

MUTATIONS IN THE NONSTRUCTURAL PROTEIN 5A GENE AND RESPONSE TO INTERFERON IN PATIENTS WITH CHRONIC HEPATITIS C VIRUS 1b INFECTION

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Abstract Background. A region associated with sensitivity to interferon has been identified in the nonstructural protein 5A (NS5A) of hepatitis C virus (HCV) genotype 1b. The region spans amino acid residues 2209 to 2248 (NS5A₂₂₀₉₋₂₂₄₈) of HCV-J, a strain of HCV-1b whose complete genomic sequence has been identified. We examined whether the NS5A₂₂₀₉₋₂₂₄₈ sequence present before therapy could be used as a predictor of the response to interferon therapy in patients with chronic HCV-1b infection.

Methods. We retrospectively analyzed 84 patients with chronic HCV-1b infection who had received interferon alfa (total dose, 516 million to 880 million units) for six months. Pretreatment serum samples were analyzed. The amino acid sequence of NS5A₂₂₀₉₋₂₂₄₈ was determined by direct sequencing of the HCV genome amplified by the polymerase chain reaction (PCR) and was compared with the established sequence for HCV-J.

Results. A complete response, as evidenced by the absence of HCV RNA in serum on nested reverse-transcription PCR for six months after therapy, did not occur in

any of the 30 patients whose NS5A₂₂₀₉₋₂₂₄₈ sequences were identical to that of HCV-J (wild type). Five of 38 patients (13 percent) with 1 to 3 changes in NS5A₂₂₀₉₋₂₂₄₈ (intermediate type) had complete responses, as did all 16 patients with 4 to 11 amino acid substitutions (mutant type), indicating that the mutant type was significantly associated with a complete response ($P < 0.001$). Although baseline serum HCV RNA levels, as measured by a branched-chain DNA assay, were lower in patients with the mutant type of NS5A₂₂₀₉₋₂₂₄₈ than in those with the other types ($P < 0.001$), multivariate analyses revealed that the number of amino acid substitutions in NS5A₂₂₀₉₋₂₂₄₈ was the only variable associated with an independent effect on the outcome of interferon therapy (odds ratio, 5.3; 95 percent confidence interval, 1.6 to 18; $P = 0.007$).

Conclusions. In patients with chronic HCV-1b infection, there is a substantial correlation between responses to interferon and mutations in the NS5A gene. (N Engl J Med 1996;334:77-81.)

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HEPATITIS C virus (HCV), which has a positive-sense, single-stranded RNA genome with approximately 9400 nucleotides, causes most cases of chronic non-A, non-B hepatitis.^{1,2} Chronic hepatitis C infection can progress to liver cirrhosis and hepatocellular carcinoma over the course of 20 to 30 years.³

Interferon is the sole therapy for chronic hepatitis C, although only 25 percent of patients treated for 6 to 12 months have sustained remissions, with the eradication of HCV (complete response).⁴ The effects of interferon differ among the various HCV genotypes.⁵ Because HCV genotype 1b (HCV-1b) is resistant to interferon,⁶ the rate of complete response is only 10 to 40 percent, which is much lower than those of the other genotypes, such as HCV-2a or HCV-2b, with rates of complete response of 60 to 90 percent.⁷⁻¹⁰ HCV-1b is the most frequent variant worldwide, with a high incidence (37 to 80 percent) in Asian, American, and European countries studied to date.¹¹⁻¹⁴ Patients with HCV-1b infection have more active disease and are more likely to have progression to liver cirrhosis and hepatocellular carcinoma than patients with other HCV genotypes.^{13,15-18} Therefore, the resistance of HCV-1b to interferon is a serious problem in the management of chronic hepatitis infection. Since interferon therapy is expensive¹⁹ and

may cause serious adverse effects,⁴ it would be useful to be able to predict the efficacy of interferon in HCV-1b infection.

The HCV genome encodes structural proteins that constitute the viral particle and nonstructural proteins that are expressed only in hepatocytes.¹ Nonstructural protein 5A (NS5A) is the amino-terminal half of nonstructural protein 5; the carboxyl-terminal half (NS5B) contains RNA-dependent RNA polymerase that replicates the HCV RNA genome.¹³ The function of NS5A is not known. Recently, using comparative analysis of the full-length HCV genome,²⁰ we showed that a small region of NS5A (NS5A₂₂₀₉₋₂₂₄₈) of HCV-1b is associated with sensitivity to interferon. The numbering of the amino acid sequence, 2209 to 2248, was based on that of HCV-J, a strain of HCV-1b, whose complete genomic sequence has been determined.²¹ In the interferon-resistant strains that remained after therapy, the NS5A₂₂₀₉₋₂₂₄₈ sequence was the same as that in the prototypical HCV-1b strains (HCV-J, HC-J4,²² and HCV-JTa²³), whereas interferon-sensitive strains had multiple amino acid substitutions in this region. In that study, interferon-resistant HCV-1b sequences were determined only after interferon therapy. Thus, we did not evaluate the importance of the NS5A₂₂₀₉₋₂₂₄₈ sequences present before treatment with respect to the prediction of the efficacy of interferon. Other clinical factors that may influence the efficacy of interferon, such as serum HCV RNA concentrations¹⁶ or histologic factors affecting the liver,⁸ have also not been evaluated simultaneously with the NS5A₂₂₀₉₋₂₂₄₈ region. Therefore, we tested the hypothesis that the NS5A₂₂₀₉₋₂₂₄₈ sequence present before treatment predicts the response to interferon therapy in patients infected with HCV-1b. We also investi-

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gated the relation among the response to interferon, pretreatment NS5A₂₂₀₉₋₂₂₄₈ sequences, and other clinical factors.

METHODS

Patients

We retrospectively analyzed patients with chronic HCV-1b infection who had been treated with interferon alfa between January 1992 and December 1993. During this period, we treated 107 patients (67 men and 40 women) who were positive for anti-HCV antibodies on a second-generation assay (Ortho Diagnostic Systems, Raritan, N.J.) and for HCV RNA on the basis of a nested reverse-transcription polymerase chain reaction (PCR) targeted to the 5' noncoding region.²⁴ All patients had detectable HCV RNA with the 1b genotype, as determined by a mixed-primer PCR targeted to the core region of the HCV genome.²⁵ We studied 84 of these patients (57 men and 27 women).

To be eligible for the study, the patients had to have biopsy-proved chronic hepatitis, to have received interferon alfa for six months in a dose of 6 million to 10 million units intramuscularly three times a week (total dose, 516 million to 880 million units), and to have been followed for at least six months before and after therapy. Twenty-two patients had received recombinant interferon alfa-2a (Roferon-A, Hoffmann-LaRoche, Basel, Switzerland), 38 recombinant interferon alfa-2b (Intron A, Schering-Plough, Kenilworth, N.J.), and 24 human lymphoblastoid interferon alfa (Sumiferon, Sumitomo Pharmaceuticals, Osaka, Japan). Patients with a positive test for serum hepatitis B surface antigen, coinfection with other HCV genotypes, histologic findings of liver cirrhosis, other causes of hepatocellular injury (such as alcoholism, autoimmune hepatitis, primary biliary cirrhosis, or a history of treatment with hepatotoxic drugs), and a need for hemodialysis were excluded. The amino acid sequences of NS5A in five patients were included in our earlier report,²¹ but no other clinical or virologic information was given (these patients were identified as 12, 13, 14, 17, and 18 in the previous report and are referred to as patients 76, 78, 52, 73, and 84, respectively, in this report).

The protocol for interferon treatment of chronic hepatitis C followed the guidelines approved by National Health Insurance of Japan and was in accordance with the Helsinki Declaration of 1975, as revised in 1983. Written informed consent was obtained from all patients before they underwent liver biopsy and received interferon therapy.

Analytic Methods

The following factors were analyzed to determine whether they were related to the efficacy of interferon: age, sex, history of transfusion, duration of infection, stage of fibrosis on liver biopsy, total dose of interferon, type of interferon given, pretreatment serum alanine aminotransferase level, serum HCV RNA level, and amino acid sequence of NS5A₂₂₀₉₋₂₂₄₈ before treatment. The duration of infection was estimated as the interval from blood transfusion to interferon therapy in 28 patients with a history of blood transfusion. The remaining 56 patients had sporadic infection without any identifiable source of transmission of HCV, such as occupational exposure to blood or blood products or intravenous-drug abuse, and these patients were excluded from the analysis of the duration of infection. Liver-biopsy specimens were evaluated blindly by an independent interpreter according to the stage of fibrosis (mild, moderate, or severe).²⁶ Serum HCV RNA levels were determined by a branched-chain DNA assay²⁷ (Quantiplex HCV RNA, Chiron, Emeryville, Calif.). The limit of detection of this assay was 0.5 million genome equivalents per milliliter.

Patients were monitored monthly with serial determinations of alanine aminotransferase. Serum was tested for HCV RNA just before therapy was started and every three months thereafter with the use of nested reverse-transcription PCR targeted to the 5' noncoding region, the detection limit of which was 100 copies of viral genome per milliliter of serum.²⁸ Patients were considered to have had a complete response to interferon if serum alanine aminotransferase levels were normal for six months after therapy, with no evidence of serum HCV

Table 1. Clinical Characteristics of 84 Patients with Chronic Hepatitis C, According to Their Responses to Interferon Therapy.*

CHARACTERISTIC	NO RESPONSE (N = 63)	COMPLETE RESPONSE (N = 21)	P VALUE
No. of amino acid changes in NS5A ₂₂₀₉₋₂₂₄₈			<0.001
Median	1	5	
Range	0-3	1-11	
Serum HCV RNA at base line — millions of genome equivalents/ml†			<0.001
Median	4.4	<0.5	
Range	<0.5-40	<0.5-12	
Age — yr	49.3 ± 12.8	50.1 ± 8.4	0.80
Sex — no.			0.18
Male	40	17	
Female	23	4	
History of blood transfusion — no. (%)	23 (36)	5 (24)	0.42
Duration of infection — yr‡	22.9 ± 9.7	30.0 ± 8.1	0.14
Stage of fibrosis — no.			0.93
Mild	4	1	
Moderate	41	14	
Severe	18	6	
Total dose of interferon — millions of units	694 ± 160	723 ± 150	0.48
Type of interferon given — no.			0.36
Alfa-2a	14	8	
Alfa-2b	30	8	
Human lymphoblastoid interferon alfa	19	5	
Serum alanine aminotransferase at base line (IU/liter)§	104 ± 109	138 ± 87	0.19

*Plus-minus values are means ± SD.

†Measured by branched-chain DNA assay.

‡Estimated as the period from blood transfusion to interferon therapy in the 28 patients with a history of blood transfusion.

§Normal range, 5 to 46 IU per liter.

RNA on nested reverse-transcription PCR at the cessation of treatment and three and six months thereafter. Otherwise, patients were considered to have had no response. To ensure the optimal detection and quantitation of HCV RNA,²⁹ serum was separated from blood samples within two hours after they were obtained and then stored at -80°C without thawing until use.

Nucleotide Sequencing of the NS5A Gene

Extraction of RNA from serum and reverse-transcription PCR were performed as described previously.³⁰ The PCR primers and sequencing primers were synthesized with a DNA synthesizer (model 391, Applied Biosystems Japan, Chiba, Japan). To determine the nucleotide sequence of the NS5A region, we amplified nucleotides 6703 to 7320 (numbered on the basis of the sequence of HCV-J) of HCV complementary DNA using the outer set of primers. One microliter of the first PCR product was transferred to the second PCR reaction along with nested 5' and 3' primers. An M13 forward primer (5'TG-TAAAACGACGGCCAGT3') and an M13 reverse primer (5'CAGG-AAACAGCTATGACC3') were attached to the 5' terminal of the 5' and 3' nested primers, respectively, to facilitate direct sequencing by an automated DNA sequencer (model 373S, Applied Biosystems Japan). Both strands of the PCR products were sequenced with the Prism dye termination kit (Applied Biosystems Japan), according to the manufacturer's instructions. The sequencing primer was the M13 forward primer for the sense strand and the M13 reverse primer for the antisense strand. The resulting amino acid sequences of NS5A₂₂₀₉₋₂₂₄₈ were compared with the NS5A₂₂₀₉₋₂₂₄₈ sequence identified in HCV-J.

The sequences of the primers used for the nested PCR were as follows: 5' outer set, 5'TGGATGGAGTGC GGTTGCACAGGTA3' (nucleotides 6703 to 6727); 3' outer set, 5'TCTTTCTCCGTG-GAGGTGGTATTGG3' (nucleotides 7296 to 7320); 5' inner set, 5'TGTA AAAACGACGGCCAGT CAGGTACGCTCCGGCGGTGCA3'

sponse in the 24 patients with HCV RNA levels below 0.5 million genome equivalents per milliliter was significantly higher than the rate of 10 percent ($P < 0.001$) in the 60 patients with HCV RNA levels of at least 0.5 million genome equivalents per milliliter. However, three patients with the mutant type of NS5A₂₂₀₉₋₂₂₄₈ had complete responses despite having HCV RNA levels of at least 0.5 million genome equivalents per milliliter, and six patients with the wild type had no response despite having HCV RNA levels below 0.5 million genome equivalents per milliliter. In the patients with the intermediate type of NS5A₂₂₀₉₋₂₂₄₈ sequence, there were no significant differences between the 33 patients with no responses to interferon and the 5 patients with complete responses with respect to serum HCV RNA levels (median, 4.1 million vs. 0.8 million genome equivalents per milliliter; $P = 0.31$), the number of amino acid changes in NS5A₂₂₀₉₋₂₂₄₈ (median, 1 in both groups; $P = 0.69$) (Fig. 1), or other variables (data not shown).

We used multiple logistic-regression analysis to examine a variety of variables (Table 3) that might contribute to a complete response to interferon. Serum HCV RNA levels were stratified into three categories: values below 0.5 million genome equivalents per milliliter (the limit of detection), values of 0.5 million to 15 million genome equivalents per milliliter, and values above 15 million genome equivalents per milliliter. The number of amino acid changes in NS5A₂₂₀₉₋₂₂₄₈ was the only variable associated with an independent effect on the outcome of interferon therapy (odds ratio, 5.3; 95 percent confidence interval, 1.6 to 18; $P = 0.007$). Although serum HCV RNA levels were

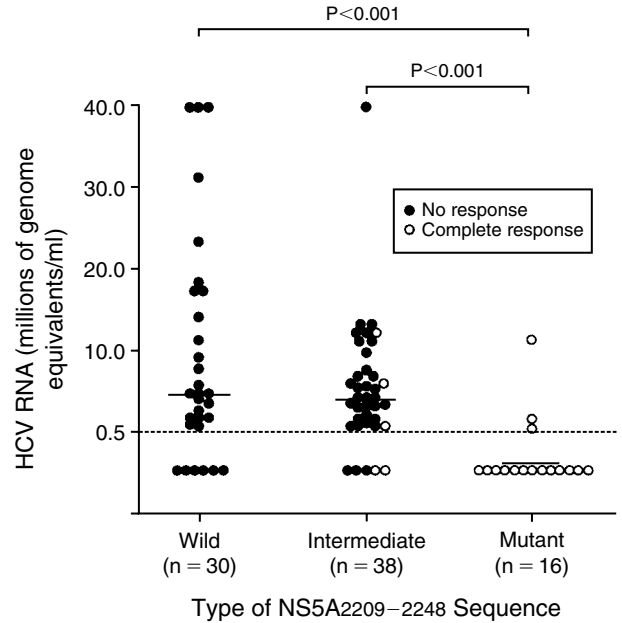


Figure 2. Serum HCV RNA Levels in Relation to the Type of NS5A₂₂₀₉₋₂₂₄₈ Sequence Present before Treatment and the Response to Interferon Therapy.

Serum levels of HCV RNA were significantly lower in patients with the mutant type of NS5A₂₂₀₉₋₂₂₄₈ than in those with the wild type or the intermediate type. The horizontal bar in each column indicates the median. The broken line indicates the limit of detection of the branched-chain DNA assay.

correlated with the response to interferon and the number of amino acid changes in NS5A₂₂₀₉₋₂₂₄₈ in univariate analyses, in multivariate analyses they were not an independent predictor of the response to interferon, even when patients with HCV RNA levels below 0.5 million genome equivalents per milliliter were compared with patients with levels above 15 million genome equivalents per milliliter ($P = 0.23$).

DISCUSSION

We found a significant correlation between the response to interferon in patients with chronic HCV-1b infection and the number of amino acid substitutions that were present before therapy in a small region of NS5A. All patients with 4 to 11 amino acid changes in the NS5A₂₂₀₉₋₂₂₄₈ sequence of HCV-1b (mutant type) had complete responses to interferon therapy, whereas all the patients with no amino acid changes (wild type) and 87 percent of those with 1 to 3 amino acid changes (intermediate type) had no responses. Thus, interferon therapy as currently offered is insufficient for patients with

Table 2. Clinical Characteristics of 84 Patients with Chronic Hepatitis C, According to the Type of NS5A₂₂₀₉₋₂₂₄₈ Sequence Identified.*

CHARACTERISTIC	WILD TYPE (N = 30)	INTERMEDIATE TYPE (N = 38)	MUTANT TYPE (N = 16)	P VALUE
Complete response — no. (%)	0	5 (13)	16 (100)	<0.001†
Serum HCV RNA at base line — millions of genome equivalents/ml‡	4.7 (<0.5–40)	4.0 (<0.5–40)	<0.5 (<0.5–11)	<0.001†
Age — yr	50.5 ± 12.6	47.8 ± 12.3	51.8 ± 8.7	0.45
Sex — no.				0.15
Male	20	23	14	
Female	10	15	2	
History of blood transfusion — no. (%)	13 (43)	11 (29)	4 (25)	0.34
Duration of infection — yr§	23.2 ± 7.9	23.4 ± 11.8	29.5 ± 9.3	0.51
Stage of fibrosis — no.				0.40
Mild	0	4	1	
Moderate	21	25	9	
Severe	9	9	6	
Total dose of interferon — millions of units	699 ± 163	701 ± 159	707 ± 152	0.99
Type of interferon given — no.				0.45
Alfa-2a	6	9	7	
Alfa-2b	14	19	5	
Human lymphoblastoid interferon alfa	10	10	4	
Serum alanine aminotransferase at base line (IU/liter)¶	122 ± 150	90 ± 49	150 ± 91	0.13

*Plus-minus values are means ± SD.

† $P < 0.001$ for the comparison of the mutant type with the wild type and with the intermediate type.

‡Measured by branched-chain DNA assay.

§Estimated as the period from blood transfusion to interferon therapy in the 28 patients with a history of blood transfusion.

¶Normal range, 5 to 46 IU per liter.

Table 3. Multivariate Analysis of the Effect of Variables on the Response to Interferon.

VARIABLE	MULTIVARIATE ODDS RATIO (95% CI)*	P VALUE
Amino acid changes in NS5A ₂₂₀₉₋₂₂₄₈ (per 1 amino acid change)	5.3 (1.6-18)	0.007
Serum HCV RNA level at base line		
>15 million genome equivalents/ml†	1.0	—
0.5 million-15 million genome equivalents/ml	0.4 (0.1-1.6)	0.19
<0.5 million genome equivalents/ml	2.9 (0.5-17)	0.23
Age (per year of age)	1.0 (0.9-1.1)	0.57
Sex (female vs. male)	1.7 (0.4-6.7)	0.47
History of blood transfusion (yes vs. no)	0.3 (0.1-5.7)	0.44
Duration of infection (per year of infection)‡	1.2 (0.7-2.0)	0.42
Stage of fibrosis (mild vs. moderate vs. severe)	0.6 (0.1-4.8)	0.59
Total dose of interferon (per million units)	1.0 (0.99-1.0)	0.53
Type of interferon given		
Human lymphoblastoid interferon alfa†	1.0	—
Alfa-2a	0.9 (0.1-7.0)	0.91
Alfa-2b	0.7 (0.1-12)	0.82
Serum alanine aminotransferase at base line (per international unit per liter)	1.0 (0.99-1.0)	0.94

*Values are the odds of having a complete response to interferon. CI denotes confidence interval.

†The reference group.

‡The odds ratio was obtained by analyzing data on 28 patients with a history of blood transfusion; three variables (the number of amino acid changes in NS5A₂₂₀₉₋₂₂₄₈, the serum HCV RNA level at base line, and the duration of infection) were used as explanatory variables for the multivariate analysis.

interferon-resistant HCV-1b with wild-type or intermediate-type NS5A₂₂₀₉₋₂₂₄₈ sequences.

Of the various clinical variables examined, the number of amino acid substitutions in NS5A₂₂₀₉₋₂₂₄₈ was the only independent predictor of the response to interferon. Among patients infected with the same genotype of HCV, those with higher serum HCV RNA levels are more resistant to interferon.^{10,14,27,31} In our study, univariate analysis confirmed the relation between serum HCV RNA levels and the response to interferon; however, multivariate analysis showed that the serum HCV RNA level was not an independent predictor. Our data suggest that serum HCV RNA levels are indirectly associated with the response to interferon through their relation to the sequence of NS5A₂₂₀₉₋₂₂₄₈ and that the NS5A₂₂₀₉₋₂₂₄₈ sequence itself is a more accurate predictor of response.

The mechanism by which NS5A₂₂₀₉₋₂₂₄₈ affects the response to interferon or serum HCV RNA level is not known. The relation between the type of NS5A₂₂₀₉₋₂₂₄₈ sequence and the HCV RNA level suggests that NS5A₂₂₀₉₋₂₂₄₈ has an important role in HCV replication. Thus, mutations in NS5A₂₂₀₉₋₂₂₄₈ may suppress the replication of HCV and increase susceptibility to interferon. It was recently reported that amino acid residues 2200 to 2250, which encompass NS5A₂₂₀₉₋₂₂₄₈, are essential for the phosphorylation of NS5A.³² Alternatively, NS5A₂₂₀₉₋₂₂₄₈ may be a direct target of antiviral proteins induced by interferon.

REFERENCES

- Houghton M, Weiner A, Han J, Kuo G, Choo QL. Molecular biology of the hepatitis C viruses: implications for diagnosis, development and control of viral disease. *Hepatology* 1991;14:381-8.
- Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 1989;244:359-62.

- Kiyosawa K, Sodeyama T, Tanaka E, et al. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 1990;12:671-5.
- Hoofnagle JH. Therapy of acute and chronic viral hepatitis. *Adv Intern Med* 1994;39:241-75.
- Simmonds P. Variability of hepatitis C virus. *Hepatology* 1995;21:570-83.
- 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. [Erratum, *J Hepatol* 1992;14:435.]
- Yoshioka K, Kakumu S, Wakita T, et al. Detection of hepatitis C virus by polymerase chain reaction and response to interferon-alpha therapy: relationship to genotypes of hepatitis C virus. *Hepatology* 1992;16:293-9.
- Mita E, Hayashi N, Hagiwara H, et al. Predicting interferon therapy efficacy from hepatitis C virus genotype and RNA titer. *Dig Dis Sci* 1994;39:977-82.
- Hino K, Sainokami S, Shimoda K, et al. Genotypes and titers of hepatitis C virus for predicting response to interferon in patients with chronic hepatitis C. *J Med Virol* 1994;42:299-305.
- Tsubota A, Chayama K, Ikeda K, et al. Factors predictive of response to interferon-alpha therapy in hepatitis C virus infection. *Hepatology* 1994;19:1088-94.
- Enomoto N, Takada A, Nakao T, Date T. There are two major types of hepatitis C virus in Japan. *Biochem Biophys Res Commun* 1990;170:1021-5.
- Nakao T, Enomoto N, Takada N, Takada A, Date T. Typing of hepatitis C virus genomes by restriction fragment length polymorphism. *J Gen Virol* 1991;72:2105-12.
- van Doorn LJ. Review: molecular biology of the hepatitis C virus. *J Med Virol* 1994;43:345-56.
- Mahaney K, Tedeschi V, Maertens G, et al. Genotypic analysis of hepatitis C virus in American patients. *Hepatology* 1994;20:1405-11.
- van der Poel CL, Cuyper HT, Reesink HW. Hepatitis C virus six years on. *Lancet* 1994;344:1475-9.
- Nousbaum J-P, Pol S, Nalpas B, Landais P, Berthelot P, Brechot C. Hepatitis C virus type 1b (II) infection in France and Italy. *Ann Intern Med* 1995;122:161-8.
- Silini E, Bono F, Cividini A, et al. Differential distribution of hepatitis C virus genotypes in patients with and without liver function abnormalities. *Hepatology* 1995;21:285-90.
- Takada N, Takase S, Takada A, Date T. Differences in the hepatitis C virus genotypes in different countries. *J Hepatol* 1993;17:277-83.
- Davis GL. Recombinant alpha-interferon treatment of non-A, non-B (type C) hepatitis: review of studies and recommendations for treatment. *J Hepatol* 1990;11:Suppl 1:S72-S77.
- Enomoto N, Sakuma I, Asahina Y, et al. Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b: sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. *J Clin Invest* 1995;96:224-30.
- Kato N, Hijikata M, Ootsuyama Y, et al. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. *Proc Natl Acad Sci U S A* 1990;87:9524-8.
- Okamoto H, Kojima M, Okada S, et al. Genetic drift of hepatitis C virus during an 8.2-year infection in a chimpanzee: variability and stability. *Virology* 1992;190:894-9.
- Tanaka T, Kato N, Nakagawa M, et al. Molecular cloning of hepatitis C virus genome from a single Japanese carrier: sequence variation within the same individual and among infected individuals. *Virus Res* 1992;23:39-53.
- Okamoto H, Okada S, Sugiyama Y, et al. Detection of hepatitis C virus RNA by a two-stage polymerase chain reaction with two pairs of primers deduced from the 5'-noncoding region. *Jpn J Exp Med* 1990;60:215-22.
- Okamoto H, Sugiyama Y, Okada S, 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.
- Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994;19:1513-20.
- Lau JY, Davis GL, Kniffen J, et al. Significance of serum hepatitis C virus RNA levels in chronic hepatitis C. *Lancet* 1993;341:1501-4. [Erratum, *Lancet* 1993;342:504.]
- Sakamoto N, Enomoto N, Kurosaki M, Marumo F, Sato C. Detection and quantification of hepatitis C virus RNA replication in the liver. *J Hepatol* 1994;20:593-7.
- Davis GL, Lau JY, Urdea MS, et al. Quantitative detection of hepatitis C virus RNA with a solid-phase amplification method: definition of optimal conditions for specimen collection and clinical application in interferon-treated patients. *Hepatology* 1994;19:1337-41.
- Enomoto N, Kurosaki M, Tanaka Y, Marumo F, Sato C. Fluctuation of hepatitis C virus quasispecies in persistent infection and interferon treatment revealed by single-strand conformation polymorphism analysis. *J Gen Virol* 1994;75:1361-9.
- Hagiwara H, Hayashi N, Mita E, et al. Quantitative analysis of hepatitis C virus RNA in serum during interferon alfa therapy. *Gastroenterology* 1993;104:877-83.
- Tanji Y, Kaneko T, Satoh S, Shimotohno K. Phosphorylation of hepatitis C virus-encoded nonstructural protein NS5A. *J Virol* 1995;69:3980-6.