Clinical significance of intrahepatic hepatitis C virus levels in patients with chronic HCV infection

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Abstract

Background—The clinical significance of a single assessment of circulating hepatitis C virus (HCV) RNA and its relation to the level of intrahepatic HCV RNA remains unclear.

Aims—To investigate the relation between intrahepatic HCV levels and clinicopathological characteristics of chronic HCV infection.

Patients—Ninety eight consecutive patients with chronic HCV infection were studied; none had received α interferon therapy. Of these, 12 patients were repeatedly negative for HCV RNA in serum by reverse transcriptase polymerase chain reaction (RT-PCR).

Methods—After diagnostic laparoscopy and liver biopsy, semiquantitative analysis of intrahepatic HCV RNA levels was carried out by limiting dilution of HCV cDNA. HCV genotypes were assessed in 96 patients by restriction fragment length polymorphism analysis of HCV cDNA.

Results—Ten out of 12 patients who were RT-PCR negative for HCV RNA in serum were RT-PCR positive in liver; however, this group had a significantly lower intrahepatic HCV level and serum aminotransferase level than the remaining 86 patients. Histological severity (cirrhosis: n=10); histological activity index; HCV genotype (genotype 1: n=41; genotype 2: n=12; genotype 3: n=36; genotype 4: n=7); mode of infection (intravenous drug abuse: n=58; post-transfusion: n=10; haemophiliac: n=4; sporadic: n=26) and alcohol abuse did not affect the intrahepatic virus level. There was no correlation between patient age, duration of infection, and intrahepatic HCV level.

Conclusions—Intrahepatic virus levels were not determined by host factors (age of patient, mode or duration of infection) or by virus factors (HCV genotype). Repeatedly negative RT-PCR for HCV RNA in serum does not indicate absence of HCV from the liver.

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infection, and there is considerable controversy as to the biochemical, virological, and histological definitions of a "past infection".

Initial, fundamental studies suggested that negative serum HCV cDNA polymerase chain reaction (PCR) results in recombinant immunoblot assay (RIBA) positive individuals correlated with the absence of inflammation in liver biopsy specimens.⁸ The serum HCV cDNA PCR result was therefore a sensitive and specific marker of liver disease in anti-HCV positive subjects, independent of serum alanine aminotransferase (ALT) values; true healthy carriers of HCV did not exist. Following these reports, it has been recommended that patients negative in serum for HCV cDNA do not undergo routine liver biopsy; furthermore, that those with persisting abnormal liver function tests should be screened for other liver diseases (for example, autoimmune chronic active hepatitis or haemochromatosis), while those with normal liver function tests ought to be followed up annually until the natural history of the disease is better documented.⁹

Many previous studies have examined the significance of serum HCV levels and have shown a wide range of clinicopathological relations¹⁰⁻¹⁸; however, there are fewer data examining intrahepatic HCV levels and their associations.¹⁹⁻²⁹ In general, previous data have not shown any correlation between HCV levels in the liver and other demographic factors such as sex, age, duration of illness, and risk factors. There is a suggestion that some genotypes, in particular genotype 1b, may be associated with higher serum and liver virus levels³⁰⁻³²; but in contrast with serum studies, intrahepatic studies have not shown any correlation between HCV RNA levels and liver injury.¹⁹⁻²⁹

To address these issues further, we determined the clinical, histological, and intrahepatic virological profile of patients with serum repeatedly negative for HCV RNA by reverse transcriptase (RT) PCR; we then compared this population with a control population of patients whose serum was repeatedly positive for HCV by RT-PCR. Concurrently, we investigated the relation between intrahepatic HCV levels and the clinicopathological characteristics of chronic HCV infection.

Patients and methods

PATIENTS

Ninety eight consecutive patients (69 male; mean age 37.8 ± 8 years) with chronic HCV infection were included in the study; none had received α interferon therapy. All patients were positive for anti-HCV antibodies by second generation enzyme immunoassay (EIA-2,

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Hepatitis C virus (HCV) is the predominant cause of post-transfusion and sporadic non-A non-B hepatitis worldwide.¹⁻⁵ Approximately 95% of HCV infected individuals can be identified by third generation anti-HCV testing.^{6,7} However, this test does not indicate active

Table 1 Comparison of demographic and virological data of serum RT-PCR positive and negative patients, all of whom have persistent infection

Host or virus parameter	Serum RT-PCR negative	Serum RT-PCR positive	p Value
Age (y)	35.2 (6)	37.8 (8)	NS
Sex (M/F)	7/3	61/25	NS
Mode of infection	6 IVDA; 1 post-transfusion; 1 haemophiliac; 2 sporadic	51 IVDA; 9 post-transfusion; 2 haemophiliac; 24 sporadic	NS
Duration of infection (median (range))	16 (8–22)	12 (4–42)	NS
Alcohol	2	13	NS
HIV	0	3	NS
Hepatitis B virus	0	1	NS
Normal ALT	7	8	<0.001
Disease state	1 Cirrhosis; 9 chronic hepatitis	9 Cirrhosis; 77 chronic hepatitis	NS
Histological activity index Riba-3 bands	6 (1–13)	8 (1–14)	NS
	9 Present (>2 bands); 1 ind	–	NS
HCV genotype	4 Genotype 1; 1 genotype 2; 4 genotype 3; 1 genotype 4	37 Genotype 1; 11 genotype 2; 32 genotype 3; 6 genotype 4	NS

Figure 1 Intrahepatic virus levels in patients repeatedly HCV RNA positive ($n=86$) or negative ($n=12$) by RT-PCR.

Abbot Laboratories, Weisbaden, Germany) and third generation recombinant immunoblot assay (RIBA-3, Chiron, Emeryville, California). Fifty eight patients had acquired their infection through abuse of intravenous drugs (IVDA), 10 had acquired infection from red cell concentrate transfusions, four were haemophiliacs transfused with infected blood prod-

Figure 2 Intrahepatic virus levels by HCV genotype. 1, genotype 1a ($n=40$); 2, genotype 1b ($n=1$); 3, genotype 2a ($n=2$); 4, genotype 2b ($n=10$); 5, genotype 3 ($n=36$); 6, genotype 4 ($n=7$).

ucts, and in 26 there were no obvious risk factors for infection.

At the time of admission to hospital, each patient was questioned about the likely duration of their infection (in the case of the IVDA group, this was calculated from the year of first injection). Serum aminotransferase was measured on the day of diagnostic laparoscopy.

A small portion of the liver biopsy specimen taken at laparoscopy was washed in ice cold normal saline, blotted on a sterile swab, and immediately frozen in liquid nitrogen and stored at -70°C for future use. The remainder of the biopsy specimen was used for histological analysis.

Serum samples were obtained, separated within three hours of collection on the day of laparoscopy, and stored at -70°C . All samples were stored for a maximum of three to six months before analysis.

HCV RNA EXTRACTION FROM SERUM SAMPLES

RNA was extracted from 0.1 or 0.5 ml of stored serum from each of the patients as previously described.^{33, 34} Briefly, the RNA was pelleted by centrifugation at 100 000 g for 90 minutes at 4°C and incubation at 37°C for two hours with 1 mg/ml proteinase K in the presence of 40 $\mu\text{g}/\text{ml}$ polyadenylic acid, 0.5% sodium dodecyl sulphate (SDS), 0.1 M NaCl, 50 mM Tris HCl (pH 8.0), and 1 mM EDTA. RNA was extracted with phenol; after centrifugation, the supernatant was re-extracted successively with phenol and chloroform-isoamyl alcohol (50:1). Nucleic acid was precipitated by the addition of one tenth volume of sodium acetate (pH 5.2) and two volumes of ethanol. The dried pellet was resuspended in 25 μl of diethyl pyrocarbonate treated water.

HCV RNA EXTRACTION FROM LIVER BIOPSY SAMPLES

RNA extraction from liver biopsy samples was carried out using a commercial modification (RNAzol, Biogenesis Ltd, Bournemouth, UK) of the single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction.³⁴

Tissue samples were homogenised with RNAzol solution (phenol derivative); RNA was extracted by centrifugation at 12 000 g for 15 minutes at 4°C with chloroform. RNA was precipitated by addition of isopropanol to the aqueous phase; the samples were stored for 15 minutes at 4°C and then centrifuged for 15 minutes at 12 000 g (4°C); the RNA precipitates formed a white-yellow pellet at the bottom of the tube. The supernatant was removed, and the RNA pellet was washed once with 75% ethanol by vortexing and centrifuged for eight minutes at 7500 g (4°C). The dried pellet was resuspended in 25 μl diethylpyrocarbonate treated water.

RT-PCR AND VIRAL GENOTYPING

RNA was reverse transcribed and amplified using nested primers matching the 5'-NCR.³⁴ Product DNAs were cleaved with restriction enzymes Hae-III/Rsa-I and Mva-I/Hinf-I.^{35–39} The fragments were

Table 2 Correlation coefficients between intrahepatic virus levels and patient age, duration of infection, serum aminotransferase level, and histological activity index

Parameter	Population median (range)	Pearson correlation coefficient	p Value
Age (y)	37 (24–65)	0.0624	0.55
Serum aminotransferase	72 (13–577)	0.091	0.391
Duration of infection (y)	12 (4–42)	–0.0661	0.592
Histological activity index	8 (1–14)	0.082	0.47

separated by agarose gel electrophoresis using 4% Metaphor agarose (FMC BioProducts). Phylogenetic comparisons of sequences in the conserved region of the genome confirm that 5'-NCR can be used to distinguish the six major genotypes.³⁸

QUANTITATION OF HCV LEVELS IN SERUM SAMPLES AND LIVER TISSUE

Liver tissue samples were quantified by limiting dilution of cDNA reverse transcribed from HCV RNA. This has been described in detail elsewhere.^{40–41} Briefly, 5 µl of HCV cDNA was diluted in a series (five for serum samples and seven for liver biopsy samples) of 10-fold steps which allowed cDNA to be quantified to within 1 log of its actual concentration. Further refinement of the quantitation by adding a specific volume of cDNA to a number of replicate PCR reactions, thus giving a Poisson distribution of positive and negative samples, and allowing the exact HCV concentration to be determined, has been shown not to be necessary to improve the accuracy of the assay if the nearest whole log only is required.

Positive controls (4×10^5 copies/ml) were run concurrently with each limiting dilution analysis to ensure reproducibility of the assay. Serum samples from healthy individuals without risk factors were examined for HCV RNA as negative controls.

The previously established efficiency of 5% for the reverse transcription reaction was assumed in this assay. Centrifugation of 0.1 ml serum provided a detection sensitivity of approximately 4000 HCV copies/ml. To increase the sensitivity of the PCR method, samples that were negative at this level of detection (less than 4000 HCV/ml) were further analysed by centrifugation of 0.5 ml (detection sensitivity: 800 HCV copies/ml) or if necessary 5.0 ml (detection sensitivity: 80 HCV copies/ml) of serum.

The limiting dilution assay has been shown to have significant reproducibility when multiple samples are tested in duplicate, using RNA

extracted on separate days from separate aliquots of sample and different batches of reagents. Likewise a significant correlation has been shown between limiting dilution and three commercial assays: bDNA1, bDNA2 (Chiron, Emeryville, California), and Roche Monitor. Furthermore, when the quantity of transcripts of genotypes 1, 2, and 3 was compared by limiting dilution, there was a statistically similar distribution of the quantity of virus. This indicates that no correction factor for different genotypes (1, 2, and 3) is required when using this assay for quantitating hepatitis C virus levels.⁴²

HISTOLOGICAL ANALYSIS

Liver biopsy specimens from all 98 patients were available for assessment by a single observer blinded to the clinical and serological data. Histological features were graded according to the classifications of Knodell *et al.*,⁴³ assessing portal, periportal, and lobular inflammation as well as fibrosis.

STATISTICAL ANALYSIS

The results were analysed by non-parametric tests where appropriate: the χ^2 or Fisher's exact test, the Mann-Whitney test, or Kruskal-Wallis probability tests.

Results

During an eighteen month period, intrahepatic HCV levels of 12 patients repeatedly negative for HCV RNA in serum by RT-PCR (on three separate occasions) were assessed and compared with 86 patients repeatedly positive for HCV RNA in serum by RT-PCR.

The relation between intrahepatic HCV levels and the clinicopathological characteristics of chronic HCV infection was then investigated.

VIRUS FACTORS

Serum RT-PCR for HCV RNA

Ten out of 12 patients RT-PCR negative for HCV RNA in serum were RT-PCR positive in the liver; however, this group had both a significantly lower intrahepatic HCV level ($p < 0.0001$) (fig 1) and a significantly lower serum aminotransferase level ($p < 0.001$) (table 1) than the remaining 86 patients. Table 1 illustrates the demographic and virological similarity between the serum RT-PCR negative and positive patients. There was no significant correlation between virus levels and any parameter.

HCV genotype

Hepatitis C virus genotypes were assessed in 96 patients by restriction fragment length polymorphism analysis of HCV cDNA. There was no significant difference in intrahepatic virus levels between patients infected with genotype 1 ($n=41$), genotype 2 ($n=12$), genotype 3 ($n=36$), and genotype 4 ($n=7$) (fig 2). The two patients who were RT-PCR negative in both liver and serum could not be genotyped.

LIVER HISTOLOGY

Ten patients had cirrhosis at diagnostic laparoscopy and liver biopsy. The remaining 88

Table 3 Qualitative histological findings and alanine aminotransferase (ALT) and intrahepatic HCV levels in serum RT-PCR HCV RNA negative patients with persistent hepatic infection

Patient no	ALT (U/l)	Log liver virus level (copies/g)	Steatosis	Portal inflammation	Lobular inflammation	Periportal fibrosis
1	37	4.72	–	+	+	–
2	11	5.42	–	–	+	–
3	69	4.10	–	+	+	+
4	32	6.57	–	+	+	–
5	35	7.68	–	+	+	+
6	37	4.44	–	+	+	+
7	56	9.28	–	+	+	+
8	40	4.49	–	–	+	–
9	105	4.22	–	+	+	+
10	19	5.58	–	–	+	+

Figure 3 Intrahepatic virus levels by disease state. 1, Chronic hepatitis; 2, cirrhosis.

patients had macroscopic and histological features of ongoing inflammatory activity in keeping with chronic HCV hepatitis. Neither the histological severity nor the histological activity index (HAI) affected the intrahepatic virus levels (fig 3; table 2).

Table 3 illustrates the descriptive histological findings in the 10 patients who were serum RT-PCR negative for HCV RNA, but RT-PCR positive in the liver.

PATIENT FACTORS

Mode and duration of infection

Intrahepatic HCV levels were unaffected by either the mode or duration of infection (fig 4; table 2).

Serum aminotransferase

There was no significant correlation between serum ALT and intrahepatic HCV levels (table 2).

Cofactors

Coinfection with hepatitis B virus (HBV) or human immunodeficiency virus (HIV), or alcohol abuse did not significantly affect intrahepatic HCV levels.

Discussion

The current pandemic of hepatitis C infection has affected 1–2% of the world's population,

Figure 4 Intrahepatic virus levels according to mode of transmission of HCV 1, IVDA ($n=58$); 2, post-transfusion ($n=10$); 3, haemophilic ($n=4$); 4, sporadic ($n=26$).

and 0.02% of the population of the UK.⁴⁴ However, it is unclear what proportion of patients have a self limiting illness, successfully eliminate HCV, and then become immune to reinfection; furthermore, the host or virus factors increasing the likelihood of successful elimination, and the baseline markers of this process are unknown. The prognostic significance of individual clinical, epidemiological, and virological parameters in this context has also not been clarified. For these reasons, we assessed the clinical, histological, and virological profile of 98 consecutive patients, presenting for staging of their liver disease by diagnostic laparoscopy and liver biopsy and further compared a unique serum HCV cDNA PCR negative population of 12 patients with a larger subpopulation of HCV cDNA PCR positive patients. Our data have shown that repeatedly negative RT-PCR for HCV RNA did not indicate complete hepatic elimination of HCV in 10 out of 12 (87.5%) of our patients; also that intrahepatic HCV levels were not determined by either host factors (age of patient, mode or duration of infection, concurrent alcohol abuse, or concurrent HBV/HIV infection) or by virus factors (HCV genotype).

For confirmation of positive anti-HCV test results, recombinant immunoblot assays were developed; RIBA-3 results are interpreted as "positive" when one or more bands are positive, and "indeterminate" when only one band is positive. A high proportion (75–80%) of RIBA-3 positive patients have viraemia as detected by RT-PCR for HCV RNA. It has been suggested that RIBA-3 positive but RT-PCR negative patients may have cleared the virus from the circulation after a previous infection, may be viraemic below the RT-PCR detection level, or may represent false positive anti-HCV reactivity. Previous studies have indicated that RT-PCR negative/anti-HCV positive patients correlate with absence of inflammation on liver biopsy specimens, and this observation probably indicates clearance of the virus.⁴⁵ In our study 10 out of 12 (87.5%) patients were RT-PCR positive for HCV RNA in liver tissue, but RT-PCR negative in serum and all had ongoing inflammation diagnosed at diagnostic laparoscopy and liver biopsy. This favours the hypothesis that these patients were viraemic below the RT-PCR detection level in serum (in our case, this was a detection sensitivity of 800 HCV copies/ml in 0.5 ml of serum); furthermore, patients RT-PCR positive for HCV RNA in serum had a significantly higher intrahepatic HCV level than patients RT-PCR negative for HCV RNA in serum. Remarkably, comparison of the serum RT-PCR negative and positive subpopulations indicated that they were statistically similar in terms of demographic, histological, and virological data; the serum RT-PCR negative subpopulation did however have a significantly lower serum ALT than the RT-PCR positive patients despite the poor correlation between liver virus level and ALT overall. Possibly, the serum RT-PCR negative patients, with their concurrent lower intrahepatic HCV level and ALT, may have a lower grade hepatic

inflammation; however, there is no significant difference in HAI between the populations. Therefore, the prognostic importance of these data is that serum RT-PCR negative patients with chronic HCV infection should be followed up indefinitely, and at present there is no indication that they are at a lower risk of severe liver disease in the future. We propose follow up studies assessing these patients for the presence of very low levels of viraemia by increasing the detection sensitivity to 80 copies HCV/ml in 5 ml of serum or even eight copies HCV/ml in 50 ml of serum.

The second important finding of this report was the absence of a significant relation between host or virus factors and intrahepatic HCV levels; thus the significance of a single assessment of intrahepatic HCV RNA in terms of diagnosis and prognosis remains unclear. Indeed, in contrast with similar serum studies, this finding corresponds to previous intrahepatic studies which have shown no correlation between HCV RNA levels and liver injury.¹⁹⁻²⁹

The largest of these studies utilised three different methodologies for quantitation of HCV RNA (Dot-Blot PRC, end point titration, and Roche Amplicor Monitor Assay), examined interassay and intra-assay variability in detail, and showed no significant association with the degree of liver injury.²⁹ There are at present no data measuring viral replication in addition to the total HCV load and its effect on liver injury. Recently, new techniques have been developed to overcome the methodological problems associated with detection of true replicative (negative or antisense) HCV RNA levels, but their ability to quantitate specifically HCV RNA over a wide range of HCV levels is still limited.⁴⁶⁻⁴⁷

There was no correlation in our study between HCV RNA levels in liver and other demographic factors such as sex, age, duration of illness, and mode of infection. These data confirm previous studies on liver virus levels but contrast with studies examining serum levels of HCV RNA where increased levels have been shown in relation to age, sex, mode, and duration of infection.¹⁰⁻¹⁸ This observed discrepancy may be explained by a combination of factors: contribution to serum HCV RNA levels from virus in other tissues (and from necrotic liver tissue), HCV RNA in serum immune complexes, technical variation between methods, different populations studied, and time dependent fluctuation in serum HCV levels. Certainly analysis based on serum studies is more difficult to interpret than that based on intrahepatic studies.

In addition, we did not show an effect of HCV genotype on intrahepatic virus levels. This conflicts with data indicating that genotype 1b generally has higher HCV RNA levels in serum and liver and therefore a higher viraemic load than other genotypes tested.³⁰⁻³² However, genotypic analysis of our data was limited by the presence of only one patient infected with genotype 1b, while 19 patients were infected with genotype 1a.

In conclusion, we have successfully shown that repeatedly negative RT-PCR for HCV

RNA in serum does not indicate complete hepatic elimination of HCV. Indeed, these patients have a similar clinical, pathological, and epidemiological profile to serum RT-PCR positive patients; their prognosis and requirements for full staging of liver disease are thus likely to be similar, and further follow up is mandatory. However, we were unable to show any significant associations between intrahepatic HCV levels and other clinicopathological parameters. Importantly, these data support the hypothesis that HCV does not cause liver disease by a cytopathic process; they also illustrate the limitations of a single assessment of HCV levels. Clearly, there is a requirement for sequential studies of chronic HCV infection in terms of molecular virological and clinical parameters, before the clinical significance of intrahepatic HCV levels is established.

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