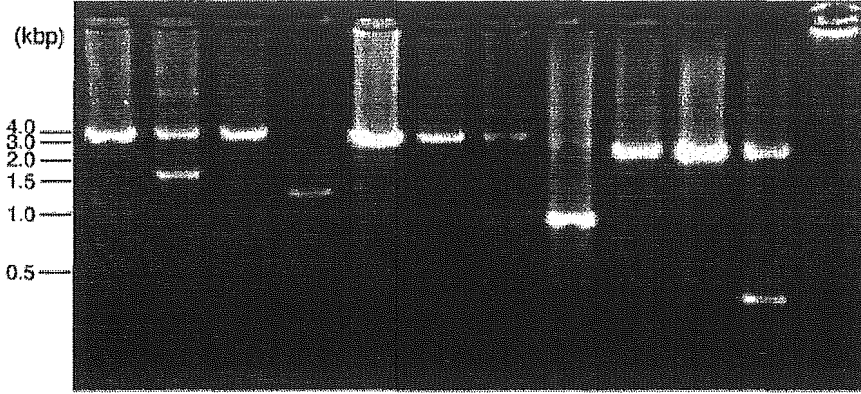


A

Primers													
cDNA synthesis		HC3481R				HC3945R				HC3945R			
1st PCR		HClongH1 X HC3481R				HC813S X HC3945R				HC813S X HC3174R			
2nd PCR		HC85F X HC3297R				HC841S X HC3759R				HC841S X HC3111AS			

Patient no.	274	295	325	373	274	295	325	373	274	295	325	373
-------------	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----



B

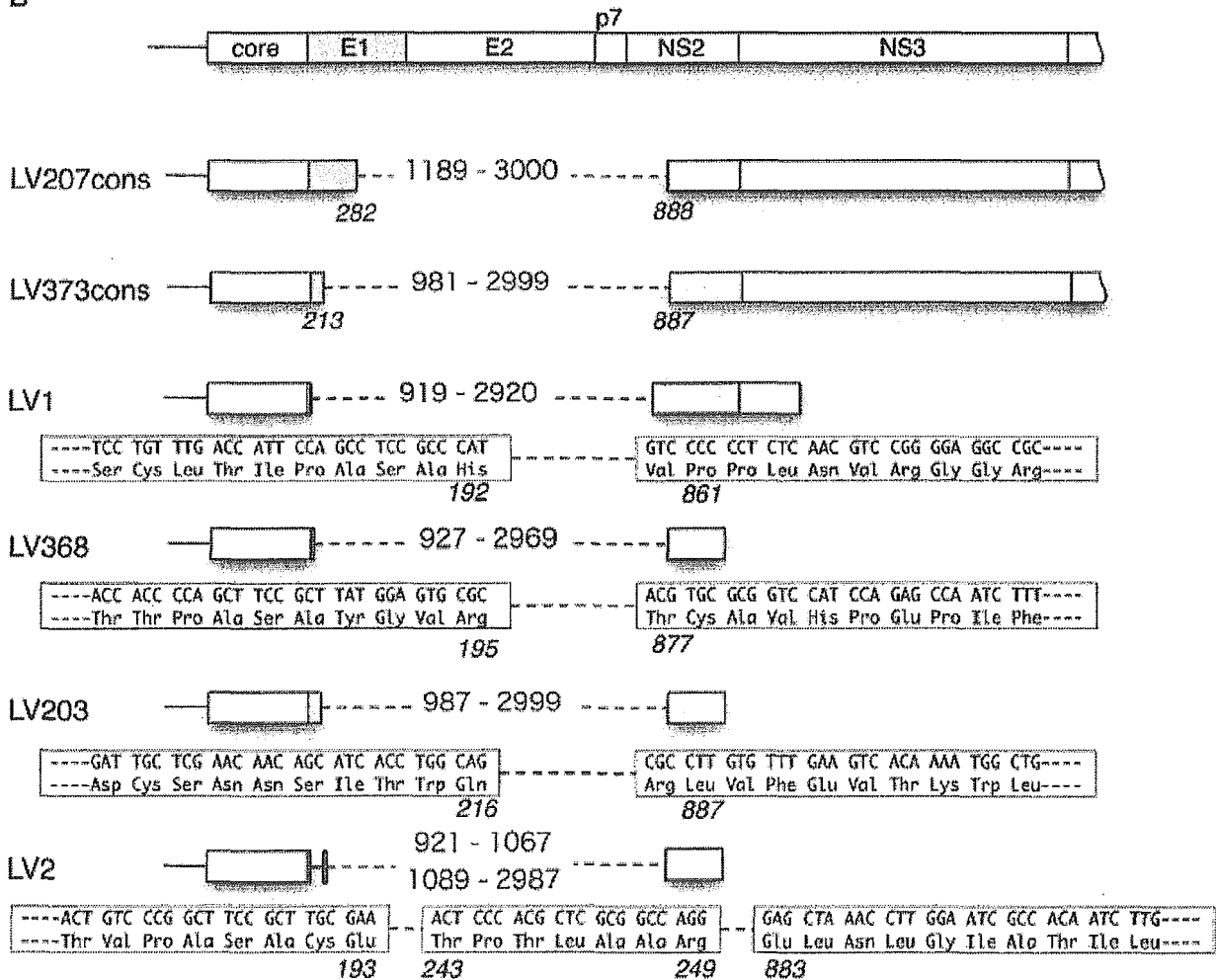


Fig. 3.

junction site. The expected length of the PCR fragment was 112 bp.

Analysis of HCV Proteins by Western Blotting

HCV cDNA expression plasmids were transfected into HEK293 cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 hr, cells were recovered and lysed in RIPA buffer containing protease inhibitor cocktails. After removing debris by centrifugation, the lysate was subjected to SDS-polyacrylamide gel electrophoresis (10–20% gradient gel, Daiichi Chemical, Tokyo, Japan), and proteins were transferred to a PVDF membrane (Millipore, Tokyo, Japan) under semi-dry conditions. The membrane was probed with the relevant antibodies. Bound antibodies were detected with anti-mouse or rabbit antibodies conjugated with HRP, and visualized by detecting the chemiluminescent signals developed using SuperSignal West Pico reagent (Pierce) with the LAS-1000 image analysis system (Fujifilm K.K., Tokyo, Japan) or exposure to Kodak Bio Max film. The membrane was rinsed in Restore reagent (Pierce) in order to remove bound antibodies, and was then probed with other antibodies.

RESULTS

Quantitation of HCV in Clinical Specimens

Table I summarizes viral parameters in 23 chronic active hepatitis C patients subsequently treated with interferon. All patients responded to IFN administration, exhibiting reduced virus titers and alanine transaminase (ALT) levels in serum; however, IFN efficacy varied (not shown). Serum and liver specimens were taken before IFN administration, and HCV viral loads were then determined by quantitation of HCV RNA by real-time RT-PCR, and by measuring HCV core antigen levels using core antigen ELISA (Table I). Correlations among viral loads were analyzed. The amount of HCV core antigen and RNA in serum and liver were well correlated ($R = 0.968$ and $R = 0.728$, respectively). In contrast, the correlation between HCV titers in serum and liver was significant but poor ($R = 0.575$ stand for core antigen and $R = 0.373$ for RNA). No obvious correlation was observed between efficacy and viral load in liver biopsy specimens.

Truncated Subgenomic HCV RNA in the Liver

In order to investigate the structure of the HCV genome in patient liver, HCV cDNAs were cloned from

two cases with high viral load (207 and 373). Figure 1A shows the strategy of LD-RT-PCR for cloning of the HCV genome. The length of LD-RT-PCR products varied between the patients. Comparison with the control cDNA, which was previously cloned from a blood donor (G14) plasma sample, indicated a 2-kb deletion occurred in the 5'-half of the HCV RNA from these patients (Fig. 1B).

The nucleotide sequences of cDNA clones from Patient 207 liver (LV207-0193-1, -3, -6 and -15) were analyzed and the deduced amino acid sequences were compared (Fig. 2A). All clones had almost identical sequences. While the open reading frames (ORFs) of two isolates were discontinued (by two stop codons in LV207-0193-6 and a one-base deletion in LV207-0193-15), LV207-0193-1 and -3 encoded a continuous 2,383 amino acid polypeptide. In the case of Patient 373, LV373-0193-10 encoded a 2,314 amino acid polypeptide. Two cDNA clones, LV373-0153-6 and -5, overlapped with LV373-0193-10, and identity in the nucleotide sequence of the overlapped region was 98%. With the exception of LV373-0153-5, which contained an in-frame stop codon, each cDNA had a contiguous ORF. The consensus HCV cDNA sequence for each patient was generated from these cDNA isolates (Fig. 2B).

Sequence comparison with authentic genotype 1b HCV (J1) [Aizaki et al., 1998] is illustrated in Figure 2B. Both consensus sequences from patient livers lacked sequences between E1 and NS2; the regions corresponded to the nucleotides 1189–3000 (amino acids 283–887), and 981–2999 (amino acids 214–886) of HCV-J1.

To confirm that the deletion was not produced during LD-RT-PCR (due to effects such as strong RNA secondary structure), non-truncated RNA transcripts were reverse-transcribed and amplified by the same protocol to give the truncated genome in Patient 207. While a deleted DNA fragment of about 7 kbp was amplified from truncated RNA transcripts of Patient 207, a full-length DNA fragment of about 9 kbp was amplified from non-truncated RNA transcripts of Donor G14, (Fig. 2C).

Deletions Found in Livers of Other Patients

In order to examine the incidence of HCV subgenomes with E1–E2 deletions, HCV genomes in other liver specimens were examined by RT-PCR with the primers listed in Table II. Two surgical samples (Patient 1 and 2) from hepatic cirrhosis patients with hepatocellular carcinoma were also subjected to this analysis. While the patterns of PCR fragments depended on the primer sets (Fig. 3A, Table II), they were classified into four

Fig. 3. Demonstration of deletions in the structural protein coding sequences of HCV genomes from patient livers. A: RNA from patient biopsies was analyzed by RT-PCR with primers for 5'-UTR to NS2 or core to NS2. Primer sets and Patient numbers are shown above the agarose electrophoresis image, and positions of the DNA markers are indicated on the left side of the image. B: Illustration shows schematic views of in-frame deletions and sequences of PCR fragments obtained by RT-PCR with the 5'-UTR and NS2 primers. cDNA isolates were designated as described in legend of Figure 2. The clones (LV1, LV368,

LV203, and LV2) of Patient 1, 368, 203, and 2 were obtained by RT-PCR using primer sets g, j, b, and f (Table II), respectively. Only truncated fragments were amplified in these cases after RT-PCR for 5'-UTR to NS2 (Table I). Structures of the truncated HCV genomes, LV207cons and LV373cons, are shown for comparison. Numbers between dot-tagged graphs represent deleted regions as they correspond to HCV-J1. Bottom lines show the corresponding amino acid positions of the deletion boundaries.

types (Table I): no amplified fragments (five cases); fragments corresponding to the non-truncated HCV genome (eight cases); fragments corresponding to both the truncated and non-truncated genomes (six cases); and fragments corresponding only to the truncated genome (four cases, including Patient 207 and 373).

RT-PCR of HCV RNA in Patient 1, 368, and 203 yielded only fragments corresponding to the truncated HCV genome. The isolated molecular clones contained in-frame deletions of amino acids extending from E1 to NS2, similarly to those isolated from Patient 207 and 373. Although cDNA from Patient 2 contained two in-frame deletions in E1 and E1-NS2, it shared the characteristics of the truncated genome; entire core, partial E1, and NS2 (Fig. 3B).

In cases with both truncated and non-truncated HCV cDNA, the predominant PCR product was non-truncated (Table I, Fig. 3A). Figure 4 shows the structures of the cDNA isolates. Deletions similar to those described above were found only in Patient 325. Patient 295, 288, 274, and 331 had out-of-frame deletions, and except for a clone from Patient 331 [LV331-(i)], sequence comparison between truncated and non-truncated HCV cDNA indicated sequence identity (3–8 nucleotides in length) in these out-of-frame deletions between the deletion donor and acceptor regions.

In Patient 207, Both Truncated and Non-Truncated HCV RNA Co-Existed for Years

E2 primers for PCR were designed to avoid PCR bias under competitive PCR conditions in order to confirm the presence of the non-truncated HCV genome in Patient 207 [Alvarez et al., 2000]. Two overlapping HCV cDNA sequences (LV0922 and LV2030) were isolated from a liver biopsy specimen from Patient 207 (Fig. 5A). The overlapping regions of the consensus sequences (LV0922cons and LV2030cons in Fig. 5B) were identical; however, the amino acid sequence identity to the truncated HCV genome (LV207cons: consensus sequence determined with isolates) was 92.1% in the E1 region, and 82.1% in the NS2 region (Fig. 5B).

Both the truncated and non-truncated genomes were detected by RT-PCR in serum from Patient 207 (Fig. 5A). The sequence of an isolate (S831) showed 99.4% nucleotide and amino acid sequence identity with LV207cons and the same in-frame deletions (Fig. 5A). The cDNA for the non-truncated genome (S2531) was also isolated from the serum by PCR with primers for E2 sequences. This cDNA was nearly identical (99.8% in nucleotide sequence) to that from the liver (LV2030cons), but differed from the truncated HCV genome identified in the liver (LV207cons) and serum (S831) (Fig. 5B).

From the RNA extracted from Patient 207 serum, a PCR fragment of the expected length was amplified using anti-sense primer for junction site at the deleted position between E1 and NS2 of the truncated genome of Patient 207. However, the PCR fragment was not amplified from the RNA of Donor G14 plasma, in which only the full-length HCV genome was detected. Simi-

larly, the PCR fragment was amplified from truncated RNA transcripts, but not amplified from non-truncated RNA transcripts (Fig. 5C).

In order to examine the persistence of both HCV genomes, a series of serum specimens consisting of samples obtained from Patient 207 were examined. Only the truncated RNA was detected by RT-PCR with primers for the core and NS2 in all samples. The sequences of PCR fragment clones were conserved at both the nucleotide and amino acid level (92–99% identity, Fig. 6A). All clones had the same deletions as observed in biopsy samples (Fig. 6A) at the E1-NS2 junction. Novel quasispecies with amino acid deletions at 13 amino acids upstream of the junction were found in March 1998 (designated as 9803). Another quasispecies was identified in March 1999. HCV E2 cDNAs of the non-truncated genome were isolated by RT-PCR from all serum samples, and were found to have conserved sequences (Fig. 6B).

Significant Difference in the Ratio of Truncated to Non-Truncated Genome Between Serum and Liver

HCV RNA from Patient 207 was measured by real-time RT-PCR for the 5'-UTR and E2 sequences. The quantity of 5'-UTR is indicative of the entire HCV genome, while that of E2 is only indicative of the non-truncated genome. The ratio of HCV E2 RNA to 5'-UTR RNA in serum was nearly constant throughout the 15-months monitoring period (Table III). Significant differences in the ratio of truncated to non-truncated genome were observed between liver and serum RNA samples; the ratio of HCV 5'-UTR to E2 RNA in liver specimens was about a hundred times of those in serum.

Expression of Truncated HCV cDNA In Vitro

HCV proteins processed from subgenomic HCV cDNA were investigated in a transient cDNA expression experiment. Chimeric cDNA for the truncated genome, LV207cont, consisted of cDNA fragments selected from 4 cDNA isolates from Patient 207 and the 3'-end of NS5B to the 3'-UTR sequence isolated from another patient (Fig. 7A). For expression of full-length HCV cDNA, chimeric cDNAs having the core-NS2 sequence of LV207cont instead of the corresponding region of J1 HCV cDNA (J1NLV) were used.

Figure 7B shows the Western blots of lysates from cells transfected with cDNA expression vectors. Anti-core monoclonal antibody detected a 19-kDa protein, thus indicating that the truncated polyprotein was correctly processed at the core-E1 junction. Each 35-kDa protein expressed from HCV cDNA, which was reactive to anti-E1 monoclonal antibody, was susceptible to endoglycosidase (Endo H), and converted to different molecular masses by enzyme treatment. The migration of deglycosylated E1 reactive peptide from the truncated HCV cDNA corresponded to the predicted molecular mass of the E1-NS2 fusion protein, 24 kDa, which differed from that of the non truncated E1 (19 kDa). The

LV325

Percent identity in nucleotide sequence

```

-----ATT GTG TAT GAG GCA GCG GAC GTG ATC ATG EAC ACC CCC GGG TGC-----CAT TCA GAG CTA ATT TTT GAA ATC ACC AAN ATC TTG CTC-----
----Ile Val Tyr Glu Ala Ala Gly Ala Ile Met His Thr Pro Gly Cys----His Ser Glu Leu Ile Phe Glu Ile Thr Lys Ile Leu Leu----

-----ATT GTG TAT GAG GCA GCG GCG GCG ATC-----
----Ile Val Tyr Glu Ala Ala Gly Ala Ile-----
                220
    
```

96.7% (245nt)

LV288

```

-----GTG GGG GAT CTT TGC GGA TCT GTC TTC CTC GTC TCC CAG CPG TTC-----CTT TCC TTC CTC ATG TTC TTC TGT GCT GCC TGG TAT ATC AAG-----
----Val Gly Asp Leu Cys Gly Ser Val Phe Leu Val Ser Glu Leu Phe-----Leu Ser Phe Leu Met Phe Phe Cys Ala Ala Trp Tyr Ile Lys-----

-----GTG GGG GAT CTT TGC GGA TCT GTC TTC CTC G-----
----Val Gly Asp Leu Cys Gly Ser Val Phe Leu-----
                286
    
```

97.3% (786nt)

LV295

```

-----GGAGGTCCTGGTAGACCTGCACC ATG AAG ACA ACT CCT-----CGA CTS TAC ATG ATG AAT AAG ACC GGG TTC ACC AAG ACG TGC-----
----Met Ser Thr Thr Pro-----Arg Leu Tyr Met Met Asn Ser Thr Gly Phe Thr Lys Thr Cys-----

-----GGAGGTCCTGGTAGACCTGCACC ATG-----
----Met Ser Thr Thr Thr Gly Phe Thr Lys Thr Cys-----
                1
                555
    
```

99.2% (1524nt)

LV274

```

-----CAG ATC GGT GGT GGA GTT TAC CTG TTG CCG CAG AGG GGC CCC AGG-----GCC GCC TTA GAG AAC CTA GCG GTC CTC AAC GCG GCG TCC GTA-----
----Glu Ile Val Gly Gly Val Tyr Leu Leu Pro Arg Arg Gly Pro Arg-----Ala Ala Leu Glu Asn Leu Val Val Leu Asn Ala Ala Ser Val-----

(i) -----CAG ATC GGT GGT GG-----
----Glu Ile Val Gly Gly-----
                33
                753
                910nt

-----GTT TAC CTG TTG CCG CAG AGG-----GGG GCA GAC ACC GCG GCG TGT GGG-----GGC CTA GTT GGT TGC ATT ATC ACT-----GGC CGC GAT GCC ATC ATC CTC CTC-----
----Val Tyr Leu Leu Pro Arg Arg-----Gly Ala Asp Thr Ala Ala Cys Gly-----Gly Leu Leu Gly Cys Phe Ile Thr-----Gly Arg Asp Ala Ile Ile Leu Leu-----

(ii) -----GTT TAC CTG TTG-----
----Val Tyr Leu Leu-----
                33
                990
                1042
                873
                not determined
    
```

LV331

```

-----GTG CCG GCG ACT AGG AAG ACT TCC GAG CCG TCG CAA CCT CGT GGA-----CAC AGG CTC AAC GCC GEA TGC AAT TGG ACT CGA GGA GAG CCG-----
----Val Arg Ala Thr Arg Lys Thr Ser Glu Arg Ser Glu Pro Arg Gly-----His Arg Leu Lys Ala Ala Cys Asn Trp Thr Arg Gly Glu Arg-----

(i) -----GTG CCG GCG ACT AGG AAG ACT TCC GAG CG-----
----Val Arg Ala Thr Arg Lys Thr Ser Glu Arg-----
                55
                642
                860nt

-----GTG CCG GCG ACT AGG AAG ACT TCC GAG CCG TCG CAA CCT CGT GGA-----AAC AAC ACG CCG CCG CCG CAA GGC AAC TGG TTC GGC TGT ACG-----
----Val Arg Ala Thr Arg Lys Thr Ser Glu Arg Ser Glu Pro Arg Gly-----Asn Asn Thr Arg Pro Pro Glu Gly Asn Trp Phe Gly Cys Thr-----

(ii) -----GTG CCG GCG ACT AGG AAG ACT T-----
----Val Arg Ala Thr Arg Lys Thr-----
                52
                544
                2652nt

-----CCGAGTACTGTTGGGTCGCGAAAGGCTTGTGGTACTGCTGATAGGGTG-----CTG GCG GGC CTT GCC TAC TAT TCC ATG GTG GGG AAC TGG-----
-----Leu Ala Gly Leu Ala Tyr Tyr Ser Met Val Gly Asn Trp-----

(iii) -----CCGAGTACTGTTGGGTCGCGAAAGGCTTGT-----
----Ala Tyr Tyr Ser Met Val Gly Asn Trp-----
                340
                1516nt
    
```

Fig. 4. Sequence comparisons between HCV genomes with and without deletions isolated from the same patient. Partial sequences of RT-PCR fragments isolated from liver biopsies are shown. cDNA fragments are labeled as described for previous figures. Nucleotide and amino acid positions are represented as the corresponding positions in HCV-J1. In these cases, both non-truncated and truncated HCV RNA were amplified from each biopsy specimen (Table I). The non-truncated and truncated RT-PCR fragments from Patient 325, 288, 295, 274, and 331 were obtained with primer sets d and d, d and d, b and b, b and e, and a and b (Table II), respectively. Numbers between tagged graphs

represent deleted regions as they correspond to HCV-J1. Bottom lines show the corresponding amino acid positions of the boundaries. The right side of the sequences shows the identities of overlapping nucleotide sequences in the truncated and non-truncated genome, and the length of the overlap is given in parentheses. The LV274-(ii) had a translocation of the NS3 sequence inserted between the core and NS2 sequences. Because we did not clone the NS3 sequence from this case, the corresponding region of HCV-J1 is italicized in the figure and the sequence identity was not determined. Sequences that coincided between the boundaries of the deletions are underlined.

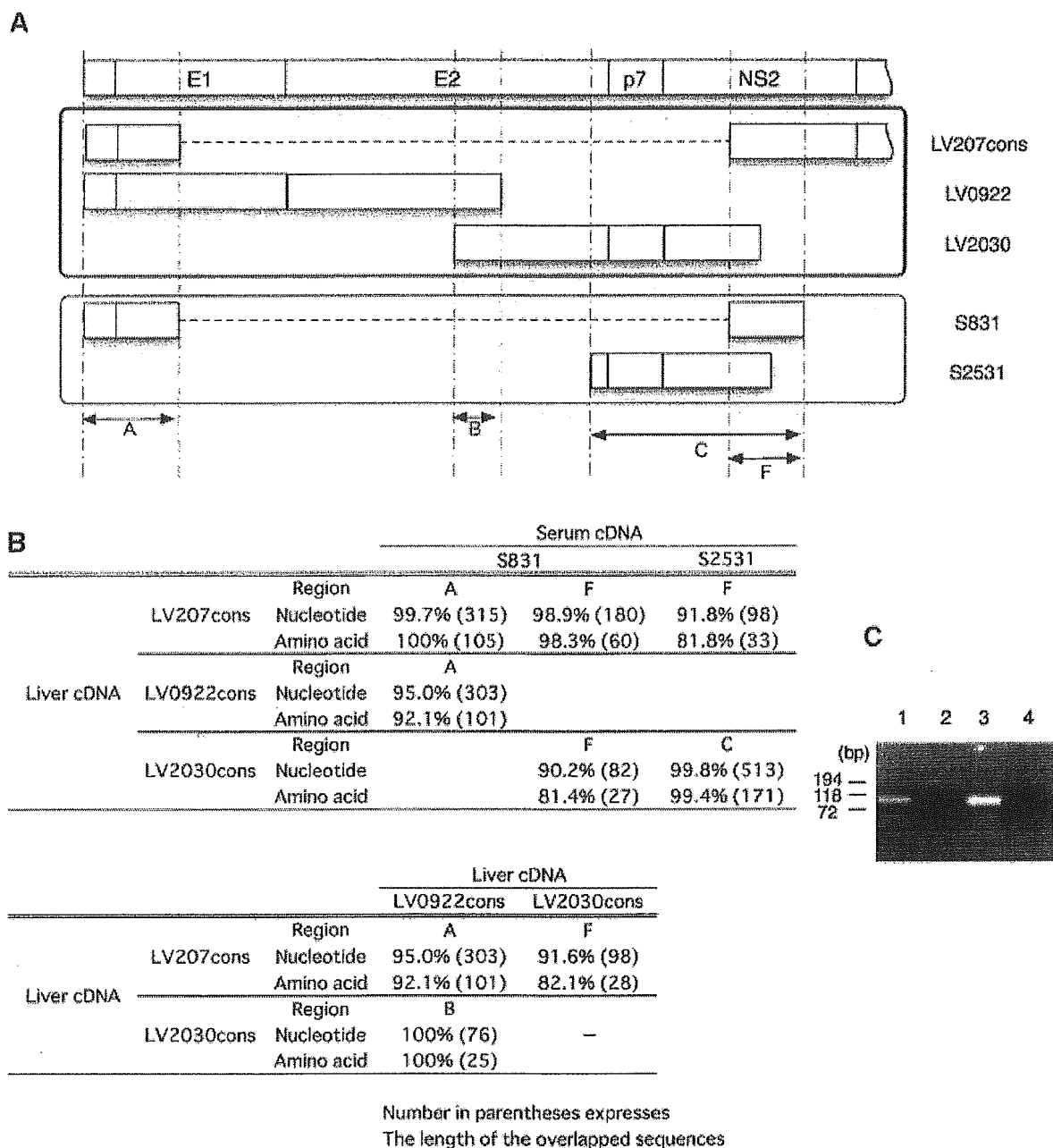


Fig. 5. Cloning of the non-truncated HCV RNA from patient 207. **A**: Schematic views of RT-PCR fragments for non-truncated HCV RNA in liver (LV0922 and LV2030) and in serum (S2531) from Patient 207, and for the truncated sequence in serum (S831). LV0922 fragment was obtained by RT-PCR with HC1b9405R for cDNA synthesis, HC841S and HC2199AS for 1st PCR, and HC948S and HC2199AS for 2nd PCR. LV2030 fragment was obtained by RT-PCR with XR58R for cDNA synthesis, HC2048SLV and LVC1392AS for 1st PCR, and HC2069S and LVC1280AS for 2nd PCR. S2531 fragment was obtained by RT-PCR with HC3174AS for cDNA synthesis, HC2430S and HC3174AS for

1st PCR, and HC2546S and HC3111AS for 2nd PCR. Arrows indicate regions being compared in the Tables (**B**), which show sequence identities between the non-truncated and truncated sequences in serum (**upper**), and those between sequences isolated from liver and liver (**lower**). **C**: Images of agarose gel electrophoresis of RT-PCR products amplified using junction site primer, from extracted RNA from Patient 207 serum (**lane 1**) and Donor G14 plasma (**lane 2**), truncated in vitro RNA transcripts of Patient 207 (**lane 3**) and non-truncated in vitro RNA transcripts of Donor G14 (**lane 4**).

protein at the 70-kDa position, which was the predicted mass of NS3, was detected by anti-NS3 polyclonal antibody. These data suggested that processing of the truncated HCV polyprotein is same as that of the full-length HCV polyprotein at the core-E1 and NS2-NS3 junctions.

DISCUSSION

Characteristics of HCV Subgenome With In-Frame Deletion

Novel truncated HCV genomes with in-frame deletions from E1 to NS2 were identified in the livers of two

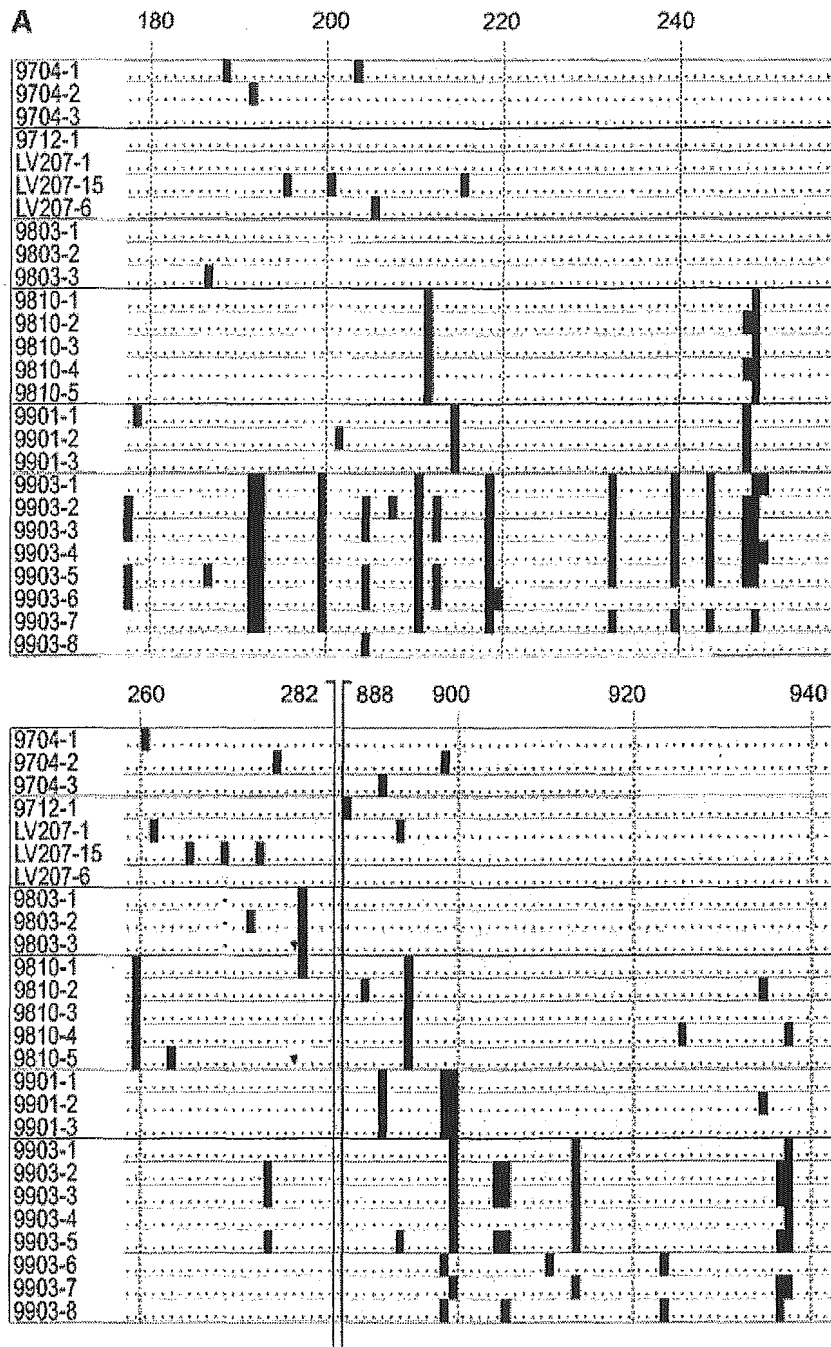


Fig. 6. Alignment of amino acid sequences of cDNA isolates from a series of serum samples from Patient 207. Comparison of nucleotide sequences of truncated genome (A) and non-truncated genome (B) was carried out using Clustal W algorithm. Numbers on top of the alignment show the corresponding amino acid positions of HCV J1. Names of the sequences indicate the date the serum was taken; 9712, for example, refers to December 1997. Bold bar represents amino acid position that differs from consensus sequence. Coinciding amino acid positions are represented by dots. All cDNA isolates from March 1998 (9803) had an amino acid deletion at the position marked by the

horizontal bar. Bold vertical bars in A show the boundaries of the deletion. Inverted triangles indicate positions of nucleotide deletions in cDNA isolates. Deduced amino acid sequences with nucleotide deletions were obtained by inserting a nucleotide at the position. Fragments of the truncated genome (A) were obtained by RT-PCR with HC3297R for cDNA synthesis, HC813S and HC3297R for 1st PCR, and HC841S and HC3174AS for 2nd PCR. Fragments of the non-truncated genome (B) were obtained by RT-PCR with HC2378R for cDNA synthesis, HC1979S and HC2378R for 1st PCR, and HC1979S and HC2300R for 2nd PCR.

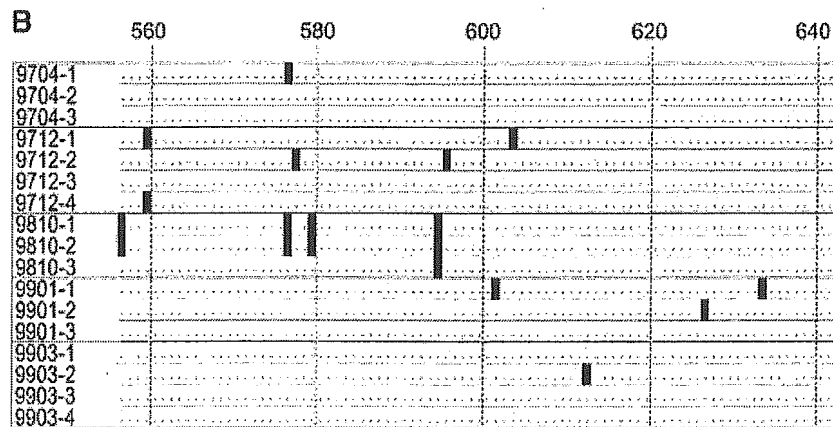


Fig. 6. (Continued)

chronic active hepatitis C patients. These HCV subgenomes encoded a single polyprotein for the entire core, five NS proteins (from NS3 to NS5B), and E1–NS2 fusion protein. This type of HCV subgenome was found in 4 of 23 chronic active hepatitis patients (16 cases with positive results by RT-PCR with primer sets tested), and in 2 hepatocellular carcinoma patients. These data suggested that HCV subgenomic RNA is generated in a certain number of chronic active hepatitis C patients.

HCV polypeptide expressed in cells with the subgenomic cDNA was processed in the same way as the authentic HCV polyprotein. The E1–NS2 fusion protein from subgenomic HCV cDNA was glycosylated and was susceptible to Endo H treatment, thus suggesting that it was located in the ER. NS2 is a membrane protein located in the ER region [Santolini et al., 1995; Kim et al., 1999]. On the other hand, localization and modification of E1 protein was governed by E2 [Cocquerel et al., 1998; Duvet et al., 1998]. These data indicate that ER retention in the E1–NS2 fusion protein is determined by NS2.

TABLE III. Quantitation of HCV RNA in a Series of Serum Samples Taken From Patient 207

Date	5'-UTR (copies/ml)	Percentage of E2/UTR
December 8, 1997	7.75×10^4 (5.75×10^3)	19.5% 0.2%
Numbers in parenthesis represents HCV quantity in liver biopsy specimen		
March 6, 1998	2.93×10^3	36.2%
March 9, 1998	1.19×10^4	22.1%
IFN treatment From March 9, 1998 to September 2, 1998		
September 29, 1998	4.17×10^4	23.8%
October 28, 1998	5.25×10^4	21.8%
December 8, 1998	2.24×10^4	26.1%
January 12, 1999	5.81×10^4	28.5%
February 10, 1999	2.57×10^4	20.3%
March 31, 1999	5.30×10^4	23.1%

Do HCV Subgenomes Replicate Autonomously in Patients?

Viral subgenomes have been isolated from viruses closely related to HCV, such as flavivirus (Murray Valley encephalitis virus) [Lancaster et al., 1998], pestivirus (classical swine fever virus; SFV [Aoki et al., 2001], and bovine viral diarrhea virus; BVDV [Tautz et al., 1994; Kupfermann et al., 1996]. The HCV subgenomes shared common structural features with these subgenomic RNAs.

The HCV subgenomes fulfilled the minimal requirements for autonomous RNA replication; the 5'-UTR, nonstructural proteins (NS3–NS5B), and the 3'-UTR, as demonstrated using artificial HCV subgenomic replicons [Lohmann et al., 1999; Blight et al., 2000]. In addition, defective genomes of DI autonomously replicate their RNA [Behrens et al., 1998]. Furthermore, sequence comparisons of the truncated and non-truncated HCV genome sequences, which were isolated from a serum series obtained from a single patient, suggested that both genomes have been replicating independently for years. These data suggest that HCV subgenomes with in-frame deletions in structural proteins replicate themselves. However, it is possible that the full-length genome is required for the replication of the subgenome, as both genomes were present for years despite the dominance of the subgenome.

The dominance of the subgenome over the full-length genome (approximately 500-fold in the liver) was indicated by real-time RT-PCR analysis for the HCV 5'-UTR and E2. The dominance of HCV subgenome suggests an advantage in RNA replication. The length of the genome is probably a key factor in viral replication. If processing velocities in translation and transcription are equal over the HCV genome, the HCV subgenome would be replicated about 20% faster than the non-truncated genome. However, other mechanisms affecting efficiency are likely present.

Heterogeneous molecular clones with out-of-frame deletions, which shared sequences with the full-length

genome, indicated that the HCV subgenome frequently arises from its full-length genome by such mechanisms found in other RNA viruses [Nagy and Simon, 1997]. However, the fact only one type of subgenome with an in-frame deletion persisted suggested that a competent subgenome for replication is selected. The NS2 in all HCV subgenomes, which preserved their NS2–NS3 protease domains [Grakoui et al., 1993a; Hijikata et al., 1993], indicated protease activity is involved in the persistence of the HCV subgenome. Furthermore, we believe that core protein is required for virus replication in vivo, because the core sequence in the HCV subgenomes was preserved among the dominant HCV subgenomes.

Comparison With HCV Subgenomes or Recombinants Described Previously

Quadri and Negro [2001] identified recently a positive-strand subgenomic RNA starting from the 5'-UTR without the 3'-UTR, and a negative-strand subgenomic RNA with the 3'-UTR lacking the 5'-UTR. Although we

did not clone the 3'-UTR from the Patient 207 sample, an HCV subgenome with same deletion was isolated from cDNA with a primer corresponding to the X-region in the 3'-UTR (data not shown), and we isolated the 3'-UTR from Patient 373 liver RNA. Based on these observations, it is considered that the subgenomic HCV RNA contains the entire 3'-UTR, rather than their proposed RNA populations.

Intergenotypic recombination has been described between genotype 2k and genotype 1b HCV at between nucleotides 3175 and 3176, about 200 nucleotides from the recombination region of the HCV subgenomes [Kalinina et al., 2002]. We did not examine the possibility of this type of recombination because the number of HCV cDNAs covering this region was too few in the present study.

HCV Subgenome and Pathogenesis

The question whether the HCV subgenome involved in the mechanism of viral persistence and pathogenesis in a similar manner as the DI particles of other viruses

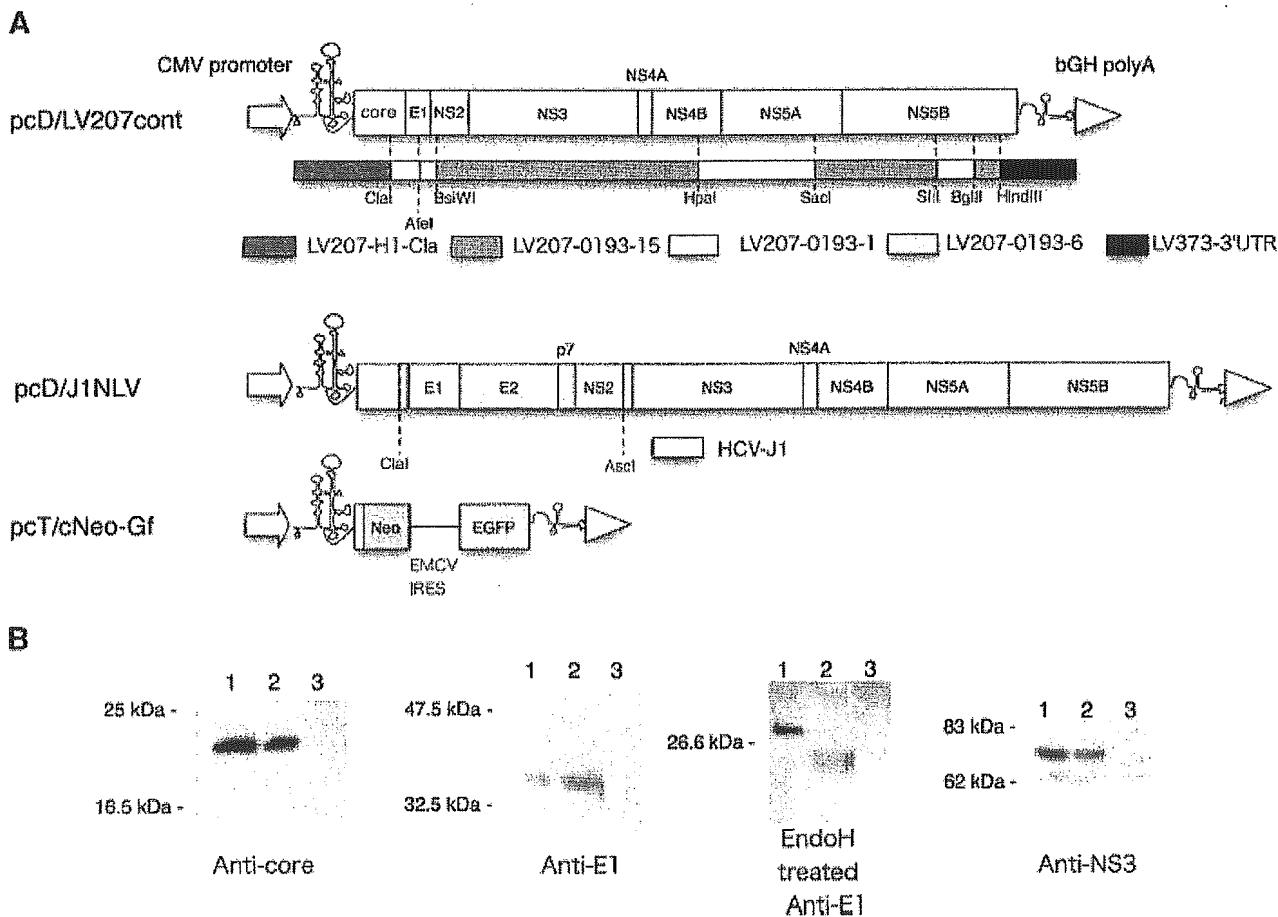


Fig. 7. Expression of HCV proteins from truncated and non-truncated HCV cDNA in mammalian cells. A: Chimeric HCV subgenomic cDNA, LV207cont, was composed of fragments from four cDNAs from patient liver (LV207-H1-Cla, 0193-15, 0193-1, and 0193-6) and a fragment from Patient 373 (LV373-3'-UTR) by using restriction sites depicted. Other chimeric cDNAs consisted of LV207cont and a fragment of HCV-J1 (J1NLV), as indicated. Dicistronic DNA constructs

of HCV core-Neo resistant fusion and EGFP genes, cNeo-EGFP, was used as a negative control for HCV protein expression. All DNA constructs were transiently expressed in HEK293 cells under the control of the CMV promoter in pcDNA3.1. B: Western blotting analysis results for core, E1 and NS3 in transfected cells are shown. The positions of pre-stained molecular weight markers are indicated on the left side of the images.

[Tautz et al., 1994; Kupfermann et al., 1996] is remained. The amounts of core protein in patients with the HCV subgenome were larger than in patients without the subgenome (not statically significant). Transgenic mice expressing core protein in liver developed steatosis and later cancer, indicating that the core protein is a potent carcinogen in mice [Moriya et al., 1997, 1998]. It was found that two HCC patients had this subgenome. These data suggested that the involvement of the truncated genome in pathogenesis; however, we must examine more cases in order to elucidate any correlations between HCV subgenome and disease, particularly for progression of the disease to HCC.

The heterogeneous nature of the HCV genomes in patients may contribute to the persistence of HCV in escaping the host defense system. Particularly, the deletion of E1/E2 proteins may have a great impact on host immune response to the virus; E1/E2 is believed to be a target molecule for neutralizing antibodies, which block the binding of virions to virus receptor [Beyene et al., 2002]. The function of the truncated HCV genome in the life cycle of HCV is uncertain, but we believe the presence of this subgenomic RNA in both the liver and serum is important for illustrating that much about the nature of HCV remains unknown.

Added in Proof

Wakita et al. recently reported that a man-made HCV subgenomic RNA lacking E1/E2 replicated *in vitro*. [Wakita et al., 2005, *Nature Medicine*, published online 12 June]

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Impact of daily high-dose IFN α -2b plus ribavirin combination therapy on reduction of ALT levels in patients with chronic hepatitis C with genotype 1 and high HCV RNA levels

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Abstract

The possibility of delaying progression to hepatocellular carcinoma in chronic hepatitis C patients with genotype 1 and high viral titers with baseline ALT levels of ≥ 50 IU/L was examined by administration of IFN plus ribavirin combination therapy using ALT normalization as index and IFN monotherapy as control. The rate of sustained ALT normalization (ALT normal at 24 weeks after the end of treatment) was 28.1% with combination therapy and 10.5% with IFN monotherapy ($P=0.001$). Furthermore, the number of patients with sustained viral response (SVR) and with sustained ALT normalization in non-SVR patients was also significantly higher in the combination therapy versus monotherapy group. Mean ALT values during treatment and for 6 months after the end of treatment were significantly lower with combination therapy versus monotherapy even in virological nonresponders, as well as significantly lower during the post-treatment observation period in patients who relapsed after the end of treatment. Since increase in the rate of sustained ALT normalization and SVR were successfully achieved, inhibition of progression to hepatocellular carcinoma should be studied with long-term IFN and ribavirin combination therapy.

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Keywords: Chronic hepatitis C; IFN α -2b; Ribavirin; ALT; Hepatocellular carcinoma prevention

1. Introduction

With the aging of the chronic hepatitis C patient population in Japan, a rapid increase in the incidence of hep-

atocellular carcinoma (HCC) is being observed [1]. Deaths due to HCC number over 30,000 per year [1], and prevention of progression to HCC is now an urgent issue. Many reports on the efficacy of interferon (IFN) in preventing progression to HCC in patients with chronic hepatitis C have been published by Japanese researchers [2–8]. At first, normalization

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of serum alanine aminotransferase (ALT) levels while on IFN therapy was thought to inhibit progression to HCC [3]. However, the results of long-term follow-up studies clearly indicate that sustained viral response (SVR) and/or sustained normalization of ALT after the end of treatment are necessary for the long-term inhibition of progression to HCC [4,6–8]. Results indicating that such inhibition is possible if ALT levels are maintained long-term within about twice the upper limit of normal (80 IU/L) have also been reported [9,10].

The standard of treatment of chronic hepatitis C worldwide is pegylated-IFN (PEG-IFN) in combination with ribavirin. The addition of ribavirin has been shown radically to increase the rate of eradication of HCV [11–13], and this is thought to result in increased inhibition of progression to HCC in patients with chronic hepatitis C. The efficacy of PEG-IFN plus ribavirin in the prevention of histologic progression using fibrosis as index has already been reported although these studies did not directly examine the effect on inhibition of progression to HCC [14,15]. Based on an average follow-up period of 20 months, one-stage improvement in METAVIR score was observed in 73% of patients on 1-year administration of PEG-Intron 1.5 µg plus ribavirin. One-stage exacerbation was observed only in 8%. Ribavirin alone has almost no effect on reducing HCV levels, but is reported to normalize ALT levels during treatment [16,17], and similar effects may be expected with combination therapy. The focus worldwide is on antiviral efficacy, and there are very few reports of detailed examination of the effect of combination therapy on liver function [18].

Recently, the efficacy of combination therapy with ribavirin in the context of inhibition of progression to HCC has started to be investigated although the number of patients involved is small compared with the numbers enrolled in clinical studies of IFN in Japan. Yang et al. [19] reported that the 7-year cumulative HCC rate is 1.4% in patients receiving IFN plus ribavirin, which is much lower than the 10.2% reported in patients receiving IFN alone, although the difference was not statistically significant due to the small sample size. It has been reported elsewhere that factors contributing to inhibition of progression to HCC include absence of liver cirrhosis before the start of treatment and sustained viral response although the data include both combination therapy and monotherapy cases [20]. The above Japanese data indicate that prevention of progression to HCC can be expected with sustained normalization of ALT, although the presence of this factor does not necessarily indicate that liver histology is normal [18]. Hence we tested the hypothesis that combination therapy consisting of IFN α -2b plus ribavirin for 24 weeks in difficult-to-treat HCV genotype 1 patients leads not only to the eradication of HCV but ultimately to the prevention of histological progression with increased normalization of liver function.

2. Materials and methods

2.1. Patient selection

Two randomized comparative clinical studies of IFN α -2b plus ribavirin combination therapy versus IFN α -2b alone were initiated in Japan in 1998 in chronic hepatitis C patients; one in difficult-to-treat genotype 1 and high viral titer (> 100 kcopies/mL) patients [21] and the other in nonresponders and relapsers to previous IFN therapy [22]. From these two studies, data on patients with ALT levels \geq 50 IU/L (i.e. about 1.5 times the mean upper limit of normal) at the start of treatment were extracted and the effects of treatment on ALT improvement were retrospectively analyzed. In both studies, IFN α -2b (Intron A; Schering Plough, Kenilworth, NJ) was administered at doses of 6 or 10 MIU six times per week for 2 weeks followed by 6 MIU three times per week for 22 weeks. Patients in the combination treatment groups additionally received ribavirin (Rebetol, Schering Plough, Kenilworth, NJ) at a dose of 600 mg/day (three capsules) and 800 mg/day (four capsules) in those weighing <60 kg and \geq 60 kg, respectively, for 24 weeks. Patients in the control groups took ribavirin placebo capsules. In both studies, patients were randomized to either treatment.

The studies were approved by the institutional review boards of each study site and all patients provided written informed consent to participate. Inclusion criteria were as follow: (1) HCV RNA-positive and ALT abnormal in tests conducted within 12 weeks prior to the start of treatment; (2) HCV genotype 1 or if genotype 2 nonresponder or relapser to previous IFN treatment; (3) age between 20 and 64 years; (4) hemoglobin \geq 12 g/dL and platelets \geq 100,000 mm⁻³ in the most recent test conducted within 12 weeks prior to the start of treatment; (5) available for hospitalization for 4 weeks after the start of treatment; and (6) contraception possible both during and for 6 months after the end of treatment. Exclusion criteria were as previously reported [23]. The database for this retrospective study included information on sex, age, body weight, histological stage and activity index, IFN treatment history, HCV RNA levels, aspartate aminotransferase, ALT, hemoglobin, white blood cells (WBC), red blood cells, platelets, and creatinine.

2.2. Study design

Pretreatment ALT levels were classified into three grades: 50 to <100 IU/L, 100 to <150 IU/L, and \geq 150 IU/L. The effect of timing of initial ALT normalization on sustained ALT normalization was examined as well as the association between virological efficacy and improvement in liver function. ALT was measured before and 1–4, 6, 8, 12, 16, 20, and 24 weeks after the start and 2, 4, 8, 12, 16, 20, and 24 weeks after the end of treatment. The judgment of ALT normalization and less than two times of upper normal ALT levels were made based on the normal values at each study site (median 37.5 IU/L, range 21–50 IU/L), and the timing of

initial ALT normalization was recorded as the day when the judgment of normal ALT was made for the first time during treatment. HCV RNA was measured before and 4, 12, and 24 weeks after the start and 24 weeks after the end of treatment. HCV RNA was measured by qualitative Amplicor assay (Mitsubishi Kagaku BCL, Tokyo, Japan), and genotype determined before the start of treatment by reverse transcriptase polymerase chain reaction (Mitsubishi). Evaluation of liver histology was conducted by a single evaluator based on liver tissue samples taken within 48 weeks prior to the start of treatment.

2.3. Definition of response

ALT normalization at 24 weeks after the end of treatment was considered “effective” and was the primary endpoint of this examination. Separately, the association with virological efficacy was also examined. HCV RNA negativity by qualitative assay at 24 weeks after the end of treatment was defined as sustained viral response. Virological relapsers were patients who were HCV RNA-negative by qualitative assay at the end of treatment but who became HCV RNA-positive after the end of treatment. Nonresponders were patients who were never HCV RNA-negative during or after treatment.

2.4. Statistical analysis

After confirming the absence of interaction in efficacy by the Breslow–Day test, comparison of sustained ALT normalization rate by pretreatment ALT levels was conducted using the Mantel–Haenszel test. The log-rank test was used to analyze the timing of initial ALT normalization, and *t*-test or Wilcoxon test was used for mean ALT values during the treatment and posttreatment observation periods. Significance level was two-sided 5%. All calculations were performed by SAS program version 6.12 (SAS Institute, Cary, NC).

3. Results

3.1. Patient characteristics

The study included 167 patients given combination therapy and 105 assigned monotherapy. Main patient characteristics are shown in Table 1. Mean age was 48–49 years and the majority of patients had extremely high HCV RNA levels exceeding the upper limit of quantitation of 850 kcopies/mL. No imbalance in patient background was observed between the two treatment groups.

3.2. ALT normalization rate

Sustained ALT normalization rate was 28.1% (47/167) in the combination therapy group and 10.5% (11/105) in the monotherapy group; combination therapy was significantly superior to monotherapy ($P=0.001$; Fisher’s direct probability test). Sustained ALT normalization rate taking into account baseline ALT levels is shown in Fig. 1. In this re-

Table 1
Patient demographics at baseline

	IFN + ribavirin	IFN alone	<i>P</i> -value
<i>n</i>	167	105	–
Sex (M/F)	135/32	81/24	0.538 (F)
Age (years), mean (S.D.)	47.9 (10.1)	49.1 (9.3)	0.391 (T)
HCV levels (kcopies/mL)			
<500	37	25	0.677 (MH)
500 to <850	46	21	
≥850	84	59	
ALT levels (IU/mL)			
50 to <100	87	48	0.228 (MH)
100 to <150	41	26	
≥150	39	31	
IFN treatment history			
Naïve	39	17	0.060 (MH)
Relapser	82	53	
Nonresponder	40	35	

F, Fisher’s exact test; T, *t*-test; MH, Mantel–Haenszel test.

spect, combination therapy was again significantly superior to monotherapy ($P=0.001$; Mantel–Haenszel test). Sustained ALT normalization rate was also significantly superior in the combination therapy group in patients whose pretreatment ALT was 100 to <150 IU/L ($P=0.001$; Fisher’s direct probability test). In the combination group, the frequency of patients with ALT levels sustained within twice the upper limit of normal (i.e. an index of inhibition of progression to HCC [9,10]) was 34.1% (29/85), 44.7% (17/38), and 25.0% (9/36) in those whose pretreatment ALT was 50 to <100 IU/L, 100 to <150 IU/L, and ≥150 IU/L, respectively; in the monotherapy group the frequency was 22.9% (11/48), 0% (0/23), and 14.3% (4/28), respectively (Fig. 2). Combination therapy was hence significantly superior to monotherapy ($P=0.001$; Mantel–Haenszel test).

3.3. Association between virological efficacy and sustained ALT normalization

The patients judged to have sustained ALT normalization were divided into SVR and non-SVR patients based on virological efficacy and the effect of the addition of ribavirin to IFN assessed (see Table 2). The sustained ALT normalization

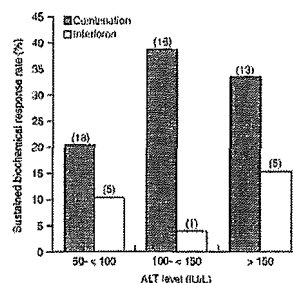


Fig. 1. Rate of sustained biochemical response to treatment by baseline ALT levels. The rate of sustained ALT normalization was significantly better with combination therapy than with interferon monotherapy (Mantel–Haenszel test: $P<0.01$). Numbers of patients in the parenthesis.

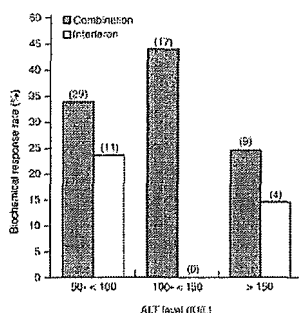


Fig. 2. Rate of patients with ALT levels sustained at less than twice the upper limit of normal (Mantel–Haenszel test: $P < 0.01$). ALT levels were measured ≥ 4 times during the post-treatment follow-up period. Numbers of patients in the parenthesis.

Table 2
Rate of sustained ALT normalization by virological response at end of follow-up and baseline ALT levels

ALT level (IU/L)	IFN + ribavirin		IFN alone	
	SVR	Non-SVR	SVR	Non-SVR
50 to <100	80% (8/10)	13% (10/77)	–	10% (5/48)
100 to <150	75% (6/8)	30% (10/33)	–	4% (1/26)
≥ 150	90% (9/10)	14% (4/29)	100% (2/2)	10% (3/29)
Total	82% (23/28)	17% (24/139)	100% (2/2)	9% (9/103)

rate in non-SVR patients was 17.3% (24/139) in the combination therapy group and 8.7% (9/103) in the monotherapy group. The results of the Mantel–Haenszel test taking ALT levels into account showed that combination therapy was significantly superior to IFN alone ($P = 0.034$). Logistic regression analysis determined the factors for sustained ALT normalization in both treatment groups, and are shown in Table 3. The risk for not achieving sustained ALT normalization in nonresponders to previous IFN treatment was four times higher than in IFN-treatment-naïve patients and relapsers. Among non-SVR patients with sustained ALT normalization, low pretreatment WBC count was the only significant influencing factor (data not shown).

3.4. Effect of timing of initial ALT normalization

The timing of initial ALT normalization with respect to pretreatment ALT levels is shown in Fig. 3. Log-rank test

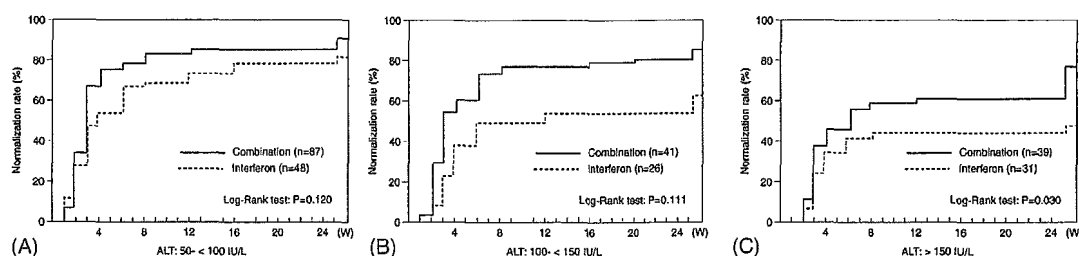


Fig. 3. Initial timing of ALT normalization by pretreatment ALT levels. No significant difference was observed between the combination and monotherapy groups in the timing of initial ALT normalization in patients with low pretreatment ALT levels (50–100 IU/L). In patients with pretreatment ALT levels of ≥ 100 IU/L, timing of initial ALT normalization was significantly earlier in patients receiving combination therapy than in patients receiving monotherapy.

Table 3
Multiple logistic regression analysis of factors associated with sustained ALT normalization

Variables	Odds ratio (adjusted)	95% CI	P-value
IFN nonresponder vs. relapser/naïve	0.250	0.093–0.669	0.0148
ALT	1.005	1.001–1.009	0.0116
WBC	1.000	0.999–1.000	0.0416
Serum creatinine	7.959	0.915–69.213	0.0598
IFN relapser vs. nonresponder/naïve	0.558	0.261–1.193	0.1277
Platelet	1.056	0.972–1.148	0.1931

indicated that the timing of normalization was significantly earlier in the combination group of patients with ALT levels ≥ 100 IU/L. ALT normalization rate at the end of treatment was directly correlated to pretreatment ALT levels: 83.9% (73/87), 80.5% (33/41), and 61.5% (24/39) of those whose baseline ALT was 50 to <100 IU/L, 100 to <150 IU/L, ≥ 150 IU/L, respectively, in the combination group and 79.2% (38/48), 53.8% (14/26), and 45.2% (14/31), respectively, in the monotherapy group showed normalization. ALT normalization rate at the end of treatment was significantly lower in the monotherapy group versus the combination group ($P = 0.015$; Mantel–Haenszel test).

3.5. Change in ALT levels by virological efficacy

Figs. 4 and 5 show mean ALT values during and after treatment in relapsers and nonresponders, respectively. No significant difference was observed between the combination and monotherapy groups in pretreatment ALT levels in either relapsers or nonresponders. When change in ALT levels during treatment in the monotherapy and combination therapy groups was compared, no difference in effect of HCV RNA-negativity during treatment was observed among relapsers. However, when the two treatment groups were compared at all time points after the end of treatment, ALT values were significantly lower in the combination therapy group ($P < 0.001$; Wilcoxon test). Furthermore, the mean value for all measurement time points was < 80 IU/L in this treatment group. Moreover, in virological nonresponders all-time point ALT values were significantly lower in the combination group compared

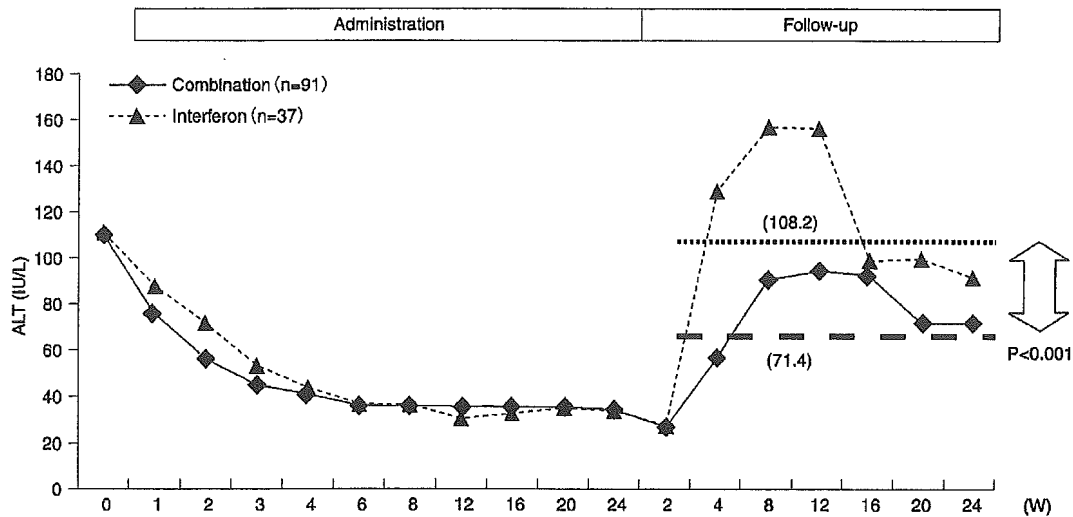


Fig. 4. Changes in ALT levels by viral response: relapsers. No difference was observed between patients receiving combination therapy and monotherapy during the treatment period. After the end of treatment, whereas ALT levels averaged within twice the upper limit of normal in patients receiving combination therapy, a period of marked increase in ALT levels was observed in patients receiving monotherapy. Mean ALT level over the entire period was significantly lower in the combination vs. monotherapy group.

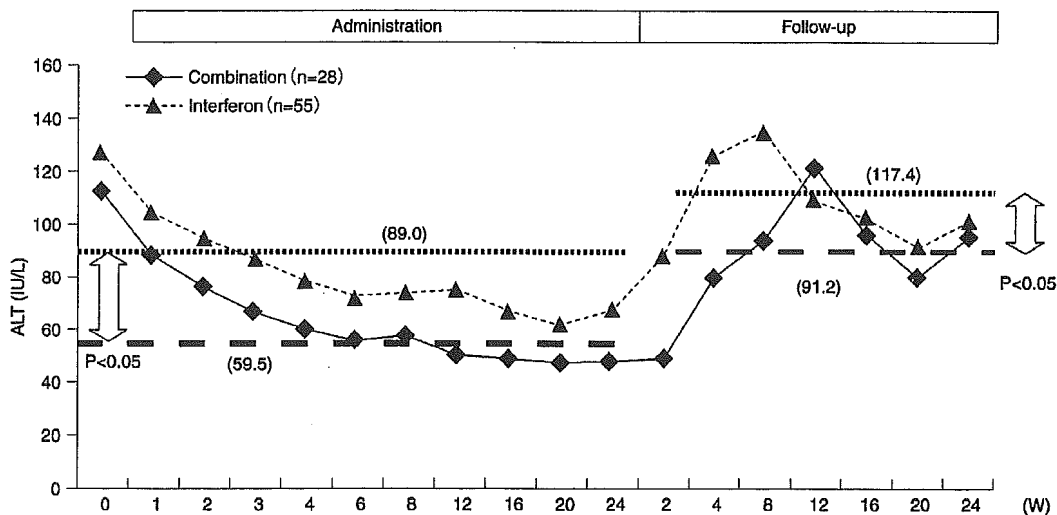


Fig. 5. Changes in ALT levels by viral response: nonresponders. Mean ALT level over the entire period was significantly lower in nonresponder patients receiving monotherapy than in patients receiving combination therapy.

with in the monotherapy group ($P=0.036$; Wilcoxon test). The mean value for all measurement time points during treatment was <80 IU/L.

4. Discussion

Pretreatment liver function affects progression to liver cirrhosis [24] and gender, alcohol consumption, ALT levels, and histological activity index are factors influencing progression to this condition in HCV-infected patients. This is perhaps related to the observation by Takimoto et al. [8] that achieving SVR in patients with high ALT levels is relatively easy. In the

current study, SVR rate was observed to increase in correlation with higher pretreatment ALT levels in both the combination and monotherapy groups (data not shown). On the other hand, Yabuuchi et al. [7] have shown that ALT concentrations in patients with sustained ALT normalization, even in those who become HCV RNA-positive, are significantly lower than in SVR patients and virological nonresponders. In the present study, ALT normalization during treatment occurred at a higher rate in correlation with lower baseline ALT levels, but earlier timing of ALT normalization was not necessarily associated with sustained ALT normalization. However, sustained ALT normalization was achieved more easily in patients with high pretreatment ALT levels regardless of

treatment group. In contrast to non-Japanese reports [25,26], we found that achieving sustained ALT normalization was difficult in nonresponders and relapsers. Older age, longer disease duration, and difference in IFN dose may have been causative factors regarding this result; the reason could not be clarified in this study.

When IFN was first introduced for the treatment of chronic hepatitis C in Japan, the ALT normalization rates reported in genotype 1 patients ranged at about 18–32% [2,3,6]. Many of the patients included in the present study were nonresponders and relapsers to previous treatment, but nevertheless the observed sustained ALT normalization rate (15%) was not very different from those reported previously. In large-scale Japanese clinical studies, the incidence of sustained ALT normalization in non-SVR patients with genotype 1 was about 7–16% [2,4,6,7]. The incidence is estimated about 10% at maximum with IFN monotherapy. The present study not only indicates that addition of ribavirin improves SVR rate but also increases the incidence of sustained ALT normalization including in non-SVR patients compared with IFN monotherapy. Hence sustained liver function normalization was improved by about 30%. Furthermore, ribavirin add-on therapy significantly boosted the number of patients whose ALT was maintained within twice the upper limit of normal, which may reduce the risk of progression to HCC.

An issue that is gaining increasing importance for the future is the method of prevention of progression to HCC in virological nonresponders [27]. In Japan, the long-term rate of hepatocarcinogenesis is considered not greatly different between virological nonresponders to IFN therapy and untreated patients [5,8]. The 5-year incidence of cancer in virological nonresponders has been reported variously at 5–14% [6,7]. Yearly incidence rates of about 1–2% [4,8,28] including a high 5.1% [5] have also been reported. In studies conducted in the USA and Europe, the 5-year incidence rate of liver cirrhosis and HCC in virological nonresponders was 27.8% and 27%, respectively [29,30]. Furthermore, the incidence of progression to HCC in patients with advanced histology followed for 5–7 years was 9.6% [30]. We observed significantly lower on-treatment ALT levels in combination therapy patients versus those on monotherapy, which remained significantly lower after the end of treatment. The timing of ALT flare-up was also delayed in the combination group. This result cannot be explained by differences in change of HCV levels in the two groups since these were not significant (data not shown). However, whether the sustained low ALT levels associated with IFN plus ribavirin combination therapy will lead to less HCC will only be revealed with longer follow-up. Since improved liver function by continued ribavirin monotherapy in virological nonresponders to IFN plus ribavirin has been reported [31], long-term residual effects of ribavirin even after the end of treatment are a possibility. A large-scale clinical study of the effect of long-term treatment with PEG-IFN on prevention of progression to HCC in virological nonresponders to PEG-IFN plus ribavirin combination therapy is ongoing [32]. Long-term IFN monotherapy

was reported to enhance ALT normalization in patients without HCV-RNA negative after previous IFN therapy [33].

On the other hand, in patients who relapsed after the end of treatment, mean ALT after the end of treatment was significantly lower in combination versus monotherapy patients. Relapse was observed delayed at 4 weeks after the end of treatment in the combination group (data not shown), suggesting a contribution of HCV to difference in the pattern of change in ALT levels. Viral relapse rate is known to be lowered by long-term combination therapy [12,13,34], and there is much expectation for increased efficacy by this regimen. The results of studies designed to test the hypothesis that time to onset of HCC is prolonged by combination therapy are awaited.

The present study was limited in that while it conclusively demonstrates that combination therapy with ribavirin increases the rate of sustained ALT normalization compared with IFN monotherapy, it was not powered to show prevention of progression to HCC in the long term. Large-scale clinical trials are necessary to examine this postulate. In particular, it is important to determine whether the period of prevention to progression to HCC is extended in virological nonresponders to combination therapy.

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Characterization of hypervariable region in hepatitis C virus envelope protein during acute and chronic infection

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Summary. Hepatitis C virus (HCV) causes persistent infection in most patients. To clarify the mechanisms underlying establishment of this persistent infection, nucleotide sequences of the E1/E2 region were characterized in 5 patients with acute and chronic HCV infection. We used direct DNA sequencing methods to identify the major sequence of HCV in each patient. Each HCV genome displayed a high frequency of nucleotide sequence variation in the hypervariable region (HVR) of E2. However, patient-specific conserved nucleotide sequences were identified in the E1/E2 region during the course of infection and conserved the higher-order protein structure.

In the acute phase HCV infection, amino acid substitution in HVR-1 as the monthly rate of amino acids substitution per site (%) between each point exceeded 10.2%. In the chronic phase HCV infection, a significantly lower rate of amino acid substitution was observed in patients. The host immune responses to HVR-1 of each HCV isolates from all clinical courses were characterized using synthetic peptides and ELISA. One chronic patient serum (genotype 1b) did not react to all to its own HVR-1 peptides, however another patient (genotype 2b) reacted to all clinical course. These results indicated that HVR-1 might not always exhibit

Note: DDBJ/EMBL/GenBank accession numbers of E1/E2 sequences reported in this paper are AB107929-AB107949.

neutralizing epitopes of HCV infection. The sequence variation in HVR-1 may instead indicate the existence of various clones in acute phase infection and the adaptation of these clones is thought to have caused persistent and chronic infection in each patient.

Introduction

Hepatitis C virus (HCV) possesses a genome of single-strand RNA with positive polarity (about 9.6 kb), and is classified in the family *Flaviviridae*, genus *Hepacivirus* [24]. HCV is the major causative agent of post-transfusion-associated non-A, non-B hepatitis, and it is estimated that 170 million people are infected worldwide. Persistent HCV infections often progress to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [3, 25]. Previous reports have suggested that variability of the HCV genome is likely to play crucial roles in facilitating escape from host immune surveillance [9, 12, 29]. In particular, high degrees of sequence variability have been observed in regions coding the E2 envelope protein, designated as hypervariable regions (HVR)-1 [13, 28] and 2 [22]. HVR-1 has been suggested as a dominant neutralizing epitope for HCV infection in chimpanzees [6]. Despite the confirmed presence of HCV-specific antibodies and cytotoxic T lymphocytes [1, 7], HCV causes frequently persistent infection. These results suggest that variation occurring in neutralizing epitopes within HVR-1 could produce escape variants able to elude the host immune system. Recent reports have indicated that the evolution of viral quasispecies may predict clinical course in viral hepatitis [8].

Although HCV preferably infects hepatocytes, as confirmed by the existence of negative-strand RNA [15], the mechanisms of adsorption into hepatocytes and transcription and replication of viral RNA in the cell remain unclear. The possibility of low-density lipoprotein (LDL)-receptor has been suggested as a virus receptor for HCV infection [2, 18]. CD81 belongs to a family of molecules called tetraspanins, characterized by four transmembrane domains forming two extracellular loops [17], and interacts with E2 protein as a putative viral receptor [23]. So far, six hepatocyte-binding regions have been defined in the E1/E2 region using synthetic peptides [11]. Inhibition of natural killer cells through engagement of CD81 by E2 protein has been reported [5]. Moreover, no polymorphisms in CD81 amino acid (a.a.) sequences on peripheral blood mononuclear cells (PBMCs) have been observed between healthy volunteers and patients during HCV infection [10].

The mechanisms of adaptation and selection allowing HCV to establish chronic infection during the first phase of acute infection remain unclear. The present study characterized patient-specific conserved original nucleotide sequences of the E1 and E2 regions, and deduced amino acid (a.a.) substitutions during the course of HCV infection for acute and chronic phase using direct DNA sequencing methods and humoral immunity of patients to HVR-1 peptides during the course of chronic HCV infection.

Materials and methods

Patients and sera

Two patients displaying acute infection with hepatitis C virus by transfusion (patients A and B; Table 1, Fig. 1) were selected retrospectively, along with three randomly selected patients with chronic hepatitis C in which high levels of serum alanine aminotransferase (ALT) were maintained for more than six months after first medical examination (patients C–E; Table 1; Fig. 1). All serum samples were utilized to determine nucleotide sequences of the HCV E1 and E2 regions during disease progression, and deduced a.a. sequences were predicted. These selected sera were aliquoted and stored below -80°C until characterization. Two patients were infected with HCV by transfusion: patient A (a 58-year-old woman) when she donated a kidney; and patient B (a 55-year-old man) during hip joint surgery. Patients A and B were followed up for 11 and 13 years, respectively. In patients A and B, serum ALT levels remained abnormal during the entire follow-up period. Patients C (54-year-old man), D (26-year-old man), and E (67-year-old man) displayed histological evidence of chronic active hepatitis C.

Informed consent was obtained from all patients in accordance with the Helsinki Declaration.

Detection of anti HCV antibody and HCV RNA

Second-generation enzyme-linked immunosorbent assay (Ortho Diagnostic Systems, Raritan, NJ) was used to detect HCV antibody in sera from the five patients during disease progression. Serum HCV RNA was extracted using the acid guanidium thiocyanate-phenol-chloroform (AGPC) method [4], and detected by reverse transcription and nested polymerase chain reaction (PCR) using primers for the 5'-noncoding region of the HCV genome [19]. Results

Table 1. Clinical evaluation of patients and time points of characterization. Randomly selected patients with hepatitis C were analyzed

Patients	HCV genotype	Age (years)	Sex	Points ^a	Duration ^b (months)
(Acute)					
A	1b	58	F	1 to 2 2 to 3	3 7
B	1b	55	M	1 to 2 2 to 3	4 8
(CH)					
C	1b	54	M	1 to 2 2 to 3	12 3
D	1b + 2a	26	M	1 to 2 2 to 3	8 7
E	2a	67	M	1 to 2 2 to 3	10 11

^aPoints, points of analysis

^bDuration, duration between points of analysis

Acute: acute infection with hepatitis C virus by transfusion, with ALT and viral RNA levels rapidly decreased immediately after infection, then subsequently increased

CH: chronic hepatitis patient, with high levels of ALT maintained for more than six months after first medical examination