J Korean Med Sci 2000; 15: 53-8
ISSN 1011-8934

Mutations of Hepatitis C Virus 1b NS5A 2209-2248 Amino Acid Sequence Is Not a Predictive Factor for Response to Interferon-alpha Therapy and Development of Hepatocellular Carcinoma

Genetic changes between codons 2209 and 2248 of NS5A of genotype 1b hepatitis C virus (HCV-1b) have been reported to be associated with the sensitivity to interferon-alpha (IFN-α). The present study was performed to analyze such relationship in Korean patients with chronic hepatitis C and HCV-1b (n=19), including 12 chronic hepatitis C patients treated with IFN-α, 3 chronic hepatitis C patients without treatment as controls, and 4 patients with hepatocellular carcinoma (HCC). Two serum samples, before and after the treatment, were analyzed for the mutations by reverse transcription-polymerase chain reaction, cloning and sequencing. The mutations were identified in 32% (6/19), including five intermediate type (1-3 mutations) and one mutant type (4 or more). In 12 patients treated with IFN-α, the number of amino acid substitutions in $NS5A_{2209-2248}$ was not associated with outcome of the treatment. Two HCV isolates with $NS5A_{2209-2248}$ mutations from HCC patients were intermediate type. These results do not support that the $NS5A_{2209-2248}$ determines interferon sensitivity of HCV-1b and that the mutations is associated with development of HCC.

Key Words: *Hepatitis C, Chronic; Genotype; Viral Nonstructural Proteins; Interferon-Alpha; Carcinoma, Hepatocellular*

Si Hyun Bae[*,‖,¶], **Young Min Park**[*,‖,¶], **Duck Gi Yoo**[‖], **Jong Young Choi**[*,‖,¶], **Byung Hun Byun**[*,‖,¶], **Jin Mo Yang**[†,‖], **Chang Don Lee**[‡,‖], **Sang Bok Cha**[§,‖], **Doo Ho Park**[*,‖], **Boo Sung Kim**[*,‖]

Department of Internal Medicine, Kangnam St. Mary's Hospital*, St. Paul Hospital†, Yijung-bu St. Mary's Hospital‡, Our Lady of Mercy Hospital§, College of Medicine, The Catholic University of Korea; ‖WHO Collaborating Center for Reference and Research on Viral Hepatitis of Korea; ¶Molecular Hepatology Laboratory, Institute of Human Molecular Genetics, Catholic Research Institutes of Medical Science, Seoul, Korea

Received: 9 September 1999
Accepted: 26 October 1999

Address for correspondence
Young Min Park, M.D.
Department of Internal Medicine, Kangnam St. Mary's Hospital, 505 Banpo-dong, Seocho-gu, Seoul 137-040, Korea
Tel: +82.2-590-2705, Fax: +82.2-536-9559
E-mail: ympark@cmc.cuk.ac.kr

INTRODUCTION

Hepatitis C virus (HCV) has a positive-sense, single-stranded RNA genome and contains a large translational open reading frame (ORF), which encodes a polyprotein of about 3,000 amino acids (1, 2). The ORF consists of two structural and five non-structural protein-encoding gene regions (3). HCV causes various liver diseases, including acute hepatitis, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (4). More than half of patients with HCV infection progress to chronic hepatitis and 20% go on to cirrhosis and hepatocellular carcinoma (4, 5).

Interferon-alpha (IFN-α) is still the choice of treatment for chronic hepatitis C (6), although sustained remission rate is only 20-30%. Generally, it has been known that the HCV-1b type is more resistant to IFN-α than other genotypes (7-9), but its molecular mechanism was unclear. Recently, Enomoto et al. identified a 40-amino acid stretch in $NS5A_{2209-2248}$, the carboxy-terminal of nonstructural protein NS5A of HCV-1b, in which the number of amino acid mutations well correlated to IFN-α responsive. They called $NS5A_{2209-2248}$ an interferon sensitivity-determining region (ISDR) (10, 11), which modulates the sensitivity of HCV-1b to IFN-α. In addition, it is also conceivable by Enomoto's results that the wild HCV-1b type might be closely associated with the development of HCC, compared to the mutant types.

However, due to inconsistent results obtained in Europe (12-14), the biologic significance of mutations in $NS5A_{2209-2248}$ of HCV-1b is still controversial. Therefore, in the present study, we evaluate the same issue for Korean patients with HCV-1b infection.

PATIENTS AND METHODS

Patients

Fifteen patients with chronic hepatitis C and four patients with hepatocellular carcinoma were included (the

mean age, 39 years old (range 25-70); 12 males and seven females). All cases were positive for antibody to HCV (Abbott Laboratories, Abbott Park, Ill., U.S.A.). No cases were positive for hepatitis B surface antigen. Twelve patients with chronic hepatitis C were treated with IFN-α (Intermax-alfa, LG Biotech™, Taejeon, Korea) three times a week for six months at a dose of 3 megaunits (MU) in six patients and 6 MU in six patients after informed consents and three controls were not treated.

According to the outcome, 12 patients with IFN-α therapy were divided into three groups: responder, who showed normal serum aminotransferase level and undetectable serum HCV RNA persistently after therapy (group I, n=4); breakthrough, who showed the reappearance of HCV RNA and elevated aminotransferase levels after initial response during IFN-α administration (group II, n=4); and complete non-responder, who showed no evidence of response during the IFN-α administration (group III, n=4). The clinical and serological features of each patient group at admission are shown in Table 1.

Methods

Extraction of HCV RNA and reverse transcription

RNA from sera was extracted by acid-guanidium-phenol method as described previously (15). Briefly, 100 μL of serum was mixed with 400 μL of guanidinium buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.9; 0.5% sarkosyl and 1% 2-mercaptoethanol). After water saturated-phenol extraction and isopropanol precipitation, the pellet was dissolved in 6 μL diethylpyrocarbonate (DEPC)-treated water.

After heating at 65℃ for 5 min, an aliquot (1 μL) of RNA solution was mixed with 4 μL reverse transcription reaction solution, containing 50 units of Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT; BRL, Gaithersburg, MD, U.S.A.), 10 units RNase inhibitor (Promega Corp., Madison, WI, U.S.A.), 50 pg random hexamer (Takara, Kyoto, Japan), 50 mM Tris-HCl (pH 7.5), 75 mM KCl, 3 mM $MgCl_2$, and 10 mM dithiothreitol (DTT). This mixture was incubated at 37℃ for 60 min, heated at 95℃ for 5 min, and then snap-frozen.

Amplification of $NS5A_{2209-2248}$ gene fragment

A nested-polymerase chain reaction (PCR) was performed by using a Thermocycler (Perkin-Elmer Co., CA, U.S.A.) in two steps; 35 reaction cycles (94℃, 60 seconds; 55℃, 60 seconds; 72℃, 60 seconds) in each step. Primer sequences were as follows (16): outer sense primer nucleotides 6703-6727, 5'-TGGATGGAGTGCGGTTGCACAGGTA-3'), outer antisense primer (nucleotides 7296-7320, 5'-TCTTTCTCCGTGGAGGTGGTATTGG-3'), inner sense primer (nucleotide 6722-6741, 5'-CAGGACCAGTCAGGTACGCTCCGGCGTGCA-3'), and inner antisense primer (nucleotide 7275-7294, 5'-CAGGAAACAGCTATGACCGGGGCCTTGGTAGGTGGCA-3').

The first PCR reaction (50 μL volume) included 1 μL of cDNA and 49 μL reaction solution, containing 400 mmol of each outer primer, 2.5 U of *Taq* polymerase (AmpliTaq; Cetus Corp., Emeryville, CA, U.S.A.), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, and 200 μM each of dNTP. The second-step PCR reaction (50 μL volume) included 1 μL of the first PCR product and 49 μL reaction solution. The nested amplicons, 120 base pairs (nucleotide positions 6954-7073 of HCV-J), were subjected to electrophoresis on 3% agarose gel (BRL), stained with ethidium bromide and observed under U.V. light.

Cloning and sequencing of $NS5A_{2209-2248}$ region

After ligation of each amplicon into pGEM-T easy vector, *E. coli* was transformed and cultured overnight in the media containing β-Gal/IPTG. The plasmid DNA was purified and concentrated by the Wizard™ PCR minipreps (Promega Corp., Madison, WI, U.S.A.). The DNA concentration was measured by a spectrophotometer. The nucleotide sequence of $NS5A_{2209-2248}$ was determined by an automated sequencing method (A.L.F., Pharmacia Biotech, Uppsala, Sweden). The amino acid sequence was analyzed by DNASISWIN VERSION 2.01 (Hitachi Software, Japan).

Table 1. Demographic features of each patient group

	Chronic hepatitis C				Hepatocellular carcinoma
	(Group I) Responder	(Group II) Breakthrough	(Group III) Complete non-responder	(Group IV) Untreated Control	(Group V)
No. of cases	4	4	4	3	4
Age (yr)	40±12	44±14	36±11	42±13	67±6
Sex (M/F)	4/0	1/3	3/1	2/1	2/2
Albumin (g/dL)	4.3±0.2	4.3±0.3	4.2±0.4	4.3±0.2	3.3±0.5
Total bilirubin (mg/dL)	1.1±0.4	0.9±0.2	1.1±0.3	0.9±0.3	2.4±1.1
Alanine aminotransferase (IU/L)	155±43	55±21	139±100	89±62	51±33

The number of mutations in $NS5A_{2209-2248}$ region was determined by comparing the amino acid sequence with a reference sequence, HCV-J, a wild-type HCV-1b reported by Kato et al. (17). According to the method suggested by Enomoto et al. (11), each case was classified into one of three types as follows: wild type if no mutation, intermediate if 3 or less mutations, and mutant if 4 or more mutations.

Genotyping of HCV RNA

Genotype of HCV-RNA was determined by a reverse transcription-polymerase chain reaction (RT-PCR) method using genotype-specific primer sets derived from NS5 region (18).

RESULTS

Mutations in $NS5A_{2209-2248}$ were identified in 6/19 (32%) patients, including 4 of 12 chronic hepatitis B patients and 2 of 4 hepatocellular carcinoma patients; five intermediate types and one mutant type. Thirteen cases (68%) were infected with HCV-1b carrying wild type $NS5A_{2209-2248}$ (Table 2, Fig. 1-2).

Among 4 HCV isolates with $NS5A_{2209-2248}$ mutations from chronic hepatitis C patients, one case with intermediate type had single mutation (2212K→R) and two cases with intermediate type contained two mutations (2218H→R and 2227I→V; 2220D→G and 2239G→S). One mutant type contained four mutations (2209P→L,

Table 2. Incidence of mutation types in $NS5A_{2209-2248}$ among each patient group

	Chronic hepatitis C				Hepatocellular carcinoma
	(Group I) Responder	(Group II) Breakthrough	(Group III) Complete non-responder	(Group IV) Untreated control	(Group V)
Type	n=4	n=4	n=4	n=3	n=4
Wild (n=10)	3	3	2	3	2
Intermediate (n=5)	1	1	1	0	2
Mutant (n=1)	0	0	1	0	0

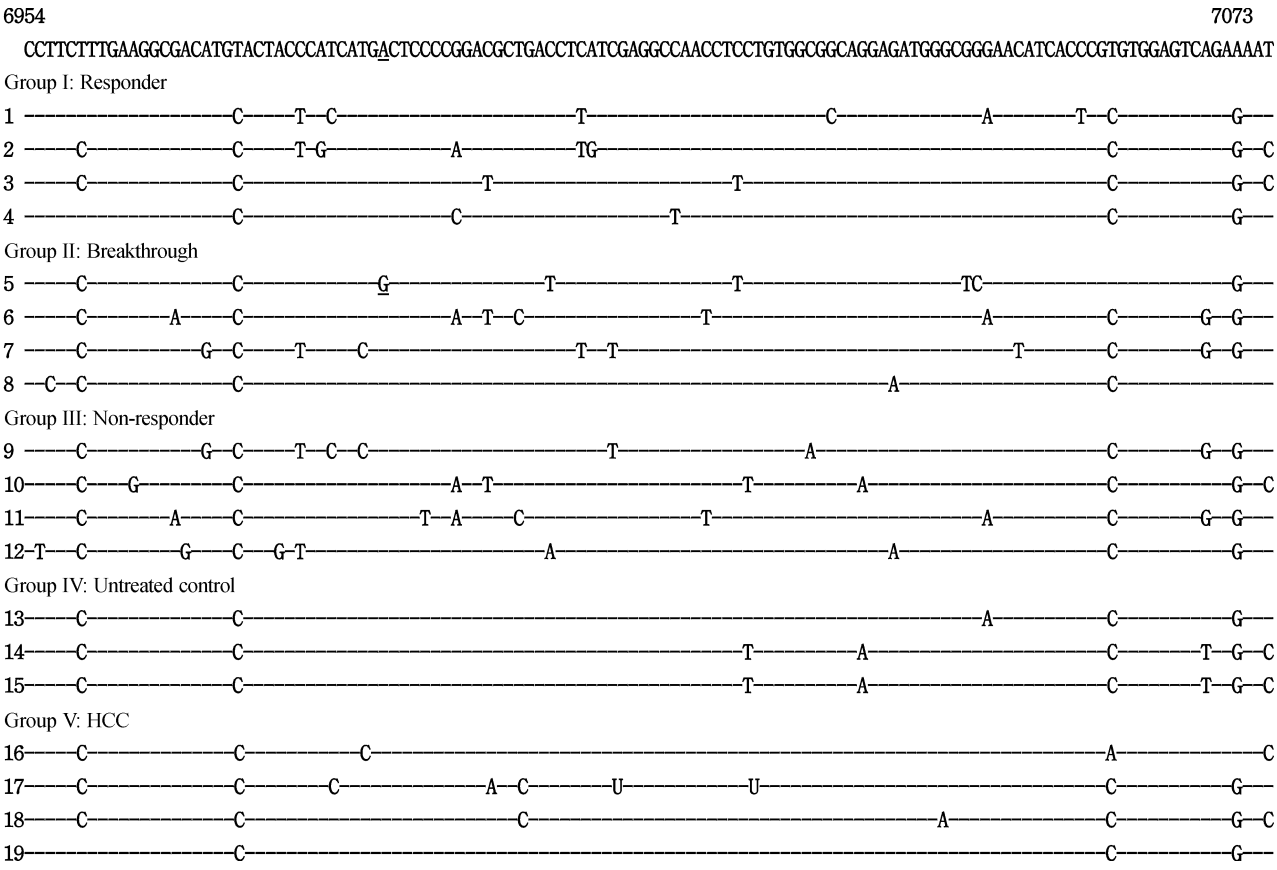

Fig. 1. Alignment of nucleotide sequences of the $NS5A_{2209-2248}$ region of HCV-1b isolates from responder (Group I), non-responder (Groups II and III) to IFN-α therapy, control (Group IV) and HCC cases (Group V). Prototype sequence (top line) corresponds to HCV-J nucleotide 6954-7073 and amino acid 2209-2248 of NS5A region.

	2209 2248 PSLKATCTTHHDSPDADLIEANLLWRQEMGGNITRVESEN	Response to IFN-α 6 months therapy ALT normal	HCV-RNA negative
Group I : Responder			
1	----------	yes	yes
2	----------R----------V----------	yes	no
3	----------	yes	no
4	----------	yes	no
Group II : Breakthrough			
5	----------G----------S----------	no	no
6	----------	no	no
7	----------	no	no
8	----------	no	no
Group III : Non-responder			
9	----------	no	no
10	-----R----------	no	no
11	----------	no	no
12	L------A----A----------E----------	no	no
Group IV : Untreated control			
13	----------	no	no
14	----------	no	no
15	----------	no	no
Group V: HCC			
1	----------		
2	----------E----------		
3	----------D----------		
4	----------		

Fig. 2. Alignment of amino acid sequences of the $NS5A_{2209-2248}$ region of HCV-1b isolates from responder (Group I), non-responder (Groups II and III) to IFN-α therapy, control (Group IV) and HCC cases (Group V). Prototype sequence (top line) corresponds to HCV-J amino acid 2209-2248 of NS5A region. *Reference sequences are cited from an article reported by Enomoto et al. (1996).

2214T→A, 2218H→A, 2225D→E) (Fig. 2).

There was no relationship between mutations in $NS5A_{2209-2248}$ and IFN-α response. Three of eight wild types and one of three intermediate types were responder, while five wild types, two intermediate types and one mutant type were non-responder (Table 2).

Two HCV isolates with $NS5A_{2209-2248}$ mutations from hepatocellular carcinoma patients were intermediate type with single mutation (2253D→E and 2299G→D).

DISCUSSION

It has been suggested that HCV genotype, serum HCV RNA concentration and histologic severity are useful factors in predicting response to IFN-α (14, 16, 19, 20). HCV-1b appears to be correlated with high viral concentration, severe histologic findings, and less sensitivity to IFN-α. Only about 10 to 20 percent of chronic hepatitis C patients infected with HCV-1b were reported to be sensitive to IFN-α, while 40 to 60 percent of those with HCV-2a or -2b could be treated with the same regimen (21).

Recently, it was suggested, through analysis of full-length sequences of HCV-1b in three non-responders before and after treatment, that the mechanism of HCV-1b sensitivity to IFN-α may be associated with genetic alterations in $NS5A_{2209-2248}$ (10). This possibility was supported by other Japanese groups (16, 20) (Table 3). It could also be supported by the observation that the NS5A binds protein kinase R, a cellular protein induced by IFN-α (13, 19, 22, 23).

However, our results and reports from European groups (12-14) and one Japanese group (24) were not consistent with the previous suggestion (Table 3). The inter-relationship between the number of mutations in $NS5A_{2209-2248}$ amino acid sequence and the response to IFN-α was not significant. Squadrito et al. suggested four different possibilities to explain this discrepancy (14). First, differences in the total IFN-α doses may modify the impact of NS5A mutations in the response to treatment; this hypothesis cannot be ruled out, but it is dif-

Table 3. Data comparison on the incidence of responder in each strain of HCV-1b to interferon therapy

	Enomoto et al. (11) n=84	Kurosaki et al. (16) n=22	Chayama et al. (20) n=103	Komatsu et al. (24) n=10	Khorsi et al. (12) n=43	Zeuzem et al. (13) n=22	Squadrito et al. (14) n=48	Bae et al. n=12
Wild	0/30	0/10	8/46	3/8	3/13	0/11	0/14	3/8
Intermediate	5/38	0/6	9/38	1/2	13/28	1/10	6/28	1/3
Mutant	16/16	4/6	14/19	0	1/2	0/1	1/6	0/1
Race	Japan	Japan	Japan	Japan	French	Europe	Europe	Korea
Significance	$p<0.001$	$p<0.001$	$p=0.0036$	$p>0.05$	$p=0.01$	$p>0.05$	$p>0.05$	$p>0.05$
Source	N Engl J Med 1996;334:77	Hepatology 1997;25:750	Hepatology 1997;25:745	J Med Virol 1997;53:361	J Hepatol 1997;27:72	Hepatology 1997;25:740	Gastroenterology 1997;113:567	

Upper cases denote references

ficult to imagine the mechanisms involved at this point. Second, the criteria used to define the long-term response to IFN may differ. In particular, results of serum HCV RNA and aminotransferase activities can be dissociated. Third, host-related factors such as HLA phenotype may be involved in the response to therapy. Fourth, this discrepancy might point to the heterogeneity of isolates classified in the same HCV type but detected in different geographical areas.

The incidence of the mutant type in the present study was lower than that of Japanese groups (11, 16, 20), while similar to that of European groups (12-14). However, the incidence of the intermediate type was higher in Europe than in Japan and Korea (Table 4). Therefore, the overall mutation rate in $NS5A_{2209-2248}$ of HCV-1b from Korean patients seems to be less common than that from Japanese and European patients (32, 54, and 70%, respectively). In addition, the mutation in the 2218th amino acid, His-to-Arg, was identified only in one of 19 Korean patients, while frequently in other groups, suggesting that geographic factors should be considered in the analysis of mutations in HCV-1b.

Regarding to the development of HCC, HCV-1b has been suggested to be strongly associated with hepatocarcinogenesis (25). Although the wild HCV-1b type might play a more important carcinogenic role than the mutant type, our result does not support such possibility. To clarify our results, a larger scale study should be performed.

ACKNOWLEDGEMENT

This study was supported by Sungyi Foundation of The Catholic University of Korea for medical research.

REFERENCES

1. Choo QL, Richman KH, Han JH, Berger K, Lee C, Dong C, Gallegos C, Coit D, Medina-Selby R, Barr PJ. *Genetic organization and diversity of the hepatitis C virus. Proc Natl Acad Sci USA 1991; 88: 2451-5.*
2. 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.*
3. Brechot C. *Hepatitis C virus: molecular biology and genetic variability. Dig Dis Sci 1996; 41: 6S-21S.*
4. Seeff LB. *Natural history of hepatitis C. Hepatology 1997; 26: 21S-8S.*
5. Muller R. *The natural history of hepatitis C: clinical experiences. J Hepatol 1996; 24: 52-4.*
6. Ahmed A, Keeffe EB. *Treatment strategies for chronic hepatitis C: update since the 1997 National Institutes of Health Consensus Development Conference. J Gastroenterol Hepatol 1999; 14(suppl): S12-8.*
7. Martinot-Peignoux M, Marcellin P, Pouteau M, Castelnau C, Boyer N, Poliquin M, Degott C, Descombes I, Le Breton V, Milotova V. *Pretreatment serum hepatitis C virus RNA levels and hepatitis C virus genotype are the main and independent prognostic factors of sustained response to interferon alfa therapy in chronic hepatitis C. Hepatology 1995; 22: 1050-6.*
8. Tsubota A, Chayama K, Ikeda K, Yasuji A, Koida I, Saitoh S, Hashimoto M, Iwasaki S, Kobayashi M, Hiromitsu K. *Factors predictive of response to interferon-alpha therapy in hepatitis C virus infection. Hepatology 1994; 19: 1088-94.*
9. Bell H, Hellum K, Harthug S, Maeland A, Ritland S, Myrvang B, von der Lippe B, Raknerud N, Skaug K, Gutigard BG, Skjaerven R, Prescott LE, Simmonds P. *Genotype, viral load and age as independent predictors of treatment outcome of interferon-alpha 2a treatment in patients with chronic hepatitis C. Construct group. Scand J Infect Dis 1997; 29: 17-22.*
10. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Izumi N, Marumo F, Sato C. *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.*
11. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C. *Mutations in the nonstructural protein 5A gene and response to*

interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 1996; 334: 77-81.

12. Khorsi H, Castelain S, Wyseur A, Izopet J, Canva V, Rombout A, Capron D, Capron JP, Lunel F, Stuyver L, Duverlie G. *Mutations of hepatitis C virus 1b NS5A 2209-2248 amino acid sequence do not predict the response to recombinant interferon-alfa therapy in French patients. J Hepatol 1997; 27: 72-7.*
13. Zeuzem S, Lee JH, Roth WK. *Mutations in the nonstructural 5A gene of European hepatitis C virus isolates and response to interferon alfa. Hepatology 1997; 25: 740-4.*
14. Squadrito G, Leone F, Sartori M, Nalpas B, Berthelot P, Raimondo G, Pol S, Brechot C. *Mutations in the nonstructural 5A region of hepatitis C virus and response of chronic hepatitis C to interferon alfa. Gastroenterology 1997; 113: 567-72.*
15. Chomczynski P, Sacchi N. *Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162: 156-9.*
16. Kurosaki M, Enomoto N, Murakami T, Sakuma I, Asahina Y, Yamamoto C, Ikeda T, Tozuka S, Izumi N, Marumo F, Sato C. *Analysis of genotypes and amino acid residues 2209 to 2248 of the NS5A region of hepatitis C virus in relation to the response to interferon-beta therapy. Hepatology 1997; 25: 750-3.*
17. Kato N, Ootsuyama Y, Ohkoshi S, Nakazawa T, Mori S, Hijikata M, Shimotohno K. *Distribution of plural HCV types in Japan. Biochem Biophys Res Commun 1991; 181: 279-85.*
18. Mori S, Kato N, Yagyu A, Tanaka T, Ikeda Y, Petchclai B, Chiewsilp P, Kurimura T, Shimotohno K. *A new type of hepatitis C virus in patients in Thailand. Biochem Biophys Res Commun 1992; 183: 334-42.*
19. Gale M Jr, Blakely CM, Kwieciszewski B, Tan SL, Dossett M, Tang NM, Korth MJ, Polyak SJ, Gretch DR, Katze MG. *Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: molecular mechanisms of kinase regulation. Mol Cell Biol 1998; 18: 5208-18.*
20. Chayama K, Tsubota A, Kobayashi M, Okamoto K, Hashimoto M, Miyano Y, Koike H, Kobayashi M, Koida I, Arase Y, Saitoh S, Suzuki Y, Murashima N, Ikeda K, Kumada H. *Pretreatment virus load and multiple amino acid substitutions in the interferon sensitivity-determining region predict the outcome of interferon treatment in patients with chronic genotype 1b hepatitis C virus infection. Hepatology 1997; 25: 745-9.*
21. Tong MJ, Reddy KR, Lee WM, Pockros PJ, Hoefs JC, Keeffe EB, Hollinger FB, Hathcote EJ, White H, Foust RT, Jensen DM, Krawitt EL, Fromm H, Black M, Blatt LM, Klein M, Lubina J. *Treatment of chronic hepatitis C with consensus interferon: a multicenter, randomized, controlled trial. Consensus Interferon Study Group. Hepatology 1997; 26: 747-54.*
22. Gale MJ Jr, Korth MJ, Tang NM, Tan SL, Hopkins DA, Dever TE, Polyak SJ, Gretch DR, Katze MG. *Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. Virology 1997; 230: 217-27.*
23. Gale MJ Jr, Korth MJ, Katze MG. *Repression of the PKR protein kinase by the hepatitis C virus NS5A protein: a potential mechanism of interferon resistance. Clin Diagn Virol 1998; 10: 157-62.*
24. Komatsu H, Fujisawa T, Inui A, Miyagawa Y, Onoue M. *Mutations in the nonstructural protein 5A gene and response to interferon therapy in young patients with chronic hepatitis C virus 1b infection. J Med Virol 1997; 53: 361-5.*
25. Romeo R, Rumi MG, Del Ninno E, Colombo M. *Hepatitis C virus genotype 1b and risk of hepatocellular carcinoma. Hepatology 1997; 26: 1077.*