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Circulating HCV-RNA, HCV genotype, and liver histology in asymptomatic individuals reactive for anti-HCV antibody and their follow-up study

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Abstract: The present study was aimed to clarify the virologic status, liver histologies, and the results of follow-up liver tests in symptom-free individuals with anti-HCV antibodies and normal liver tests. Forty-nine individuals with normal liver tests and positive second generation anti-HCV antibody assay were entered into this study. Cases with hepatitis C viremia were evaluated for HCV genotype, amount of circulating HCV-RNA, and liver histology and were followed-up for more than one year. Of the forty-nine individuals, 36 had hepatitis C viremia, indicated by polymerase chain reaction (PCR) assay. Liver histology was as follows: 3 had non-specific changes, 25 had chronic persistent hepatitis (CPH), and 8 had chronic active hepatitis (CAH). Twenty-four cases with CPH and CAH developed an elevated AST and/or ALT concentration (> 30IU/l) between 12 and 32 months of follow-up. The amount of circulating HCV-RNA ranged from 10^2 to 10^7 copies/50 µl serum. The distribution of HCV genotypes was nearly the same as that for symptomatic CAH. These data suggest that the histological examination and follow-up examination are very important for following symptom-free individuals with hepatitis C viremia because there are some candidates for interferon therapy among them. There are few individuals who will remain healthy among asymptomatic HCV carriers.

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Key words: asymptomatic HCV carrier – HCV genotype – HCV-RNA – liver histology – follow-up study

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Second generation anti-HCV antibody tests incorporating structural and nonstructural proteins or peptide epitopes have been widely used recently for both screening and confirmatory assays. Although the second generation tests have good sensitivity and specificity, some seropositive individuals have not exhibited viremia by the polymerase chain reaction (PCR) assay (1) and/or have been non-reactive by second generation recombinant immunoblot assay (RIBA-2: Chiron, Emeryville, CA, USA) (2, 3).

There has been an increase in the reported prevalence of asymptomatic individuals with positive anti-HCV antibodies as a consequence of the use of second generation anti-HCV antibody tests to screen blood donors and individuals appearing for a routine medical examination. Therefore, it is important to determine the degree of underlying and subsequent liver injury and the precise virologic status of patients with anti-HCV antibodies and/or serum HCV-RNA and normal liver tests. This report describes the detection of HCV-RNA, amount of circulating HCV-RNA by PCR assay, genotypes of infecting HCV, liver histology, and their follow-up studies in individuals with initially normal liver tests who are reactive for anti-HCV antibody assay.

Material and methods

HCV-seropositive individuals with normal liver tests

Forty-nine individuals (15 males, 34 females) ranging in age from 11 to 69 years (mean±SD: 42.8±14.7) had normal serum transaminases and a positive second generation ELISA (Ortho Diagnostic Systems, Raritan, NJ, USA) specific for the putative core NS3 and NS4 proteins of HCV. Thirty of the 49 individuals were blood donors, and 19 were found to be positive for anti-HCV antibodies upon medical examination between April 1991 and March 1993. All subjects had normal liver tests when they received liver tests one year prior to entry, and none had a history of increased transaminase concentration during the previous 12 months. No subjects had serologic evidence of infection with hepatitis A or B viruses as determined by assays for IgM-anti-HA, HBsAg and anti-HBcAb. Heavy drinkers and individuals with ultrasonographic evidence of fatty liver were excluded from this study. All study subjects fulfilled the following criteria: 1) positive second generation anti-HCV, 2) normal serum concentrations (≤30IU/l) of both aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and 3) a platelet count greater than 150,000/μl. Serum samples collected on the same day as the liver biopsy were used for quantitation and for typing of the HCV-RNA. The 36 subjects with proven hepatitis C viremia underwent follow-up liver tests every 1 to 2 months, and all were followed for 12 to 32 months (mean±SD: 19.2±5.2 months).

Patients were divided into two groups: A) negative serum HCV-RNA by PCR (n=13) and B) positive serum HCV-RNA by PCR (n=36). Written informed consent was obtained from each patient.

Oligonucleotides

Oligonucleotides were designed from the 5′-highly conserved sequence in the noncoding region of the HCV genome (HC-J1) isolated by Okamoto et al. (4). The sequence and the position of the primers used in reverse-transcriptase (RT)-PCR were as follows:
A, 5′-ACTCGCAAGCACCCTATGAG-3′ (276–295);
S1, 5′-CGACACTCCACCATAGATCA-3′(3–22);
S2, 5′-GAGCCATAGTGGTCTGCGCA-3′ (118–137);

RT-PCR

HCV-RNA was detected in serum samples using RT-nested PCR. Total RNA was extracted from 150 μl of serum by the acid guanidinium thiocyanate-phenol chloroform method (5). Single-stranded cDNA was synthesized using primer A with 25 units of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL Gaithersburg, MD, USA) in 25μl of a reaction mixture containing 50mN tris-HCl (pH 8.3) 75mM KCl, 3mM $MgCl_2$, 10mM DTT, 0.5mM of each dNTP and 20 units of ribonuclease inhibitor (Takara Shuzo, Kyoto, Japan) at 37°C for 90 min. One fifth of the cDNA mixture (5 μl) was subjected to PCR in 55 μl of a reaction mixture containing 10mM tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin, 0.2mM of each dNTP, 30 ng of primers A and S1, and 1 unit of Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA). The PCR conditions were 94°C for 5 min; 35 cycles of 95°C for 80 sec, 55°C for 60 sec, 72°C for 60 sec; and 72°C for 7 min. A 3 μl aliquot of the PCR product was reamplified under identical conditions for 25 cycles with primers A and S2. The resulting PCR product was analyzed by 1% agarose gel electrophoresis and visualized with ethidium bromide staining.

Quantification of serum HCV-RNA

The amount of HCV-RNA in serum was determined by a competitive RT-PCR assay (6). A HCV-cDNA corresponding to nucleotides 3 to 295 of the HC-J1 sequence cloned into the Eco RV site of the pBluescript KS (-) vector (Stratagene, La Jolla, CA, USA) and a 31 base pair (bp) Nhe I -Aat I fragment was removed by restriction enzyme digestion. The resulting plasmid, pHCV-Δ2, was subcloned and used as a template for *in vitro* transcription. To synthesize the competitive RNA, pHCV-Δ2 was linearized with Xba I and transcribed with T3 RNA polymerase (Seikagaku Kogyo, Tokyo, Japan). The transcript was purified by acid guanidium thiocyanate-phenol-chloroform extraction and ethanol precipitation. The RNA concentration was determined by measuring the optical density at 260 nm.

For the competitive RT-PCR assay, serum RNA was mixed with serial dilutions of the competitor HCV-RNA, reverse transcribed and amplified by PCR, as described above. The expected size of the PCR product derived from the sample RNA, and the competitor RNA was 178 bp and 147 bp, respectively. The amount of HCV-RNA in the serum was estimated to equal the concentration of the competitor RNA at the dilution point where the intensity of the two bands on an ethidium bromide stained agarose gel was nearly equal. The quantitative limit of circulating HCV-RNA was 10^2 to 10^7 copies /50 μl of serum.

Typing of HCV by PCR

HCV-cDNAs were classified into four subgroups by PCR, as described by Okamoto et al. (7) with slight

modification. Briefly, HCV-RNA was reverse transcribed and amplified by first round PCR using universal primers designed from a conserved sequence in the core region. The first PCR product then was reamplified by a second round of PCR with a universal primer (sense) and a mixture of four type-specific primers (antisense). We used the type I specific antisense primer, 5′CGATAGGCTGACGTCTACCT-3′, instead of the original one. HCV type was determined by analysis of the size of the second PCR product. Okamoto's type I, II, III and IV correspond to 1a, 1b, 2a and 2b, respectively, in the international classification scheme (8).

Second-generation recombinant immunoblot assay

All subjects were examined by the four-antigen recombinant immublot assay (RIBA-2) developed by Chiron (2). Sera reactive with two or more of the four RIBA antigens were considered positive, specimens reactive with just one antigen were designated indeterminate, and specimens that did not react were considered negative .

Liver histology

All subjects underwent percutaneous liver biopsy using a Surecut needle (Tochigi Seiko Inc., Tochigi, Japan) within one month following the initial visit to our outpatient department. Specimens were fixed in formalin, stained with hematoxylin and eosin and Masson's trichrome for light microscopy. The diagnosis of chronic persistent hepatitis (CPH) and chronic active hepatitis (CAH) were based on standard criteria. CAH cases with bridging fibrosis were described as CAH-c̄BF. The term nonspecific change (NSC) was used to describe specimens with a mild inflammatory reaction in some, but not all, portal areas and some spotty necrosis in the lobule or portal area.

Statistical analysis

Wilcoxon's rank-sum test, with Bonferroni's correction, was used to compare titers of HCV-RNA. Results are expressed as the mean±standard deviation (SD). All confidence intervals are 95%.

Results

Laboratory data, liver histology, RIBA-2, and HCV-RNA

Thirteen of the 49 individuals were in group A and 36 were in group B (Table 1). Four subjects were men and 9 were women in group A, ranging in age from 19 to 68 years (mean±SD: 40.3±14.9). Twelve

Fig. 1. Liver biopsy from specimen case No. 7 (41-year-old female) in group B. Mild inflammatory cell infiltration (arrows) with well preserved lobular architecture is observed. Note the lack of fibrosis. (Stain: hematoxylin and eosin, original magnification: ×800)

of the 13 cases without hepatitis C viremia were indeterminate, and one was positive by the RIBA-2 assay. Of the 12 subjects with indeterminate RIBA-2, five subjects had normal liver histology, six showed NSC, and one was compatible with CPH. Their serum AST and ALT levels were 11 to 16 IU/l and 3 to 20 IU/l, respectively. The liver histology of the RIBA-2 positive case (60 year-old man) was compatible with CPH. Serum AST and ALT concentration of this case was 12 IU/l and 25 IU/l, respectively.

Eleven patients in group B were men and 25 were women, with ages ranging from 11 to 69 years (mean±SD: 43.6±14.8). Serum AST and ALT concentration ranged from 13 to 29 IU/l and 11 to 30IU/l. respectively. Three of the 36 asymptomatic HCV carriers had NSC on biopsy (Fig. 1), 25 had CPH, 6 had CAH, and 2 had CAH-c̄BF. Three of 36 patients

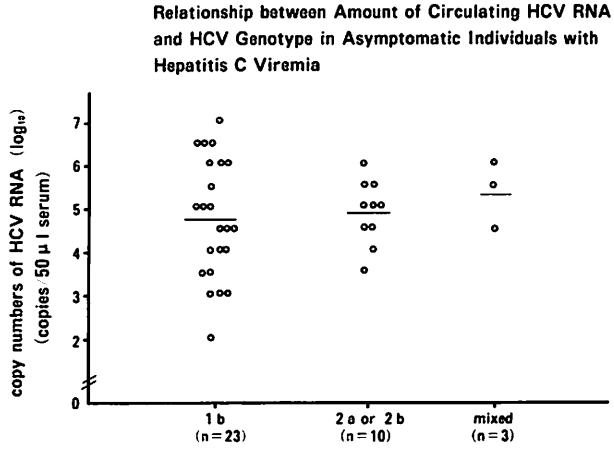

Fig. 2. Relationship between amount of circulating HCV-RNA and HCV genotype in asymptomatic individuals with hepatitis C viremia.

Table 1. Laboratory data, HCV genotype, amount of circulating HCV-RNA and liver histology in asymptomatic HCV carriers

Patient number	Age (yr)	Sex	AST/ALT	Genotype	CRT-PCR Copy numbers /50μl serum	Liver histology
1	21	M	13/11	2a	$10^{3.5}$	NSC
2	41	F	23/27	Ib	10^{7}	NSC
3	52	F	16/14	Ib	10^{4}	NSC
4	11	M	24/23	1b	$10^{5.5}$	CPH
5	24	F	13/11	Ib	$10^{3.5}$	CPH
6	24	F	16/12	Ib+2a	$10^{5.5}$	CPH
7	24	M	21/26	2a	$10^{4.5}$	CPH
8	25	F	19/13	2b	$10^{4.5}$	CPH
9	30	F	19/20	1b	10^{5}	CPH
10	30	F	22/23	2b	10^{5}	CPH
11	32	M	20/21	Ib	10^{2}	CPH
12	34	F	23/14	2a	10^{5}	CPH
13	35	F	18/19	Ib	$10^{4.5}$	CPH
14	36	F	27/27	2a	$10^{5.5}$	CPH
15	37	M	24/11	2b	10^{5}	CPH
16	39	F	19/18	Ib	10^{3}	CPH
17	42	F	23/22	1b	10^{6}	CPH
18	46	F	13/14	Ib	10^{6}	CPH
19	46	F	25/19	2a	10^{4}	CPH
20	47	F	23/25	1b	$10^{6.5}$	CPH
21	50	F	22/29	Ia+1b	$10^{4.5}$	CPH
22	53	F	21/22	Ib	10^{4}	CPH
23	55	M	22/25	Ib	10^{6}	CPH
24	58	F	18/16	Ib+2a	10^{6}	CPH
25	58	M	25/29	1b	$10^{6.5}$	CPH
26	59	F	19/15	2a	10^{6}	CPH
27	62	M	29/30	1b	$10^{4.5}$	CPH
28	69	F	23/15	Ib	$10^{6.5}$	CPH
29	43	F	23/19	1b	10^{5}	CAH
30	43	F	16/11	Ib	10^{3}	CAH
31	45	F	18/22	2a	$10^{5.5}$	CAH
32	59	M	27/30	1b	$10^{4.5}$	CAH
33	67	M	27/11	Ib	$10^{3.5}$	CAH
34	68	F	26/22	1b	10^{5}	CAH
35	43	M	23/25	Ib	10^{3}	CAH c BF
36	63	F	21/12	Ib	10^{4}	CAH c BF

NSC=nonspecific change; CPH=chronic persistent hepatitis; CAH=chronic active hepatitis; CAHcBF=chronic active hepatitis c bridging fibrosis.

had a history of hepatitis, 9 had a history of blood transfusion, one was a drug abuser, and one had been tattooed.

Three patients with hepatitis C viremia had NSC, however, the remaining 33 patients with hepatitis C viremia had chronic hepatitis, suggesting that few carriers were free of pathology among symptom-free individuals with hepatitis C viremia. Patient No. 2 in group B had the highest amount of serum HCV-RNA and has been followed for 20 months with AST and ALT concentrations remaining less than 30 IU/l.

Amount of circulating HCV-RNA, HCV genotype, and liver histology

The HCV-RNA serum titers were widely distributed. One case of NSC had 10^7 copies of HCV-RNA/50 μl serum. The copy number of HCV-RNA ranged from 10^2 to $10^{6.5}$ in CPH and from 10^3 to $10^{5.5}$ in CAH. The average copy number (mean±SD) of HCV-RNA was $10^{5.0+1.1}$/50μl serum in CPH and $10^{4.2+1.0}$/50μl serum in CAH and CAH-cBF. The amount of circulating HCV-RNA was greater in CPH than in CAH; however, there was no significant difference in HCV-RNA copy number between cases of CPH and CAH.

Classification of the genotype of the 36 asymptomatic individuals with hepatitis C viremia was as follows: 23 patients had genotype 1b (63.9%), 7 patients had genotype 2a (19.4%), 3 patients had genotype 2b (8.3%), and mixed genotypes were detected in 3 patients (8.3%) (Fig. 2). Of the 25 CPH patients with viremia, 14 had genotype 1b, 5 had genotype 2a, 3 had genotype 2b, and 3 were mixed (Table 1). The average amount of circulating HCV-RNA in

Table 2. Cases associated with abnormal liver tests during the follow-up period of the 36 asymptomatic HCV carriers

Patient number	Follow-up periods (months)	Peak values of AST/ALT (IU/l) in the follow-up periods		Appearance of abnormal AST and/or ALT
4	12	31/45	(12M)*	12 months later**
6	19	42/76	(3M)	3
7	21	25/35	(2M)	2
8	32	66/126	(23M)	19
10	24	30/42	(7M)	7
11	19	24/40	(17M)	17
14	17	25/36	(2M)	2
16	18	37/58	(15M)	15
19	13	36/49	(11M)	11
20	19	31/35	(13M)	13
21	23	65/116	(9M)	4
22	13	32/30	(6M)	6
23	23	25/31	(8M)	8
24	20	39/51	(18M)	4
25	21	30/43	(4M)	4
26	21	68/157	(11M)	9
27	32	69/102	(31M)	26
30	20	30/39	(7M)	7
31	20	28/36	(5M)	5
32	22	51/72	(2M)	1
33	18	32/16	(5M)	5
34	13	22/31	(4M)	4
35	22	36/35	(13M)	13
36	32	36/43	(15M)	15

* Numbers in parentheses represent the month with the highest concentration of aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT) levels in the follow-up period.

** Months with more than 30IU/l AST and/or ALT after the first visit.

each genotype was as follows (mean±SD):$10^{4.7+1.4}$ copies/50μl serum for genotype 1b, $10^{4.9+0.7}$ copies for genotype 2a or 2b, and $10^{5.3}$ copies for the mixed genotype, respectively (Fig. 2.) There were no significant differences in the amount of circulating HCV-RNA among these three groups.

Follow-up study of asymptomatic patients with hepatitis C viremia

The follow-up period of the 36 patients with hepatitis C viremia ranged from 12 to 32 months. Twenty-four of the 36 patients with hepatitis C viremia (66.7%) developed transiently or persistently elevated AST and/or ALT concentrations during the follow-up period (Table 2). The time of appearance of an elevated AST and/or ALT concentration ranged from 1 to 26 months following the start of the follow-up study. Four patients had an ALT concentration greater than 100IU/l during the follow-up period and were prescribed interferon therapy for 6 months. Three of the 4 cases had a good response to interferon with resolution of the abnormal concentrations of AST and ALT. However, 8 cases of CPH, 1 of CAH, and 3 with NSC histology have been associated with normal serum concentrations of AST and ALT during the follow-up period.

Of the 13 patients without HCV-RNA by PCR, 2 cases showing CPH were followed-up for 19 and 24 months, respectively. One patient showed a transiently abnormal serum ALT concentration (AST/ALT: 29/38) 8 months later; however, the other one patient has been asymptomatic during the follow-up period. The remaining 11 patients did not undergo liver tests following liver biopsy. The follow-up PCR study was done for the 2 cases of CPH and 4 of 6 cases showing NSC 15 to 29 months later. All patients showed negative for HCV-RNA in their sera.

Discussion

We have studied the virologic and histologic features of asymptomatic individuals with serum anti-HCV antibodies in a longitudinal study of symp-

tom-free subjects with hepatitis C viremia. Approximately 74% (36/49) of the asymptomatic individuals with anti-HCV antibodies by second-generation assay had hepatitis C viremia. All but 3 of the 36 viremic patients had histologic evidence of chronic hepatitis. The present results indicate that symptom-free individuals with anti-HCV antibodies have a high likelihood of chronic hepatitis and hepatitis C viremia. Our results confirm the report by Esteban et al. (3) in which the necessity of liver biopsy for blood donors with anti-HCV antibodies confirmed by RIBA-2, irrespective of ALT concentrations, was emphasized. However, we observed one individual with positive RIBA-2 but undetectable HCV-RNA: histopathology of a liver biopsy specimen was compatible with CPH. This result suggested that false negative HCV-RNA assays due to a low serum concentration of HCV-RNA may occur even with the PCR assay (9).

The amount of circulating HCV-RNA in the serum of our patients ranged from 10^2 to 10^7 copies / 50μl, averaging $10^{5.0+1.2}$ copies/50μl in the 25 cases of CPH and $10^{4.2+1.0}$ copies in the 8 cases of CAH and CAH-cBF. A competitive RT-PCR assay using synthetic mutant HCV-RNA as a competitor is useful for the quantitation of serum HCV-RNA (10, 11). It has been reported that the amount of circulating HCV-RNA was increased in proportion to the severity of the liver histopathology in patients with symptomatic chronic hepatitis and liver cirrhosis with hepatitis C virus infection (10, 11). Naito et al. (12) have reported that the quantity of HCV-RNA in the serum of asymptomatic HCV carriers was less than that of symptomatic HCV carriers, being particularly low in healthy HCV carriers. However, Lau et al. (13) have reported that serum HCV-RNA levels in patients with CPH were higher than in those with CAH and liver cirrhosis. Our present data demonstrate that the amount of HCV-RNA did not correlate with liver histology or prognosis in symptom-free patients with hepatitis C viremia. From the present analysis, we can not determine why the amount of circulating HCV-RNA in symptom-free individuals with hepatitis C viremia showing CPH was greater than in individuals with CAH.

The distribution pattern of HCV genotype in patients with HCV infection in Japan has been reported to vary somewhat based on the district (4, 14). In an analysis of 122 cases of symptomatic CAH seen in our clinic, genotype la was noted in 2 cases (1.6%), 1b in 92 (75.4%), 2a in 17 (13.9%), 2b in 6 (4.9%), and mixed genotypes were observed in 5 (4.1%) (unpublished data). The percentage of asymptomatic patients with genotypes 1b, 2a, and 2b was 64%, 19%, and 8%, respectively, in the present study. Recently, Silini et al. (15) have reported that HCV genotype 2a infection was noted frequently in asymptomatic individuals with hepatitis C viremia and in patients with mild liver disease. However, our data showed no significant difference in the frequency of various HCV genotypes when patients with symptomatic chronic hepatitis C infection were compared to asymptomatic patients with hepatitis C viremia by Student's paired *t* test analysis.

The three cases of nonspecific histologic changes lacked associated elevation of AST and ALT concentrations during the follow-up period. These results indicated that apparently "healthy HCV carriers" with nearly normal liver histology are relatively infrequent among asymptomatic HCV carriers. Recently, Brillanti et al. (16) also have reported four cases of HCV healthy carriers. In addition, we even found a few cases with advanced chronic hepatitis among asymptomatic individuals with detectable HCV-RNA.

We can't explain why some patients with CPH remain asymptomatic despite having considerable amounts of HCV-RNA in the serum. We speculated that asymptomatic patients may have had chronic hepatitis prior to entry and/or had developed transient elevation of serum transaminases in the past. Furthermore, Navas et al. (17) have reported that plus and minus strand HCV-RNA was detected in peripheral blood mononuclear cells and liver tissue in patients with normal ALT concentration and normal liver histology. As Brillanti et al. (16) and Navas et al. (17) have pointed out, one also should consider the possibility of infection with avirulent or low virulent HCV strains, tolerance to HCV infection, and other possibilities.

Our follow-up study demonstrated that 24 (66.7%) of the 36 asymptomatic individuals with hepatitis C viremia developed elevated AST and/or ALT concentrations from 1 to 26 months following the first liver tests. The elevation of serum AST and ALT concentrations during the follow-up period was mild in most patients, and a few patients showed a transient significant elevation of AST and/or ALT. Therefore, the label "asymptomatic" may not precisely characterize individuals with normal ALT concentrations during a fixed follow-up interval.

In conclusion, our results indicate that it is important periodically to monitor symptom-free individuals with hepatitis C viremia because some will have underlying CAH and a high rate of progressive liver damage. There are few truly healthy HCV carriers among asymptomatic subjects with hepatitis C viremia. The level of viral replication is not always low in healthy HCV carriers and patients with CPH compared with CAH. Interferon therapy should

therefore be considered for patients with persistently abnormal liver tests during follow-up observation.

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