16	ETR	1
16	E5 E6	female	58	1b	24	528	0 48	8.7E+06 5.6E+07	85 32	ETR	4
17	E7 E8	male	41	1b	24	880	0 48	1.0E+03 6.4E+08	319 26	ETR	0
18	E9 E10	female	59	1b	24	580	0 48	4.7E+06 1.3E+07	52 37	ETR	0
19	E11 E12	female	45	1b	24	740	0 101	3.2E+07 3.4E+07	60 36	ETR	0
20	E13 E14	female	59	1b	24	656	0 72	3.2E+07 6.1E+08	83 34	ETR	3
21	E15 E16	female	36	1b	24	793	0 48	5.6E+06 1.1E+06	57 30	ETR	0
22	E17 E18	male	56	1b	24	580	0 48	1.1E+09 2.5E+08	100 43	ETR	3
23	E19 E20	female	56	1b	24	600	0 48	6.5E+07 1.5E+05	75 20	ETR	1
24	E21 E22	male	40	1b	24	810	0 94	1.5E+08 4.9E+08	68 38	ETR	1
25	N1 N2	female	56	1b	24	528	0 48	1.4E+07 6.1E+07	59 78	NR	0
26	N3 N4	female	33	1b	24	528	0 48	1.1E+07 1.8E+07	59 198	NR	0
27	N5 N6	male	58	1b	24	793	0 48	1.3E+08 1.6E+08	213 173	NR	5
28	N7 N8	female	63	1b	24	528	0 48	2.1E+06 8.1E+06	135 80	NR	0
29	N9 N10	female	52	1b	24	528	0 48	3.4E+07 6.3E+05	184 119	NR	1
30	N11 N12	male	56	1b	24	528	0 48	2.8E+07 1.3E+08	123 127	NR	3
31	N13 N14	male	43	1b	24	528	0 48	1.8E+03 3.8E+05	77 58	NR	8
32	N15 N16	male	58	1b	24	656	0 48	2.4E+07 5.2E+07	114 133	NR	1
33	N17 N18	male	48	1b	24	580	0 48	1.6E+07 4.4E+07	48 114	NR	7
34	N19 N20	female	62	1b	24	580	0 72	3.0E+07 2.7E+08	98 129	NR	3
35	N21 N22	male	42	1b	24	880	0 72	3.1E+07 1.8E+07	74 93	NR	1

Serum samples were collected before and after withdrawal of the IFN therapy in NR and ETR patients, while collected only before the therapy in SVR patients. In most cases, IFN was administered 6 times a week for initial 4 weeks, and 3 times a week for 20 weeks thereafter.