Chronic hepatitis C virus infections: predictive value of genotype and level of viraemia on disease progression and response to interferon α

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Abstract

The effects of hepatitis C virus genotype and viraemia on disease outcome in patients with chronic hepatitis C virus infection were studied. Patients infected with genotype 1 tended to develop more severe disease, and to respond less well to interferon (IFN) treatment, but no pre-treatment variable successfully predicted either the severity of the disease or the response to IFN. Failure to eliminate the virus during the first three months of therapy, however, predicted a failure to derive long term benefit from the current IFN regime. Hence pretreatment variables cannot be used to determine whether individual patients will respond to IFN, but observations during the first three months of therapy can be used to decide which patients will not respond to prolonged therapy. In these patients consideration should be given to changing the IFN dosing regime or using alternative treatments.

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The hepatitis C virus (HCV) is the major cause of blood borne non-A, non-B hepatitis.¹⁻³ After exposure to HCV, 50% of patients develop chronic hepatitis.^{4,5} Although many of those chronically infected with HCV do not develop serious liver disease,⁶ some develop a rapidly progressive hepatitis, and overall 20% of patients develop cirrhosis.^{7,8} Interferon α (IFN α) is effective in a proportion of patients with chronic HCV infections: 50% respond to therapy with an improvement in liver function tests and disappearance of HCV by polymerase chain reaction (PCR), but only half of these show a sustained response.⁹⁻¹⁴ The reasons for this variation in disease activity and response to IFN are unknown.

Several strains of HCV have now been identified and classified according to the sequence of various genomic regions. Unfortunately, different research groups have used different viral regions to classify HCV, and many classification systems have been developed.¹⁵⁻²² For this study we have adopted the phylogenetic classification as described by Simmonds *et al*,^{16,17} which distinguishes three major types of HCV infection by sequence analysis of the 5' non-coding region, and has recently incorporated sequences from Africa, South Africa,

and Hong Kong as types 4, 5, and 6 respectively. Analysis of the coding regions of the viral genome (core, NS-3, NS-5) divides most of the major types into a number of subtypes. This compares to the description by Okamoto *et al*, whereby HCV types I, II, III, IV, V, and VI correspond to types 1a, 1b, 2a, 2b, 3a, and 3b respectively. A number of studies have suggested that the viral genotype and level of viraemia may influence both the response to IFN and the severity of the hepatitis.²³⁻²⁶ Pozzato *et al*²⁷ showed that patients infected with HCV type 1b (characterised by Kato *et al*²¹) had more severe liver disease and a poorer response to IFN than patients with other viral strains, but other groups have suggested that different genotypes may also be associated with severe disease.^{16,24,25,28}

A number of different approaches have been used to assess disease severity in HCV infection, including single and serial transaminase measurements²⁹ and histological indices of fibrosis and inflammation, based on the Knodell scoring system.³⁰ To determine the relevance of these measurements to the rate of progression of HCV infection, we examined serial liver function tests and Knodell scores in patients in whom the rate of progression of fibrosis could be assessed. The influence of HCV genotype and viraemia on disease progression were then assessed in these patients. In order to examine the factors predicting a response to IFN, we assessed the pretreatment HCV genotype as well as the early response to IFN in a group of chronically infected patients who took part in a controlled clinical trial of lymphoblastoid IFN (Wellferon).

Methods

PATIENTS

Patients from St Mary's Hospital²⁹ who took part in a multicentre, cross over study of IFN α (Wellferon) in non-A, non-B hepatitis were reassessed. All patients underwent a pretrial liver biopsy and serial liver function test analysis for six weeks before treatment. Patients were then randomised to receive IFN α for 12 months or no treatment for 12 months followed by 12 months' therapy. All patients ultimately received treatment for one year. Patients were started at a dose of 5 MU thrice weekly for two months. The dose was then reduced to 3 MU thrice weekly for two months, then 1.5 MU thrice weekly for two months, and finally to 0.5 MU thrice weekly for six months. Each reduction in dose was

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		Nucleotide position
Primer set 1		
Outer	5' ATA GAT CAC TCC CCT GTG	3' (+) -311 to -292
	5' CGG TTG GTG TTA CGT TTG	3' (-) +29 to +47
Inner	5' CTT CAC GCA GAA AGC GTC T	3' (+) -280 to -262
	5' GAT GCA CGG TCT ACG AGA CC	3' (-) -20 to 0
Primer set 2		
Outer	5' AGT ATG AGT GTC GTG CAG CC	3' (+) -249 to -230
	5' TCA GGC AGT ACC ACA AGG CC	3' (-) -65 to -45
Inner	5' GGG AAT TCC ATA GTG GTC TGC GGA ACC	3' (+) -211 to -185
	5' TTT CTA GAC CCA ACA CTA CTC GGC TAG	3' (-) -92 to -66

Figure 1: Locations of primers used for nested reverse transcription-polymerase chain reaction (RT-PCR). Nucleotide positions marked relative to start of open reading frame (position 0). (+)-sense; (-)-anti-sense. Note that a restriction site is included in the primer to facilitate subsequent cloning of the PCR product.

dependent upon a sustained fall in the transaminase activity and an increase beyond the normal range after dose reduction was controlled by returning to the previous effective dose. The maximum period of treatment was 52 weeks. The IFN was given by intramuscular or deep subcutaneous injection by the patients themselves or by medical personnel.

Informed consent was obtained from each patient and the study was approved by the local ethics committee.

In 15 patients, the date of infection could be reliably determined (history of hepatitis, blood transfusion, or sharing needles during intravenous drug use). These patients were used for the analysis of the rate of progression to fibrosis. A further nine patients with a well defined date of infection were obtained from patients attending the viral hepatitis clinic.

ASSESSMENT OF DISEASE SEVERITY

We used the rate of development of cirrhosis, determined by liver biopsy, as the most clinically useful parameter of disease severity. Severe disease was classified as infection for less than 10 years with progression to cirrhosis and mild disease as infection for more than 10 years with no evidence of cirrhosis. Twenty four patients with HCV infections of known onset were investigated but four patients (all from the trial) with cirrhosis who had been infected for longer than 10 years were regarded as uninformative and were not classified so that a total of 20 patients was studied.

DETECTION, GENOTYPING, AND QUANTITATION OF HCV RNA

HCV RNA was detected in stored serum (samples had been spun down within four hours of collection and stored at -20°C) by the polymerase chain reaction (PCR) using primers from the 5' non-coding region as shown (Fig 1). A proportion of sera were negative with primer set 1, which we believe was due to a mismatch at the 3' terminal nucleotide of the primer used for reverse transcription. These sera underwent PCR using primer set 2. Patients were regarded as negative if no product was seen with both primer sets. PCR was performed as described.³¹

Direct sequencing of the product was performed using the TAQUENCE kit

(Cambridge Bioscience, Cambridge, UK) as recommended by the manufacturers. The sequence of the 5' non-coding region was then used to classify HCV into genotypic groups as described.¹⁶

HCV viraemia was assessed semiquantitatively by the addition of a competitive internal mutant RNA template at known concentration (3.3×10^5 copies per ml) as described.³² PCR of the mutant RNA yields a fragment size of 93 base pairs (bp) compared with the wild type which produced a fragment size of 279 bp with primer set 1. Initially PCR conditions were optimised to obtain a gradient of band intensities corresponding to the presence of 10^3 – 10^7 copies of mutant RNA per reaction (that is, 10^3 – 10^7 copies per ml as the reaction volume was 10 μl). The serum HCV RNA titre was then obtained from the number of copies of competitive HCV RNA template at the point of band equivalence divided by three, which corresponds to the difference in size of the PCR products. The quantitative PCR assay was able to detect serum levels of between 3.3×10^2 and 6.6×10^8 viral genomes per ml of serum in a group of HCV infected patients.³² In this study viraemia was classified as high if the predominant band was wild type, indicating a concentration of greater than 3.3×10^5 copies HCV/ml serum, or low if the mutant band predominated or was equivalent to wild type.

CALCULATION OF AVERAGE DAILY AST VALUES

Liver function tests were analysed using a SMA II auto analyser. The average daily AST value was estimated by dividing the area under the AST-date curve by the duration of the observation period.

Results

ASSESSMENT OF DISEASE SEVERITY

Of the 20 patients assessed for disease severity, 11 had serial AST estimates over a period of at least six months. Since the AST value fluctuates in chronic HCV infections, we analysed the mean daily AST activity (see methods) to overcome the effects of day to day variation. The mean daily AST activity from the three patients with rapidly progressive disease (severe disease) was 114.8 IU/ml and was not significantly different ($p > 0.05$, Fisher's exact test) from the mean daily AST activity (97.8 IU/ml) in the eight patients with mild disease. To assess whether the Knodell score³⁰ on a single liver biopsy specimen correlated with disease progression, pretreatment specimens from the 11 patients in the Wellferon trial with hepatitis of known severity were evaluated using the Knodell scoring system. In eight patients with mild disease the Knodell scores were 2, 3, 4, 4, 7, 7, 7, and 7, compared with 4, 7, and 7 in the three patients with severe disease, indicating no major difference between the groups. It therefore seems unlikely that the rate of progression of liver disease in patients with HCV infection can be

Comparison of genotypic group in relation to severity of disease. Mild disease – infection for greater than 10 years with no evidence of cirrhosis; severe disease – infection for less than 10 years with cirrhosis

Disease severity	Genotypic group		
	G1	G2	G3
Mild	5	4	5
Severe	4	1	1

Figure 2: The percentage of patients with abnormal aspartate transaminase (AST) activity before, during, and after interferon therapy grouped according to genotype.

accurately predicted from AST activity or examination of a single liver biopsy specimen.

Since conventional markers of disease severity do not predict the rate of progression to cirrhosis, we regard them as poor markers of disease severity, and we restricted our analysis of disease severity to the 20 patients whose rate of disease progression could be accurately determined (see methods). Six patients had severe disease (that is, they progressed to cirrhosis within 10 years of infection) and 14 patients had mild disease. On genotype analysis, severe disease was present in 44.4% (four of nine) of patients infected with HCV type 1, compared with 18.2% (two of 11) of patients infected with HCV type 2 or 3 (Table). This difference was not significant by Fisher's exact test. Further analysis of patients infected with type 1 HCV showed that one of four patients with severe infections and one of five patients with mild infections were infected with HCV genotype 1b.

Severe disease was present in 33.3% (four of 12) of patients with high levels of viraemia ($>3.3 \times 10^5$ gnomes per ml of serum) compared with 25% (two of eight) of patients with low levels. This difference was not statistically significant. Hence, neither HCV genotype nor the level of viraemia predict the severity of HCV infection, although severe disease may be more common in patients infected with genotype 1.

Figure 3: The percentage of patients who remained viraemic during therapy grouped according to genotype. In one patient infected with HCV type 1, who had been polymerase chain reaction (PCR) -ve at three months, there was no post therapy sample for PCR analysis.

ANALYSIS OF RESPONSES TO IFN

Pretreatment variables

Serum samples from the 29 patients who had received 1 year's treatment with IFN and in whom full follow up information was available were analysed by PCR. Several patients (four) were consistently PCR negative and were excluded from further analysis. HCV infection was probably not the cause of non-A, non-B hepatitis in these four patients. The effect of HCV genotype on the response to IFN is shown in Figures 2 and 3. In nine of the 14 HCV type 1 patients we were able to subtype into subtypes 1a and 1b, but in the remaining five patients insufficient serum was available. Overall, seven patients (28%) had normal transaminase activities during therapy but this was sustained in only four of these patients. In a further two patients liver function returned to normal at the end of treatment, thus leading to a long term biochemical response in six (24%; two genotype 1, one genotype 1a, two genotype 2, and one genotype 3) of the treated patients. HCV RNA disappeared during treatment in 17 patients (68%) but out of the 16 patients in whom post-treatment samples were available (in one type 1 infected patient no post-treatment serum was available), only four patients (16%; 2 genotype 1a, 2 genotype 2) remained PCR negative. Hence, most patients do not show any sustained response to IFN. There was no significant difference in the frequency of response to IFN in each of the pretreatment HCV genotypic groups ($p > 0.05$, Fisher's exact test).

During therapy variables

It was noted that in those patients who ultimately responded to therapy the effects of treatment were apparent during the first few months of treatment. We therefore examined the predictive value of a reduction in AST and the disappearance of viral RNA on the long term response to IFN. Figure 4 shows the effects of IFN on the daily mean AST activity. In the six patients who derived long term benefit from treatment (that is, normal AST after therapy) four had normal liver function tests during the first three months of treatment. Of these six patients, four remained PCR positive after treatment despite an apparent biochemical response to IFN. However, all of the long term responders (normal liver function tests and no detectable viraemia after therapy) were PCR negative three months after therapy was begun. Hence, persistence of viral RNA after three months' therapy is always associated with treatment failure.

The effect of IFN therapy on the mean daily AST was most marked in patients infected with HCV of genotypes 2 and 3 (Fig 4). The reduction in AST activity during treatment in patients infected with genotype 1 was significantly less than that seen in patients infected with the other genotypes (mean daily AST in patients with genotype 1 fell from 116.2 before treatment to 86.2 at three months, compared with a reduction from 102.7 to 48.65 in

Figure 4: The mean daily serum aspartate transaminase (AST) activity measured before, during, and after interferon (IFN) therapy in patients infected with genotype 1 (A), 2 (B), and 3 (C). Filled circles represent PCR negative serum. The value of 40 IU/ml represented the upper limit of the normal range. The figures in bold are the mean serum AST values before, during and after IFN therapy. In one patient the mean AST value after therapy was 34 but a recent AST was 106 suggesting that the mean value did not represent a biochemical response.

patients with genotypes 2 and 3 $p=0.02$, paired t test).

It is interesting to note that the pretreatment mean daily AST was significantly higher in those patients infected with type 1b than in those infected with type 1a ($p<0.01$, unpaired t test), but the fall in AST on treatment was not significantly different.

Discussion

It is estimated that over 100 million people worldwide are infected with HCV. Although some patients do not develop significant liver damage,⁵ others suffer from irreversible hepatic disease. The factors that influence disease activity in patients with chronic HCV infection are poorly understood. A number of studies have suggested that different HCV genotypes may be associated with different patterns of disease^{25 27-29 33} and the identification of those genotypes leading to an aggressive clinical course would be helpful to the clinician.

The natural history of chronic HCV is characterised by fluctuating transaminase activities, which makes this parameter unsatisfactory for an assessment of disease severity. We believe that the rate of progression to hepatic fibrosis is the most clinically relevant variable, and we have used this indicator as a marker of disease severity. We found that

there was no relationship between the rate of progression of fibrosis and either serial AST measurement or histological index (Knodell score³⁰) on a single liver biopsy specimen. Hence AST activity, even when corrected for day to day variation, can not be used to predict the rate of progression of fibrosis in chronic HCV infections and neither can the Knodell score on a single liver biopsy specimen. This observation may be due to the fluctuating nature of the hepatic damage in HCV: presumably the development of fibrosis depends upon the rate of liver damage relative to the rate of regeneration and repair. This complex relationship makes it impossible to assess the severity of the disease using conventional markers of hepatic damage. Using a more clinically relevant marker of disease severity (rate of progression to cirrhosis), we find that patients infected with HCV genotype 1 tend to have a higher frequency of severe disease than those infected with other genotypes. However, this difference was not statistically significant and it should be noted that most patients infected with genotype 1 had mild disease. As only two of the nine patients infected with HCV type 1 had type 1b (one mild and one severe), we were unable to make a useful comparison between type 1b and other genotypes. Hence HCV genotype may play a minor role in determining the outcome of infection, but other factors are clearly involved.

Fluctuating viraemia (as measured by the RNA concentration in serum³⁴) is also a feature of chronic HCV infection, and it has been suggested that the level of viraemia is related to the histological grade of liver disease.³⁵ Since the level of viraemia varies, it seems unlikely that a single measurement will be able to predict disease outcome, and our data show that this is indeed the case. In this study, we used a PCR based quantitation method³² which has a sensitivity of 3.3×10^2 virus particles per ml compared with the lower limit of detection of the widely used branched DNA assay of 3.5×10^5 virus particles per ml. Previous methods involving either serial dilutions of cDNA or the use of a shortened cDNA template make assumptions on the efficiency of the reverse transcription and PCR sensitivity, and thus make comparison between samples unreliable. Using a shortened RNA template in a competitive PCR assay, the concentration of serum RNA can be estimated by identifying the point of equivalence of the PCR products. This comparison is not influenced by the efficiency of either reverse transcription or PCR. Our results show a tendency towards severe disease in patients found to have high levels of viraemia. However, we used a relatively crude quantitation assay (greater than or less than 3.3×10^5 genomes per ml) and it is possible that a more sensitive assay would have shown a significant difference. Indeed, if the HCV RNA concentration is to be useful in clinical practice, sophisticated quantitative methods may well be required.

Assessing the response to IFN therapy is difficult. The most important end points are death or development of cirrhosis but, clearly, detection of such long term effects is not practicable at present. It is therefore necessary to use biochemical and virological markers of improvement such as a return to normal of liver function tests or loss of viraemia. Transient loss of viraemia and normalisation of serum AST activity is likely to be associated with slower disease progression but the most important response to IFN is a permanent return to normal of AST and loss of viraemia. Analysing both transient and sustained responses to IFN, we found no significant difference in response rates between patients infected with different HCV genotypes, although the number of patients studied was small and we may not have detected a small difference in disease outcome. Our data suggest that the HCV genotype should not be used to determine which patients receive therapy. Other groups have suggested that patients infected with HCV type 1 respond poorly to IFN,²⁷ and that those patients infected with HCV type 2 may have a favourable response.²⁵ Our study confirms this trend towards a more favourable outcome with HCV genotype 2 but shows that patients infected with other genotypes do benefit from therapy. It was interesting to note that patients infected with HCV of genotype 1 showed a significantly smaller reduction in mean AST activity than patients infected with other viral genotypes. This suggests that this HCV strain

may be either more cytotoxic than other strains or, perhaps, less sensitive to the effects of IFN.

All of the six patients who ultimately responded to IFN long term were PCR negative after three months' treatment. A number of patients who were PCR negative during therapy ultimately failed to eliminate the virus and thus the clearance of virus on treatment, as assessed by PCR, does not predict long term response to IFN α . However, a patient who fails to clear the virus while receiving therapy will not have a sustained response. This may be a valuable marker that correctly identifies those patients who will not benefit from further therapy. Patients who remain PCR positive after three months of IFN treatment should be considered for alternative therapies – perhaps high dose IFN or alternative antiviral agents.

- 1 Choo Q-L, Kuo G, Weiner A, Overby L, Bradley D, Houghton M. Isolation of a cDNA clone derived from a blood-borne viral hepatitis genome. *Science* 1989; **244**: 359–62.
- 2 Choo Q-L, Richman K, Han J, Berger K, Lee C, Dong C, *et al.* The genetic organization and diversity of the hepatitis C virus. *Proc Natl Acad Sci USA* 1991; **88**: 2451–5.
- 3 Houghton M, Choo Q, Kuo G. Chiron Corporation. European Patent App 88310922.5 1988; Publication number 0318216.
- 4 Stevens C, Taylor P, Pindick J, Choo Q-L, Bradley D, Kuo G, *et al.* Epidemiology of hepatitis C virus. *JAMA* 1990; **263** (1): 49–53.
- 5 Alter MJ, Margolis HS, Krawczynski K, Judson FN, Mares A, Alexander WJ, *et al.* The natural history of community-acquired hepatitis C in the United States. *N Engl J Med* 1992; **327** (27): 1899–905.
- 6 Seeff LB, Buskell-Bales Z, Wright EC, Durako SJ, Alter HJ, Iber FL, *et al.* Long-term mortality after transfusion-associated non-A, non-B hepatitis. *N Engl J Med* 1992; **327** (27): 1906–11.
- 7 Hopf U, Moller B, Kuther D, Stemerowicz R, Lobeck H, Ludtke-Handjery A, *et al.* Long-term follow-up of posttransfusion and sporadic chronic hepatitis NANB and frequency of circulating antibodies to hepatitis C virus (HCV). *J Hepatol* 1990; **10**: 69–76.
- 8 Realdi G, Alberti A, Rugge M, Rigoli A, Tremolada F, Schivazappa L, *et al.* Long-term follow-up of acute and chronic non-A, non-B post-transfusion hepatitis: evidence of progression to liver cirrhosis. *Gut* 1982; **23**: 270–5.
- 9 Davis G, Balart L, Schiff E, Lindsay K, Bodenheimer H, Perrillo R, *et al.* Treatment of chronic hepatitis C with recombinant interferon alpha. *N Engl J Med* 1989; **321** (22): 1501–6.
- 10 Davis G. Recombinant α -interferon treatment of non-A, non-B (type C) hepatitis: review of studies and recommendations for treatment. *J Hepatol* 1990; **11**: S72–7.
- 11 Di Bisceglie A, Martin P, Kassianidis C, Lisker-Melman M, Murray L, Waggoner J, *et al.* Recombinant interferon alpha therapy for chronic hepatitis C. A randomized, double-blind, placebo-controlled trial. *N Engl J Med* 1989; **321** (22): 1506–10.
- 12 Di Bisceglie A, Hoofnagle J. Therapy of chronic hepatitis C with α -Interferon: The answer? or more questions? *Hepatology* 1991; **13** (3): 601–3.
- 13 Jacyna M, Brooks M, Loke R, Main J, Murray-Lyon I, Thomas H. Randomised controlled trial of interferon alpha (lymphoblastoid interferon) in chronic non-A, non-B hepatitis. *BMJ* 1989; **298**: 80–2.
- 14 Shindo M, Di Bisceglie AM, Hoofnagle JH. Long-term follow-up of patients with chronic hepatitis C treated with α -interferon. *Hepatology* 1992; **15** (6): 1013–6.
- 15 Cha T-A, Beall E, Irvine B, Kolberg J, Chien D, Kuo G, Urdea MS. At least five related, but distinct, hepatitis C viral genotypes exist. *Proc Natl Acad Sci USA* 1992; **89**: 7144–8.
- 16 Chan S-W, McOmish F, Holmes EC, Dow B, Peutherer JF, Follett E, *et al.* Analysis of a new hepatitis C virus type and its phylogenetic relationship to existing variants. *J Gen Virol* 1992; **73**: 1131–41.
- 17 Simmonds P, McOmish F, Yap PL, Chan S-W, Lin CK, Dusheiko G, *et al.* Sequence variability in the 5' non-coding region of hepatitis C virus: identification of a new virus type and restrictions on sequence diversity. *J Gen Virol* 1993; **74**: 661–8.
- 18 Okamoto H, Sugiyama Y, Okada S, Kurai K, Akahane Y, Sugai Y, *et al.* Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. *J Gen Virol* 1992; **73**: 673–9.
- 19 Enomoto N, Takada A, Nakao T, Date T. There are two major types of hepatitis C virus in Japan. *Biochem Biophys Res Commun* 1990; **170** (3): 1021–5.
- 20 Houghton M, Weiner A, Han J, Kuo G, Choo Q-L.

- Molecular biology of the hepatitis C viruses: implications for diagnosis, development and control of viral disease. *Hepatology* 1991; 14 (2): 381-8.
- 21 Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, *et al.* Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc Natl Acad Sci USA* 1990; 87: 9524-8.
 - 22 Dusheiko G, Simmonds P. Sequence variability of hepatitis C virus and its clinical relevance. *Journal of Viral Hepatitis* 1994; 1: 3-15.
 - 23 Chemello L, Alberti A, Rose K, Simmonds P. Hepatitis C serotype and response to interferon therapy. *N Engl J Med* 1994; 330: 143.
 - 24 Kanai K, Kako M, Okamoto H. HCV genotypes in chronic hepatitis C and response to interferon. *Lancet* 1992; 339: 1543.
 - 25 Yoshioka K, Kakumu S, Wakita T, Ishikawa T, Itoh Y, Takayanagi M, *et al.* Detection of hepatitis C virus by polymerase chain reaction and response to interferon- α therapy: relationship to genotypes of hepatitis C virus. *Hepatology* 1992; 16: 293-9.
 - 26 Kobayashi Y, Watanabe S, Konishi M, Yokoi M, Kakehashi R, *et al.* Quantitation and typing of serum hepatitis C virus RNA in patients with chronic hepatitis C treated with interferon- β . *Hepatology* 1993; 18 (6): 1319-25.
 - 27 Pozzato G, Moretti M, Franzin F, Crocy L, Tiribelli C, Masayu T, *et al.* Severity of liver disease with different HCV clones. *Lancet* 1991; 338: 509.
 - 28 Takada N, Takase S, Enomoto N, Takada A, Date T. Clinical backgrounds of the patients having different types of hepatitis C virus genomes. *J Hepatol* 1992; 14: 35-40.
 - 29 McOmish F, Chan S-W, Dow BC, Gillon J, Frame WD, Crawford RJ, *et al.* Detection of three types of hepatitis C virus in blood donors: investigation of type-specific differences in serologic reactivity and rate of alanine aminotransferase abnormalities. *Transfusion* 1993; 33 (1): 7-13.
 - 30 Knodell R, Ishak K, Black W, Chen T, Craig 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.
 - 31 Dourakis S, Brown J, Kumar U, Karayiannis P, Kernoff P, Chiba J, *et al.* Serological response and detection of viraemia in acute hepatitis C virus infection. *J Hepatol* 1992; 14: 370-6.
 - 32 Kumar U, Thomas H, Monjardino J. Serum HCV RNA levels in chronic HCV hepatitis measured by quantitative PCR assay; correlation with serum AST. *J Virol Methods* 1994; 47: 95-102.
 - 33 Dusheiko G, Schmilovitz-Weiss H, Brown D, McOmish F, Yap P-L, Sherlock S, *et al.* Hepatitis C genotypes: An investigation into type-specific differences in geographic origin and disease. *Hepatology* 1994; 19 (1): 13-8.
 - 34 Gunji T, Kato N, Mori S, Ootsuyama Y, Hijikata M, Imawari M, *et al.* Correlation between serum level of hepatitis C virus RNA and disease activities in acute and chronic hepatitis C. *Int J Cancer* 1992; 52: 726-30.
 - 35 Hagiwara H, Hayashi N, Mita E, Naito M, Kasahara A, Fusamoto H, *et al.* Quantitation of hepatitis C virus RNA in serum of asymptomatic blood donors and patients with type C chronic liver disease. *Hepatology* 1993; 17 (4): 545-50.