Quantitation of hepatitis C viral RNA in liver and serum samples using competitive polymerase chain reaction

M Sugano, Y Hayashi, S Yoon, M Kinoshita, T Ninomiya, K Ohta, H Itoh, M Kasuga

Abstract

Aims—To investigate whether the amount of hepatitis C viral RNA (HCV-RNA) in liver and serum can predict the effectiveness of interferon treatment in patients with chronic hepatitis C.

Methods—The amount of HCV-RNA in frozen liver tissues and sera of 22 patients with chronic hepatitis C was determined before and after interferon alfa treatment by the competitive reverse transcriptional polymerase chain reaction method.

Results—Patients with small amounts of HCV-RNA in serum before treatment showed a significantly more effective response to interferon treatment. After treatment, HCV-RNA disappeared from both the liver and serum of all patients who sustained complete response ($n=11$); in contrast, HCV-RNA decreased but persisted in the liver of all those who relapsed after cessation of treatment ($n=11$).

Conclusions—The elimination of hepatitis C virus from the liver as well as from the serum seems to be essential for sustained remission. The quantitation of HCV-RNA in liver biopsy specimens obtained after treatment would be a highly accurate predictor of whether relapse is likely to occur. (*J Clin Pathol* 1995;48:820-825)

Keywords: Hepatitis C, interferon treatment, competitive RT-PCR, liver tissue, liver biopsy, pathology.

Interferon alfa has been used as an effective treatment for chronic hepatitis C and has been shown to normalise aminotransferase levels. Although approximately 80% of patients respond to this treatment, half of them suffer later relapse.^{1,2} The mechanisms of relapse after withdrawal of interferon have not been clarified. The current antibody test has no clinical value in predicting relapse or in monitoring the effects of treatment.³⁻¹⁰ Recently a new approach, the genetic amplification method of polymerase chain reaction (PCR), has been applied successfully for the detection of hepatitis C viral RNA (HCV-RNA) in serum, and has provided new insights into the potential mechanisms involved in its pathogenesis. Shindo *et al*¹¹ have shown disappearance of HCV-RNA from the serum of the majority (81%) of patients responding to a 6 month course of interferon alfa; all respondents also showed a decrease in HCV-RNA levels.¹¹ The dis-

appearance of HCV-RNA from serum, however, is not predictive of a sustained response to interferon.¹²⁻¹⁴ Our findings showed that half of those who responded (negative HCV-RNA in serum at the end of therapy) later relapsed, with the reappearance of HCV-RNA in serum. These findings suggest that hepatitis C virus was reduced to undetectable level in the serum but was only suppressed in the liver of patients who relapsed after treatment.

To evaluate the antiviral effect of interferon on hepatitis C virus, we determined the amount of HCV-RNA in both the liver and serum of chronic hepatitis C patients who responded to interferon alfa. A highly sensitive quantitative assay by the competitive reverse transcriptional polymerase chain reaction (RT-PCR) procedure was used.^{15,16} The amount of HCV-RNA was measured in both the liver tissues and sera obtained before and after treatment, and the results of paired samples were analysed.

Methods

MATERIALS

The 22 cases investigated were selected from 72 Japanese patients with chronic hepatitis C referred to our hospital for interferon treatment. All patients had abnormal serum alanine aminotransferase (ALT) and were positive for antibodies to hepatitis C virus, determined by second generation enzymed linked immunosorbent assay (Ortho), and for serum HCV-RNA, determined by the RT-PCR assay. Before treatment, diagnosis of the biopsy sample in each case confirmed chronic aggressive hepatitis. The patients underwent a 6 month course of treatment with recombinant interferon alfa (9-10 million units (MU) intramuscularly daily for 2-6 weeks and three times weekly thereafter), or with natural interferon alfa (6 MU intramuscularly daily for 2 weeks and three times weekly thereafter).

Of the 72 patients, 56 (77.8%) responded with normal ALT levels and disappearance of HCV-RNA from serum at the end of the treatment, and 16 patients (22.2%) did not respond. Twenty three of the 56 patients who showed a 6 month sustained normalisation of ALT without serum HCV-RNA after interferon withdrawal are referred to as long term complete responders; 33 patients who relapsed, with an abnormal increase of ALT and reappearance of HCV-RNA within 6 months of interferon withdrawal, are referred to as short term complete responders.

Faculty of Medicine,
Kobe University,
Kobe,
Japan:

2nd Department of
Internal Medicine
M Sugano
S Yoon
M Kasuga

1st Department of
Pathology
Y Hayashi
T Ninomiya
K Ohta
H Itoh

Diagnostics Division,
Otsuka
Pharmaceutical
Co Ltd.
Japan
M Kinoshita

Correspondence to:
Dr Yoshitake Hayashi,
1st Dept of Pathology,
Faculty of Medicine,
Kobe University,
7-5-1 Kusunoki-cho,
Chuo-ku, Kobe 650,
Japan.

Accepted for publication
18 January 1995

Figure 1 Outline of quantitative assay of hepatitis C virus (HCV) RNA by a competitive reverse transcriptase-polymerase chain reaction method. Different amounts of synthetic mutant HCV-RNA and target HCV-RNA in serum (or liver) samples were amplified in the same tube. After digestion with *Kpn-I* and electrophoresis on an agarose gel, amplified DNA fragments were separated into three bands and viewed under an ultraviolet lamp.

Paired biopsied specimens of liver and serum samples were obtained twice, before and after interferon treatment, from 22 patients: 11 long term responders and 11 short term responders. Each liver sample was divided into two fragments: a larger one was embedded in paraffin and stained for histological diagnosis, and the other, about 2–5 mm in length, was snap frozen and stored at -80°C for assay by competitive RT-PCR. Histological diagnoses were performed by routine microscopic examination of the sections stained with a haematoxylin-eosin, and where necessary, with Masson's trichrome. Biopsy specimens were evaluated as showing chronic persistent hepatitis (CPH), moderate chronic aggressive hepatitis (CAH2A), severe chronic aggressive hepatitis (CAH2B), or cirrhosis (LC),¹⁷ and graded according to the histological activity index for numerical scoring of Knodell *et al.*¹⁸ Serum materials were also frozen and stocked for measuring HCV-RNA by competitive RT-PCR.

Figure 2 Nucleotide sequences and positions of wild-type (OAL-7, 8, SMD-11) and mutant-type primers (SMD-9, 10). A mutant fragment with a restriction enzyme (*Kpn-I*) digestive site at nucleotide number 145, referred to as TAM 15, was amplified by PCR with OAL-7 and OAL-8; templating HCV-cDNA was prepared by priming with SMD-11.

QUANTIFICATION OF HCV-RNA

The RT-PCR assay, as described¹⁹ with minor modifications, was done first to detect HCV-RNA in the serum of all 72 patients before treatment. HCV-RNA was quantified by the competitive RT-PCR assay according to the methods of Becker-Andre and Hahlbrock²⁰ or of Hagiwara *et al.*,² with minor modifications, using synthetic mutant HCV-RNA which was synthesised from the 5' non-translating region of HCV-RNA sequences (fig 1).

SITE DIRECTED MUTAGENESIS AND IN VITRO TRANSCRIPTION

HCV-RNA in patients' serum was reverse transcribed with SMD-11 primer. Reverse transcription was carried out at 37°C for 60 min in a mixture (final volume 20 μl) containing RNA sample, 5* reaction buffer, 50 mM DTT, 8 μl each of dNTP and 20 pmol of primer, 16 U RNase inhibitor (BRL), and 108 U reverse transcriptase from Moloney murine leukaemia virus (MoMLV) reverse transcriptase (BRL). PCR was separately used to amplify a 125 base pair (bp) fragment with OAL-7 and SMD-10 primers and a 206 bp fragment with SMD-9 and OAL-8 primers.²¹ A mutant strand with a novel restriction endonuclease (*Kpn-I*) site was generated by annealing the 25-mer mutagenic primers (referred to as SMD-9 and SMD-10) followed by extension with Taq DNA polymerase (fig 2). The 306 bp fragment of the mutant cDNA was force-subcloned into pCR 1000 vector (Stratagene) (referred to as pTAM15) and digested with *EcoRI* and *HindIII*. The *EcoRI-HindIII* fragment was subcloned into KS+Blu-script (Stratagene). The plasmid was linearised with *HindIII*, and served as a template for T7 RNA polymerase to generate sense transcripts. The templates were digested with ribonuclease-free deoxyribonuclease (fig 3). The mutant RNA transcripts were purified by phenol-chloroform extraction and ethanol precipitation.²²

RNA PREPARATION AND RT-PCR ASSAY

The snap frozen tissue was weighed, resolved, and mixed with 500 μl guanidine solution. After addition of different amounts of mutant HCV-RNA and extraction twice with phenol-chloroform, 2.5 volumes of absolute ethanol were added to the aqueous phase. The mixture was left at -80°C overnight, after which the RNA was pelleted by centrifugation (20 min, 18 500 g 4°C). The pellet was washed with 80% ethanol, dried, and dissolved in 11 μl distilled water. The RNA solution was heated at 75°C for 5 min with 20 pmol of antisense primer and cooled at 0°C . The reverse transcriptase reaction was performed on a final volume 20 μl as described above. Serum (50–100 μl) was also mixed with 500 μl guanidine solution and subjected to the same procedures.

PCR of 35 and 35 cycles was carried out to amplify a 144 bp fragment of the 5' non-translating region of HCV-RNA as described.²³ The amplification profile was as follows: de-

Figure 3 pTAM15 is an EcoRI-Hind III fragment, consisting of TAM15 and multicloning site of pCR 1000 at both ends, was inserted into a vector KS(+) Blue script. This plasmid was linearised with Hind III, and served as templates for T7 RNA polymerase to generate sense transcripts.

naturation at 92°C for 60 s; annealing at 55°C for 45 s; extension at 72°C for 120 s. Negative controls (normal liver extracts, no template, or no AMV transcriptase) and positive controls were included in all experiments.

ANALYSIS OF PCR PRODUCTS

Ten microlitres of each PCR product was treated with 1 µl Kpn-I (Takara). After digestion, 10 µl of the solution was analysed by electrophoresis on a 3% agarose gel containing 0.5 mg/ml ethidium bromide. The gels were viewed and photographed on an ultraviolet transilluminator.

PCR products derived from target HCV-RNA in sera and liver tissues were 144 bp long. Digestion with Kpn-I selectively cuts PCR

Figure 5 Data are plotted as log (serum HCV-RNA) copy/ml v log (liver HCV-RNA) copy/mg tissue and histological activity index (HAI) score v log (liver HCV-RNA) copy/mg tissue in the 22 patients before interferon treatment. HCV = hepatitis C virus.

products derived from mutant HCV-RNA into two fragments of 82 and 62 bp in length. A representative result of competitive RT-PCR assay is shown in fig 4. The amount of HCV-RNA in serum or liver tissue was determined by comparing the signal intensities of the three bands on an agarose gel. Target HCV-RNA of $>10^2$ copies were quantified.

Results

The genomic, histological, and biochemical data of the long term and short term responders are listed in tables 1 and 2, respectively.

ANALYSIS OF QUANTITIES OF HCV-RNA IN SERUM AND LIVER TISSUE

The amount of HCV-RNA in serum correlated well with that in liver tissue from each patient in the long term and short term responder groups before interferon treatment ($r=0.585$, fig 5), but this correlation disappeared after the treatment, and HCV-RNA persisted only in the liver tissue of the short term responders (table 2). Before interferon treatment, the quantity of HCV-RNA in sera and liver tissue of the long term responders was smaller than that in the short term responders (sera: 5.0 (SD 0.9) and 6.3 (1.1) logarithmic HCV-RNA copy/ml, respectively; liver tissue: 6.8 (1.0) and 7.4 (1.3) logarithmic HCV-RNA copy/mg tissue, respectively), but these differences were statistically not significant (table 1 and table 2). However, the majority of the patients with

Figure 4 A representative result of competitive reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Mixture of 1 mg liver tissue and different amounts of mutated HCV-RNA were tested. After 35 cycles of amplification and Kpn I digestion, the products were analysed by electrophoresis on a 3% agarose gel. The top band (144-bp) represents amplified DNA fragments derived from target HCV-RNA. At the equivalent point of signal intensities between the upper two bands, the amounts of target HCV-RNA are the same as the copy numbers of the competitor (No 1: 10^2 = copies/ml; No 8: 10^8 = copies/ml). Numbers are the copies of synthetic mutated HCV-RNA.

Table 1 Serological and histological data for the long term complete responder group

Case No	Serum ALT (IU/l)		Histology		HAI scores		Serum HCV-RNA [log (copy/ml)]		Liver HCV-RNA [log (copy/mg tissue)]	
	Before	After	Before	After	Before	After	Before	After	Before	After
1	77	17	CAH2A	CPH	6	3	4.0	<2	6.2	<2
2	149	17	CAH2A	CPH	6	6	4.3	<2	6.0	<2
3	326	33	CAH2A	CAH2A	10	11	4.3	<2	6.9	<2
4	91	25	CAH2A	CAH2A	10	8	5.0	<2	6.1	<2
5	85	7	CAH2A	CPH	12	3	5.0	<2	7.9	<2
6	244	33	CAH2B	CAH2B	14	10	5.5	<2	6.6	<2
7	191	41	CAH2B+LC	CAH2B	15	10	5.5	<2	8.2	<2
8	162	13	CAH2A	CPH	12	7	6.0	<2	5.8	<2
9	156	22	CAH2B	CAH2B	15	8	6.0	<2	5.8	<2
10	118	38	CAH2B	CAH2B	12	10	6.0	<2	7.2	<2
11	468	20	CAH2B	CAH2A	13	6	6.5	<2	8.3	<2

ALT=alanine transaminase; HAI=histological activity index; HCV=hepatitis C virus; CPH=chronic persistent hepatitis; CAH2A=moderate chronic aggressive hepatitis; CAH2B=severe chronic aggressive hepatitis; CAH2B+LC=severe chronic aggressive hepatitis with cirrhosis.

Table 2 Serological and histological data for the short term complete responder group

Case No	Serum ALT level (IU/l)		Histology		HAI scores		Serum HCV-RNA [log (copy/ml)]		Liver HCV-RNA [log (copy/mg)]	
	Before	After	Before	After	Before	After	Before	After	Before	After
12	141	221	CAH2B	CAH2B	15	15	3.5	<2	3.9	2.3
13	96	15	CAH2B+LC	CAH2B+LC	16	15	5.3	<2	8.0	2.0
14	148	16	CAH2A	CAH2A	12	10	6.0	<2	6.9	4.0
15	52	23	CAH2A	CPH	6	5	6.0	<2	8.1	7.1
16	137	95	CAH2B	CAH2A	15	10	6.5	<2	7.9	5.0
17	100	16	CAH2A	CPH	10	4	7.0	<2	7.8	2.2
18	24	8	CAH2A	CAH2A	8	8	7.0	<2	7.9	4.1
19	43	12	CAH2A	CAH2A	10	4	7.0	<2	8.1	6.3
20	350	43	CAH2B+LC	CAH2B+LC	17	15	7.5	<2	6.3	5.8
21	111	19	CAH2A	normal liver	8	2	8.0	<2	7.9	4.2
22	63	12	CAH2A	CAH2A	8	6	8.0	<2	8.3	6.9

ALT=alanine transaminase; HAI=histological activity index; HCV=hepatitis C virus; CPH=chronic persistent hepatitis; CAH2A=moderate chronic aggressive hepatitis; CAH2B=severe chronic aggressive hepatitis; CAH2B+LC=severe chronic aggressive hepatitis with cirrhosis.

serum HCV-RNA under 10⁶ copies/ml were long term responders (10/14; 71.4%), whereas most of the patients with serum HCV-RNA over 10⁶ copies/ml were short term responders (7/8, 87.5%); 7/10 patients with liver HCV-RNA under 10⁷ copies/mg tissues were long term responders (70%), and 8/12 patients with liver HCV-RNA over 10⁷ copies/mg tissue were short term responders (66.7%). Treatment was significantly more effective in patients with serum HCV-RNA under 10⁶ copies/ml (p<0.05, by Fisher's test).

After treatment, HCV-RNA was not detected in the sera of either group, signifying that interferon had eliminated it from the serum. In the liver tissue, HCV-RNA was not detected in

the long term responders (table 1); in contrast, HCV-RNA decreased but persisted in the short term responders (4.8 (1.9) logarithmic HCV-RNA copy/mg tissue) (table 2). The amount of HCV-RNA in liver tissue of the two groups before and after treatment is shown in fig 6.

ANALYSIS OF HISTOLOGICAL ACTIVITY INDEX SCORE

Histological damage was estimated by the histological activity index score (for each of the following factors: (1) periportal and/or bridging necrosis; (2) intralobular degeneration and focal necrosis; (3) portal inflammation; (4) fibrosis of liver tissue) before and after interferon treatment. Before treatment, the difference in scores between the long term responders and the short term responders was not significant, at 11.0 (3.2) v 10.8 (3.5). There was no correlation between the points of the histological activity index score and the amount of HCV-RNA in liver tissue (fig 5). Interferon treatment had more effect in the long term responders than in the short term responders on the index score at the end of treatment (7.3 (3.7) v 9.6 (4.6), but the difference was not statistically significant (tables 1 and 2).

Discussion

Interferon alfa is useful in eliminating HCV-RNA from patients with chronic hepatitis C; however, relapse occurs in many cases. Some seem to have only a transient flare in amino-transferase activity, but in the majority, hepatitis

Figure 6 Quantitation of HCV-RNA in liver tissue in long term (LCR) and short term (S-CR) complete responder groups before and after interferon treatment. HCV=hepatitis C virus.

recurs with persistent biochemical abnormality. Recently the RT-PCR assay for the detection of HCV-RNA has shown that a relapse of hepatitis is always accompanied by the appearance of HCV-RNA in serum, indicating transient suppression of viral replication.^{13,23} In our study of the 72 patients who underwent a 6 month course of interferon treatment, 56 (77.8%) showed disappearance of HCV-RNA from the serum at the end of treatment, 23 (31.9%) showed continued normalisation of aminotransferase levels and HCV-RNA-free serum after treatment, and 33 (45.8%) relapsed, with the reappearance of HCV-RNA in serum within 6 months. The treatment in the relapsed patients may only have produced a decrease in the amount of virus or an inhibition of viral replication,^{21,14} indicating that the virus was not eradicated from the liver, one of its major reservoirs. Although HCV-RNA was not detected in serum after the 6 month course of interferon, whether or not it was eliminated and whether or not treatment should be prolonged remained unresolved. We therefore investigated the quantification of HCV-RNA in serum and liver by competitive RT-PCR assay to evaluate the antiviral effect of interferon and to gauge whether such quantification serves as a predictor of sustained response.

In this study, we have shown that before treatment the amount of HCV-RNA in serum correlated well with that in the liver in all patients. After treatment, HCV-RNA disappeared from the liver and serum of the long term responders, while in the short term responders it decreased but persisted at a lower level in the liver. These results indicate that sustained response after interferon withdrawal (long term responder group) is associated with complete elimination of the virus from the liver—or at least with a decrease in the virus load to levels below those measured with our competitive RT-PCR method—and also that the liver is a possible major reservoir for the hepatitis C virus. In this respect, Balart *et al*¹ reported that biochemical relapse occurred in four of 11 “responding” patients in whom disappearance of HCV-RNA from liver tissues was shown by current RT-PCR methods, but that this disappearance could not be used to predict sustained remission. They suggested several possible explanations for this phenomenon: (1) sampling variations, or too small a biopsy sample to evaluate the amount of virus; (2) failure of their PCR method to detect minute amounts of virus in liver tissues; (3) other organs or tissues acting as reservoirs for the virus.¹ In relation to this last possibility, Gil *et al*¹² suggested that a relapsed patient without HCV in the liver and serum after interferon treatment had positive strand HCV-RNA in peripheral blood mononuclear cells, which suggests that replication of hepatitis C virus may be occurring in these cells. In our study of a larger number of consecutive cases, all relapsed patients with HCV-RNA in liver tissue were from the short term responder group, and those without HCV-RNA in serum and liver were long term responders. This discrepancy between our findings and those of Gil remains

unresolved, but we consider that the relapsed patients without HCV-RNA in sera and liver tissues are the exception and that our competitive RT-PCR method is more sensitive for detecting HCV-RNA in liver biopsy specimens.

In our studies and those of others,^{1,10,12,13} the disappearance of HCV-RNA from sera after interferon treatment was not an accurate gauge of its complete elimination. Although prolonged treatment with interferon α seems necessary for sustained remission,^{24,25} factors predicting a beneficial response have remained unclear. For establishing an effective schedule of treatment, it is necessary to make a clear and early identification of factors affecting individual sensitivity to interferon. In the samples before treatment, the quantity of HCV-RNA in both serum and liver of the long term responders was smaller than in the short term responders, and in particular most of the cases with under 10^6 copies/ml of HCV-RNA in serum were long term responders. This suggests that interferon treatment is likely to be more effective in patients with a smaller amount of HCV-RNA as measured by our competitive PCR method. To evaluate whether histology could predict individual sensitivity to interferon, we applied the histological activity index scoring system to estimate the histological changes in liver biopsy specimens before and after treatment, and found that the scores before treatment could not predict effectiveness, but that after treatment histological damage was less pronounced in the longer term responders.

We have shown in this study that interferon treatment has a strong antiviral effect which eliminates HCV-RNA from the sera of the majority of patients and promotes sustained complete remission when the virus is also eliminated from the liver; relapse occurs, however, when HCV-RNA in serum quantitated by our competitive RT-PCR method appears to have a significant correlation with the response to interferon in most patients. The quantitation of HCV-RNA, particularly in liver biopsy specimens obtained after treatment, would therefore be a highly accurate predictor of whether or not the patient is likely to relapse.

This work was supported in part by a Grant in Aid for Scientific Research (C 05807014) from the Ministry of Education, Science and Culture, Japan. We are grateful to Ms Mari Kohmoto for technical assistance with competitive PCR assays.

- 1 Balart LA, Perrillo R, Roddenberry J, Regenstein F, Shim K-S, Shieh YSC, *et al*. Hepatitis C RNA in liver of chronic hepatitis C patients before and after interferon α treatment. *Gastroenterology* 1993;104:1472-7.
- 2 Hagiwara H, Hayashi N, Mita E, Takehara T, Kasahara A, Fusamoto H, *et al*. Quantitative analysis of hepatitis C virus RNA in serum during interferon α therapy. *Gastroenterology* 1993;104:877-83.
- 3 Kuo G, Choo QL, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, *et al*. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science* 1989;244:362-4.
- 4 McGuinness PH, Bishop GA, Lien A, Wiley B, Parsons C, McCaughan GW. Detection of serum hepatitis C virus RNA in HCV antibody-seropositive volunteer blood donors. *Hepatology* 1993;18:485-90.
- 5 Chaudhary RK, Maclean C. Detection of antibody to hepatitis C virus by second-generation enzyme immunoassay. *J Clin Pathol* 1993;99:702-4.
- 6 Hagiwara H, Hayashi N, Mita E, Hiramatsu N, Ueda K, Takehara T, *et al*. Detection of hepatitis C virus RNA in serum in chronic liver disease. *Acta Hepatol Jpn* 1991;32:762-6.

- 7 Nalpas B, Thiers V, Pol S, Driss F, Thepot V, Berthelot P, *et al.* Hepatitis C viremia and anti-HCV antibodies in alcoholics. *J Hepatol* 1992;14:381-4.
- 8 Sakamoto N, Sato C, Haritani H, Mackawa S, Kurosaki M, Enomoto N, *et al.* Detection of hepatitis C viral RNA in sporadic acute non-A, non-B hepatitis by polymerase chain reaction. *J Hepatol* 1993;17:28-33.
- 9 Yuki N, Hayashi N, Hagiwara H, Takehara T, Oshita M, Kasahara A, *et al.* Serodiagnosis of chronic hepatitis C in Japan by second-generation recombinant immunoblot assay. *J Hepatol* 1993;17:170-4.
- 10 Yuki N, Hayashi N, Hagiwara H, Takahara T, Katayama K, Kasahara A, *et al.* Quantitative analysis of antibodies to hepatitis C virus during interferon- α therapy. *Hepatology* 1993;17:960-5.
- 11 Shindo M, Di Bisceglie AM, Cheung L, Shih JW-K, Cristiano K, Feinstone SM, *et al.* Decrease in serum hepatitis C viral RNA during alpha-interferon therapy for chronic hepatitis C. *Ann Intern Med* 1991;115:700-4.
- 12 Gil B, Qian C, Riezu-Boj JI, Civeira MP, Prieto J. Hepatic and extrahepatic HCV RNA strands in chronic hepatitis C: different patterns of response to interferon treatment. *Hepatology* 1993;18:1050-4.
- 13 Hagiwara H, Hayashi N, Mita E, Ueda K, Takehara T, Kasahara A, *et al.* Detection of hepatitis C virus RNA in serum of patients with chronic hepatitis C treated with interferon- α . *Hepatology* 1992;15:37-41.
- 14 Kakumu S, Yoshioka K, Wakita T, Ishikawa T, Takayanagi M, Higashi Y. A pilot study of Ribavirin and interferon beta for the treatment of chronic hepatitis C. *Gastroenterology* 1993;105:507-12.
- 15 Gilliland G, Perrin S, Blanchard K, Bunn HF. Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. *Proc Natl Acad Sci USA* 1990;87:2725-9.
- 16 Kato N, Yokosuka O, Hosoda K, Ito Y, Ohto M, Omata M. Quantification of hepatitis C virus by competitive reverse transcription-polymerase chain reaction: increase of the virus in advanced liver disease. *Hepatology* 1993;18:16-20.
- 17 De Groote J, Desmet VJ, Gedigk P, Korb G, Popper H, Poulsen H, *et al.* A classification of chronic hepatitis. *Lancet* 1968;i:626-8.
- 18 Knodell RG, Ishak KG, Black WC, Chen TS, Graig R, Kaplowitz N, *et al.* Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology* 1981;1:431-5.
- 19 Ito W, Ishiguro H, Kurosawa Y. A general method for introducing a series of mutations into cloned DNA using the polymerase chain reaction. *Gene* 1991;102:67-70.
- 20 Becker-André M, Hahlbrock K. Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). *Nucleic Acids Res* 1989;17:9437-46.
- 21 Kinoshita M, Shin S, Aono T. A sensitive and quantitative method for the determination of number of HPV 16 DNA copies by using the competitive polymerase chain reaction. *Genet Anal Tech Appl* 1993;10:116-21.
- 22 Okamoto H, Okada S, Sugiyama Y, Yotsumoto S, Tanaka T, Yoshizawa H, *et al.* The 5'-terminal sequence of the hepatitis C virus genome. *Jpn J Exp Med* 1990;60:167-77.
- 23 Shindo M, Di Bisceglie AM, Hoofnagle JH. Long-term follow-up of patients with chronic hepatitis C treated with α -interferon. *Hepatology* 1992;15:1013-6.
- 24 Picciotto A, Varagona G, Valle F, Coviello DA, Lapertosa G, Celle G. Interferon therapy in chronic hepatitis C. Evaluation of a low dose maintenance schedule in responder patients. *J Hepatol* 1993;17:359-63.
- 25 Iino S, Hino K, Kuroki T, Suzuki H, Yamamoto S. Treatment of chronic hepatitis C with high-dose interferon α -2b. A multicenter study. *Dig Dis Sci* 1993;38:612-28.