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Abstract

Recently, factors predicting the response to interferon (IFN) therapy against hepatitis C virus (HCV) have received much attention. To evaluate the usefulness of the quantitation of intrahepatic HCV RNA as a predictive marker of the response to IFN therapy, we compared the amount of intrahepatic HCV RNA with serum levels in 16 patients. Eleven patients who had 10(10) copies/g or more of intrahepatic HCV RNA had increased level of serum alanine aminotransferase (ALT) after IFN therapy, while 4 of 5 patients who had less than 10(10) copies/g of intrahepatic HCV RNA achieved sustained normalization of serum ALT level and were designated as complete responders. Four complete responders possessed significantly less HCV RNA in the liver parenchyma than partial and nonresponders ($P = 0.010$, Mann-Whitney U-test), but the amount of HCV RNA in the serum was not significantly different between those groups. In conclusion, the results suggest that the quantitation of intrahepatic HCV RNA is a better indicator of the response to IFN therapy than serum HCV RNA.

KEYWORDS: hepatitis C virus, interferon, liver tissue, quantitation, polymerase chain reaction

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Quantitation of Hepatitis C Virus RNA in Liver Tissue as a Predictive Marker of the Response to Interferon Therapy in Chronic Hepatitis C

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Recently, factors predicting the response to interferon (IFN) therapy against hepatitis C virus (HCV) have received much attention. To evaluate the usefulness of the quantitation of intrahepatic HCV RNA as a predictive marker of the response to IFN therapy, we compared the amount of intrahepatic HCV RNA with serum levels in 16 patients. Eleven patients who had 10^{10} copies/g or more of intrahepatic HCV RNA had increased level of serum alanine aminotransferase (ALT) after IFN therapy, while 4 of 5 patients who had less than 10^{10} copies/g of intrahepatic HCV RNA achieved sustained normalization of serum ALT level and were designated as complete responders. Four complete responders possessed significantly less HCV RNA in the liver parenchyma than partial and non-responders ($P = 0.010$, Mann-Whitney U-test), but the amount of HCV RNA in the serum was not significantly different between those groups. In conclusion, the results suggest that the quantitation of intrahepatic HCV RNA is a better indicator of the response to IFN therapy than serum HCV RNA.

Key words: hepatitis C virus, interferon, liver tissue, quantitation, polymerase chain reaction

Hepatitis C virus (HCV) has been identified as the major causative agent of non-A, non-B posttransfusion hepatitis (1, 2). As HCV is thought to have a positive-sense single-stranded RNA genome, the presence of the virus can be diagnosed by reverse transcription-polymerase chain reaction (RT-PCR) (3, 4). Utilizing RT-PCR, it has been revealed that more than 95% of non-A, non-B posttransfusion hepatitis are caused by HCV (5), and competitive RT-PCR has made it possible to quantify the amount of HCV RNA in liver

tissue and the serum (6).

In our previous report (6), it was shown that the level of intrahepatic HCV RNA is about 1,000-fold higher than serum levels, and this suggests that intrahepatic HCV RNA is more sensitive to HCV infection. However, regional difference of laparoscopic findings (7) or histological severity of hepatocellular injury have been reported in the liver of patients with chronic hepatitis C. Balart *et al.* (8) reported that intrahepatic HCV RNA could not be detected in 5 cases despite of the existence of HCV RNA in serum, and they suggested that sampling variation or small sample size may contribute to this discrepancy. To examine whether a quantitative assay of one biopsy specimen accurately reflects the quantity of intrahepatic HCV RNA, we compared the levels of HCV RNA in different biopsy specimens from the same patient.

Although interferon (IFN) therapy is widely used in the treatment as a major therapy of chronic hepatitis C, the response rate is still not satisfactory. Trials of IFN therapy have revealed that IFN therapy is not equally effective in all patients with chronic hepatitis C, and several factors influencing the response to IFN therapy have been reported. In this regard, it has been reported that the patients with HCV genotype II respond poorly to IFN therapy compared with those infected with HCV genotype III or IV (9-11). Arase *et al.* (12) reported that genotype, histological severity, treatment schedule and the quantity of circulating HCV RNA are the best predictive factors of the response to IFN therapy. Kobayashi *et al.* (11) investigated the relationship between the response to IFN therapy and clinical features, such as serum alanine aminotransferase (ALT) response, the quantity of circulating HCV RNA, and hepatitis C virus genotype, and concluded that the quantity of circulating HCV RNA was the best predictive factor of the response to IFN therapy.

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Since the liver is regarded as a major organ, if not only, as a site of HCV replication (6, 13), the quantity of intrahepatic HCV RNA may predict the efficacy of anti-viral therapy more accurately than circulating HCV RNA, but this possibility has not been fully examined (14-16). In this study, we compared the relationships between the quantity of intrahepatic and circulating HCV RNA and the response to IFN therapy.

Subjects and Methods

Patients. To examine regional differences of HCV RNA in the liver parenchyma, 14 patients with hepatitis C infection were subject to preliminary experiment. A pair of liver specimens was obtained from different sites of the right lobe of the liver with Silverman's needle under laparoscopy, and each was separately processed for the quantitation of intrahepatic HCV RNA.

We studied 16 Japanese patients who received IFN therapy to clarify the value of the quantitation of intrahe-

patic HCV RNA. All patients were positive for HCV antibody (the second generation EIA kit, Kokusai-Shiyaku, Kobe, Japan) and negative for HBs antigen. There was no evidence of drug, alcoholic abuse, or an autoimmune hepatitis in those patients. Six patients had a history of blood transfusion and one patient had a needle-stick accident 10 months before the liver biopsy.

All patients had undergone laparoscopic liver biopsies and they were histologically classified as chronic persistent hepatitis (CPH), chronic aggressive hepatitis, moderate (CAH2A), chronic aggressive hepatitis, severe (CAH2B), or liver cirrhosis (LC). Fifteen patients underwent a liver biopsy at 1 to 10 weeks before IFN therapy, and one patient (patient 9 in Table 1) at 24 weeks before IFN therapy.

Informed consent was obtained from all patients. Liver tissue was stored at -70°C and serum was stored at -20°C until use. The weight of the tissue sample was from 4.6 to 44 mg.

IFN treatment schedule. All of the patients

Table 1 Clinical data of the patients and quantity of hepatitis C virus RNA in liver tissue and the serum

Patient	Sex	Age	BT	ALT	Histology	Genotype	HCV RNA		Interferon therapy			R ^e
							Tissue ^a	Serum ^b	Type	Duration ^c	Total dose ^d	
1	M	62	-	112	CAH2B	II	11	7	$\alpha 2a \rightarrow \alpha 2b$	65	1218	N
2	M	46	-	142	CAH2A	II	11	7	$\alpha 2a$	26	774	N
3	F	61	-	104	LC	II	10	8	$\alpha 2a$	26	258	N
4	F	56	-	91	LC	II	10	8	$n\alpha$	24	480	N
5	F	68	-	59	CAH2A	II	10	8	$n\alpha$	26	258	N
6	M	74	34	71	CAH2B	II	10	7	$n\alpha$	25	216	N
7	F	48	25	84	CPH	III	10	7	$\alpha 2a$	14	441	N
8	M	48	-	55	CAH2B	III	8	5	$\alpha 2a \rightarrow \alpha 2b$	24	480	N
9	F	60	29	35	CAH2B	II	11	8	$\alpha 2b$	24	354	P
10	F	63	-	36	CAH2B	II	11	7	$\alpha 2a$	23	702	P
11	M	53	30	55	CAH2B	II	10	8	$n\alpha$	24	475	P
12	M	55	27	162	CAH2B	II	10	7	$\alpha 2a \rightarrow n\alpha$	24	585	P
13	F	39	1 ^f	464	CPH	II	9	7	$\alpha 2a$	24	324	C
14	F	45	27	17	CPH	II	9	7	$\alpha 2a$	13	414	C
15	M	53	-	86	CPH	IV	9	7	$\alpha 2a$	23	711	C
16	F	34	-	203	CAH2B	II	9	6	$\alpha 2a$	27	510	C

a: Quantity of hepatitis C virus RNA in liver tissue (log copies/g)

b: Quantity of hepatitis C virus RNA in the serum (log copies/0.5ml)

c: Duration of IFN therapy (week)

d: Total dose of IFN (MU)

e: Response to IFN therapy. N, non-responder; P, partial responder; C, complete responder

f: Needle stick accident 10 months before IFN therapy

BT, Blood transfusion; ALT, Serum alanine aminotransferase at the start of IFN therapy (IU/l); HCV RNA, Hepatitis C virus RNA before IFN therapy; CPH, Chronic persistent hepatitis; CAH2A, Chronic aggressive hepatitis, moderate; CAH2B: Chronic aggressive hepatitis, severe; LC, Liver cirrhosis; IFN, Interferon; $\alpha 2a$, recombinant $\alpha 2a$; $\alpha 2b$, recombinant $\alpha 2b$; $n\alpha$, natural α ; F, Female; M, Male.

in this study received intramuscular injections of IFN- α at various total doses and durations as described in Table 1. Fifteen patients received injection daily for 2 weeks and then three times per week for 11 to 63 weeks. Another patient received injection three times per week for 24 weeks from the start. Serum ALT levels were monitored once a month before IFN therapy and were followed-up after cessation of IFN therapy.

Response to IFN therapy. We divided 16 patients into three groups according to the response to IFN therapy: four complete responders who achieved sustained normalization of ALT levels after IFN therapy, four partial responders whose ALT levels were normalized during IFN therapy but relapsed after cessation of IFN therapy, and eight non-responders whose ALT levels were not normalized during and after IFN therapy. The four complete responders showed sustained normalization of ALT levels at least 8 months and up to 14 months after cessation of IFN.

Quantitation of HCV RNA. A pair of primers for quantitation of HCV RNA were as follows: NCR32 (sense), 5'-CTGTGAGGAACTACTGTCTT-3' (nt 28-47), NCR36 (anti-sense), 5'-AACACTACTC-GGCTAGCAGT-3' (nt 229-248), derived from the sequence of the 5'-noncoding region (NCR) of the HCV genome (17, 18).

A mutant HCV RNA fragment was generated as described elsewhere (6) which corresponded to nt 13-251 of the 5'-NCR of HCV with U substituted for A at nt 138 which creates a novel *Bam*HI site on its cDNA fragment.

A pair of primers for beta-actin mRNA were as follows: ACT 1 (sense), 5'-CCAGCCATGTACGTTG-CTAT-3' (corresponding to nt 429-448 of the human beta-actin cDNA sequence), ACT 4 (anti-sense), 5'-CA-GCACTGTGTTGGCGTACA-3' (nt 919-938) (19). It was designed to span an intron to distinguish genomic DNA from cDNA of mRNA. Oligonucleotides used as primers in this study were synthesized on a DNA synthesizer (Applied Biosystems Japan, Tokyo, Japan).

HCV RNA in liver tissue and in the serum was quantified by competitive RT-PCR (6). Samples from 11 patients (patient nos. 1-3, 7-10, 12, 13, 15) were quantified previously. In brief, RNA was extracted from the patient's liver biopsy specimen or the serum. RNA extracted from 50 μ l of the patient's serum or 1.5 ng of total liver tissue RNA was reverse-transcribed along with a dilution series of a known quantity of the mutant RNA in a solution containing 1 \times PCR buffer (50 mM of KCl,

10 mM Tris-HCl [pH 9.0], 0.1 % of Triton X-100) (Promega Biotec, Madison, WI, USA), 3.0 mM of MgCl₂, 0.1 mM dNTPs (Perkin-Elmer Cetus, Norwalk, CT, USA), 1 mM of dithiothreitol, 4 units of ribonuclease inhibitor, RNasin (Promega), 1 mM of anti-sense primer, and 2.5 units of Rous-associated virus 2 reverse transcriptase (Takara, Ohtsu, Japan). Sequentially, RT products were supplemented with PCR mixture containing 1 \times PCR buffer, 0.1 mM of dNTPs, 1 mM sense primer, and 0.5 unit of Taq DNA polymerase (Takara), and amplified by PCR for 55 cycles in a DNA thermal cycler (Perkin-Elmer Cetus).

The PCR products were digested with *Bam*HI (Takara) and electrophoresed on 3 % agarose gels (2.25 % NuSieve, 0.75 % SeaKem LE) (FMC Bio-products, Rockland, ME, USA). Then they were stained with ethidium bromide and visualized by UV fluorescence. The amount of HCV RNA in a sample was estimated by determining the point where the wild-type and mutant PCR product were equivalent when compared with known amounts of mutant RNA.

To examine if the liver tissue RNA was intact, RT-PCR was performed with primers for beta-actin as an internal control.

HCV genotyping. HCV genotype was assayed by RT-PCR using mixed type-specific primers for the core region, as described by Okamoto *et al.* (20). Oligonucleotides used as primers were synthesized as described above. Conditions of reverse-transcription and amplification by PCR were the same as described above.

Statistical analysis. Mann-Whitney U-test was used for statistical analysis and $P < 0.05$ was considered to be statistically significant.

Results

A pair of liver specimens obtained from 12 of 14 patients contained the same amount of HCV RNA and those from the other 2 patients differed within one grade of HCV RNA on a logarithmic scale.

The clinical and virological data of 16 patients who were subject to IFN therapy are shown in Table 1. Three of 4 CPH patients were complete responders. On the other hand, only 1 of 8 CAH2B patients was a complete responder. All complete responders were treated with IFN- α_{2a} , while the other patients treated with IFN- α_{2a} were partial or non-responders. There were no significant differences in the duration and total dose of IFN therapy

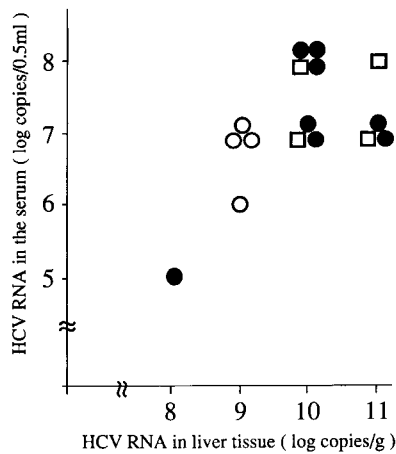


Fig. 1 Correlation of the quantity of hepatitis C virus (HCV) RNA in liver tissue and the serum. ○ = complete responder; □ = partial responder; ● = non-responder

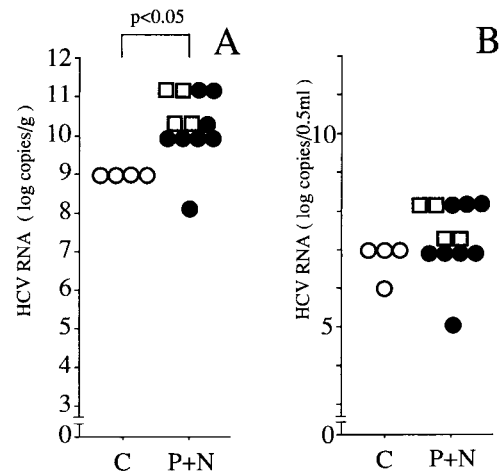


Fig. 2 Comparison of quantity of hepatitis C virus (HCV) RNA in liver tissue (A) and the serum (B) between complete responders and partial or non-responders. C = complete responders (○); P + N = partial (□) or non-responders (●)

in those two groups.

The quantity of intrahepatic and circulating HCV RNA ranged from 10^8 to 10^{11} copies/g in liver parenchyma and from 2×10^5 to 2×10^8 copies/ml in the serum respectively. One gram of liver tissue contained 10^2 to 10^4 times as many copies as 1 ml of the serum (Fig. 1).

Although, the quantity of intrahepatic HCV RNA was significantly lower in complete responders than partial or non-responders ($P = 0.010$) (Fig. 2), the difference in the quantity of circulating HCV RNA between complete responders and partial or non-responders was not statistically significant. The difference in the quantity of HCV RNA was not significant between the 4 partial responders and 8 non-responders.

The serum ALT level before, during, and after IFN therapy was compared between the groups with less than 10^{10} copies/g and 10^{10} copies/g or more of intrahepatic HCV RNA (Fig. 3). The serum ALT level was improved during IFN therapy in both groups and there was no relationship between the serum ALT level and the pre-treatment quantity of HCV RNA before and during the IFN therapy. In contrast, the group with less HCV RNA maintained significantly lower levels of serum ALT after the IFN therapy despite the one non-responder.

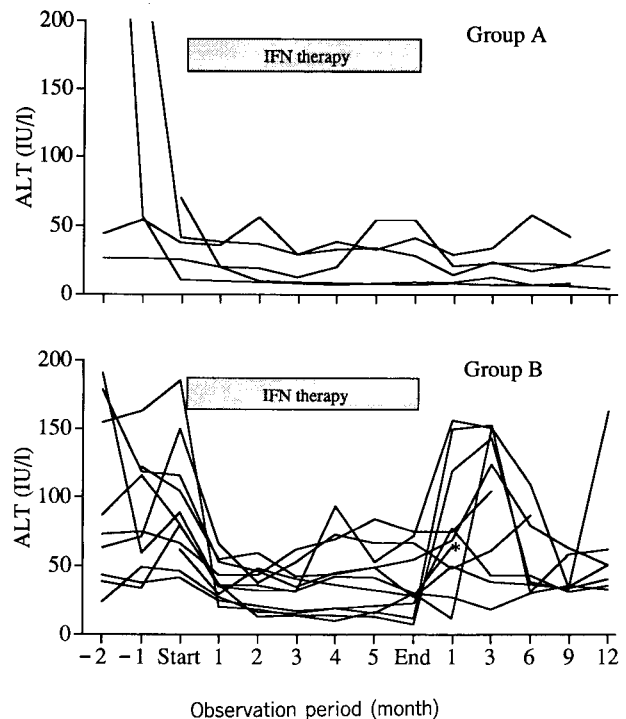


Fig. 3 Comparison of serum alanine aminotransferase (ALT) level between the groups with low quantity (Group A) and high quantity (Group B) of hepatitis C virus (HCV) RNA during observation period. * indicates a case in which interferon was re-started two months after the cessation. Group A = five patients who possessed less than 10^{10} copies/g of intrahepatic HCV RNA; Group B = eleven patients who possessed 10^{10} copies/g or more of intrahepatic HCV RNA.

Discussion

Since the identification of the HCV genome, the diagnosis of hepatitis C has become possible. Studies about the natural course of hepatitis C revealed that this disease progresses to liver cirrhosis and hepatocellular carcinoma in approximately 21 years and 29 years after blood transfusion, respectively, and only rare cases spontaneously remit (21). About three-fourths of the patients with hepatocellular carcinoma in Japan were positive for anti-HCV antibody (22).

The introduction of IFN in the treatment of chronic hepatitis C was a breakthrough in this previously untreatable disease (23, 24), but its response rate is still not satisfactory. The normalization of serum ALT levels has been observed in approximately 50 % of patients, but about half of them relapse after cessation of IFN therapy. In other words, only about 30 % of all achieve sustained normalization of ALT levels (10, 24, 25). Several studies indicate the factors which influence the response to IFN therapy (8-12, 14).

Multifarious effects of IFN has been revealed, such as an activation of natural killer cells (26), induction of cytotoxic T lymphocytes by activation of helper T lymphocytes (27), induction of several kinds of cytokines. Protein kinase (28) and 2-5A synthetase (29) which are induced by IFN, have been thought to play an important role in the suppression of viral replication, and both of them are activated by double-stranded RNA which appears during the viral replication process (30).

The HCV genome has been analyzed and similarities of flavivirus and pestivirus have been reported (31). These three kinds of virus genomes consist of positive-strand RNA and, in the latter two viruses, the negative-strand RNA is supposed to be a replicative intermediate (32). We have established the qualitative assay of negative-strand HCV RNA in liver tissue and have suggested the presence of double-stranded HCV RNA in liver tissue which is formed during the replication of HCV (13).

This evidence suggests that IFN exerts its anti-viral effect in patients with chronic hepatitis C by suppressing HCV replication in the liver. So the quantity of intrahepatic HCV RNA might determine the outcome of IFN therapy as well as the influence on the pathogenesis of hepatitis C.

In several trials of IFN therapy to chronic hepatitis C,

the amount of circulating HCV RNA is reported to be one of the predictive factors of its efficacy (10-12, 14, 33). We have reported that the quantity of intrahepatic HCV RNA in 1 g of liver tissue is about 10^3 times as much as that of circulating HCV RNA in 1 ml of the serum (6). Therefore, the quantity of circulating HCV RNA reflects the quantity of intrahepatic HCV RNA. However, there is a discrepancy in some cases as shown in Fig. 1. Hijikata *et al.* (34) reported that the serum of patients with hepatitis C contained immune complex including HCV, and this reduced the infectivity of the serum to chimpanzee. This immune complex in the serum may account for the difference between the amount of intrahepatic and circulating HCV RNA and reduce the predictive power of circulating HCV RNA for IFN efficacy.

Before evaluating the usefulness of quantitation of intrahepatic HCV RNA, we examined the reliability of quantitation of intrahepatic HCV RNA estimated from one biopsy specimen. A pair of liver tissue specimens obtained from 14 patients contained practically equal amounts of HCV RNA. The regional difference of quantity of HCV RNA is thought to be minimal, and the quantity obtained from one tissue specimen could represent the amount of intrahepatic HCV RNA with reasonable accuracy.

Although complete responders had less HCV RNA both in liver tissue and in the serum than partial or non-responders, only the amount of HCV RNA in liver tissue was significantly different. In this study, a patient who had the least amount of intrahepatic HCV RNA, genotype III, was a non-responder. This suggests that other factors, such as histological stage or sequence diversity of HCV, alter the response to IFN therapy. Okada *et al.* (35) reported that responders to IFN therapy showed little or no diversity in the sequence of complementary DNA clones of HCV, while non-responders showed significant diversity.

Tsubota *et al.* (36) reported that the histological stage is one of the predictive factors of efficacy of IFN therapy. In this study, the rate of complete response was more in CPH patients than CAH or LC patients. But this fact does not reduce the usefulness of quantitation of intrahepatic HCV RNA. Kato *et al.* (37) reported that CPH patients had less HCV RNA than CAH and LC patients. So the good response to the IFN therapy in CPH patients may have been due to the small quantity of HCV RNA in liver tissue and the serum.

Gil *et al.* (15) reported that all complete responders

were negative in detection of intrahepatic HCV RNA after IFN therapy. Hosoda *et al.* (38), who performed follow-up biopsies 3 to 5 years after IFN therapy, revealed histological improvement in most of the complete responders. They also reported that some of the complete responders may have even been cured of chronic hepatitis. When IFN therapy successfully eradicates HCV from the infected liver, the activity of hepatitis decreases due to loss of target antigen to which host immunity is directed (39).

In this study, patients with 10^{10} copies/g or more of HCV RNA relapsed after the cessation of IFN therapy, while those with less than 10^{10} copies/g of HCV RNA maintained near normal ALT level for at least 8 months. The serum ALT level was improved during the IFN therapy independent of the amount of HCV RNA. Current IFN therapy is not sufficient to clear HCV from the liver of patients with higher levels of HCV RNA, even though it is enough to suppress its replication during the therapy.

In conclusion, we believe that the quantity of intrahepatic HCV RNA is a useful marker to predict the response to IFN therapy.

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