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Original article

Identification of novel HCV deletion mutants in chronic hepatitis C patients

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Background: The HCV genome consists of a positive 9.6 kb single-strand of RNA. Nucleotide substitutions in the HCV genome are common and a 2 kb deletion has been reported.
Methods: A total of 117 chronic hepatitis C (CHC) patients who were treated with pegylated interferon plus ribavirin combination therapy were enrolled in this study. Total RNA was extracted from the patients' sera and reverse transcription and PCR were performed. Statistical analysis was performed to evaluate the effects of HCV deletion mutants on treatment with combination therapy.
Results: By amplifying entire HCV genomes using long-distance PCR, novel large deletion mutants were identified. Sequence analysis revealed that these deletions extended approximately 6 kb from the core/E2 region to the NS5A region and that there are three kinds of deletions that are identical at their 3′ and 5′ extremities. The subgenome virus particles appeared to coexist with full-genome virus particles in the sera of CHC patients despite lacking essential components for HCV viral replication. These short fragments were detected in 26 of 117 patients and were associated with significantly higher HCV RNA levels (P=0.018) and poor response to combination therapy (P=0.043). Moreover, the existence of HCV deletion mutants was significantly associated with virological relapse following combination therapy (P=0.046, OR=3.4).
Conclusions: HCV deletion mutants may affect the HCV life cycle and reduce the antiviral effects of interferon therapy for CHC.

Introduction

More than 170 million people are chronically infected with HCV [1–3], and chronic HCV infection may lead to chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [4–8]. HCV is an enveloped single-stranded RNA virus in the *Flaviviridae* family. The 9.6 kb viral genome includes a large open reading frame encoding a large viral polyprotein that is post-translationally processed into ≥10 proteins essential for viral replication [9–11]. Because of the lack of proofreading ability of the viral RNA-dependent RNA polymerase, genomic mutations in RNA viruses occur more frequently than in DNA viruses, facilitating escape from strong selective pressure imposed by host immunological mechanisms and antiviral therapy [12,13]. As a result of high virion production in chronically HCV-infected patients, approximately 10^{-3}–10^{-4} errors per site per year occur in HCV genomes. Such vigorous mutation has resulted in the diversification of virus genomes into six genotypes as well as the typical presence of quasispecies in infected patients [10,14,15].

Several viral and host factors are known to be associated with outcome of interferon (IFN) therapy. HCV genotype, HCV RNA level and HCV amino acid substitutions in the core region and the IFN sensitivity determining region (ISDR) are known as predictive factors for sustained virological response (SVR). Patients with genotype 1b and high virus titres tend to respond poorly to IFN therapy [16–19]. One or more amino acid (aa) substitutions in the ISDR is associated with SVR in HCV genotype 1b patients [20], and the presence of aa70 and/

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or aa91 substitutions in the HCV core region is an independent predictor of virological response (for example, early virological response, SVR and non-virological response) [21–24]. Host factors, such as patient age, gender and liver fibrosis stage are also known to be associated with response to IFN therapy. Several recent genome-wide association studies have revealed a strong association between genetic variation in the interleukin 28B (IL28B) locus and response to IFN therapy [25–27].

HCV deletion mutants were recently identified in sera or liver biopsy specimens of chronically infected HCV patients [28–30] and were detected in approximately 20% of chronic hepatitis C patients independently of HCV genotype [29]. Deletions occurred in various regions between E1 and NS2 of the HCV genome [29]. Despite various large in-frame deletions, these mutants were still able to produce HCV-related proteins, but the consequences of these deletions have not been examined [30]. It has previously been reported that deletion mutants in the envelope region are associated with poor response to IFN-α2b and ribavirin (RBV) combination therapy in patients with recurrent hepatitis C after liver transplantation [28]. HCV RNA in all of these patients decreased by <2 $\log_{10}$ within the initial 2 weeks of the combination therapy [28].

In this study, we report a novel large deletion of the HCV genome that spans from the core to the NS5A region and examine the clinical characteristics of patients with this deletion and the relationship between the presence of the deletion and the outcome of pegylated (PEG)-IFN and RBV combination therapy.

Methods

Patients and human serum samples

A total of 353 adult Japanese patients infected with HCV genotype 1b provided written informed consent to participate in the present study at Hiroshima University Hospital in Hiroshima, Japan. Among these patients, 117 who were treated with PEG-IFN plus RBV combination therapy between August 2004 and June 2008 were selected as study subjects based on the following criteria: high viral load before therapy (>5.0 $\log_{10}$ IU/ml [31]); not coinfected with HIV or HBV; no other liver diseases, such as alcoholic liver disease, autoimmune hepatitis or decompensated cirrhosis (patients with total ethanol intake >100 kg in their lifetime were excluded [32]); and no coexisting conditions, such as post-transplantation, poorly controlled diabetes mellitus, decompensated renal disease, pre-existing psychiatric disease, seizure disorders, cardiovascular disease, haemophilia or autoimmune diseases. All selected patients were followed until 6 months after the completion of combination therapy. Overall, 45 of the 117 patients had received prior antiviral treatments (IFN monotherapy or IFN plus RBV combination therapy), but no patients had received PEG-IFN monotherapy or PEG-IFN plus RBV combination therapy. The experimental protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Hiroshima University Hospital ethical committee.

Laboratory tests

White blood cell (WBC) count, haemoglobin, platelet count (Plt), total bilirubin (TBil), aspartate aminotransferase, alanine aminotransferase and γ-glutamyl transpeptidase (γ-GTP) were measured prior to the start of therapy. HCV RNA levels were analysed with the COBAS® Amplicor HCV monitor test (version 2.0; Roche Diagnostics, Indianapolis, IN, USA) prior to treatment, at weeks 1, 2 and 4, and again 24 weeks after the end of treatment.

Virological responses to the combination therapy were defined as follows: rapid virological response, undetectable HCV RNA by a qualitative PCR test 4 weeks after initiation of therapy; early virological response, undetectable HCV RNA 12 weeks after the start of therapy; SVR, continuously undetectable HCV RNA and normalized alanine aminotransferase levels by 24 weeks after the end of treatment; virological response, HCV RNA became negative during treatment; non-virological response, HCV RNA never became negative; virological relapse, HCV RNA became negative during therapy but was positive again 24 weeks after the end of treatment.

Extraction of HCV RNA and reverse transcription

HCV RNA was extracted from serum samples by Sepa Gene RV-R (Sanko Junyaku Co., Ltd, Tokyo, Japan) and dissolved with 8.8 μl of RNase-free water. Reverse transcription (RT) reactions were performed with 20 μl of the reaction mixtures and contained random primer (Takara Bio Inc., Shiga, Japan), RT buffer and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) according to the manufacturer's instructions.

Amplification of HCV genomes

To amplify the HCV genomes, nested-PCR was performed using complementary DNA (cDNA) from patient sera. Following RT, we performed nested-PCR with two programmes using LA Taq (Takara Bio Inc.) according to the manufacturer's instructions. Long-distance RT nested-PCR was performed as follows: the first amplification was performed with a step cycle programme of 94°C for 1 min, 55°C for 1 min and 72°C for 6.5 min for 30 cycles, using primers cc7 and 5A02k1 (Additional file 1). For the second PCR, with 1 μl of 50× diluted first PCR product as a template, amplification was performed with an initial denaturation for

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2 min at 94°C followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 6.5 min and final extension at 72°C for 7 min using primers HCV-J146 and 5A05k1R (Additional file 1). For amplification of HCV deletion mutants, short distance RT nested-PCR was performed using HCV-J146 as the forward primer and 5A05k1R or 1delR to approximately 5delR as the reverse primer (Additional file 1). Except for an extension time that was reduced to 1 min in each cycling step and final extension in the second PCR, the PCR conditions were the same as for long-distance RT nested-PCR. Amplified products were separated in 1% or 2% agarose gel, and purified with the QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany). To avoid PCR artefacts, nested-PCR was performed using a plasmid including the HCV full genome obtained from HCV carrier and cDNA product extracted from the serum of chronic hepatitis B patient.

Quantification of full genome HCV RNA

To quantify full-genome HCV RNA levels, real-time PCR was performed using cDNA from patient sera at weeks 0 and 4 using primers HCV-J3801F and HCV-J3950R (Additional file 1) under the following thermal cycling conditions: a pre-cycling period of 1 min at 95°C followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min.

DNA sequencing and sequence analysis

The extracted DNA fragments were sequenced using amplification primers and BigDye Terminator version 3.1 (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems).

Detection of amino acid substitutions in core and NS5A regions

To determine the nucleotide sequences in the HCV core region, cDNA was amplified by nested-PCR using the following protocol in 115 patients: initial denaturation at 95°C for 5 min, 35 cycles of denaturation for 30 s at 94°C, annealing of primers for 1 min at 57°C, and extension for 1 min at 72°C, followed by final extension at 72°C for 7 min. Primers cc11 and e14 were used for the first-round PCR, and cc9 and e14 for second-round PCR (Additional file 1) as described previously [21,22,28–30,33]. Nested-PCR with the same protocol was performed to determine the nucleotide sequences of the HCV ISDR region. Primers IM11 and 5A02k1 were used for first-round PCR and 5A05k1 and IM10 for second-round PCR (Additional file 1). After amplification, the final PCR products were separated in 2% agarose gel and purified with the QIAquick gel extraction kit (Qiagen GmbH). Sequence analysis was performed by ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems). Nucleotide sequences were compared with those of the HCV-J genotype 1b reference sequence (GenBank accession number D90208) [34].

Determination of genotype in IL28B

Several IL28B single nucleotide polymorphisms (SNPs) are associated with viral clearance [25–27]. We genotyped SNP rs8099917 in 95 patients using TaqMan® Pre-Designed SNP Genotyping Assays as recommended by the manufacturer (Applied Biosystems) [35].

Statistical analyses

Statistical analysis was performed using one-way analysis of variance followed by Student's *t*-test. Baseline characteristics of the patients were compared and the differences among subgroups were assessed by χ^2 test with Yate's correction, Fisher's exact probability test and Mann–Whitney U test. All *P*-values <0.05 by two-tailed test were considered statistically significant. Univariate and multivariate logistic regression by stepwise backward elimination of factors with $P \leq 0.05$ in univariate analysis was used to identify independent predictors of treatment response. Statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

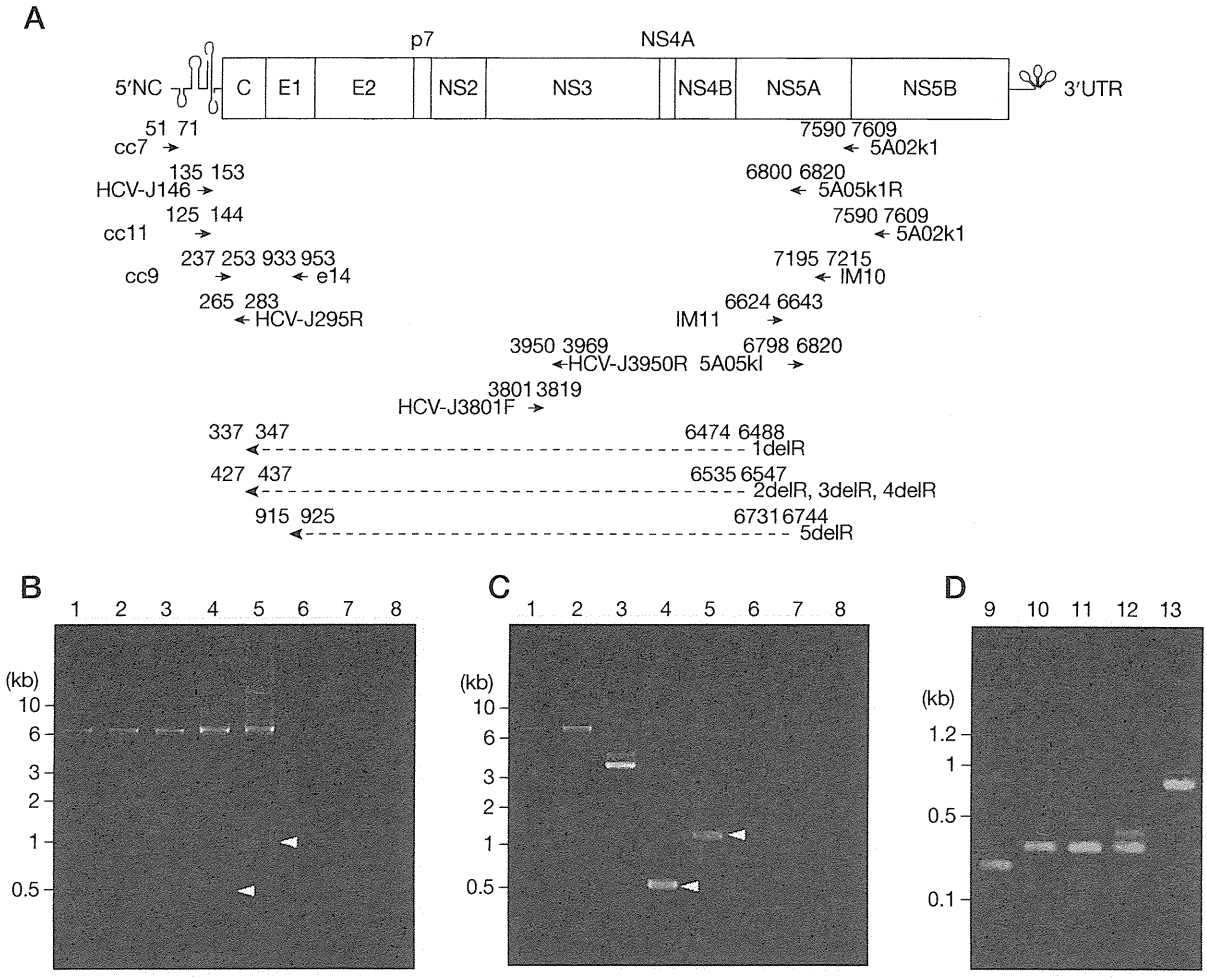
Detection of HCV deletion mutants in patients with chronic HCV infection

After electrophoresis of the long-distance RT nested-PCR products, we observed very short 0.6 to approximately 0.9 kbp DNA fragments (Figure 1A) compared with the expected size of approximately 6,700 bp for a complete HCV genome, suggesting the presence of a large 5,800–6,100 bp deletion. These short fragments were detected in 26 patients. Representative electrophoresis patterns are shown in Figure 1A (lanes 4 and 5). To verify that the short fragments were not artefacts, short-distance RT nested-PCR was performed using primers HCV-J146 and 5A05k1R (Additional file 1). Electrophoresis results imply that long deletion mutants coexist with intact viral genomes (Figure 1B). We designed specific primers to detect each deletion (Additional file 1), and in each sample, a single major band of the expected size was observed, showing specific amplification of deletion mutants (Figure 1C).

Sequence analysis of HCV deletion mutants

To identify the deleted region, sequence analysis was performed by amplifying these fragments with specific primers. In patients 1–4, the presence of an approximately 6,100 bp deletion spanning from the core to NS5A was identified. In patient 5, an approximately 5,800 bp deletion from E1 to NS5A was observed (Figure 2A and 2B). In-frame deletion was observed

Figure 1. HCV deletion mutants were detected by long-distance reverse transcription PCR



(A) Map of the primers used for HCV amplification. (B) HCV deletion mutants were detected as short (approximately 0.6–0.9 kbp) DNA fragments by long-distance reverse transcription PCR in sera of patients infected with HCV genotype 1b. PCR products were separated on 1% agarose gels. White arrows indicate HCV deletion mutants. Lanes 1–3: sera of patients who were not carrying HCV deletion mutant. Lanes 4 and 5: sera from patients who were carrying HCV deletion mutant. Lane 6: HCV expression plasmid. Lane 7: serum from a chronic hepatitis B patient. Lane 8: negative control (H2O). (C) HCV full genomes detected in sera of the same patients (1% agarose gel; see (B) for lane descriptions). Arrows indicate HCV deletion mutants. (D) Selective detection of HCV deletion mutants using specific reverse primers. The reverse primers used for lanes 1–5 in (B) and (C) were 1delR–5delR, respectively (Additional file 1). Reverse primers overlapping deleted regions were specifically designed for each sample (2% agarose gel). Lanes 9–13: the other sera from patients who were carrying HCV deletion mutant. NC, non-coding; UTR, untranslated region.

only in one fragment from patient 1, whereas others had frame-shift deletions. All of the deletions occurred between similar sequences in the 3′ side of the core or E1 region and the 5′ side of the NS5A region.

Relationships between HCV deletion mutants and antiviral responses to combination therapy

We investigated the relationship between the presence of deletion mutants and the response to PEG-IFN and RBV combination therapy. HCV RNA levels in SVR patients were significantly lower than those in non-SVR patients at each time point after the beginning of treatment (Figure 3A). No significant differences were observed in the decline of HCV RNA levels between patients with and without deletion mutants (Figure 3B), but patients with the long-distance HCV deletion mutants showed significantly poorer response to combination therapy (Table 1).

Clinical significance of the large deletion mutants

To assess the proportion of deletion mutants in serum HCV RNA, we performed real-time PCR. We used HCV-J146 and HCV-J295R primers designed for amplification of 5′-untranslated region (UTR)

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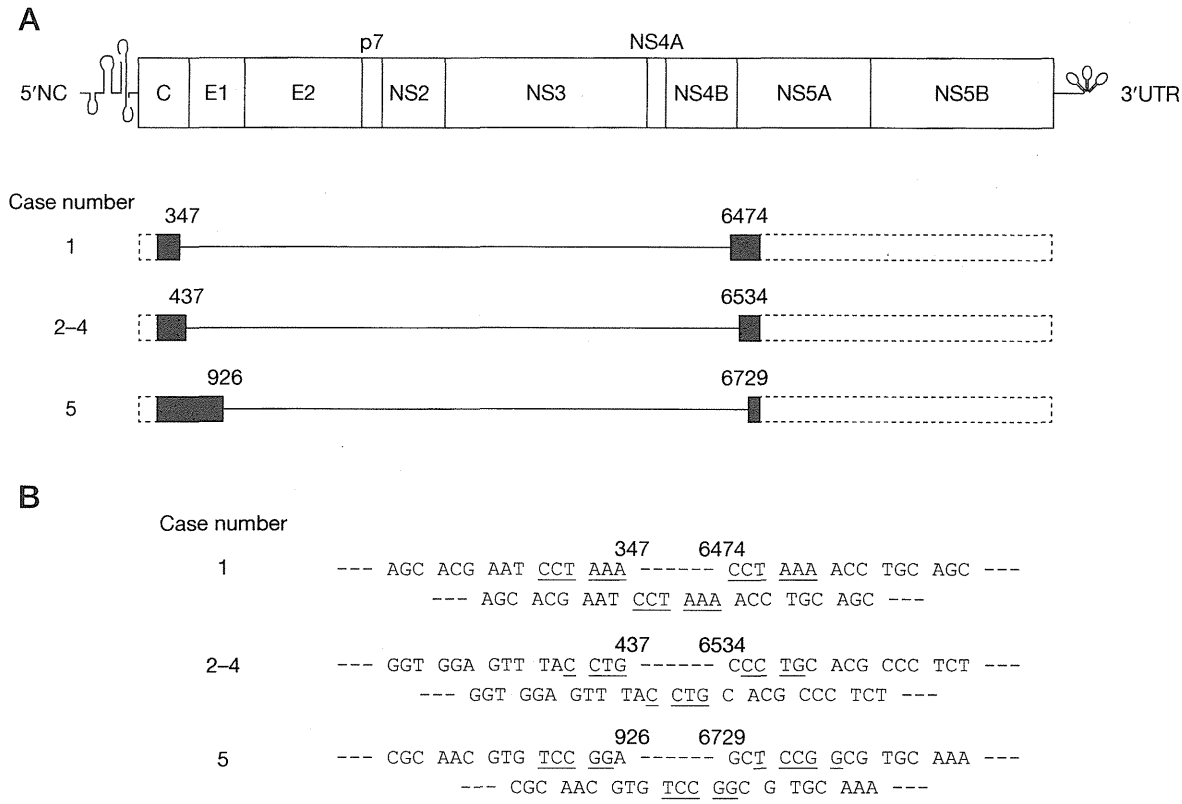
Figure 2. Structure of novel HCV deletion mutants



(A) Schematic presentation of HCV deletion mutants in the sera of chronic HCV patients. Black boxes represent complete sequences and black lines represent deleted regions. (B) Details of nucleotide sequences of HCV deletion mutants. Underlined bases indicate regions of repeated sequence. NC, non-coding; UTR, untranslated region.

to quantify total HCV RNA, and HCV-J3801F and HCV-J3950R primers for specific amplification of full-genome HCV RNA (Additional file 1). Deletion mutant HCV RNA level was calculated by subtracting the full-genome HCV RNA level from the total HCV RNA level. In total, 26 patients had the long deletion mutant prior to starting therapy. The level of deletion mutant RNA was 5–50-fold higher than that of HCV full genomes (data not shown). The presence of deletion mutants significantly reduced the SVR rate for combination therapy (OR 2.3, P=0.043), and the serum HCV RNA titres in the carriers of HCV deletion mutants were significantly higher than in non-carriers before treatment (P=0.018; Table 1). By contrast, well-known predictive factors for IFN response to combination therapy, such as early virological response, rapid virological response, the presence of substitutions in the core regions or NS5A-ISDR, and IL28B SNP alleles, were not significantly associated with the presence of the large deletion mutants (Table 1).

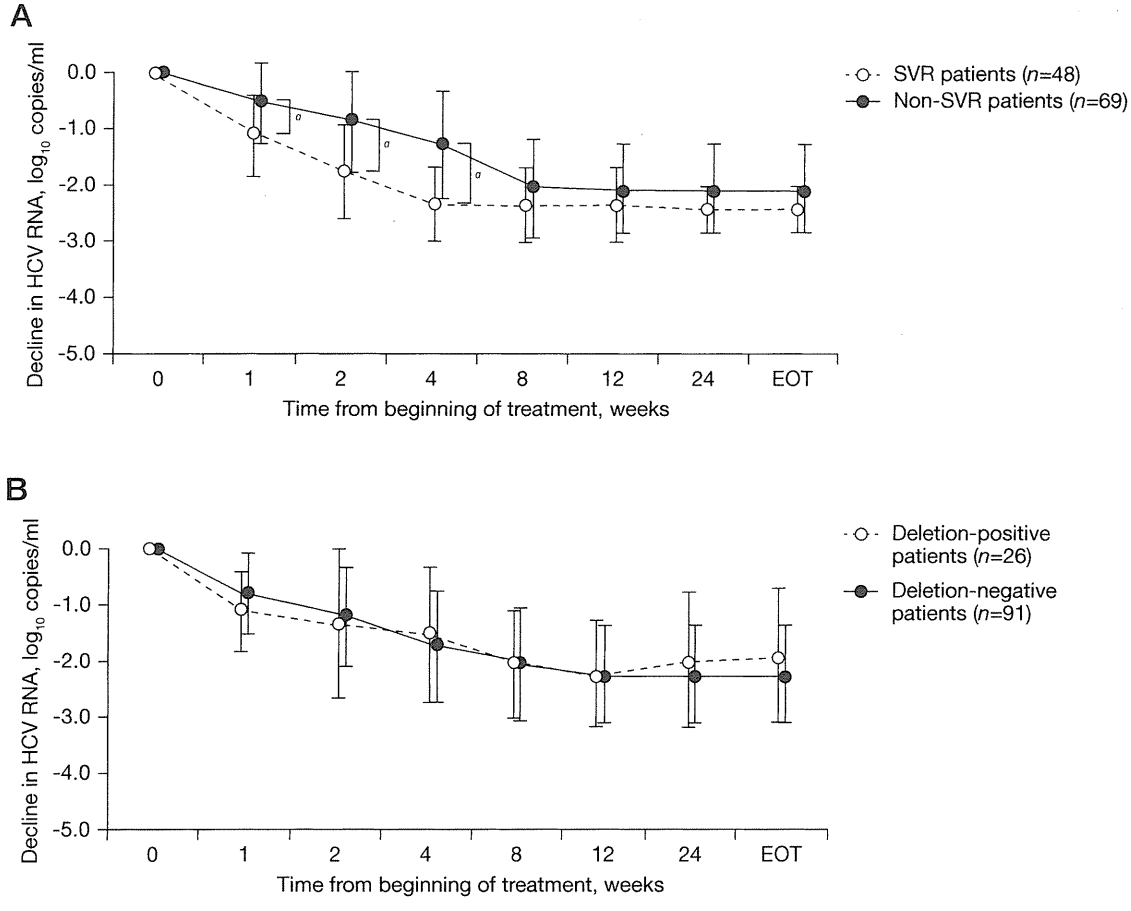
Clinical and virological predictive factors for SVR in combination therapy

Several clinical background characteristics, including age, WBC, Plt, TBil, and γ-GTP, were significantly different between SVR and non-SVR patients in univariate analysis, and *IL28B* rs8099917 genotype was marginally significant (Table 2). Viral factors, including core substitutions at aa70 and aa91, were also significantly associated with SVR (OR=2.0, P=0.007 and OR=1.7, P=0.008, respectively). By contrast, HCV RNA titre and the number of ISDR substitutions were not significantly different (Table 2). Following multivariate analysis, aa70 substitutions (P=0.023) and high γ-GTP (P=0.025) were independent predictive factors for non-SVR, whereas the presence of long deletion mutants was not (Table 2).

Clinical and virological predictive factors for virological relapse after combination therapy

Virological response was obtained in 84 patients at the end of the combination therapy (Table 1). To identify predictive factors for virological relapse after

Figure 3. Reduction of HCV titres by pegylated interferon plus ribavirin combination therapy



(A) Changes in HCV titre at each time point grouped by response to combination therapy. (B) Changes in HCV titre at each time point grouped by the presence of large deletion mutants. Statistical analysis was performed using the Mann-Whitney U test ($^aP<0.01$). EOT, end of treatment; SVR, sustained virological response.

combination therapy, clinical and virological backgrounds were compared between SVR patients and virological relapsers. As shown in Table 3, five factors, including age, WBC, Plt, TBil and the presence of long deletions, were significant in univariate analysis, whereas *IL28B* SNP genotype (rs8099917) and aa70 and aa91 substitutions were not. Multivariate regression analysis revealed that the presence of deletion mutants (OR=3.4, P=0.046) was an independent predictive factor only for virological relapse (Table 3).

To assess the reduction of full-genome HCV RNA, RT-real time PCR was performed using HCV-J3801F and HCV-J3950R primers in 36 virological relapse cases. Full-genome HCV titres in patients without deletion mutants were significantly more reduced than in patients with deletion mutants after 4 weeks of combination therapy (P=0.009; Figure 4).

Discussion

Deletions within viral genomes have been reported for many RNA and DNA viruses, including the closely related Murray Valley encephalitis virus [36]. RNA viruses notably tend to change rapidly and to generate new or defective strains [37]. In this study, we identified novel HCV deletion mutants in 26 of 117 (22.2%) chronic hepatitis C patients, and these mutants contained both in-frame and out-of-frame deletions. In previous reports, deletions were observed in the E1-NS2 region of the HCV genome, and the length of deletions were less than 1 kb [28–30]. The deletion mutants identified in this study have much longer deletions than previously reported. The lengths of deletions were found to be approximately 6 kb, including the core-NS5A region (Figure 2A). Most of these deletions were out-of-frame

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Table 1. Comparison of clinical characteristics between patients with or without deletion mutants prior to treatment with pegylated interferon plus ribavirin combination therapy

Characteristic	Deletion-positive (*n*=26)	Deletion-negative (*n*=91)	*P*-value
Age, years	61.8 ±10.4	58.3 ±12.5	0.216
Gender, male:female	16:10	43:48	0.267
Body mass index, kg/m^2	23.1 ±2.7	23.0 ±3.0	0.812
White blood cell count, cells/mm^3	5,181 ±1,914	5,192 ±1,482	0.646
Haemoglobin, g/dl	14.2 ±1.2	13.8 ±1.6	0.332
Platelet count, ×10^4 cells/mm^3	15.4 ±5.2	15.5 ±9.0	0.451
Total bilirubin, mg/dl	0.7 ±0.2	0.8 ±0.3	0.633
Aspartate aminotransferase, IU/l	52 ±32	54 ±32	0.607
Alanine aminotransferase, IU/l	56 ±37	62 ±40	0.251
γ-Glutamyl transpeptidase, IU/l	64 ±54	51 ±34	0.523
HCV RNA, $\log_{10}$ copies/ml	6.5 ±0.2	6.3 ±0.4	0.018
Past interferon therapy, yes:no	12:14	33:58	0.492
Non-SVR:SVR	20:6	49:42	0.043
Non-EVR:EVR	8:18	29:62	1.000
Non-RVR:RVR	18:8	68:23	0.441
VR:non-VR	19:7	65:26	1.000
ISDR mutation, 0:≥1	14:12	52:37	0.822
Core 70, wild:mutant	12:14	56:33	0.173
Core 91, wild:mutant	10:16	46:43	0.270
IL28B, GG:GT/TT	17:7	52:19	0.797

Total *n*=117. Continuous data are reported as mean ±SD. EVR, early virological response; ISDR, interferon sensitivity determining region; RVR, rapid virological response; SVR, sustained virological response; VR, virological response.

Table 2. Comparison of clinical characteristics between patients grouped by achievement of sustained virological response with pegylated interferon plus ribavirin combination therapy

Factor	SVR (*n*=48)	Non-SVR (*n*=69)	Univariate analysis, *P*-value	Multivariate analysis	
				OR (95% CI)	*P*-value
Age, years	54.9 ±14.5	62.0 ±9.2	0.008	-	0.089
Gender, male:female	24:24	35:34	1.000	-	-
Body mass index, kg/m^2	23.0 ±3.1	23.1 ±2.8	0.691	-	-
White blood cell count, cells/mm^3	5,592 ±1,568	4,911 ±1,536	0.022	-	0.062
Haemoglobin, g/dl	13.9 ±1.6	13.8 ±1.4	0.704	-	-
Platelet count, ×10^4 cells/mm^3	17.5 ±10.3	14.1 ±6.2	0.005	-	0.468
Total bilirubin, mg/dl	0.7 ±0.3	0.8 ±0.3	0.010	-	0.253
Aspartate aminotransferase, IU/l	48 ±23	58 ±36	0.221	-	-
Alanine aminotransferase, IU/l	61 ±35	61 ±42	0.752	-	-
γ-Glutamyl transpeptidase, IU/l	41 ±28	62 ±44	0.003	0.984 (0.971, 0.998)	0.025
HCV RNA, $\log_{10}$ copies/ml	6.3 ±0.4	6.4 ±0.3	0.302	-	-
Deletion, positive:negative	6:42	20:49	0.043	-	0.291
ISDR mutation, 0:≥1	24:23	42:26	0.338	-	-
Core 70, wild:mutant	35:12	33:35	0.007	3.268 (1.163, 9.174)	0.023
Core 91, wild:mutant	30:17	26:42	0.008	-	0.184
IL28B, TT:GT/GG	39:8	37:20	0.059	-	0.241

Total *n*=117. Continuous data are reported as mean ±SD. ISDR, interferon sensitivity determining region; SVR, sustained virological response.

deletions, preventing production of HCV-related proteins. Interestingly, 4 or 5 bp sequence repeats are observed upstream of the deletion site and at the end of the deletion sequence in each case (Figure 2B).

Anti-HCV therapy has been reported to enhance selection of resistant strains. Therefore we compared the prevalence of HCV deletions between treatment-naive patients and patients previously treated with IFN. Overall, 45 of 117 patients had experienced prior IFN therapy, and 26.7% of them carried HCV deletion mutants, whereas the prevalence was 19.4% in treatment-naive patients. Thus, there was no significant difference

Table 3. Comparison of clinical characteristics between relapsers and patients who achieved sustained virologic response following treatment with pegylated interferon plus ribavirin combination therapy

Factors	Relapse (n=36)	SVR (n=48)	Univariate	Multivariate analysis	
			P-value	OR (95% CI)	P-value
Age, years	62.8 ±9.2	54.9 ±14.5	0.007	-	0.081
Gender, male:female	18:18	24:24	1.000	-	-
Body mass index, kg/m^2	23.1 ±2.8	23.0 ±3.1	0.709	-	-
White blood cell count, cells/mm^3	4,920 ±1,461	5,592 ±1,568	0.096	-	0.197
Haemoglobin, g/dl	13.8 ±1.4	13.9 ±1.6	0.603	-	-
Platelet count, ×10^4 cells/mm^3	14.3 ±5.7	17.5 ±10.3	0.039	-	0.546
Total bilirubin, mg/dl	0.9 ±0.3	0.7 ±0.3	0.006	-	0.051
Aspartate aminotransferase, IU/l	61 ±42	48 ±22	0.309	-	-
Alanine aminotransferase, IU/l	67 ±50	61 ±35	0.910	-	-
γ-Glutamyl transpeptidase, IU/l	58 ±48	41 ±28	0.179	-	-
HCV RNA, log copies/ml	6.4 ±0.3	6.3 ±0.4	0.165	-	-
Past interferon therapy, yes:no	18:17	15:31	0.112	-	-
ISDR mutation, 0:≥1	20:16	24:23	0.825	-	-
Core 70, wild:mutant	22:14	35:12	0.236	-	-
Core 91, wild:mutant	16:12	30:17	0.118	-	-
IL28B, TT:GT/GG	30:4	39:8	0.753	-	-
Deletion, postitive:negative	13:26	6:42	0.017	3.393 (1.022, 11.266)	0.046

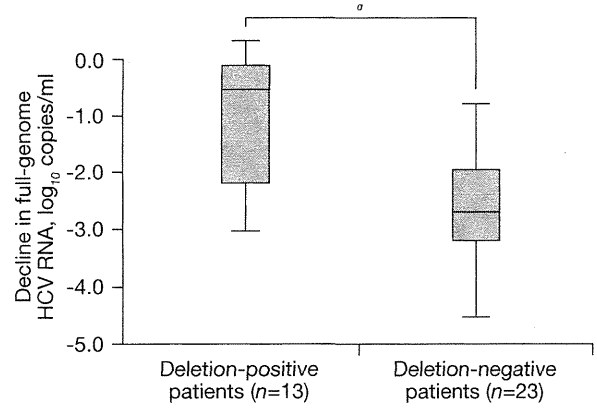
Total n=84. Continuous data are reported as mean ±SD. ISDR, interferon sensitivity determining region; SVR, sustained virological response.

(P=0.36) in the prevalence of HCV deletion mutants between IFN-naive and IFN-experienced patients.

We speculate that there are two potential roles for the deletion mutants in escaping the human immune system. One role involves the inhibition of the intracellular immune system by HCV deletion mutants. There are several RNA recognition systems for eradicating RNA viruses in human hepatocytes [38–44]. When HCV infects human hepatocytes, several RNA sensors, such as retinoic acid inducible gene-I (RIG-I) and Toll-like receptors, recognize and trigger a response to HCV RNA [39–41,43,44]. In particular, RIG-I recognizes the 3′-UTR of HCV RNA and activates an intracellular antiviral response that induces type-1 IFN and IFN-stimulated gene (ISG) expression to limit the spread of HCV infection [43]. Although the 3′-UTR region was intact in the HCV deletion mutants identified in this study, HCV RNA derived from deletion mutants could be recognized by RIG-I and could induce superfluous production of ISGs in chronic hepatitis C patients. As higher ISG expression is negatively associated with HCV eradication in combination therapy [45,46], the existence of deletion mutants might lead to higher ISG expressions of ISG in human hepatocytes and inhibit intracellular IFN responses during combination therapy.

Another potential role is to function as decoys. Generally, HCV-specific antibodies to several HCV-related proteins interact with HCV particles to limit the spread of infection and to destroy HCV particles directly through complement activation. When the deletion mutants were present in human serum, HCV-specific antibodies could interact with both mature HCV particles as well as deletion mutants, in which case humoral immune responses could not function adequately and may fail to eradicate the mature HCV particles. Thus, we speculate that these deletion mutants may function as decoys against the human

Figure 4. Reduction of full genome HCV titres 4 weeks after the start of treatment in virological relapse cases



Full-genome HCV RNA levels were quantified by real-time PCR using primers amplifying the NS3 region. Log reduction of full-genome HCV RNA was compared between patients with and without HCV deletion mutants. Statistical analysis was performed using the Mann–Whitney U test (^aP<0.05).

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immune system, although further studies are needed to discern the role of these deletion mutants.

Previous studies using the HCV subgenome replication system *in vitro* demonstrated that E1-NS2 deletion mutants that preserved essential machinery for HCV replication were able to replicate the subgenome virus particles independently [47,48] or by recruiting coexisting full-genome virus particles as helper viruses [49,50]. In many cases the defective subgenome virus particles interfered with the replication of the helper virus [51–53]. The subgenome virus particles identified in this study appeared to coexist with full-genome virus particles in the sera of chronic HCV patients despite lacking essential components for viral replication.

It was previously reported that the subgenomes were associated with the response to IFN and RBV combination therapy and that defective interfering particles may contribute to this association [30]. Similarly, in this study, we demonstrated that the existence of subgenome HCV particles were significantly associated with SVR independently of ISDR and core substitutions. In addition, we demonstrated that the existence of subgenome HCV particles affects serum HCV RNA titres prior to IFN treatment. The serum HCV RNA titres were usually evaluated based on the amplification of the 5′-UTR region by real-time RT-PCR, whereas the long deletions of the HCV genome occurred in the core-NS5A region. Thus, the serum HCV RNA titres represent both full- and subgenome HCV genomes. Yagi *et al.* [30] have demonstrated that the number of HCV subgenomes was approximately 500-fold greater than that of full genomes in the livers of HCV patients. We were concerned that all sera of SVR patients with subgenome particles contained low amounts of full-genome HCV. These findings suggest that independent measurement of the amount of full- and subgenome HCV might be important for understanding early viral response against IFN therapy as well as the prediction that IFN affects the existence of subgenome virus particles, although further study is needed.

We also identified a significant association between the presence of HCV long deletion mutants and virological relapse after combination therapy in virological response patients. In the analysis of virological response patients, the incidence of virological relapse in patients with long deletion mutants was significantly higher than in those without deletion mutants (P=0.017). The mechanism underlying this difference is currently unclear. According to recent reports, RBV administration in combination with either IFN or PEG-IFN to patients with HCV genotype 1 reduces the relapse rate [16,17,54,55]. Thus, it was suggested that the role of RBV in combination therapy could be to enhance the antiviral effects of IFN treatment and to repress virological relapse after IFN treatment. We speculate that long deletion mutants might repress the antiviral effects of IFN by interfering with RBV-associated signals.

Recently, polymorphisms in the *IL28B* locus (rs8099917 and rs12979860) were shown to be strongly associated with the IFN response [25–27]. The SVR rate in patients homozygous for the major allele was significantly higher than in those with the minor allele. Thus, we hypothesized that the incidence of the deletion mutants in patients with the minor allele might be elevated, but no association between IL28B genotype and the presence of long deletion mutants was observed.

In conclusion, we identified novel HCV genome deletion mutants that are much longer than those previously reported. These deletion mutants might assist HCV replication and/or interfere with the eradication of HCV with IFN therapy. Independent measurement of full-genome and subgenome titres may improve the prediction of the effects of IFN therapy.

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Disclosure statement

The authors declare no competing interests.

Additional file

Additional file 1: A table listing of primers for HCV amplification can be accessed via http://www.intmedpress.com/uploads/documents/AVT-12-OA-2462_Kohno_Add_file_1.pdf

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

IL28B polymorphism is associated with fatty change in the liver of chronic hepatitis C patients

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Abstract
Background Several single nucleotide polymorphisms (SNPs) within the *interleukin 28B* (*IL28B*) locus are associated with sustained viral response in chronic hepatitis C (HCV) patients who were treated with pegylated interferon (PEG-IFN) plus ribavirin (RBV) combination therapy. Recently, an association between γ-GTP level and IL28B genotype was identified. In this study, the relationship between IL28B genotype and liver steatosis was analyzed.
Methods One hundred fifty-three patients who underwent liver biopsy before PEG-IFN plus RBV combination therapy were enrolled. The level of liver steatosis was measured using a BIOREVO BZ-9000 microscope, and the proportion of fatty change and clear cell change were calculated using Dynamic cell count BZ-H1C software. IL28B SNP genotype (rs8099917) was determined using the Invader Assay.
Results Vesicular change was significantly associated with body mass index (BMI), HCV RNA titer, serum aspartate aminotransferase, γ-GTP, IL28B genotype and liver fibrosis level ($P < 0.05$). Clear cell change was significantly associated with serum aspartate aminotransferase, γ-GTP and IL28B genotype by univariate logistic regression analysis ($P < 0.05$). Under multiple logistic regression, IL28B genotype ($OR_{adj} = 8.158$; 95% CI 2.412–27.589), liver fibrosis ($OR_{adj} = 2.541$; 95% CI 1.040–6.207) and BMI ($OR_{adj} = 1.147$; 95% CI 1.011–1.301) were significant independent factors for vesicular change and IL28B genotype ($OR_{adj} = 3.000$; 95% CI 1.282–7.019) for clear cell change.
Conclusion In this study, a new quantitative method to objectively evaluate hepatic steatosis was described. IL28B genotypes were significantly associated with both vesicular and clear cell changes of livers in chronic hepatitis C patients.

Keywords HCV · Core substitution · IL28B · Fatty change · SNP

Abbreviations

HCV	Hepatitis C virus
IFN	Interferon

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PEG-IFN	Pegylated interferon
RBV	Ribavirin
ISDR	IFN-sensitivity determining region
IL28	Interleukin 28
SNP	Single nucleotide polymorphism
BMI	Body mass index
γ-GTP	Gamma glutamyl transpeptidase
aa	Amino acid
HOMA-IR	Homeostasis model assessment of insulin resistance

Background

Hepatitis C virus (HCV) is one of the most serious global health problems, affecting more than 170 million people worldwide [1–3]. Chronic HCV infection leads to the development of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) [4–8]. To attempt to eradicate the virus and prevent the development of advanced liver diseases and HCC, interferon is administered to chronic hepatitis C patients, with success in a subset of patients in which marked biochemical and histological improvements can be obtained [9, 10]. However, patients with a high virus titer and those who are infected with genotype 1b, which is the major genotype affecting about 70% of Japanese patients, show poor response to interferon monotherapy. Less than 20% of patients treated with interferon monotherapy show sustained virological response [11–14]. With the advent of pegylated interferon (PEG-IFN) and ribavirin (RBV) combination therapy, the eradication rate of the virus has improved. However, the eradication rate of genotype 1b with high viral load still remains only 40–50% [15–17].

Several viral and host factors have been identified that are predictive of the outcome of PEG-IFN plus RBV combination therapy. HCV genotype, HCV RNA level and HCV amino acid (aa) substitutions in the core region and the interferon-sensitivity determining region (ISDR; aa positions 237–276 of the NS5A region) have been reported as viral factors for achieving sustained viral response. On the other hand, host factors, such as patient age, gender, liver fibrosis stage, liver steatosis and homeostasis model assessment of insulin resistance (HOMA-IR), are also known to be associated with IFN response. Okanoue et al. demonstrated that the frequency of liver steatosis and HOMA-IR were significantly lower in patients who achieved sustained virological response than those who did not [18–23].

Recently, genome-wide association studies (GWAS) have examined the association between human genetic variation and the response to IFN treatment, and several common polymorphisms in the interleukin 28B (IL28B) locus were found to predict successful HCV clearance with IFN therapy [24–27]. IL28B single nucleotide polymorphisms (SNPs) (rs12979860, rs8099917, and rs12980275) are strongly associated with sustained virological response in patients who undergo PEG-IFN plus RBV combination therapy. In a recent study, the serum γ-GTP level was also found to be associated with the IL28B SNP (rs8099917) genotype in patients infected with HCV genotype 1b [28]. Serum γ-GTP levels were significantly lower in patients homozygous for the major allele (TT) than in patients with the minor allele (GG or GT) [28]. On the other hand, the serum γ-GTP level is well known to be related to liver steatosis; therefore, it was hypothesized that the levels of liver steatosis may be associated with the IL28B genotype. In the present study, a quantitative method to evaluate fatty change was established, and the association between fatty change and host or viral factors was analyzed.

Patients and methods

Patients

Three hundred fifty-three adult Japanese patients infected with HCV genotype 1b provided written informed consent to participate in the present study at Hiroshima University Hospital, Hiroshima, Japan. Among these patients, 153 who underwent liver biopsy or hepatic resection from December 2001 to August 2009 before commencing antiviral therapy were selected for the study based on the following exclusion criteria: (1) not co-infected with other viruses, such as human immunodeficiency virus or hepatitis B virus; (2) no other liver diseases such as hemochromatosis, auto-immune hepatitis or decompensated cirrhosis; (3) no co-existing diseases such as de-compensated renal disease, pre-existing psychiatric disease, seizure disorders, cardiovascular disease, hemophilia, autoimmune diseases or post-transplantation. The experimental protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Hiroshima University Hospital ethics committee. Written informed consent was obtained from each patient. Patients whose ethanol intake was more than 20 g/day were defined as alcoholic [29, 30]. Baseline characteristics of the 153 patients are shown in Table 1.

Evaluation of liver steatosis

Liver tissues with hematoxylin–eosin stain were used in this study. Liver fibrosis and activity stages were diagnosed by a pathologist at Hiroshima University Hospital according to the criteria of Desmet et al. [31]. The level of liver steatosis was measured using a BIOREVO BZ-9000 microscope (Keyence, Osaka, Japan), and the proportion of

Table 1 Clinical backgrounds of patients

Characteristics	
Age (years)[a]	60 (15–76)
Gender (M:F)	83:70
BMI (kg m^{-2})[a]	22.7 (16.2–39.4)
Alcohol (+:−)	47:103
Laboratory data	
Platelet count ($\times 10^3$ μl^{-1})[a]	99 (23–759)
Prothrombin activity (%)[a]	99 (12–166)
Total bilirubin (mg dl^{-1})[a]	0.7 (0.4–14.7)
Aspartate aminotransferase (IU l^{-1})[a]	46 (14–2617)
Alanine aminotransferase (IU l^{-1})[a]	51 (2–2707)
Lactate dehydrogenase (IU l^{-1})[a]	202 (43–899)
Gamma glutamyl transpeptidase (IU l^{-1})[a]	43 (12–295)
Albumin (g dl^{-1})[a]	4.3 (2.9–5.3)
Total cholesterol (mg dl^{-1})[a]	174 (100–312)
Triglycerides (mg dl^{-1})[a]	100 (33–2466)
Glucose (mg dl^{-1})[a]	101 (68–334)
HCV RNA (log IU ml^{-1})[a]	6.3 (4.2–7.9)
Core 70 (wild:mutant)	77:39
Core 91 (wild:mutant)	54:62
ISDR (0:≥1)	38:76
IL28B (TT:TG:GG)	116:33:4
Liver histology	
Fibrosis (F0–1:F2–3)	42:111
Inflammatory activity (A0–1:A2–3)	59:94

[a] Median (range)

fatty change was calculated using Dynamic cell count BZ-H1C software (Keyence). The average of the observed area was 3.03 mm^2 (0.74–6.77 mm^2). The distribution of brightness values in liver tissue images was evaluated using a brightness distribution histogram as shown in Fig. 1a. The brightness was divided into 256 gradation sequences. Regions with a brightness value over 60% between minimum and maximum brightness were considered to represent fatty change areas in the liver tissue. The area of interest was calculated, and <5 μm^2 of the extracted areas were excluded as noise as determined by particle elimination technology (Dynamic cell count BZ-H1C software) (Fig. 1b). The remaining highlighted noise, including glycogen nuclei and vascular spaces, was excluded as much as possible by visual observation. After two exclusion steps, the remaining noise spaces were sufficiently narrow (far <5% of total fatty changed spaces) that they could be considered negligible. The observed area of the sample was also calculated with BZ-H1C software (Fig. 1c). All threshold values of vesicular change or clear cell change ratio were defined based on the approximate median values of each change.

Determination of amino acid sequences in the HCV core region and ISDR

HCV RNA was extracted from 100 μl of stored serum samples by SepaGene RV-R (Sanko Junyaku Co., Ltd, Tokyo, Japan), dissolved in 20 μl of H_2O and converted to cDNA by RT with random primers and MMLV reverse transcriptase (Takara Shuzo, Tokyo, Japan). The cDNA was then amplified by nested PCR to determine the nucleotide sequences in the HCV core region. The PCR protocol was as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation for 30 s at 94°C, annealing of primers for 1 min at 57°C and extension for 1 min at 72°C, followed by a final extension at 72°C for 7 min. The primers for the first round PCR were cc11 (forward, 5′-GCC ATA GTG GTC TGC GGA AC-3′) and e14 (reverse, 5′-GGA GCA GTC CTT CGT GAC ATG-3′), and the primers for the second-round PCR were cc9 (forward, 5′-GCT AGC CGA GTA GTG TT-3′) and e14 (reverse), as described previously [19, 21, 32, 33]. To determine the nucleotide sequences of the HCV ISDR region, we also performed nested PCR with the same protocol as in the HCV core region. The primers used were IM11 (forward, 5′-TTC CAC TAC GTG ACG GGC AT-3′) and 5A02KI (reverse, 5′-CCC GTC CAT GTG TAG GAC AT-3′) for the first round PCR, and 5A05KI (forward, 5′-GGG TCA CAG CTC CCA TGT GAG CC -3′), and IM10 (reverse, 5′-GAG GGT TGT AAT CCG GGC GTG C-3′) for the second round PCR. After amplification, the final PCR products were separated in 2% agarose gel and purified with the QIAquick gel extraction kit (QIAGEN GmbH, Hilden, Germany). Sequence analysis was performed by ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Amino acid sequences were compared with the genotype 1b HCV-J reference sequence (Gene Bank accession no. D90208) [34]. Arginine and leucine were considered wild type for amino acids 70 and 91, respectively, and the most frequent amino acid substitutions were glutamine or histidine at aa 70 and methionine at aa 91.

Determination of genotype in IL28B

In Asian populations, the genotype of rs8099917, a SNP within the *IL28B* locus, has been reported to be strongly correlated to nearby SNPs rs12979860 and rs12980275 because of strong linkage disequilibrium [35]. In this study, rs8099917 was used to represent the genotype of the *IL28B* SNP. Genotypes of rs8099917 were determined using TaqMan® Pre-Designed SNP Genotyping Assays as described previously [28].

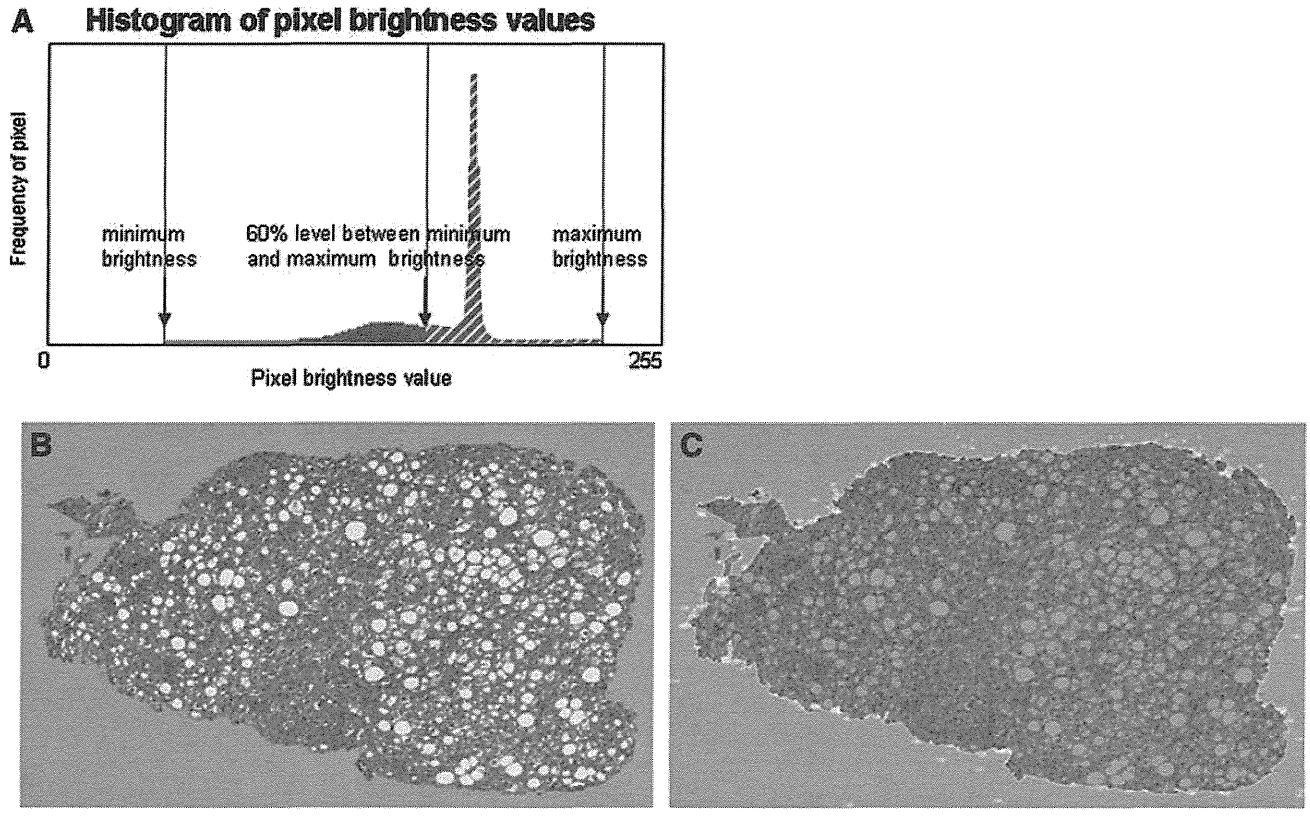

Fig. 1 Evaluation of fatty change in liver tissue. **a** The distribution of brightness in liver tissue images was represented with a brightness distribution histogram using BZ-H1C software. The relative cumulative frequency of pixels with brightness values over 60% between minimum and maximum brightness was interpreted as the proportion of fatty change in the liver tissue. **b** The area of the fatty change is depicted in *yellow*. **c** The area inside the *yellow line* is defined as the observed area (color figure online)

Statistical analysis

In univariate analysis, the clinical backgrounds of the patients in the two groups were compared, and differences were assessed by chi-square test with Yate's correction for categorical variables and by Mann–Whitney *U* test for continuous variables. All thresholds were determined based on median values. All *P* values <0.05 by two-tailed test were considered statistically significant. To analyze the association between clinical characteristics and fatty change, multivariate logistic regression analysis was performed. To identify significant independent predictive factors, variables with statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) in univariate analysis were used as the starting model for forward stepwise logistic regression model using the likelihood ratio test. Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Patterns of histological change in the liver

Comparing the liver tissues between chronic hepatitis C and non-alcoholic fatty liver disease, we identified two different histological change patterns. As shown in Fig. 2, histological change in patients with non-alcoholic fatty liver disease consisted mainly of vesicular change (Fig. 2a), but histological change in the chronic hepatitis C patients consisted of both vesicular change and clear cell change (Fig. 2b), suggesting that clear cell change was the distinguishing change in chronic hepatitis C. Accordingly, we further analyzed liver specimens from patients with these diseases to determine the best criteria to distinguish clear cell change areas from vesicular change areas. Most of the vesicular change areas were found to be more than 200 μm^2 (data not shown). Therefore, we defined areas with <200 μm^2 of fatty change as areas of clear cell change (Fig. 3). Although narrow noise spaces (<200 μm^2), including small vacuoles or hepatocyte nucleus, remained after these automatic steps, the remaining noise spaces were then excluded as much as possible by visual observation. As shown in Fig. 4, the distribution of vesicular change was associated with clear cell change.

Association between vesicular change and clinical background

To analyze the relationship between fatty change and clinical background, we compiled the clinical backgrounds

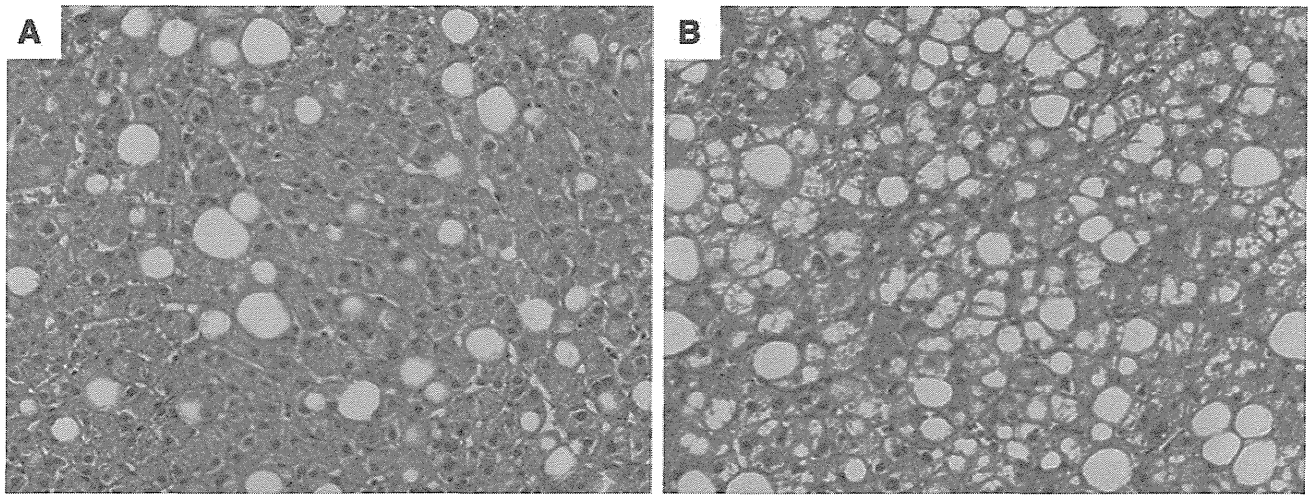

Fig. 2 Different fatty change patterns between NAFLD and chronic hepatitis C. The fatty change pattern was compared between NAFLD (**a**) and chronic hepatitis C (**b**). The liver tissues were stained with H&E

of 153 participating patients (Table 1). Because the minor allele frequency of the IL28B rs8099917 SNP was only 0.14 and the IL28B rs8099917 GG genotype was observed in only four patients, the IL28B rs8099917 genotypes were split into two groups (TT vs. TG/GG) in all analyses. The relationship between vesicular change and clinical backgrounds was examined. The proportion of vesicular change (vesicular change ratio) was defined as the ratio of the area of vesicular change and the observed area (= area of vesicular change/observed area). As shown in Table 2, in univariate analysis the greater vesicular change ratio was significantly associated with γ-GTP, *IL28B* genotype, aspartate aminotransferase, body mass index (BMI), liver fibrosis stage and HCV RNA at the point of liver biopsy ($P < 0.05$). The association between IL28B genotype and vesicular change was examined using multivariate analysis, and *IL28B* genotype, liver fibrosis stage and BMI were retained in the final model (Table 2). These results indicate that the vesicular change was not only associated with *IL28B* genotype, but also associated with BMI. To confirm the association between *IL28B* genotypes and vesicular change, multiple regression analysis was also performed using continuous values. *IL28B* genotype, HCV RNA titer and liver fibrosis stage were identified as independent factors for vesicular change ($P = 0.003$, $P = 0.022$, $P = 0.047$, respectively).

Association between clear cell change and clinical background

Next, the relationship between clear cell change and clinical background was analyzed. The proportion of clear cell change (clear cell change ratio) was defined as the ratio of the area of the clear cell change and the observed area (= area of clear cell change/observed area). We divided the study subjects into two groups according to the clear cell change ratio. As shown in Table 3, in univariate analysis the greater clear cell change ratio was significantly associated with aspartate aminotransferase, IL28B genotype and γ-GTP at the point of liver biopsy ($P < 0.05$). In multivariate analysis, the IL28B genotype was found to be a significant independent predictor, and liver inflammatory activity and gender were marginally associated (Table 3). By using continuous values in multiple regression, the *IL28B* genotype was also identified as an independent factor for clear cell change ($P < 0.001$). These results suggest that the *IL28B* genotype is significantly associated with clear cell change in livers of chronic hepatitis C patients.

The association between fatty degeneration and IL28B genotype in the non-obese group

To analyze the association between fatty change and IL28B genotype, the study subjects were divided into two groups. Thirty-six patients whose BMI was more than 25 kg m^{-2} were assigned to the obese group, and the other 114 patients were assigned to the non-obese group. In the obese group, no association between fatty change and IL28B genotype was observed (Table 4). On the other hand, each measure of fatty change (vesicular change, and clear cell change) was significantly associated with IL28B genotype in the non-obese group ($P = 0.001$, $P = 0.009$, respectively, Table 4).

Discussion

It is generally difficult to compare evaluations of liver fatty change because such studies depend on the respective assessments of individual pathologists [36, 37]. In the

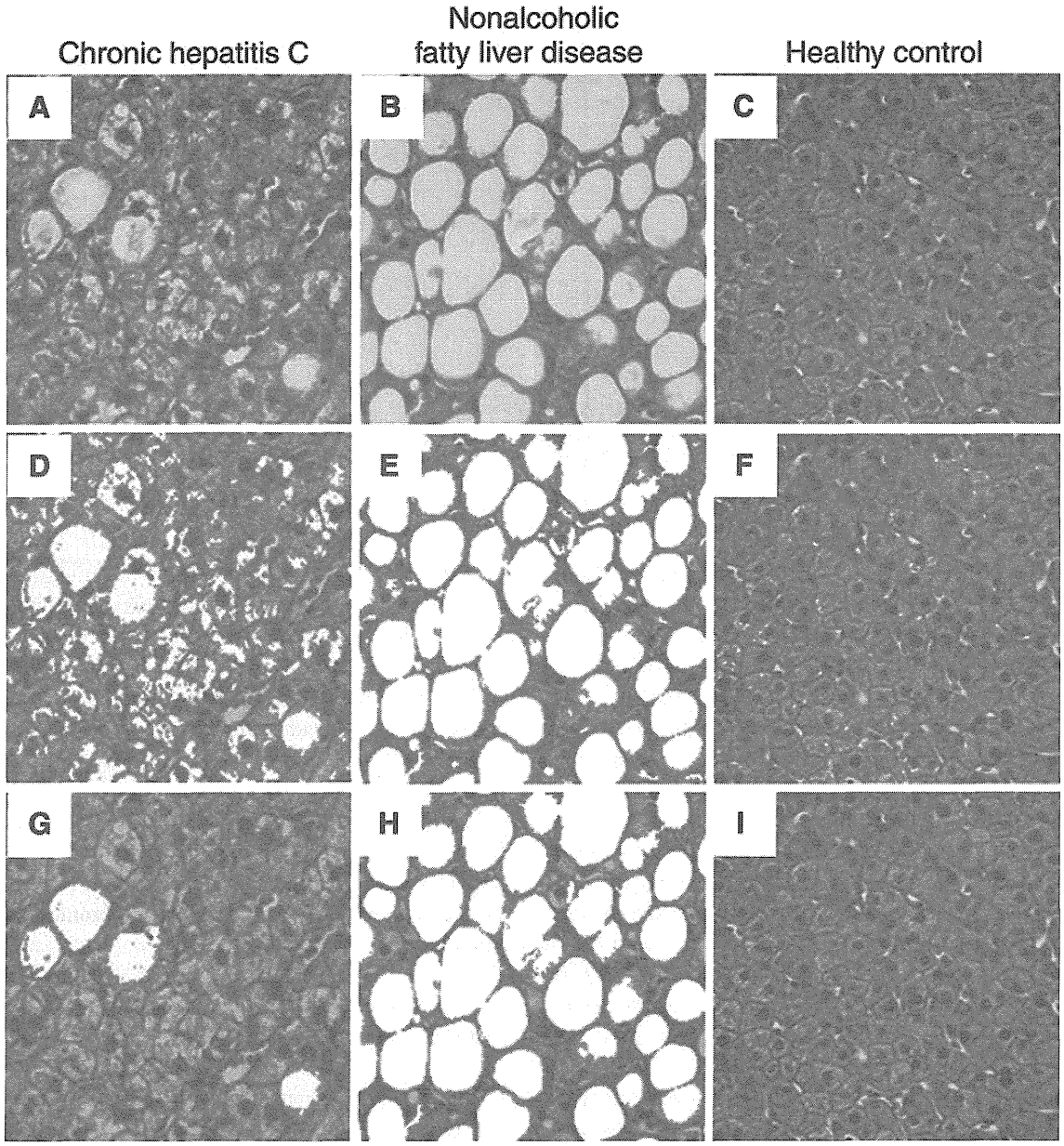

Fig. 3 Discrimination between vesicular and clear cell change. The evaluation results of three cases are shown. The *upper panels* are H&E staining of liver specimen from NAFLD (**a**), chronic hepatitis C (**b**) and a healthy control (**c**). In the *middle panels*, the fatty change area is depicted in *yellow* (**d–f**). In the *lower panels*, the vesicular change area is preferentially depicted with *yellow* using a criteria of $<200\ \mu m^2$ of fatty change (**g–i**) (color figure online)

present study, we established criteria by which the degree of liver fatty change can be evaluated objectively. It is well known that the proportions of fatty change differ by location in liver tissues obtained by liver biopsy or operation. For quantitative evaluation of fatty change, an efficient algorithm was devised using the BIOREVO BZ-9000 microscope and Dynamic cell count BZ-H1C software. Using this method, fatty changes were analyzed in whole tissue specimens in chronic hepatitis C patients. With this algorithm, it was possible to evaluate fatty changes of whole tissues in the slides and obtain the same results by different operators. Furthermore, vesicular changes and clear cell changes could be evaluated separately by the distribution of brightness in liver tissues.

The main limitation of this study is that some selection biases and confounders such as alcohol consumption and the definition of fatty degeneration might affect the internal validity of the study. The study subjects were selected based on a history of liver biopsy or liver resection and infection with HCV genotype 1b. As levels of steatosis have been shown to differ among HCV genotypes, the present study was restricted to HCV genotype 1b, which

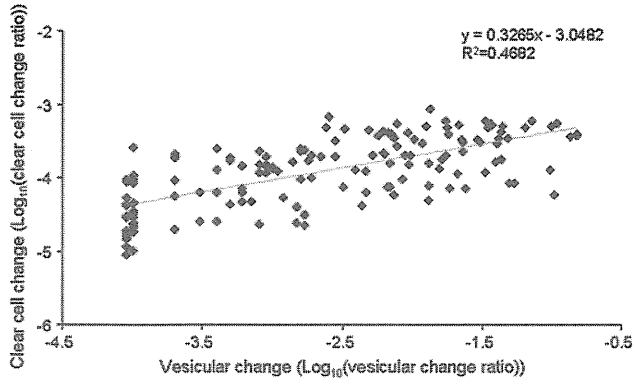

Fig. 4 Association between vesicular and clear cell change. The vesicular and clear cell change ratios were performed with logarithmic transformation, and the distributions were compared. The association between the distribution of vesicular and clear cell change are shown by a scatter diagram

accounts for more than 70% of chronic hepatitis C cases in Japan and is the best studied with respect to IL28B polymorphisms. Because ethanol intake of more than 20 g/day is associated with progressive liver damage [29, 30], patients whose daily alcohol intake exceeded this threshold were defined as alcohol positive. To evaluate fatty degeneration objectively, the distribution of brightness in liver tissue images was used. The brightness was calculated with Dynamic cell count BZ-H1C software and divided into 256 gradation sequences, and the fatty degeneration area was determined with the fixed brightness range. To evaluate the association between fatty degeneration and other factors, statistical analysis using continuous values may be more precise than using thresholds, assuming a large sample size with homoskedastic, normally distributed data. When the data were analyzed using continuous values in univariate

Table 2 Association between vesicular change and clinical background

	Vesicular change ratio		Univariate analysis	Multiple logistic regression analysis		
	<0.002 ($N = 76$)	≥0.002 ($N = 77$)	*P* value	*P* value	Adjusted odds ratio	95% CI
Age (years)[a]	60 (22–76)	61 (15–76)	0.774			
Gender (M:F)	44:32	39:38	0.368**			
BMI (kg m^{-2})[a]	22.0 (16.2–30.6)	23.5 (17.7–39.4)	0.009	0.033	1.147	1.011–1.301
Alcohol (+:−)	26:49	21:54	0.379**			
Platelet count ($\times 10^3$ μl^{-1})[a]	144 (23–759)	143 (49–327)	0.436			
Prothrombin activity (%)[a]	99 (56–124)	100 (12–166)	0.727			
Total bilirubin (mg dl^{-1})[a]	0.7 (0.4–14.7)	0.7 (0.4–2.1)	0.922			
Aspartate aminotransferase (IU l^{-1})[a]	42 (20–2617)	53 (14–250)	0.008	0.317		
Alanine aminotransferase (IU l^{-1})[a]	48 (11–2707)	61 (2–327)	0.093	0.318		
Lactate dehydrogenase (IU l^{-1})[a]	197 (129–899)	204 (43–473)	0.286			
Gamma glutamyl transpeptidase (IU l^{-1})[a]	34 (12–187)	55 (14–295)	<0.001	0.110		
Albumin (g dl^{-1})[a]	4.4 (2.9–5.3)	4.2 (3.1–5.1)	0.069			
Total cholesterol (mg dl^{-1})[a]	174 (100–312)	174 (122–263)	0.975			
Triglycerides (mg dl^{-1})[a]	97 (33–339)	113 (35–2466)	0.141			
Glucose (mg dl^{-1})[a]	98 (68–334)	103 (70–237)	0.533			
HCV RNA (log IU ml^{-1})[a]	6.4 (4.2–7.9)	6.2 (4.2–7.5)	0.031	0.068		
Core 70 (wild:mutant)	37:12	40:27	0.075**	0.230		
Core 91 (wild:mutant)	21:28	33:34	0.495**			
ISDR (0:≥1)	15:33	23:43	0.687**			
IL28B (TT:TG or GG)	65:11	51:26	0.005**	0.001	8.158[b]	2.412–27.589
Fibrosis (F0–1:F2–3)	36:40	23:54	0.026**	0.041	2.541[c]	1.040–6.207
Inflammatory activity (A0–1:A2–3)	24:51	17:60	0.168**			

Univariate analysis was performed with Mann–Whitney *U* test and **chi-square test

Multiple logistic regression analysis was performed using variables that were significant ($P < 0.05$) or marginally significant ($P < 0.10$) in univariate analysis

[a] Median (range)

[b] IL28B genotypes were coded as 0 or 1 depending on whether the subject carried the minor allele

[c] Fibrosis was coded as 0 for patients with mild fibrosis (F0–1) and 1 for patients with severe fibrosis (F2–3)

Table 3 Association between clear cell change and clinical background

	Clear cell change ratio		Univariate analysis	Multiple logistic regression analysis		
	<0.03 ($N = 77$)	≥0.03 ($N = 76$)	P value	P value	Adjusted odds ratio	95% CI
Age (years)[a]	60 (22–76)	61 (15–76)	0.408			
Gender (M:F)	47:29	36:41	0.061**	0.057		
BMI (kg m^{-2})[a]	23.0 (16.2–32.3)	22.7 (17.6–39.4)	0.477			
Alcohol (+:−)	27:47	20:56	0.179**			
Platelet count ($\times 10^3$ μl^{-1})[a]	152 (23–759)	131 (49–327)	0.175			
Prothrombin activity (%)[a]	99 (56–166)	100 (12–136)	0.829			
Total bilirubin (mg dl^{-1})[a]	0.7 (0.4–14.7)	0.7 (0.4–2.1)	0.815			
Aspartate aminotransferase (IU l^{-1})[a]	42 (14–2617)	56 (15–250)	<0.001	0.824		
Alanine aminotransferase (IU l^{-1})[a]	48 (11–2707)	65 (2–327)	0.018	0.809		
Lactate dehydrogenase (IU l^{-1})[a]	197 (123–899)	209 (43–473)	0.193			
Gamma glutamyl transpeptidase (IU l^{-1})[a]	37 (12–187)	47 (13–295)	0.049	0.928		
Albumin (g dl^{-1})[a]	4.3 (2.9–5.1)	4.2 (3.0–5.3)	0.376	0.200		
Total cholesterol (mg dl^{-1})[a]	174 (122–270)	173 (100–312)	0.161			
Triglycerides (mg dl^{-1})[a]	102 (33–2466)	97 (35–517)	0.861			
Glucose (mg dl^{-1})[a]	97 (68–334)	104 (70–284)	0.092	0.456		
HCV RNA (log IU ml^{-1})[a]	6.3 (4.2–7.9)	6.2 (4.2–7.5)	0.060	0.101		
Core 70 (wild:mutant)	35:14	42:25	0.325**			
Core 91 (wild:mutant)	20:29	34:33	0.290**			
ISDR (0:≥1)	14:34	24:42	0.421**			
IL28B (TT:TG or GG)	64:12	52:25	0.016**	0.011	3.000[b]	1.282–7.019
Fibrosis (F0–1:F2–3)	33:43	26:51	0.220**			
Inflammatory activity (A0–1:A2–3)	25:50	16:61	0.081**	0.066		

Univariate analysis was performed with Mann–Whitney U test and **chi-square test

Multiple logistic regression analysis was performed using variables that were significant ($P < 0.05$) or marginally significant ($P < 0.10$) in univariate analysis

[a] Median (range)

[b] IL28B genotypes were coded as 0 or 1 depending on whether the subject carried the minor allele

Table 4 Association between fatty degeneration and IL28B genotype

	IL28B		Univariate analysis
	TT	TG or GG	P value
Obesity group (BMI ≥25 kg m^{-2}, $N = 36$)			
Vesicular change ratio (<0.002:≥0.002)	12:19	2:3	1.000*
Clear cell change ratio (<0.033:≥0.033)	20:11	2:3	0.357*
Non-obesity group (BMI <25 kg m^{-2}, $N = 114$)			
Vesicular change ratio (<0.002:≥0.002)	52:30	9:23	0.001
Clear cell change ratio (<0.033:≥0.033)	48:34	10:22	0.009

Univariate analysis was performed with chi-square test and *Fisher's exact test

analysis using the Mann–Whitney U test, the IL28B genotype was found to be significantly associated with clear cell change and vesicular change ($P = 0.001$, $P < 0.001$, respectively). When continuous values were used in multiple regression, the association between IL28B genotype and fatty degeneration was also observed. *IL28B* genotype, HCV RNA titer and liver fibrosis stage were identified as independent factors for vesicular change ($P = 0.003$, $P = 0.022$, $P = 0.047$, respectively), and the *IL28B* genotype was identified as an independent factor for clear cell change ($P < 0.001$). Genotypes of rs738409 within the Patatin-like phospholipase domain-containing 3 (PNPLA3) locus were also recently reported to be associated with hepatic steatosis in chronic hepatitis C patients

(odds ratio 1.90–2.55) [38–40]. It would be interesting to analyze the effect of the PNPLA3 genotype on fatty degeneration in the present study; however, given the small number of study subjects and the small odds ratios reported in these studies, this study lacks the statistical power to verify the association between hepatic steatosis and PNPLA3.

Chronic HCV infection is known to be associated with fatty change of the liver, and the incidence of fatty change in chronic hepatitis C patients is higher than in those with other chronic liver dysfunctions [36, 41]. The mechanisms of fatty change in chronic hepatitis C patients are still unclear, but the induction of liver steatosis was observed in the presence of the HCV core protein in vitro and in vivo. In a transgenic mouse study, all the male and approximately 50% of the female mice developed liver steatosis by the age of 6 months [42]. Furthermore, the HCV core transfected cells were shown to activate the deposition of lipid [36, 37, 41, 43]. Thus it was hypothesized that chronic HCV infection might be associated with fatty change and the effect of clinical background. As shown in Tables 2 and 3, hepatic histological changes, including both vesicular and clear cell change, were significantly associated with BMI, aspartate aminotransferase, γ-GTP and HCV RNA levels in univariate analysis, whereas clear cell change was not associated with BMI. As shown in Fig. 2, clear cell change was strongly related to HCV infection, whereas it was rarely observed in the liver tissues of patients with non-alcoholic fatty liver disease. These findings suggest that vesicular change is associated with obesity or other lipid depositions, and that clear cell change is associated with chronic HCV infection.

Recently, several groups have reported significant associations between several linked SNPs in the *IL28B* locus and HCV eradication with IFN therapy based on genome-wide association analysis. The sustained virological response rate in chronic hepatitis C patients homozygous for the major allele (genotypes rs8099917 TT or rs12980275 CC or rs12979860 CC) was significantly higher than in patients heterozygous or homozygous for the minor allele [24–27]. Serum γ-GTP levels and liver histological fibrosis and inflammation levels in chronic hepatitis C patients with the favorable TT or CC SNP genotypes were also significantly lower than in those with the minor genotypes [28]. Although other host factors, such as liver steatosis or insulin resistance, have been demonstrated to be associated with virological response [23, 44], this study demonstrates that SNPs in the *IL28B* locus might affect steatosis in chronic hepatitis C patients. As the influence of the *IL28B* genotype was observed with both clear cell change and vesicular change (Tables 2, 3), and these associations became more remarkable in the non-obese group (Table 4), it is tempting to speculate that differences in IL28B expression might cause an aberration of lipid metabolism in chronic hepatitis C patients. An association between IL28B genotype at rs12979860 and hepatic steatosis in chronic hepatitis C patients was demonstrated in a previous report [45]. The results of this study were very similar given the strong association between the rs8099917 and rs12979860 genotypes [35], but the methods used to evaluate hepatic steatosis were quite different. In the previous report, hepatic steatosis was subjectively evaluated by pathologists using the Brunt classification, whereas in the present study, hepatic steatosis was evaluated objectively using a quantitative method, and two different classes of steatotic change, clear cell change and vesicular change were analyzed separately.

In conclusion, the relationship between clinical background and fatty change in liver tissue was analyzed using an operator-independent method, and significant associations between fatty change level and *IL28B* genotypes or HCV RNA level were identified. These findings suggest that these factors are connected to an aberration of lipid metabolism in chronic hepatitis C patients.

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Conflict of interest None.

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