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The Prediction of Interferon-α Therapeutic Effect by Sequence Variation of the HCV Hypervariable Region 1

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Abstract

Interferon-α (IFN-α) has been used to treat hepatitis C virus (HCV)-induced hepatitis, but it has been effective in only about half of the treated patients, with recurrence appearing in the other half. As a consequence of the possible complications associated with IFN-α and the high cost of treatment, it has become extremely important to select the proper patients for IFN-α treatment. In our previous study, we found that the quasispecies in the hypervariable region (HVR) 1 of HCV were various and that a new quasispecies can appear in non-responders and/or lead to deterioration in the patients' condition. The preliminary data we obtained in the process of our previous research led us to believe that the quasispecies of HVR 1 has something to do with the effect of IFN-α. Thus, in this investigation, we tried to determine the predictive factors of IFN-α therapy. Thirty patients with HCV infection were treated with IFN-α. Among them, 15 patients recovered after six months IFN-α treatment, but the remaining 15 patients showed no response after six months IFN-α treatment. We cloned HVR 1 DNA by reverse transcription-polymerase chain reaction (RT-PCR) and examined the quasispecies of HVR 1. As the quasispecies of HVR 1 in non-responders varied more than in the complete remission group, we concluded that the sequence variation in HVR 1 of HCV can be used to predict the effect of IFN-α.

Key Words: Hepatitis C virus, hypervariable region, quasispecies, interferon

INTRODUCTION

Hepatitis C virus (HCV) is the first virus to be verified not by classical biological method but by molecular biological technique. Its identification was initially made by cloning cDNA from HCV RNA in the plasma of a chimpanzee with chronic non-A, non-B hepatitis.[1,2] Acute post-transfusion hepatitis due to HCV-caused chronic hepatitis in more than 50% of the cases over a one-year period, while cirrhosis occurred in about 20% of HCV-infected patients after 10 to 20 years.[3,4] Moreover, it is also known that HCV is related to the pathogenesis of hepatocellular carcinoma.[5-9]

Interferon-α (IFN-α) has been used to treat HCV-induced hepatitis, but it has been effective in only about one-half of patients.[10,11] In addition, interferon is difficult to use, its treatment is costly, and it carries the possibility of complications. As a result, much research has gone into determining the predictive factors of interferon therapy. It has been reported that differing responses to IFN-α treatment in HCV infection may be influenced by the HCV subtype,[12,13] by HCV RNA titer at the beginning of IFN-α therapy,[14,15] by the complexity of quasispecies in the hypervariable region (HVR) 1,[16-18] or by the sequences of the interferon sensitivity determining region (ISDR) of the nonstructural 5A (NS5A).[19,20] By contrast, other reports have shown quite the opposite results concerning the complexity of quasispecies[21] and ISDR sequences.[22-25]

Among the factors mentioned above, HVR 1, in which many mutations are frequently found, corresponds with 27 amino acid residues located at the

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amino terminus of envelope gene 2 and nonstructural gene 1 (E2/NS1). It has been proposed that the mutation at HVR 1 is an adaptive process to escape from the host immune system to maintain continuous propagation,[26,27] and that the appearance of the sequence variation in HVR 1 is related to the therapeutic response to IFN-α.[28] However, since these earlier research studies were accomplished using an indirect technique, that is, using single-strand conformational polymorphism (SSCP) or by using a small number of samples, they could only show the tendency in the relationship between HVR 1 quasispecies and the effect of IFN-α.

In this study, we determined the quasispecies of HVR 1 in HCV found in pretreatment serum samples taken from HCV-infected patients who had received a 6-month course of IFN-α therapy. The relationship between the quasispecies and therapeutic results was analyzed.

MATERIALS AND METHODS

Specimens

Serum samples were collected from HCV-infected patients who had been admitted to Severance Hospital in Seoul, Korea. Sera were screened for anti-HCV antibody with enzyme immunoassay (EIA) and were confirmed with recombinant immunoblot assay (RIBA, Lucky Biotech., Chungwon, Korea). Histological and clinical findings indicated that all of the patients had chronic hepatitis.

Classification of patients according to IFN-α response

All patients were treated using IFN-α (Intermax α®, Lucky Biotech., Taejon, Korea) with 6 million units daily for one week, and then three times a week for 6 months. After 6 months IFN-α therapy, we classified patients into two groups: i) the IFN-α responder group in which the ALT (alanine aminotransferase) level became normal, and ii) the non-responder group in which the ALT level was above normal during IFN-α therapy and/or the ALT level was normal during IFN-α therapy and then became elevated again during IFN-α therapy. Each group was comprised of 15 patients and they were selected on the basis of genotypes after IFN-α therapy.

All sera were prepared in sterilized conditions and were stored at $-70^{\circ}C$ until use.

Preparation of HCV cDNA

HCV RNA was extracted from serum with the modified method described by Poel et al.[29] After ethanol precipitation, each RNA pellet was dissolved in 10 μl of DEPC (diethylpyrocarbonate)-treated distilled water and used for cDNA preparation.

cDNA synthesis was performed using the Reverse Transcription System (Promega Co., Madison, WI, USA) and oligo-d(T) primers were used for this process.

Polymerase chain reaction (PCR)

The primer sequences for HCV typing and HVR are listed in Table 1. These primers were synthesized by 381A DNA synthesizer (Applied Biosystem, Foster City, CA, USA).

PCR amplification was performed in 50 μl of reaction volume containing 10 mM Tris-HCl pH 8.3, 50 mM potassium chloride, 2.5 mM magnesium chloride, 15 pmole of each primer, 100 μM of each deoxyribonucleotide triphosphates, 0.01% gelatin and 1.5 units of *Taq* DNA polymerase with an overlay of mineral oil. The reaction was carried out using the modified method described by Saiki et al.[30] in a Mini Cycler™ (MJ Research Inc., Watertown, MA, USA). Each reaction cycle included denaturation at 94°C for 30 sec, primer annealing at 55°C for 30 sec and primer extension at 72°C for 30 sec. The first PCR was performed for 30 cycles with outer primers and 1 μl of the PCR products was used as a template for the second PCR of 30 cycles using inner primers. For genotyping of HCV, a mixture of type-specific primers was used in PCR using the procedure of Okamoto et al.,[31] and the subtypes were classified according to the method of Simmonds et al..[32]

PCR products were subjected to agarose gel electrophoresis in Tris-acetate-EDTA buffer and visualized in the presence of ethidium bromide under ultraviolet transillumination.

DNA sequencing

Amplified cDNAs of HVR in HCV were isolated

Table 1. Nucleotide Sequences of Primers

Region	Primer direction	Sequence (5' to 3')	Nucleotide No.*
Core†	outer sense (1a-2b)	CGCGCGACTAGGAAGACTTC	480→499
	(3a)	CGCGCGACGCGTAAAACTTC	480→499
	outer antisense	ATGTACCCCATGAGGTCGGC	781→762
	inner sense (1a-2b)	AGGAAGACTTCCGAGCGGTC	489→508
	(3a)	CGTAAAACTTCTGAACGGTC	489→508
	inner antisense (1a)	GGATAGGCTGACGTCTACCT	537→518
	(1b)	GAGCCATCCTGCCCACCCCA	632→613
	(2a)	CCAAGAGGGACGGGAACCTC	662→643
	(2b)	ACCTCGTTTCCGTACAGAG	611→592
	(3a)	GCTGAGCCCAGGACCGGTCT	576→557
HVR	outer sense	CACCGCATGGCTTGGGATATGATG	1331→1354
	outer antisense	CAACAGGGCTTGGGGTGAAGCA	1927→1906
	inner sense	ATGGCTTGGGATATGATGATGAAC	1336→1359
	inner antisense	AAGCAGTCGACTGGACCACACAC	1910→1888

* The nucleotide No. indicates the nucleotide sequence number.
† Core region primers for HCV typing are the same as those of Okamoto et al.[31] and classified HCV subtypes according to the method of Simmonds et al.[32]

Table 2. Characteristics of HCV before IFN-α Treatment

		Sex/Age	Subtype	ALT	No. of quasispecies			Sex/Age	Subtype	ALT	No. of quasispecies
A	1	M/24	1a	133	2	B	1	F/51	1a	191	5
	2	M/27	1b	232	2		2	M/55	1b	295	6
	3	F/56	1b	70	2		3	M/53	1b	45	6
	4	M/26	1b	232	3		4	M/67	1b	362	7
	5	M/61	1b	156	3		5	M/44	1b	126	6
	6	F/58	1b	130	3		6	F/59	1b	209	6
	7	F/64	1b	75	3		7	F/60	1b	35	7
	8	M/66	1b	85	4		8	M/63	1b	332	7
	9	M/31	1b	553	3		9	M/65	1b	79	6
	10	F/42	1b	99	1		10	M/34	1b	74	7
	11	M/36	1b	198	3		11	F/62	1b	156	4
	12	M/54	2a	174	3		12	F/62	2a	108	5
	13	M/52	2a	126	2		13	M/56	2a	77	7
	14	F/58	2a	172	2		14	F/65	2a	76	7
	15	F/35	1a,1b,3a	75	5		15	M/48	1a,1b,2b	168	8

All samples were diagnosed by enzyme immunoassay (EIA) and recombinant immunoblot assay (RIBA). They were confirmed to be chronic hepatitis by pathology before IFN-α treatment.
A: complete remission group after 6-month IFN-α treatment.
B: no response group after 6-month IFN-α treatment.

from agarose gel and purified with DNA PrepMate™ (Bioneer, Korea). They were then subcloned by inserting the cDNA into a pMOSBlue T-vector (Amersham Pharmacia Biotech., Uppsala, Sweden). Plasmid containing cDNA of HVR of HCV were selected by α-complementation.

Ten clones from each patient's plates were randomly selected and then plasmid prepared from each clone was used as a template for DNA sequencing. DNA sequences were determined by using the mod-

ified method of Sanger's dideoxy method.[33] A T7 Sequencing kit (Amersham Pharmacia Biotech., Sweden) was used for this process.

Statistical analysis

The Student's t-test was performed to compare results between the two groups. We determined the p-values between the two groups with regard to age, ALT, and the number of quasispecies of HVR 1.

RESULTS

Characteristics of patients

We divided A group as the IFN-α responder group and B group as the IFN-α non-responder group. Both groups were composed of 15 patients (9 males and 6 females), respectively.

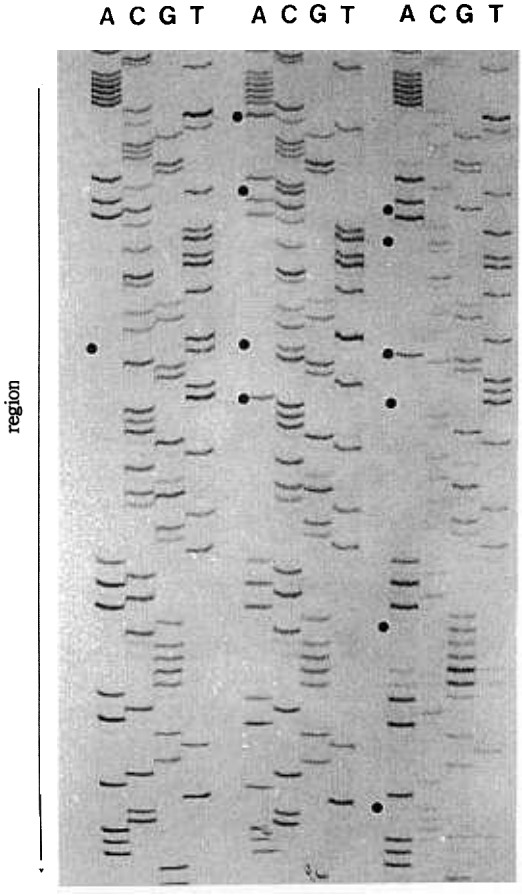

Fig. 1. Nucleotide sequences of the cDNA fragments corresponding to HVR 1 of HCV. The bold-face marks "●" indicated the mutated sequences (This figure is taken from the Archives of Thesis in Medical Science, YUMC Vol(2) p258, 1994.).

Classification of HCV genotypes in patients

When the type-specific PCR amplification of the core region of HCV was performed, four different sizes of PCR products were observed. Among the A group samples, the number of patients with types 1a, 1b, and 2a were 1, 10, and 3, respectively, and 1 sample was a mixed type of 1a, 1b, and 3a. The B group samples showed the same results except for 1 sample, which was a mixed type of 1a, 1b, and 2b (Table 2).

Identication of HVR 1 nucleotide sequences in HCV cDNA

The sequence diversity of HVR 1 of HCV in the same patient's serum (No. 2 in Fig. 3) is shown in Fig. 1. Nucleotide sequences of HVR 1 of HCV cDNA in 10 cloned plasmid DNA samples obtained from one individual serum donor in group A and are from group B are shown in Fig. 2 and 3.

Each of 10 cloned cDNA of HCV among the 15 group A patients showed from 1 to 5 different amino acid sequences at the beginning of the experiment (Fig. 2), while those among group B patients showed from 4 to 8 amino acid sequences (Fig. 3). As the sample number for each subtype was not high, we ignored the differences between subtypes.

Statistical analysis

Both groups were composed of 9 males and 6 females. Student's t-test was performed to compare the results between the 2 groups.

In age, group A was 46.0 ± 15.0 and Group B was 56.3 ± 9.1. ALT levels were 167.3 ± 119.7 and 155.5 ± 104.3, respectively. Age and ALT were not statistically different in p-value < 0.05 between the 2 groups.

The number of quasispecies of HVR 1 were 2.73 ± 0.96 in group A and 6.27 ± 1.03 in goup B, which showed a statistically significant difference between groups.

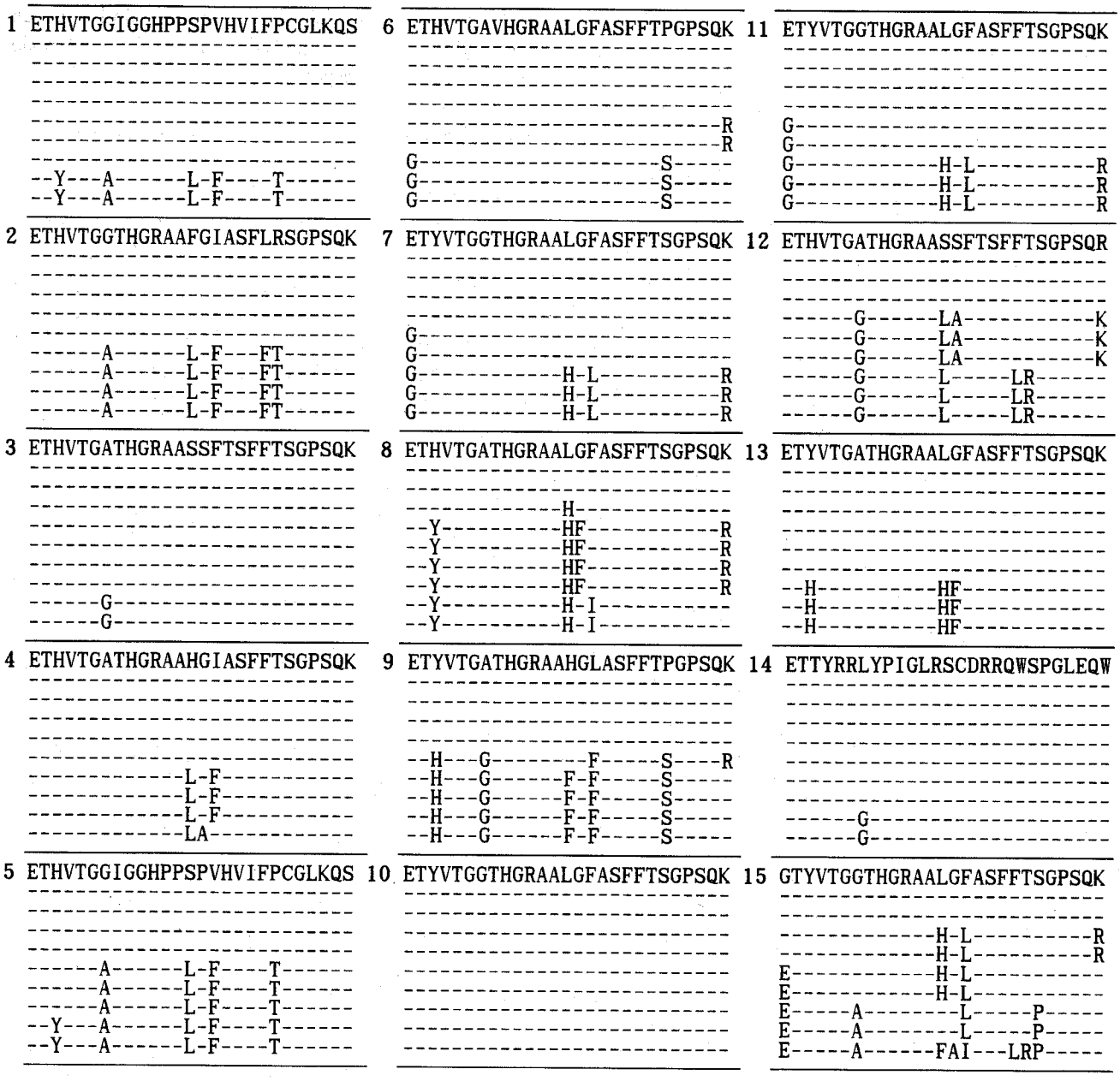

Fig. 2. HVR sequence diversity of HCV. These 15 patients showed a response during 6-month IFN-α treatment.

DISCUSSION

HCV, like other RNA viruses, tends to produce mutated forms due to the incomplete ability of its RNA replication. Therefore, HCV with various sequences exists in individual subjects during the same period of time, and theses are defined as quasispecies.[34] The highest variability in HCV sequences appears in 27 amino acid residues in the N-terminal part of E2/NS1, which is known as HVR 1.[35] It has been reported that HVR 1 is important in its clinical implications because the variability of HVR 1 enables the HCV to escape from the host's immune system,[36] and that the incidence of the newly mutated sequences of HVR 1 is related to the effect of IFN-α[28] and the resistance to antiviral therapy.[16,17]

Recently, trials on interferon and ribavirin in HCV-infected and relapsed patients have been performed,

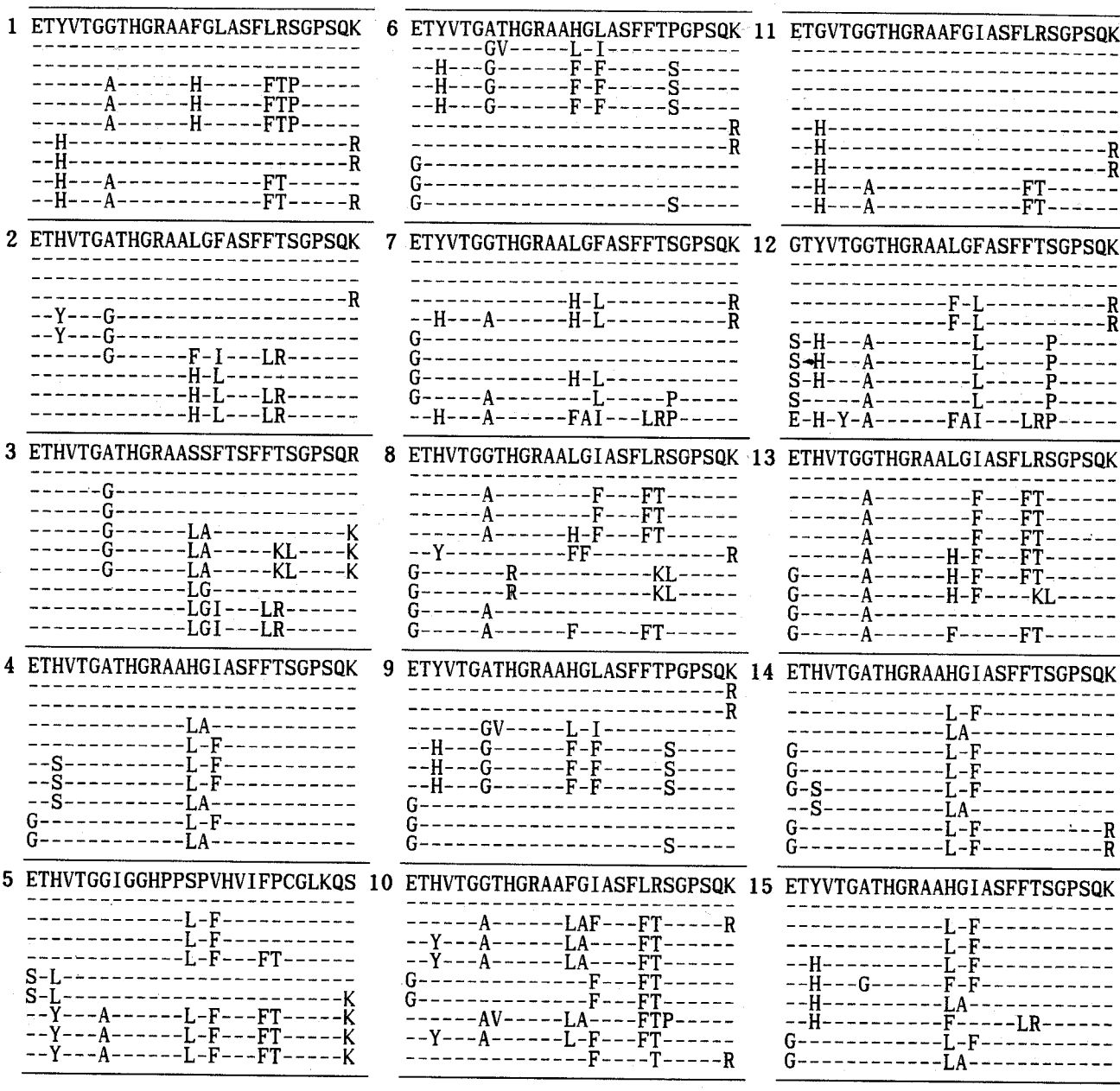

Fig. 3. HVR sequence diversity of HCV. These 15 patients showed no response during 6-month IFN-α treatment.

and as a result interferon is regarded as the drug of choice for HCV treatment. However, interferon is inconvenient to use and has the possibility of causing complications. In particular, interferon is so costly that its use in HCV-infected patients could not be universal. Many researchers have tried to determine the predictive factors for interferon therapy, and some have been suggested. HCV RNA titer, HCV subtypes, ALT levels, as well as nucleotide sequences of ISDR or HVR 1 have been proposed as predictive markers, but most of them are still controversial.[12-25] In our previous study, we found that the IFN-α responder group tended to have less quasispecies than the IFN-α non-responder group,[28] and we hypothesized that HVR 1 quasispecies could be a predictive factor for IFN-α therapy.

In this study, HCV-infected patients who underwent IFN-α therapy were divided into two groups:

Table 3. Age, ALT, and No. of Quasispecies of HVR1

Group	No.	M : F	Age	ALT	No. of quasispecies
A	15	9 : 6	46.0±15.0	167.3±119.7	2.73±0.96*
B	15	9 : 6	56.3±9.1	155.5±104.3	6.27±1.03*

A: complete remission group after 6-month IFN-α treatment.
B: no response group after 6-month IFN-α treatment.
The data of age, ALT, and No. of quasispecies are shown in average±S.D values.
* means statistically different in $p<0.01$.

IFN-α responder and IFN-α non-responder (Table 2). We observed that IFN-α was effective only in about 50% of HCV-infected patients, and this result was very similar to earlier reports.[3,4,37-39] Normalization of the ALT level in the IFN-α responder group appeared after 2 to 5 months of IFN-α therapy. However, among the IFN-α non-responder group, the ALT level was also normalized, but it was reelevated during treatment in two patients.

Our previous data showed that sex, age, and ALT level had no correlation between the IFN-α responder and IFN-α non-responder groups, but a statistical difference was seen in the number of quasispecies of HVR 1 between the two groups (Table 3). These results suggested that the number of quasispecies of HVR 1 can be used to predict the IFN-α therapeutic effect in HCV-infected patients. There have been several reports which examined the relationship between HVR 1 quasispecies and the effect of interferon. Most of them examined a small number of samples,[27,40] or used an indirect method such as SSCP,[18] in which they were unable to obtain statistically significant data.

Recently, the mutation at HVR 1 has been suggested to be an adaptive process by which HCV escapes from the host immune system for its continuous propagation.[26,27] It is probable that fewer quasispecies in infected HCV is a reflection on the beginning of adaptation to a host environment, that more quasispecies in HCV is a reflection on the completion of the adaptive process, and that consequently IFN-α is more effective in patients with fewer quasispecies of HVR 1. In this study, we performed DNA sequencing for HVR on 10 cloned HCV cDNA obtained from the patient sera of each IFN-α responder and IFN-α non-responder. Though we couldn't confirm all kinds of quasispecies, 10 clones seemed sufficient to determine the relationship between the effect of IFN-α and quasispecies.

Although many researchers have tried to find the predictive factors for IFN-α therapy in treating HCV-infected patients, many discrepancies still remain on the recommendation of certain predictive factors. However, HVR 1 quasispecies applied in this investigation were not controversial as a predictive factor for IFN-α therapy as demonstrated by statistical analysis. Therefore, we concluded that determination of the quasispecies of HVR 1 before interferon therapy may be useful in predicting the effect of IFN-α.

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