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Monitoring Antibody Titers to Recombinant Core-NS3 Fusion Polypeptide is Useful for Evaluating Hepatitis C Virus Infection and Responses to Interferon-alpha Therapy

To evaluate the clinical feasibility of the antibody titer against a chimeric polypeptide (named Core 518), in which a domain of Core and NS3 of hepatitis C virus (HCV) was fused, ELISA was performed in a total of 76 serum samples. Each serum was serially diluted using two-fold dilution method with distilled water into 10 concentrations. They were all positive for second generation anti-HCV assay (HCV EIA II; Abbott Laboratories). Genotyping RT-PCR, quantitative competitive RT-PCR, and RIBA (Lucky Confirm; LG Biotech) were also assayed. Anti-Core 518 antibody was detected in $\times$12800 or higher dilutions of sera from 35 of 43 chronic hepatitis C (81.4%) and nine of 16 hepatocellular carcinoma sera (56.3%), one of four cirrhosis (25%), 0 of four acute hepatitis C, and one of nine healthy isolated anti-HCV-positive subjects (p=0.0000). The anti-Core 518 antibody titers were well correlated with the presence of HCV RNA in serum (p=0.002). The anti-Core 518 antibody titers decreased significantly in nine of ten responders to IFN-α treatment. Monitoring anti-Core 518 titers may be helpful not only for differentiating the status of HCV infection among patients with various type C viral liver diseases, but also for predicting responses to IFN-α treatment.

Key Words : *Viral fusion proteins; Hepatitis C-like viruses; Hepatitis C antibodies*

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INTRODUCTION

Hepatitis C virus (HCV) infection results in various liver diseases, including acute hepatitis, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (1, 2). Since the discovery of HCV (3) and the introduction of antibody assay for screening HCV infection in 1989 (4), detecting antibodies to recombinant HCV antigens in serum has became the standard for screening HCV infection, even though several supplemental assays such as Recombinant immunoblot assay (RIBA), RT-PCR, and bDNA have been introduced (5). Because of the low concentration of various types of HCV antigen, no diagnostic methods to detect antigens are available. Although RT-PCR method to detect HCV RNA in serum is the most sensitive direct method for diagnosing HCV infection (6), the reliability of this method is mainly dependent on the examiners or laboratories (7). The bDNA method is an alternative method to address the drawbacks of RT-PCR (8), but it is still impractical because of its high cost. RIBA has been used to confirm the test results of anti-HCV antibody screening assays (9). However, its use has been limited due to its high cost and inefficiency. Other studies have suggested that measuring anti-HCV antibody titer may be useful for the evaluation of chronic hepatitis C (10). Based on the report that both Core and NS3 peptides contain strong antigenic epitopes compared to those of other recombinant peptides (11, 12), a fusion protein of a domain of Core with that of NS3, named Core 518, was made and included in the third generation anti-HCV EIA kit (HCD3.0, LG Biotech, Taejon, Korea) to improve the sensitivity of antibody detection (13). In the present study, we measured the antibody titers in serum samples from patients with various type C viral liver disease to analyze its clinical significance by using ELISA which utilizes Core

518 peptide as an antigen.

MATERIALS AND METHODS

Serum samples

A total of 76 sera were included in this study. All were positive for second generation anti-HCV antibody assay, and disease spectrum consisted of acute hepatitis C (n=4), chronic hepatitis C (n=43), cirrhosis (n=4), and hepatocellular carcinoma (n=16). Patients were administered to the Department of Internal Medicine, Kangnam St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul, Korea, between 1995 and 1997. According to the clinical or pathological features, patients were diagnosed as acute hepatitis C if anti-HCV antibody is negative during the peak of the elevation in serum aminotransferase value but HCV RNA is detectable, and as chronic hepatitis C if serum aminotransferase values are repeatedly abnormal during the observation period longer than 6 months in patients positive for both anti-HCV antibody and HCV RNA in serum. Pathologic diagnosis was performed in patients treated with interferon-α (IFN-α). Hepatocellular carcinoma was diagnosed by the computerized tomogram of liver or ultrasonogram-guided tumor biopsy in cases with elevated serum α-fetoprotein and hepatic mass. In addition, 9 healthy anti-HCV-positive subjects were also included. They had no previous history of IFN-α treatment for chronic hepatitis C. During the observation period longer than 12 months, anti-HCV antibody was positive repeatedly, but serum aminotransferase values were normal in the sequential tests. None of them showed any evidence of cirrhosis and abnormal features on hepatic ultrasonogram. The mean age of the subjects, including 48 males and 28 females, was 54 years old (range, 19-79 years). After getting informed consent from each patient, 20 patients with chronic hepatitis C and 1 acute hepatitis C patient were treated with recombinant IFN-α 2a (Intermax-alpha®, LG Biotech, Taejeon, Korea), subcutaneously injected three times a week for 6 months, using 3 to 6 million units. Complete response to this protocol was determined by persistent normalization of serum aminotransferase values and permanent loss of serum HCV RNA, when observed for at least 6 months or longer after the withdrawal of administration.

Serologic tests

Hepatitis B surface antigen (HBsAg), anti-HBs, hepatitis B e antigen (HBeAg), anti-HBe, and total antibody to hepatitis B core antigen (anti-HBc antibody) were tested by commercially available RIA kits (Abbott Laboratories, Chicago, IL, USA). Anti-HCV was tested by a second generation EIA kit (Abbott Laboratories).

Core 518 peptide

Recombinant Core 518, which is a fusion protein expressed in *E. coli*, was purified by affinity column chromatography. A domain of Core antigen (amino acid numbering from 2 to 102) was fused with a domain of NS3 (amino acid numbering from 1284 to 1457) (14).

ELISA and titration of anti-HCV antibody

To prepare reaction plates, Core 518 polypeptide (0.3 μg/mL in 50 mM sodium borate buffer, pH 9.0) was dispensed into a 96-well plate (Immulon Type I; Dynatech, U.S.A.), incubated at 4℃ overnight, and then treated with a blocking solution, a phosphate buffered saline (PBS, pH 7.4) containing 0.2% gelatin and 0.02% thimerosal, at 37℃ overnight. From each 10 μL serum per case, 10 diluted test samples were prepared with PBS containing 0.2% gelatin, 1 mM EDTA, 1% Triton X-100, and 0.02% thimerosal by a two-fold dilution method as follows: ×100, ×200, ×400, ×800, ×1600, ×3200, ×6400, ×12800, ×25600, and ×51200. Each 200 μL diluted sample was added into the prepared plate wells in duplicate, incubated at 37℃ for 1 hr and pipetted from the wells. Each well was treated five times with a washing solution, PBS containing 0.05% Tween-20 and 0.02% thimerosal. And then 200 μL of a second antibody (anti-human IgG; Behringer Mannheim, U.S.A.), which was diluted to 1:8000 in PBS, was added to each well, incubated at 37℃ for 1 hr, and washed again. Two hundred μL of substrate solution (13 mg 3,3',5,5'-Tetra-methylbenzidine in 25 mL 0.1 M sodium acetate buffer, pH 5.6, warmed at 40℃ for 30 min, and 33 μL of 30% hydrogen peroxide was added immediately before use) was added to each well and incubated at room temperature in a dark place. For color development, each 200 μL *o*-phenylene diamine (Sigma) solution, for which one tablet was diluted in 15 mL citrate buffer before use, was added to the wells, incubated at room temperature for 30 min, and then the reaction was stopped by adding 200 mL of 4N H_2SO_4. Finally, the optical density (OD) was read at 492 nm using a spectrophotometer (MR7000, Dynatech). An OD reading of 0.5 units or higher was considered to be positive. Among ELISA-positive dilutions in 10 serially diluted test samples per serum, the highest dilution showing ELISA-positive was considered to be the corresponding antibody titer in each serum.

Recombinant immunoblot assay

RIBA was tested according to an instruction manual supplied with a commercially available kit (Lucky Confirm®,

LG Biotech). A strip of RIBA contains two control bands for determining cut-off, one positive control band, one blank band for detecting false results, and 5 different antigens: NS5, 897, E1E2NS4, Core 518, and Core 14. Results interpreted by the guide supplied with the product were classified into 3 patterns; reactive, intermediate, and nonreactive.

RT-PCRs for detecting serum HCV RNA, Genotyping, and QC RT-PCR

By using the same RT-PCR methods described above (15, 16), HCV RNA was detected in serum using primer sets derived from the 5'-noncoding region (15). Genotypes of HCV RNA were determined using each genotype-specific primer set derived from the NS5 region (16), and QC RT-PCR was performed using primer sets deduced from the 5'-noncoding region (16).

Statistical Analysis

Using a statistics software program (SPSSWIN, version 6.0), Fischer's exact test and nonparametric tests, such as the Mann-Whitney U test and the Kruskal-Wallis test, were performed to compare the results.

RESULTS

Anti-Core 518 antibody titers in each group

By using a quantitative ELISA with a serial dilution method as mentioned above, antibody titer to Core 518 was ×12800 or higher in 35/43 chronic hepatitis C (81.4%), in 9/16 hepatocellular carcinoma (56.3%) and in 1/4 cirrhosis (25%). None of 4 acute hepatitis C samples (0%) and only one of nine healthy anti-HCV-positive subjects (11%) had serum anti-Core 518 titer of × 12800. On the other hand, antibody titer to Core 518 was × 6400 or lower in 8/43 chronic hepatitis C (18.6%), while in 7/16 hepatocellular carcinoma (43.6%), in 3/4 of cirrhosis (75%), in 3/4 acute hepatitis C (75%), and 5/9 healthy anti-HCV-positive subjects (56%) showed ELISA-positive in × 6400 or lower dilutions. ELISA-negative sera were also identified in 3/9 (33%) anti-HCV-positive healthy subjects, 1/4 (25%) acute hepatitis C patients, and 1/43 (2.3%) chronic hepatitis C patients (p=0.0000) (Fig. 1).

Detection of HCV RNA and anti-Core 518 antibody titers

HCV RNA was detectable in 82.9% (63/76). Anti-Core 518 titer was ×12800 or higher in 69.8% of HCV RNA-positive sera (44/63) and in 15% of HCV RNA-negative sera (2/13). Five ELISA negative cases consisted of 2/63 (3.2%) HCV RNA-positive and 3/13 (23%) HCV RNA-negative. Seven of 13 HCV RNA-negative cases (54%) showed ELISA-positive at ×3200 or lower dilutions, while 7/63 HCV RNA-positive sera (11.6%) did (p=0.002) (Fig. 2).

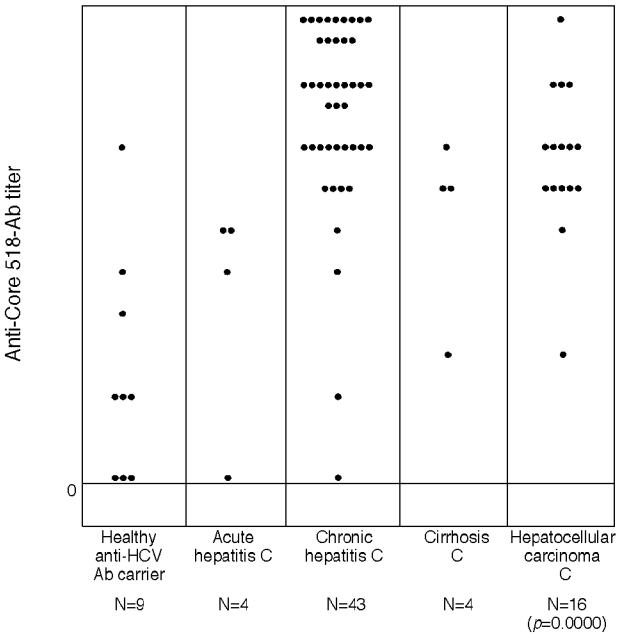

Fig. 1. Comparison of anti-Core 518 titers in each group.

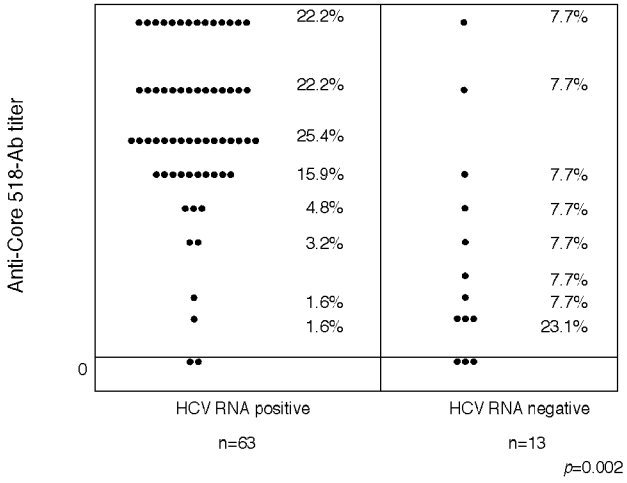

Fig. 2. Comparison of anti-Core 518 titers between HCV RNA-positive and negative sera.

Quantitation of HCV RNA and anti-Core 518 antibody titers

By QC RT-PCR, serum titer of HCV RNA was mea-

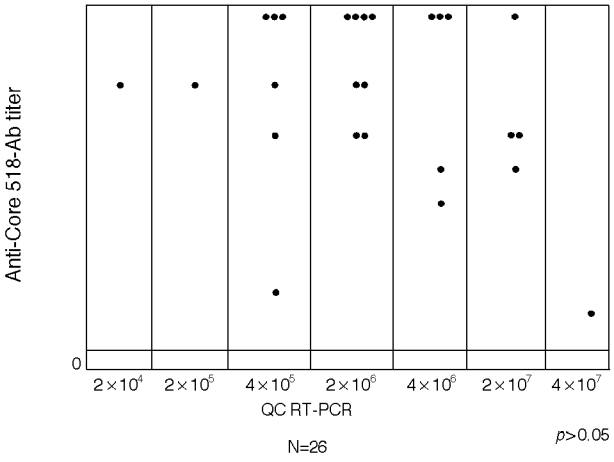

Fig. 3. Relationship between anti-Core 518 titer and HCV RNA quantity.

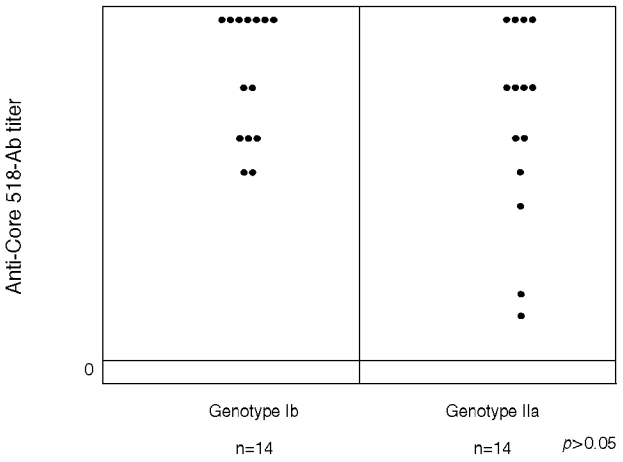

Fig. 4. The comparison of anti-Core 518 titers between HCV genotype Ib (n=14) and IIa (n=14).

Table 1. Results of anti-Core 518 titers in relation to pathologic findings, HCV genotypes, HCV RNA titers, and RIBA

Case No.	Pathology	Anti-Core 518 Ab titer	HCV genotype	HCV RNA titer (copy number / serum mL)	RIBA (*Confirmatory test)
1	CAH	×12800	Ib	2×10^7	reactive
2	CAH	×12800	Ib	2×10^6	reactive
3	CAH	×25600	Ib	2×10^6	intermediate
4	CAH	×51200	Ib	2×10^6	reactive
5	CAH	×51200	Ib	4×10^5	reactive
6	CAH	×25600	Ib	4×10^5	reactive
7	CAH	×51200	Ib	2×10^7	reactive
8	CAH	×51200	Ib	4×10^6	reactive
9	CAH	×51200	Ib	4×10^6	reactive
10	CAH	×51200	Ib	2×10^6	reactive
11	CAH	×12800	IIa	2×10^6	nonreactive
12	CAH	×3200	IIa	4×10^6	reactive
13	CAH	×25600	IIa	2×10^5	reactive
14	CAH	×51200	IIa	4×10^6	reactive
15	CAH	×51200	IIa	4×10^5	reactive
16	CPH	×51200	Ib	4×10^5	reactive
17	CPH	×6400	Ib	2×10^7	nonreactive
18	CPH	×200	IIa	4×10^7	nonreactive
19	CPH	×12800	IIa	4×10^5	reactive
20	CPH	×25600	IIa	2×10^4	reactive
21	HCC	×12800	Ib	2×10^7	not tested
22	HCC	×6400	Ib	4×10^6	not tested
23	HCC	×400	IIa	4×10^5	not tested
24	HCC	×25600	IIa	2×10^6	not tested
25	HCC	×51200	IIa	2×10^6	not tested
26	HCC	×51200	IIa	2×10^6	not tested
27	HCC	×6400	IIa	not tested	not tested
28	HCC	×25600	IIa	not tested	not tested

CAH: chronic active hepatitis, CPH: chronic persistent hepatitis, HCC: hepatocellular carcinoma. *LG Biotech, Taejon, Korea

sured in 26 sera, including 21 chronic hepatitis C and 5 hepatocellular carcinoma. No correlation between quantity of HCV RNA and antibody titer was observed (Fig. 3 and Table 1).

Genotypes of HCV RNA and anti-Core 518 antibody titers

Genotyping RT-PCR of HCV RNA was performed in 28 sera, including 21 chronic hepatitis C and 7 hepatocellular carcinoma C. By Simmond's classification (17), 14 belonged to genotype Ib and 14 belonged to genotype IIa. No significant difference in the antibody titers could be observed between these two genotypes, although ELISA-positive sera at × 3200 or lower diluents could be identified only in the sera with genotype IIa (Fig. 4 and Table 1).

RIBA and anti-Core 518 antibody titers

RIBA was tested in 20 sera from chronic hepatitis C. There were 16 reactive (80%), one intermediate (5%), and three nonreactive samples (15%). Of the 16 serum samples showing reactive results in RIBA, 15 (94%) had anti-Core 518 titer of ×12800 or higher levels, and one (6%) was ELISA-positive at ×3200 or lower diluents. In three sera showing RIBA-nonreactive, anti-Core 518 antibody titer was measured of ×12800, ×6400, and ×200, respectively. And one RIBA-intermediate case was ELISA-positive at ×25600 (Table 1).

Changes in anti-Core 518 antibody titers after treatment with IFN-α 2a

Complete response to IFN-α 2a treatment could be obtained in 52% (11/21), consisting of ten chronic hepatitis C patients (10/20, 50%) and one acute hepatitis C patient.

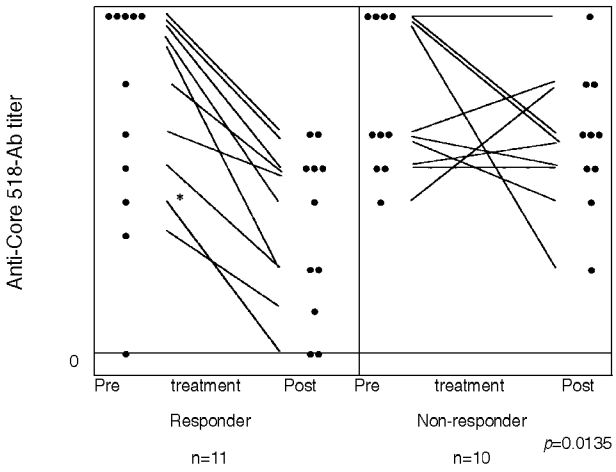

Fig. 5. Changes in anti-Core 518 titers before and after IFN-α 2a treatment in responders (n=11) and non-responders (n=10).

Compared with the results before the treatment, anti-Core 518 antibody titer decreased in 90% (9/10) of responders one year after treatment, while among ten non-responders, antibody titer did not change in three, decreased in four, and increased in three (p=0.0135) (Fig. 5).

DISCUSSION

Many recombinant HCV peptides, such as C100-3 (NS4 region) (18, 19), sp75 (Core region) (20), C11 (Core region) (21), CP14 (Core region) (22), S29-1/S4 (Core region) (23), and HCV-E2 (E2 region) (24), have been used to measure antibody titers against corresponding antigens. From those studies, it has been suggested that monitoring antibody titers can be a new and useful marker in evaluating HCV infection.

For example, IgM antibody reactivity towards the immunologically active region sp75 of the core protein of HCV showed a striking correlation with the presence of HCV RNA in serum (19). In that study, none of the HCV RNA-negative sera were IgM anti-sp75 reactive, while 73% of the HCV RNA-positive sera also contained IgM anti-sp75 antibody. Another study also identified a close association between the anti-c11 antibody titers and the presence of HCV RNA (20). In this study, we observed a significant correlation between the presence of HCV RNA in serum and anti-Core 518 titers.

Anti-Core 518 titers from 8/9 healthy anti-HCV carriers were much lower than those from patients with chronic hepatitis C or hepatocellular carcinoma C. They were all negative for HCV RNA by RT-PCR, while one healthy anti-HCV carrier with higher anti-Core 518 titer had HCV RNA in serum. These suggest that, in addition to the detection of HCV RNA, the anti-Core 518 titer may be useful for differentiating healthy anti-HCV carriers from patients with chronic hepatitis C.

All patients with acute hepatitis C had much lower anti-Core 518 antibody titers, compared to patients with chronic hepatitis C, whose anti-Core 518 titers were usually higher than $\times$12800 (81.4%). These discrepancies may indicate that the humoral immune response may be delayed during acute hepatitis C and become evident after the establishment of chronic infection.

On the other hand, 18.6% of the chronic hepatitis C had anti-Core 518 antibody titers equal to or lower than $\times$ 6400. Among these, one case had no antibody response to Core-518 peptide. Few patients with cirrhosis and hepatocellular carcinoma showed such low antibody titers in serum. Unlike cases among healthy anti-HCV carriers or patients with acute hepatitis C, it may be possible that some patients are infected by a genetic variant of HCV, which has a different immune specificity.

There was no relationship between anti-Core 518 titers and serum HCV RNA titer. Although 21% of cases with genotype IIa had anti-Core 518 titers lower than $\times$6400, no significant difference in anti-Core 518 titers could be seen between sera containing genotype Ib and IIa.

Reportedly, the titration of anti-C100-3 was helpful in the evaluation of chronic hepatitis C patients (19), and changes in anti-S29-1/S4 titers after interferon treatment correlated with virus clearance (23). Other studies also reported that titration of antibodies to an HCV core protein is an effective method for evaluating virus clearance after interferon therapy (25-27). Our results were consistent with those observations. Therefore, in addition to serial detection of serum HCV RNA, monitoring the anti-Core 518 titers may be helpful for evaluating responses to IFN-α 2a therapy as well as for differentiating various diseases with HCV infection.

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