Expression of IFNAR2 mRNA in peripheral blood mononuclear cells of patients with HCV infection

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Abstract

Background : **To investigate the differences of interferon-α/β receptor 2 (IFNAR2) mRNA expression level in peripheral blood mononuclear cells (PBMCs) between different stages of hepatitis C virus (HCV) infection and to determine the correlation with the effectiveness of interferon therapy.**

Methodology : **58 patients, positive for anti-HCV antibodies, were divided into three groups depending on their clinical symptoms : acute hepatitis (4 cases), chronic hepatitis (46 cases) and liver cirrhosis (8 cases). 15 volunteers served as healthy controls. PBMCs were purified by density gradient centrifugation and IFNAR2 mRNA was amplified from these cells by a reverse transcription-polymerase chain reaction (RT-PCR) assay.**

Results : **The detection rate of IFNAR2 mRNA was 87.9% (51/58 cases) in the PBMCs of patients with HCV infection, significantly higher than that in the control group (33.3%, 5/15 cases ; $P < 0.05$). While the positive rate was 93.5% (43/46 cases) in the chronic hepatitis group, which was significantly higher than that in the liver cirrhosis group (50%, 4/8 cases ; $P < 0.05$). Furthermore, there is no significant difference in the positive rates for HCV-RNA of PBMCs among groups ($P > 0.05$). The positive rate of IFNAR2 mRNA in PBMCs was not correlated with the viral load of HCV-RNA in serum ($P > 0.05$). However, higher expression of IFNAR2 mRNA in the PBMCs did correlate with the effectiveness of interferon therapy ($P < 0.05$).**

Conclusions : **HCV infection up-regulates the expression of IFNAR2 mRNA in PBMCs. IFNAR2 mRNA expression in the chronic hepatitis group was higher than that in the liver cirrhosis group, and significantly correlated with the effectiveness of interferon therapy, which was independent of the viral load.** (Acta gastroenterol. belg., **2012**, 75, **228-233**).

Key words : hepatitis C virus infection ; peripheral blood mononuclear cells ; interferon receptor 2.

Introduction

Hepatitis C virus (HCV) infection occurs worldwide, and in more than 50-80% of patients the infection develops into chronic hepatitis, hepatic cirrhosis, or even hepatocellular carcinoma (1). Interferon (IFN) alone, or in combination with ribavirin, is currently the only approved treatment for HCV infection. When patients are treated with IFN-based therapy, achieving serum HCV-RNA negativity by week 12 (known as the early viral response, EVR) is an important predictor of a sustained virologic response. The factors that correlate with the effectiveness of IFN therapy can be mainly classified into viral factors (such as viral load, viral genotype, quasispecies) (2-5) and host factors (such as epidemiological factors including the patient's age, sex and race, liver histological damage and the immune response) (6-8). The activity of IFN is mediated by its high-affinity binding to specific cellular IFN receptors, which subsequently induce antiviral proteins. Type I IFN receptor is the most common receptor of IFN-α and IFN-β, containing at least two subunits, IFNAR1 (IFN-α receptor) and IFNAR2 (IFN-α/β receptor). IFNAR2 has been reported to play an important role in determining the IFN response and the clinical phenotype of HBV infection in the Chinese Han population (9). Some studies have also suggested that HCV patients positive for the expression of IFN receptor in hepatic tissue showed better responses to IFN therapy (10-13).

Peripheral blood mononuclear cells (PBMCs) are sites of HCV contamination, but little is known about the expression of IFN receptor in PBMCs. The detection rate of IFNAR2 mRNA was found to be higher than that of IFNAR1 mRNA in PBMCs. Yamaguchi *et al.* (14) demonstrated that the level of IFNAR2 mRNA in PBMCs correlated with that in hepatic tissue, but this was not the case for IFNAR1 mRNA. Ishii *et al.* reported that IFNAR2 in the PBMCs of patients with a high viral load of HCV genotype 1, harbored a substitution of amino acid 70 in the core region (15). In the present study, we investigated the relationship between the expression of IFNAR2 mRNA in PBMCs and different stages of HCV infection. We further explored whether IFNAR2 expression levels in PBMCs could be used to replace IFNAR2 expression levels in the liver, acting as markers for predicting the response to IFN therapy in chronic hepatitis C patients.

Materials and methods

Patients

From November 9, 2005 to December 31, 2006, a total of 58 interferon-naive patients with positive anti-HCV antibodies (an ELISA test using the third generation of the anti-HCV antibody test kit, Sino American

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Biotechnology, China) and positive HCV-RNA (≥ 1 × 10^3 copies/ml) were enrolled in the first step study. 54 patients were HCV antibody-positive more than 6 months (including 8 cases with liver cirrhosis). 4 patients were HCV antibody-negative 6 months before and became positive now. Liver biopsy was not mandatory. Patients were excluded for the following reasons : decompensated cirrhosis ; positive serum hepatitis B surface antigen, HIV coinfection ; neutrophilcount < 1500 cells/mm^3 ; platelet count < 80000 cells/mm^3 ; haemoglobin levels < 11 g/dl ; serum creatinin elevels > 1.5 mg/dl ; active alcohol or drug dependence ; pregnancy or lactation. None of the patients had any other cause of liver disease. Examination of ALT levels (an automatic biochemicsl analyzer, Hitachi, Tokyo, Japan), anti-HCV antibody levels and IFNAR2 mRNA levels in PBMCs, and an ultrasound of the liver, were performed on all patients. In the first step study, the patients were classified into three groups : acute hepatitis group (four cases), chronic hepatitis group (46 cases) and liver cirrhosis group (eight cases, classified as Child-Pugh A, without evidence of decompensation and with normal or nearly normal liver function), according to the criteria for management of hepatitis C in the National Institute of Health Consensus Development Conference Statement published in 2002 (16). 15 volunteers with no hepatitis virus infection served as a healthy controls. The serum HCV-RNA load and the ALT levels of patients were determined. To eliminate the effect of HCV genotype, only 32 genotype 1 chronic HCV patients were enrolled in the second step study (interferon in combination with ribavirin treatment, recombinant human interferon alpha-2b (IFN-α2b), 300 MU by means of a subcutaneous injection, once every other day ; ribavirin 1000 mg/day). During the period of treatment, patients who had detectable serum HCV-RNA after 24 weeks were considered non-responders (NR), while had undetectable (< 50 IU/ml)) serum HCV-RNA after 12 weeks were considered complete responders (CR), and had detectable serum HCV-RNA after 12 weeks and undetectable serum HCV-RNA after 24 weeks were considered partial responders (PR). The written informed consent was obtained from each patient. Our study was in accordance with ethical standards for human experimentation and was approved by the Ethics Committee of China Medical University.

Isolation of PBMCs

PBMCs were isolated from 4 ml of heparinized blood by density gradient centrifugation. Briefly, 4 ml of blood was diluted to 8 ml with saline, and then divided equally into two cuvettes. Then, 2 ml of lymphocyte separation medium (Shanghai Hengxin, China) was added and the diluted blood was layered onto the separation medium slowly, and centrifuged at 900 g. After 30-min incubation at room temperature, PBMCs in the mesosphere were collected with a capillary pipette and diluted to 10 ml in another centrifuge tube. The cells were then centrifuged twice, with 10-min incubation steps at room temperature following each centrifugation. The supernatant was then removed and the PBMCs were stored at -70°C prior to RNA extraction.

Reverse transcription-polymerase chain reaction (RT-PCR) for the detection of IFNAR2 mRNA

RNA was extracted from PBMCs using the guanidine isothiocyanate-phenol-chloroform method (17). This is involved initial denaturation at 95°C for 5 minutes, followed by reverse transcription at 37°C for 30 minutes. The PCR conditions were 35 cycles of denaturation at 94°C for 45s, annealing at 56°C for 45s and extension at 72°C for 60s. The extension step of the last cycle was extended to 7 minutes. GAPDH mRNA was used as an internal control. IFNAR2 mRNA was amplified using the primers : forward : 5´-GCTTTTGAGCCAGAATGCCT-3´, and reverse : 5´-CCCTCTGACTGTTCTTCAATG-3´, and the expected product size was 525 bp. GAPDH mRNA was amplified using the primers : forward : 5´-ATTCAAGGCACCGTCAAGG-3´, and reverse : 5´-GGGCCATCGATAGTCTTCTG-3´, and the expected product size was 408 bp. Amplification products were separated by 2% agarose gel electrophoresis and stained with ethidium bromide for visualization. The results were observed under 302-nm UV light, and analyzed with a 1D gel electrophoresis image analysis system (Kodak, USA). The expression level of IFNAR2 mRNA = light intensity value of IFNAR2 mRNA/each light intensity value of GAPDH mRNA.

Determination of PBMCs HCV-RNA, serum HCV-RNA load and HCV genotype

Line gene fluorescent quantitative polymerase chain reaction amplicor was used to detect the serum HCV-RNA titer (Bioer Technology, China). PBMCs HCV-RNA was detected using the nested RT-PCR detection kit for HCV (Hua Mei Biotechnology, China. The expected product size was 225 bp and the results were marked as "positive" or "negative"), according to the manufacturer's instructions. 100 µl of serum from patients was used to determine the HCV genotype, using the Bayer HCV kits and the Bayer Lipa assay.

Therapeutic effect assessment

Patients were divided into complete responders (CR), partial responders (PR) and non-responders (NR), based on their ALT level and HCV-RNA titer in response to IFN therapy.

Statistical analysis

All data were expressed as the mean ± SD and were analyzed using the SPSS 11.0 software. Differences in the expression rate of IFNAR2 mRNA between different groups were assessed using the χ^2 test. Differences in the mean values were assessed by analysis of variance, and

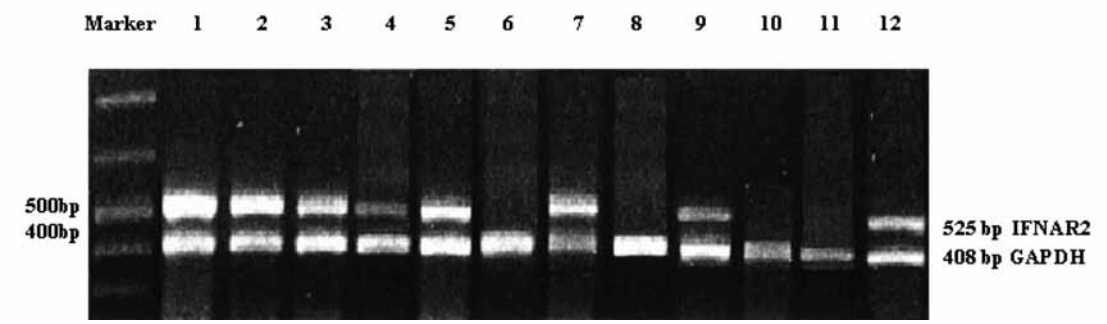

Fig. 1. — The expression of PBMC IFNAR2 mRNA in the three groups with different stages of HCV infection.
RT-PCR of the IFNAR2 gene. The GAPDH gene was amplified as a control. Two patients with acute hepatitis had the highest positive detection rate and the highest expression level of PBMC IFNAR2 mRNA. Six patients with chronic hepatitis, had higher positive detection rates and expression levels of PBMC IFNAR2 mRNA, and five patients with hepatic cirrhosis, had the lowest positive detection rates and expression levels of PBMC IFNAR2 mRNA. Lanes 1, 2 : amplification of the RNA isolated from two patients with acute hepatitis ; lanes 3-7 : amplification of the RNA isolated from five patients with chronic hepatitis ; lanes 8-12 : amplification of the RNA from five patients with hepatic cirrhosis.

correlations were determined using Pearson's correlation coefficient. $P < 0.05$ was considered statistically significant.

Results

Demographics

No pregnant woman or children were enrolled in the study. 41 males and 17 females ; The median age of the patients was 43.3 ± 15.7years (range, 28-60 years). All of the four acute hepatitis patients are genotype 2 HCV-infected. There are 32 genotype 1 and 14 genotype 2 HCV-infected patients in chronic hepatitis group. All of the eight cirrhosis patients are genotype 1 hepatitis C virus (HCV)-infected. In our second step study, only 28 among 32 genotype 1 chronic HCV patients completed anti-viral treatment for 6 months. 12 patients are CR, 8 patients are PR, and 8 patients are NR. The morbidity of low body mass index (BMI) of patients was calculated. 14 of the 16 patients (87.5%) were aged between 33 and 59 years. There was just 1 patient (6.25%) over 60 years old and one under 30 years old. The male to female ratio was 1.67 to 1 (10:6). There is no difference in serum alanie aminotransferase and BMI among NR, PR, and CR patients.

Expression of IFNAR2 mRNA in PBMCs

The positive detection rate of IFNAR2 mRNA in the PBMCs of patients with HCV infection was 87.9% (51/58), which was significantly higher than that of the healthy control subjects (33.3%, 5/15 ; $P < 0.05$). Among the patients infected with HCV, there was no significant difference in the rate and level of PBMC IFNAR2 mRNA between males and females (90.2% (37/41) vs. 82.4% (14/17), $P > 0.05$; 0.7435 ± 0.3597 vs. 0.6771 ± 0.3480, $P > 0.05$). The expression of PBMC IFNAR2 mRNA did differ between different stages of HCV infection. The positive detection rate of PBMC IFNAR2 mRNA in the acute hepatitis group was the highest among the three groups. In the chronic hepatitis group, the expression of PBMC IFNAR2 mRNA was significantly higher than that observed in the liver cirrhosis group ($P < 0.05$) (Fig. 1, Table 1).

The relationship between expression of PBMC IFNAR2 mRNA and viral factors

The positive rate of PBMC HCV-RNA is 74.14% (43/58). The level of IFNAR2 mRNA expression were also not significantly different between patients with positive PBMC HCV-RNA (0.6999 ± 0.3459) and patients with negative PBMC HCV-RNA (0.7930 ± 0.3809, $P = 0.598$). The expression of PBMC IFNAR2 mRNA was also not correlated with serum HCV-RNA load prior to treatment ($r = 0.043$, $P > 0.05$). The serum HCV-RNA load prior to treatment were also not significantly different between patients with positive PBMC HCV-RNA and patients with negative PBMC HCV-RNA ($P = 0.252$).

The relationship between expression of PBMC IFNAR2 mRNA, PBMC HCVRNA , serum HCVRNA and the effect of IFN therapy

Among the patients with chronic hepatitis C, 28 completed six months of IFN therapy. The levels of PBMC IFNAR2 mRNA were highest in the complete responders (CR) prior to treatment ($P < 0.05$). The levels of PBMC IFNAR2 mRNA in the partial responders (PR) were higher than those in the non-responders (NR) ($P < 0.05$) (Table 2). The positive rate of both PBMC HCV-RNA and the level of serum HCV-RNA were not significantly different among CR, PR, NR groups ($P = 0.802$; $P = 1.000$).

Table 1. — **The expression of PBMC IFNAR2 mRNA in different stages of HCV infection**

Group	Serum HCVRNA (copies/ml)	HCV genotype	Light intensity value of IFNAR2 mRNA/each light intensity value of GAPDH mRNA.	IFNAR2 mRNA	PBMC IFNAR2 mRNA	
					Positive detection rate	Expression value
Acute hepatitis (n = 4)	9.7×10^5	2	2515 × 233/2017 × 201	1.4456	100% (4/4)	1.0679 ± 0.3865
	7.7×10^5	2	3111 × 253/5773 × 201	0.6783		
	4.9×10^5	2	3009 × 184/2203 × 233	1.0798		
	3.2×10^6	2	3412 × 305/3372 × 289	1.0679		
Chronic hepatitis CR (n = 12)	4.5×10^5	1	4745 × 236/4603 × 232	1.0485	93.5% (43/46)	0.7465 ± 0.2982
	3.1×10^5	1	5219 × 233/5510 × 230	0.9595		
	8.7×10^6	1	4830 × 243/57362 × 49	0.8217		
	3.4×10^5	1	4523 × 243/4293 × 256	1.4490		
	7.2×10^6	1	5159 × 224/5062 × 217	1.0521		
	1.2×10^5	1	3742 × 230/3882 × 243	0.9618		
	4.5×10^5	1	4598 × 171/4937 × 186	0.8562		
	2.2×10^6	1	4495 × 215/3881 × 249	1.4410		
	4.9×10^5	1	4957 × 170/4700 × 178	1.0071		
	8.1×10^5	1	4844 × 248/3970 × 248	1.1021		
	1.6×10^5	1	3176 × 155/3251 × 165	0.9211		
	5.2×10^5	1	4113 × 164/4715 × 149	0.9601		
Chronic hepatitis PR (n = 8)	3.6×10^5	1	3458 × 172/5094 × 145	0.8052		
	7.2×10^6	1	4287 × 233/3934 × 243	1.0448		
	4.7×10^6	1	3980 × 165/4737 × 173	0.8013		
	8.4×10^6	1	4460 × 256/4511 × 241	0.6858		
	3.1×10^5	1	4287 × 213/4024 × 219	1.0362		
	4.6×10^5	1	4019 × 196/3766 × 201	1.0406		
	2.7×10^5	1	3376 × 204/3725 × 225	0.8217		
	5.5×10^5	1	5159 × 224/4890 × 233	1.0142		
Chronic hepatitis NR (n = 8)	7.3×10^6	1	3557 × 198/6260 × 213	0.5282		
	3.5×10^5	1	3382 × 245/6209 × 264	0.5055		
	4.5×10^5	1	2852 × 29/4909 × 227	0.0680		
	1.6×10^6	1	2231 × 243/4721 × 216	0.5316		
	4.8×10^5	1	2470 × 218/3810 × 282	0.5012		
	8.6×10^6	1	2443 × 176/2953 × 223	0.6543		
	3.2×10^5	1	2924 × 146/3555 × 252	0.4770		
	4.2×10^5	1	3192 × 260/5217 × 273	0.5827		
Chronic hepatitis treatment interruptions (n = 4)	3.6×10^6	1	5826 × 247/5368 × 301	0.8905		
	7.1×10^5	1	3780 × 155/4731 × 168	0.7371		
	2.9×10^6	1	NO	negative		
	1.8×10^5	1	NO	negative		
Chronic hepatitis (n = 14)	5.4×10^5	2	NO	negative		
	5.8×10^5	2	4441 × 107/4319 × 265	0.4146		
	2.9×10^6	2	3487 × 266/3033 × 302	1.0125		
	3.1×10^6	2	3145 × 198/2099 × 216	0.6798		
	2.8×10^5	2	3323 × 206/3275 × 257	0.8134		
	5.2×10^6	2	3669 × 268/6097 × 297	0.5430		
	7.0×10^6	2	2912 × 319/4207 × 248	0.8903		
	5.6×10^5	2	2817 × 105/3029 × 217	0.4485		
	8.5×10^5	2	2643 × 281/3145 × 309	0.7643		
	1.7×10^6	2	2337 × 261/2100 × 288	1.0085		
	7.7×10^5	2	2415 × 269/3929/213	0.7763		
	2.9×10^6	2	2554 × 245/3848 × 271	0.6012		
	8.8×10^5	2	2886 × 216/3650 × 298	0.5731		
	6.5×10^5	2	3001 × 240/1786 × 279	0.5069		
Cirrhosis (n = 8)	3.2×10^5	1	NO	negative	50% (4/8)*	0.4225 ± 0.4503*
	2.1×10^6	1	NO	negative		
	6.6×10^5	1	NO	negative		
	9.2×10^5	1	NO	negative		
	3.0×10^5	1	2344 × 223/2085 × 256	0.9792		
	8.3×10^6	1	4002 × 317/5003 × 341	0.7436		
	1.9×10^5	1	3621 × 361/3536 × 376	0.9831		
	4.4×10^6	1	2784 × 402/4882 × 340	0.6740		

NO : not obtained the production ; *$P < 0.05$ compared with the chronic hepatitis group.

Table 2. — **Response to IFN therapy**

Group	Serum HCV RNA (copies/ml)	Light intensity value of IFNAR2 mRNA/each light intensity value of GAPDH mRNA	IFNAR2 mRNA	PBMC IFNAR2 mRNA expression value	PBMC HCV RNA
CR (n = 12)	4.5×10^5	4745 × 236/4603 × 232	1.0485	1.0521 ± 0.2256*	+
	3.1×10^5	5219 × 233/5510 × 230	0.9595		+
	8.7×10^6	4830 × 243/5736 × 249	0.8217		+
	3.4×10^5	4523 × 243/4293 × 256	1.4490		+
	7.2×10^6	5159 × 224/5062 × 217	1.0521		+
	1.2×10^5	3742 × 230/3882 × 243	0.9618		-
	4.5×10^5	4598 × 171/4937 × 186	0.8562		-
	2.2×10^6	4495 × 215/3881 × 249	1.4410		-
	4.9×10^5	4957 × 170/4700 × 178	1.0071		+
	8.1×10^5	4844 × 248/3970 × 248	1.1021		+
	1.6×10^5	3176 × 155/3251 × 165	0.9211		+
	5.2×10^5	4113 × 164/4715 × 149	0.9601		+
PR (n = 8)	3.6×10^5	3458 × 172/5094 × 145	0.8052	0.9062 ± 0.1603*	+
	7.2×10^6	4287 × 233/3934 × 243	1.0448		+
	4.7×10^6	3980 × 165/4737 × 173	0.8013		-
	8.4×10^6	4460 × 256/4511 × 241	0.6858		+
	3.1×10^5	4287 × 213/4024 × 219	1.0362		+
	4.6×10^5	4019 × 196/3766 × 201	1.0406		+
	2.7×10^5	3376 × 204/3725 × 225	0.8217		-
	5.5×10^5	5159 × 224/4890 × 233	1.0142		+
NR (n = 8)	7.3×10^6	3557 × 198/6260 × 213	0.5282	0.4833 ± 0.2027	+
	3.5×10^5	3382 × 245/6209 × 264	0.5055		+
	4.5×10^5	2852 × 29/4909 × 227	0.0680		+
	1.6×10^6	2231 × 243/4721 × 216	0.5316		-
	4.8×10^5	2470 × 218/3810 × 282	0.5012		-
	8.6×10^6	2443 × 176/2953 × 223	0.6543		+
	3.2×10^5	2924 × 146/3555 × 252	0.4770		+
	4.2×10^5	3192 × 260/5217 × 273	0.5827		+

* $P < 0.05$ compared with the NR group.

Discussion

In the present study, both the level and positive detection rate of IFNAR2 mRNA were significantly higher in patients with HCV infection than that of the healthy controls, a result consistent with previous studies. Yatsuhashi *et al*. (18,19) demonstrated that infection with hepatitis virus up-regulated expression of the IFNAR2 gene in the liver, indicating an immune response strategy of the host to eliminate the virus. However, the exact mechanism behind increased IFNAR2 mRNA expression remains unclear, but probably cytokines such as IFN-γ that could regulate the expression of IFN receptors (20,21).

In our study, at different stages of HCV infection, the expression levels of PBMC IFNAR2 mRNA were found different. In the acute hepatitis group, the positive detection rate of PBMC IFNAR2 mRNA was 100%. Ohata *et al*. (22) showed that the level of PBMC IFNAR2 mRNA in the acute hepatitis group was higher than that in the chronic hepatitis group. Furthermore, we also observed that the positive rate of PBMC IFNAR2 mRNA in the chronic hepatitis group was significantly higher than that in the liver cirrhosis group. With the progress of disease stage, the level of PBMC IFNAR2 mRNA expression appeared to decrease. Ishimura *et al*. (23,24) previously reported that hepatic IFNAR2 mRNA expression correlated inversely with the fibrosis stage. This could explain the phenomenon that the response to IFN therapy was obviously decreased with the progress of hepatic fibrosis and further supports the importance of early treatment.

To clarify the correlation between the expression of PBMC IFNAR2 mRNA and the outcome of interferon therapy, 28 patients received anti-viral treatment. As a result, the levels of PBMC IFNAR2 mRNA detected in the complete responders (CR) and the partial responders (PR) were significantly higher than in the non-responders (NR). Therefore, we concluded that the patients with pre-therapeutic positive expression of PBMC IFNAR2 mRNA responded more positively to IFN therapy. It is well known that the genotype of HCV, the serum and PBMC loads of HCV-RNA prior to treatment are viral predictors of the IFN therapeutic outcome (25). However, our study demonstrated that the expression level of PBMC IFNAR2 mRNA did not correlate with these viral predictors, but clearly was dependent on the pathological stages of hepatic cirrhosis. HCV RNA is

probably compatible with passive adsorption or contamination, rather than viral replication in the PBMC. Hence, we infer that PBMC IFNAR2 mRNA is predictive of the response to IFN treatment. Our results are in agreement with those previously reported by Yamaguchi *et al*. (14). However, Fujiwara *et al*. (26) concluded that PBMC IFNAR2 mRNA could not replace liver IFNAR2 mRNA as a predictor of the response to IFN treatment for chronic HCV infection. Further studies are therefore required to clarify this issue by larger sample sizes.

Massirer *et al*. (27) reported that the expression of PBMC IFNAR1 mRNA was associated with the IFN response. They also found that the level of IFNAR1 mRNA was significantly reduced after three months of treatment in responders, whereas there was no change in IFNAR1 expression in non-responders during IFN therapy. Because the expression level of IFNAR2 mRNA reported by Yamaguchi *et al*. (14) was less than that of IFNAR1 mRNA in PBMCs, we proposed that future studies should focus on IFNAR2 mRNA.

Mizukoshi *et al*. (28) reported that a soluble form of IFNAR2 in serum suppressed the effectiveness of IFN. It is therefore possible that different host immune responses regulate the dynamic equilibrium between insoluble (located in the cell membrane or cytoplasm) and soluble interferon receptor.

In conclusion, HCV infection up-regulates the expression of PBMC IFNAR2 mRNA. The expression of PBMC IFNAR2 mRNA in the chronic hepatitis group was significantly higher than that in the liver cirrhosis group. The expression level of PBMC IFNAR2 mRNA ante-treatment is an independent predictor of the effectiveness of IFN therapy. Further studies are now required to verify these results using larger sample sizes and prolonged follow-up periods.

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