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Prevalence and genotyping of hepatitis C virus RNA in Turkish patients with chronic non-A, non-B liver disease*

Non-A ve Non B hepatitine bağlı kronik karaciğer hastalığı olan Türk hastalardaki hepatit C virüs prevelans ve genotipleri

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SUMMARY: *The clinical features of hepatitis C virus (HCV) infection and the distribution of HCV genotypes in 86 patients with chronic non-A, non-B (NANB) liver disease were examined in this study. The HCV infection was diagnosed using anti-HCV testing, and by the detection of HCV RNA by reverse transcriptase-polymerase chain reaction (RT-PCR). HCV genotypes were determined in 27 of HCV RNA positive patients by restriction fragment length polymorphism (RFLP) analysis of PCR products.*

HCV RNA was detected in the serum of 62 out of 86 (72.1%) patients on PCR while 68 out of 81 (84%) patients were positive for anti-HCV. PCR and anti-HCV results were concordant in 60 out of 81 (74.1%) patients. Patients with HCV RNA in serum were indistinguishable from those without HCV RNA in serum apart from serum ALT and total bilirubin levels (P<0.05).

The most common genotype was HCV 1b (81.5 %). Other genotypes detected were 1a (14.8 %) and 4 (3.7 %).

HCV infection is the major cause of chronic non-A, non-B (NANB) liver disease and HCV 1b is the predominant genotype.

Key words: Hepatitis C virus, chronic non-A, non-B liver disease, anti-HCV, HCV RNA, HCV genotype, PCR

ÖZET: *Bu çalışmada kronik NANB karaciğer hastalığı olan 86 hastada HCV enfeksiyonunun klinik özellikleri ve HCV genotiplerinin dağılımı incelendi. HCV enfeksiyonu tanısı anti-HCV testi ve RT-PCR ile HCV RNA'nın saptanması ile kondu. HCV genotipleri HCV RNA pozitif 27 hastada PCR ürünlerinin "restriction fragment length polymorphism" (RFLP) analizi ile saptandı.*

HCV RNA PCR ile 86 hastanın 62'sinde (% 72.1) saptanırken, 81 hastanın 68'i (% 84) anti-HCV pozitifti. PCR ve anti-HCV sonuçları 81 Hastanın 60'ında (% 74.1) uyumluydu. Serum ALT ve total bilirubin düzeyleri hariç serumda HCV RNA bulunan hastalar bulunmayanlardan farksızdı. Ensık izlenen genotip HCV 1b olup (% 81.5), diğerleri 1a (% 14.8) ve 4 (% 3.7)'tü.

HCV enfeksiyonu, kronik NANB karaciğer hastalığı olanlarda esas etken olup, HCV 1b predominan genotiptir.

Anahtar sözcükler: Hepatit C virüsü, kronik non-A, non-B karaciğer hastalığı, anti-HCV, HCV RNA, HCV genotipi, PCR

The identification of hepatitis C virus (HCV) by Choo et al in 1989 (1) led to the development of a recombinant based immunoassay for the detection of specific antibodies to HCV (anti-HCV) (2). Seroepidemiological studies using these assays have shown that HCV is a major causative agent of post-transfusion and community-acquired NANB hepatitis that can lead to the development of cirrhosis and hepatocellular carcinoma (2-6). The value of anti-HCV testing for viremia monitoring is limited and, a sensitive method to detect HCV RNA as a marker for viremia is therefore required. Since titres of HCV RNA are low, amplification of viral subgenomic fragments by reverse trancriptase-polymerase chain reaction (RT-PCR) is currently the most sensitive method to demonstrate HCV viremia (7). Based on variations in the nucleotide sequences of HCV, at least six genotypes have been identified (8). While some HCV genotypes have a worldwide distribution, certain genotypes are confined to certain geographical areas. In this study, the clinical features of HCV infection in a group of Turkish patients with chronic NANB liver disease were examined. The distribution of HCV genotypes in these patients was also investigated.

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Table 1. Clinical features, laboratory data and histologic findings in patients

Characteristic	*Data*
Sex (F/M)	54/32
Age (yr)	49.7 ± 12.2
Causal factors (%)	
Blood transfusion	22/84 (26.1)
Operation	50/82 (60.9)
Laboratory data	
AST (IU/L)*	82.0 ± 51.0
ALT (IU/L)*	84.1 ± 54.8
GGTP (IU/L)*	91.5 ± 99.8
ALP (IU/L)*	131.7 ± 104.4
Total protein (gm/dL)*	7.4 ± 0.8
Albumin (gm/dL)*	4.0 ± 0.7
Total bilirubin (mg/dL)*	0.9 ± 0.6
Direct bilirubin (mg/dL)*	0.3 ± 0.3
Prothrombin time (sc)*	13.0 ± 1.4
Hemoglobin (gr/dL)*	13.4 ± 1.6
Platelet count (x10 9/lt)	173.0 ± 72.8
Histologic findings (%)	
CAH	53 (61.6)
CPH	11 (12.8)
CAH and cirrhosis	7 (8.1)
Cirrhosis	5 (5.8)
Fatty liver	3 (3.5)
Clinically cirrhosis	7 (8.1)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGTP,gama glutamyl transpeptidase; ALP, alkaline phosphatase (normal value:
*: Data expressed as mean ± S.D.

MATERIAL AND METHODS

Patients

Between September 1993 and December 1995, 86 patients with chronic NANB liver disease were recruited in to the study group. The diagnosis of chronic NANB liver disease was based on the following criteria:

a) elevation of serum amino transferase level without history of alcohol intake or use of hepatotoxic drugs,

b) seronegativity for HBsAg,

c) seronegativity for anti nuclear antibody, anti smooth muscle antibody and antimitochondrial antibody. Other possible rare causes of chronic liver diseases were not investigated. All patients but seven underwent percutaneous liver biopsy.

The clinical features, laboratory data and histologic findings of these patients are summarized in Table 1. The group comprised 54 females and 32 males, mean age was 49.71 ± 12.24 yr (range= 18 to 74 yr). While 22 patients had histories of blood transfusion, a history of surgery was found in 50 patients. Liver histology showed features of chronic active hepatitis (CAH) in 53 cases, chronic persistent hepatitis (CPH) in 11 cases, CAH and cirrhosis in 7 cases, cirrhosis in 5 cases and fatty liver in 3 cases. Seven cirrhotic patients were diagnosed clinically without liver biopsy.

All serum samples were aliquoted and stored at -70 °C until they were used.

Anti-HCV serological test

Anti HCV was detected in serum samples by a third generation enzyme immunoassay (EIA) (UBI HCV 4.0, Hauppauge, NY, USA).

Detection of HCV RNA

RNA was extracted from 100 microliter of serum samples by acid guanidium thiocyanate-phenol-chloroform method; reverse transcribed and amplified by nested PCR using 5'-noncoding region (NCR) specific primers 939, 209, 940 and 211 as described previously (9). PCR products were run on a 2 % agarose gel, stained with ethidium bromide and visualized under ultra violet light. To avoid false positive results, the recommendations of Kwok and Higuchi were strictly followed (10).

Genotyping of HCV

HCV genotypes were determined in 27 of HCV RNA positive patients by restriction fragment length polymorphism (RFLP) analysis of PCR products. Restriction digests were carried out on 25 microliter of secondary PCR product for 4-16 hours after adjustment with the appropriate 10x enzyme reaction buffer. Reactions were incubated at 37 °C in the presence of 10 units each of

a) HaeIII and RsaI,

b) Hinf I and MvaI,

c) ScrFI, or

d) BstUI (at 600 C)

Digestion products were visualized under ultra violet light after electrophoresis through a 4 % metaphor agarose gel (FMC Bioproducts, Rockland, ME 199, USA) in 1x TBE (134 mM Tris CI, pH 10.0, 68 mM Boric acid, 2.5 mM EDTA) containing 0.5 microg/ml ethidium bromide (11). To confirm the genotyping results, some samples

Table 2. Comparison of serum HCV RNA detection on PCR with anti-HCV testing

	PCR result	
Anti HCV	*Positive*	*Negative*
Positive	53	15
Negative	6	7

were directly sequenced using sense and antisense primers from 5'-NCR and NS-5 regions as described previously (9).

Statistical analysis

Data were analysed with Fisher's exact chi-square and Student's t test by the use of SPSS program in a computer.

RESULTS

HCV RNA was detected in the serum of 62 out of 86 (72.1 %) patients on PCR while 68 out of 81 (84 %) patients were positive for anti-HCV. PCR and anti-HCV results were concordant in 60 out of 81 (74.1 %) patients: 53 were positive and seven were negative on both tests. Fifteen patients were anti-HCV positive and PCR negative, whereas six patients were PCR positive and anti-HCV negative (Table 2).

HCV RNA was detected in the sera of 53 out of 68 (77.9 %) patients who were anti-HCV positive and in those of 6 out of 13 (46.1 %) patients who were anti-HCV negative. The anti-HCV was negative in 7 of 22 patients who lacked HCV RNA in serum (specificity, 31 %) while it was positive in 53 of 59 patients who were positive for HCV RNA (sensitivity 89 %). The positive predictability of anti-HCV for the presence of HCV RNA was 77 % , and its negative predictability was 46 %.

Comparison between HCV RNA positive and negative patients

Patients with HCV RNA in serum were indistinguishable from those without HCV RNA in serum regarding age, gender, causal factors and biochemical findings. Significant differences between the two groups were observed only with regard to serum ALT and total bilirubin levels ($P < 0.05$) (Table 3).

Genotype distribution

Genotypes were determined in 27 (17 females, 10 males, mean age: 54.03 yrs) of 62 HCV RNA positive patients. Liver biopsy results were CAH (n=19), CAH and cirrhosis (n=3), CPH (n=2)and cirrhosis (n=2). One patient was diagnosed as cirrhosis on clinical examination. The most common genotype was HCV 1b (81.5 %). Other genotypes detected were 1a (14.8 %) and 4 (3.7 %).

Table 3. Comparison between HCV RNA positive and negative patients

	HCV RNA positive	*HCV RNA negative*	*P value*
No	62	24	
Age (yr)			
Sex (F/M)			
Causal factors (%)			
Blood transfusion	17/60 (28.3)	5/24 (20.8)	NS
Operation history	39/58 (67.2)	11/24 (45.8)	NS
Labaratory data			
AST (IU/L)	79.82 ± 43.72	96.82 ±77.48	0.03
ALT (IU/L)	82.35 ± 53.08	82.17 ± 48.67	NS
GGTP (IU/L)	90.19 ± 101.11	99.95 ± 102.88	NS
ALP (IU/L)	120.67 ± 97.46	163.40 ± 120.42	NS
Albumin (gm/dL)	4.08 ± 0.59	4.04 ± 0.76	NS
Total bilirubin (mg/dL)	1.05 ± 0.72	0.79 ± 0.38	0.02
Dir.bilirubin (mg/dL)	0.46 ± 0.54	0.31 ± 0.30	NS
Prothrombin time (sc)	13.03 ± 1.47	13.21 ± 1.43	NS
Hemoglobin (gr/dL)	13.17 ± 2.32	13.41 ± 1.78	NS
Platelet count (x10/L)	175.63 ± 78.04	166.26 ± 68.42	NS

DISCUSSION

In accordance with the findings of other studies (12, 13, 14), we found that HCV RNA could be detected in the sera of most patients with chronic NANB liver disease by RT-PCR with primers from 5'-NCR. The third generation anti HCV EIA and PCR results were concordant in 74.1% of cases.

HCV RNA was detected in 6 (46.1 %) patients who were anti-HCV negative. This result is comparable with to that of Hagiwara et al (13), although first generation serological assay was used in this study. HCV viremia levels may be responsible for this result. PCR sensitivity threshold is approximately 10 to 100 copies/ml (15), but it can rise up to 10^4, even 10^5 copies/ml depending on different laboratories. Yuki et al' s study (16) revealed that HCV antibodies were frequently induced in patients with serum HCV RNA levels of > 10^6 copies /ml and that patients with low viremia levels were poorly detected by immunoassays. We could not quantify of serum HCV RNA but viremic levels of these 6 patients may have been so low that they couldn't be detected by immunoassays.

HCV RNA was not detected in 15 (22%) patients who were anti-HCV positive. It would be more accurate to assess whether the anti-HCV results could be confirmed by recombinant immuno blot assay (RIBA) in our study but several factors may account for this result:

i) Intermittent viremia (14),

ii) Resolution of HCV infection despite persistence of anti-HCV (14),

iii) Anti-HCV cross-reaction with auto-antibodies with an epitope resembling part of HCV (17).

For example, although the first serum sample of one of our patients gave a negative result, six months later HCV RNA was detected in the new serum sample of the same patient. Intermittent viremia has been observed in this patient. Part of HCV may resemble a peptide produced by the host, such as GOR. Antibodies against this host component, such as anti-GOR can cross-react in a asassay of anti-HCV. This cross-reaction can give false-positive results in anti-HCV tests (17).

The causes of chronic liver disease may have been NANB and non-C viral agents especially in the seven anti-HCV and PCR negative patients.

When we compared the features of HCV RNA positive patients with those of negative patients, they were statistically similar to each other except for serum ALT and total bilirubin levels (18-20).

In accordance with previous reports (18, 19, 20), this study showed that HCV genotype 1b is predominant in Turkish patients. In Western Europe, HCV genotypes 1a, 1b, 2a, 2b and 3a are present in different ratios (11, 21). There is a trend for more frequent infection with type 1b in Southern and Eastern Europe (18).The distribution of HCV genotypes in Turkey has a similar HCV distribution pattern as that found in Southern and Eastern Europe (18).

In conusion HCV infection detected by HCV RNA is the major cause of chronic NANB liver disease in our patients and HCV genotype 1b is predominant.

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