

Hepatitis C and Occult Hepatitis C Infection Among Hemodialysis Patients from Central Anatolia

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Hepatitis C virus (HCV) is a common cause of infection in patients receiving hemodialysis. In this study we aimed to determine the detection of HCV RNA status in peripheral blood mononuclear cells (PBMCs) of the patients who require hemodialysis and the prevalence of occult HCV infection by real-time polymerase chain reaction (PCR) in central Anatolia. One hundred end-stage renal disease patients, over 16 years old, receiving hemodialysis treatment were enrolled in this study. Participants' demographic data were all evaluated in this cross sectional study. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), hepatitis markers, plasma HCV RNA and HCV RNA in PBMCs was measured. Occult hepatitis C (OHC) was described in patients with or without elevated liver function tests (LFTs) of unknown etiology and it is characterized by the presence of HCV RNA in PBMCs in the absence of detectable viral RNA in serum by conventional assays. We found that 19 out of the 100 patients testing positive for at least one of these tests; anti-HCV, plasma HCV RNA and PBMCs RNA. Three (%3.6) out of 84 patients in both anti-HCV and plasma HCV RNA negative group, HCV RNA in PBMCs was positive. These patients were evaluated as OHC. Detection of HCV RNA status in PBMCs of the patients, is a noninvasive method that will help to determine occult infection.

Keywords: Hepatitis C virus, hemodialysis, occult hepatitis C infection, Peripheral blood mononuclear cells.

HCV is one of the common cause of infection around the world and it is estimated that there are 210 million HCV infected persons in the world. The prevalence of HCV was reported between 1% and 2.4% in Turkey^{1,2}. The prevalence of HCV has decreased in recent years however there are differences between countries (4-70%)^{1,3}. Patients receiving hemodialysis have an increased risk of acquiring HCV infection^{4,5}.

HCV related infections can be classified as acute, chronic and occult infections. OHC is a new entity, emerged in recent years. Viral replication continues during the occult infections in hepatocytes and cells of the immune system so that antigenomic chains of HCV can be determined in the majority of patients^{6,7}. Liver biopsy is an invasive procedure so may not be practicable in many patients. Due to lymphotropic feature of HCV, sensitivity of HCV RNA in PBMCs have been investigating⁸⁻¹¹. OHC is a moderate disease that causes less damage than chronic hepatitis C (CHC) to the liver¹²⁻¹⁴. Viral replication in the extrahepatic sites can cause progression and also transmission of the disease^{10,14,15}. We designed this study to evaluate the point prevalence of OHC among hemodialysis patients.

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Patients and Methods

One hundred end-stage renal disease patients receiving hemodialysis treatment were enrolled in Kayseri, Turkey between May 2010 and April 2011. The study was designed as a cross sectional study and approved by the Ethics Committee. Selection criteria for study participants were being over 16 years old and receiving hemodialysis treatment. ALT, AST, GGT, blood lipid levels, HCV RNA in plasma and HCV RNA in PBMCs, serological markers for hepatitis B virus (HBV), HCV and human immunodeficiency virus (HIV) were measured. Serum samples were tested for anti-HCV with enzyme immunoassay (ELISA) (Architect, Abbott, Germany). The separation of PBMCs consist of a series of steps. Initially 3 mL peripheral blood sample was collected into an EDTA-containing tube from each individuals. Ficoll hypaque (FH) solution was added into a gel-free tube. Peripheral blood samples were transferred to this tube slowly and centrifuged at 1600 rpm for 15 minutes. In the middle of the tube a cloudy appearance occurred were PBMCs, 1 ml of these cells transferred to falcon tube by micropipet. 4 ml phosphate buffered saline (PBS) was added and centrifuged at 1300 rpm for 10 minutes. This washing process was repeated four times to purify the cells. Inactivated fetal calf serum (FCS) was added into the 50 ml of RPMI 1640 medium, 1 mL of this broth was transferred to eppendorf tubes, 100 μ L of 10% dimethyl sulfoxide (DMSO) was added and this mixture was transferred into the cells as a last step. PBMCs were kept at -80°C until use. Another peripheral blood sample was collected into EDTA-containing tube from each individuals was centrifuged then plasma was stored at -80°C and used for PCR. RNA isolation procedure was applied to the plasma samples (QIAGEN, QIAamp Viral RNA Mini Kit 50, Germany) and PBMCs (QIAGEN R Neasy Mini Kit 50, Germany). After the isolation of RNA from both of the plasma and PBMCs we have used the PCR method. HCV RNA was extracted from the plasma samples and PBMCs by using real-time PCR (COBAS TaqMan 48, HCV Test v2.0 For Use With The High Pure System, HCV HPS V2 kit, Germany). Measurement range of the PCR test was $25\text{-}3.91 \times 10^8$ IU/mL and the sensitivity of the test was 25 IU/mL. OHC was defined as the presence of HCV RNA in liver and patients in the PBMCs in the absence of detectable viral RNA in

serum by standard assays, can be found in anti-HCV positive or in anti-HCV negative patients with normal serum levels or elevated LFTs of unknown etiology (16). The statistical software package SPSS 15.0 was used for statistical analysis. We compared diagnostic tests with Mc Nemar Test.

RESULTS

The mean age of the patients was 58.5 ± 13.9 years and 56% were male. The mean levels of ALT and AST were 17 ± 1.1 IU / L and 16.1 ± 7.2 IU / L, respectively. Four (4%) patients were positive for HBsAg, 47 (47%) were positive for anti-HBc and 53 were (53%) positive for anti-HBs. We found that 19 out of the 100 patients testing positive for at least one of these tests; anti-HCV, plasma HCV RNA and PBMCs RNA. Out of 100 patients, 10 were positive for anti-HCV, 11 were positive for plasma HCV RNA and 7 positive for PBMCs HCV RNA (Table 1). None of these patients were tested positive for other serological markers.

Five of 10 patients whose anti-HCV positive were both negative for RNA in plasma and PBMCs. Six (6.7%) out of 90 patients whose anti-HCV were negative, HCV RNA in plasma were positive. All the patients with low levels of HCV RNA in plasma were negative in PBMCs. RNA levels were between 33-338 IU/mL. None of the anti-HCV negative patients whose test are not determined simultaneously HCV RNA positive in both plasma and PBMCs. Three (3.6%) out of 84 patients in both anti-HCV and plasma HCV RNA negative group, HCV RNA in PBMCs were positive and defined as OHC. LFT levels and other serological markers were all negative in this group of patient. HCV RNA in PBMCs were all negative in seven patients with the level of plasma HCV RNA were under 500 IU/mL. HCV RNA in PBMCs was detected in four patients, their HCV RNA results were over 500 IU/mL. The point prevalence of OHC was determined as 3.6%. We evaluated diagnostic tests. Based on our evaluation HCV prevalence was found 14 % when two test results (HCV RNA in plasma and PBMCs) were assessed, and with all three diagnostic tests the HCV prevalence was 19%.

The diagnostic tests were compared with Mc Nemar Test and found medium concordance between anti-HCV and HCV RNA in plasma and PBMCs (Table 2) .

Table 1. Positive results for anti-HCV, HCV RNA in plasma and PBMCs

Patient number	Anti-HCV	HCV RNA in Plasma (IU/mL)	HCV RNA in PBMCs (IU/mL)
1	(+)	(-)	(-)
2	(+)	(+) (67.300)	(+) (974)
3	(-)	(-)	(+) (<25)
4	(+)	(-)	(-)
5	(+)	(+) (6.830)	(+) (110)
6	(+)	(+) (211)	(-)
7	(-)	(-)	(+) (127)
8	(-)	(-)	(+) (124)
9	(-)	(+) (<25)	(-)
10	(+)	(-)	(-)
11	(+)	(-)	(-)
12	(+)	(+) (3.910.000)	(+) (38.800)
13	(-)	(+) (75)	(-)
14	(+)	(-)	(-)
15	(+)	(+) (3.910.000)	(+) (91.900)
16	(-)	(+) (48.1)	(-)
17	(-)	(+) (206)	(-)
18	(-)	(+) (33)	(-)
19	(-)	(+) (338)	(-)

Table 2. Comparison of HCV tests

		HCV RNA in PBMCs		p value
		Negative	Positive	
HCV RNA in Plasma	Negative	86 (%96.6)	3 (%3.4)	0.508
	Positive	7 (%63.6)	4 (%36.4)	
Anti-HCV				
HCV RNA in Plasma	Negative	84 (%94.4)	5 (%5.6)	1.000
	Positive	6 (%54.5)	5 (%45.5)	
Anti-HCV				
HCV RNA in PBMCs	Negative	87 (%93.5)	6 (%6.5)	0.508
	Positive	3 (%42.9)	4 (%57.1)	

DISCUSSION

HCV is an important public health problem especially in patients who are receiving hemodialysis. The prevalence of HCV infection in hemodialysis centers varies between 4% and 70% (1). In Turkey the seroprevalence was 9.8% by the end of 2009 (17). In this study when anti-HCV was performed as a diagnostic test in hemodialysis patients, similar rates were found (10%).

Anti-HCV antibodies are used for the determination of non neutralizing antibodies. More than 90% of HCV infected patients can be confirmed with serological tests¹⁸. The presence of anti-HCV

does not indicate positive HCV RNA results at the same time. In the present study five (50%) of 10 patients whose anti-HCV positive were tested negative for plasma HCV RNA. This result could be explained by intermittent viremia or low levels of HCV RNA, below the detection limit of the assay¹⁹. Heparin could be another factor may cause false negative results by affecting PCR²⁰. In this study all samples were taken before the dialysis treatment to prevent false negative test results.

In the present study six patients were positive for HCV RNA in plasma among anti-HCV negative 90 patients. None of the patients whose anti-HCV negative, were not determined positive for

HCV RNA simultaneously in both plasma and PBMCs. It is possible that OHC could occur with mutant strains. A mutation affects the translational capacity, the stage of capsule formation or the releasing phase of the virion into the bloodstream. This leads to a low level of viremia and anti-HCV. Low levels of antibodies and HCV RNA cannot be determined because of the existing sensitivity of the serological and PCR methods¹⁰. Clonal and sequence analysis have been performed in different regions of genomes in patients with OHC, however no mutations were found to prevent the detection of anti-HCV. For this reason, OHC may be related to unidentified viral and host factors¹². All patients with low levels of HCV RNA in plasma were negative in PBMCs in the present study. In recent years positive test results for HCV RNA in plasma in spite of negative results in PBMCs was attributed to different "quasispecies" in both plasma and blood cells²¹. Similar results have also been reported in literature. PBMCs from 56 viremic blood donors have also analyzed for the presence of HCV RNA and 43 out of 56 donors HCV RNA was detected in a prior study²².

In this study 3 out of 100 patients defined as OHC. This infection distributed worldwide and defined as the presence of HCV RNA in liver and in PBMCs in the absence of detectable viral RNA in serum with normal or elevated LFTs. The presence of the antigenome HCV RNA chain has been shown in both PBMCs and the liver²³. It is known as an indicator for viral replication²⁴. In the literature OHC has been demonstrated in 0-50% of patients by detecting HCV RNA in PBMCs and also 0-83 % of patients by liver biopsy¹⁰. Positive stranded HCV RNA has been detected in liver tissue in 57 out of 100 patients were both negative for anti-HCV and HCV RNA and abnormal LFTs. Viral RNA in PBMCs were positive in 70 out of 100 patients⁷. In the present study point prevalence of OHC infection was determined 3.6%.

The rate of OHC has been determined 10% in patients with SVR⁶ and 45% in patients receiving hemodialysis with abnormal LFTs and naive for hepatic viral infections²⁵. Hemodialysis patients are at higher risk for HCV. Hepatotropic viruses and also OHC was investigated for the etiology of elevated LFTs among patients receiving hemodialysis. Conversely OHC may occur in patients with normal LFTs (10, 26-28). Nine out of 276 patients with normal LFTs have been evaluated as OHC²⁶.

The presence of OHC has been studied among hemodialysis patients with elevated LFTs, though LFT abnormalities are not common in this population. LFT levels were also normal in this study.

In this present study, 19 out of the 100 patients tested positive for at least one of these tests: anti-HCV, HCV RNA in plasma and in PBMCs. Diagnostic tests were independently evaluated and the frequency of HCV infection was determined at different rates. The frequency of OHC was determined as 3.6%. When we assessed the frequency of HCV infection according to RNA in plasma and PBMCs, HCV prevalence was found 14%; with all three diagnostic tests, HCV prevalence was 19%. HCV RNA in PBMCs was negative in seven patients with HCV RNA plasma levels under 500 IU/ml. HCV RNA in PBMCs and plasma were found in four out of 14 patients positive and HCV RNA plasma levels were over 500 IU/ml. Four patients. These results may be depends on assay sensitivity or sequestered HCV RNA in blood circulation, HCV RNA in plasma levels could be below the test limit value and be detected as negative. Castillo, *et al.*,²⁹ indicated OHC in patients with the presence of HCV RNA in the liver despite negative results for anti-HCV and for serum HCV RNA; 36% of 122 patients with HCV anti-core tested positive, 57% tested positive for HCV RNA in serum after ultracentrifugation and 61% tested positive for HCV RNA in the PBMCs. 91% of the patients were positive for at least one marker with combining the results of the assays,. Testing for all these tests may also help for the diagnosis without the need of liver biopsy. If a patient's intrahepatic HCV RNA load is significantly elevated, all three HCV infection markers could be positive with high probability, while a positive result in one or two markers indicates a lower HCV-RNA load.

Viremia should be followed up by appropriate assays periodically, especially in risk groups such as the hemodialysis population because viremia could be transient³⁰. The main limitation of this research was not to evaluate viremia with recurrent blood samples. Carreno, *et al.*,¹² reported that specific methodological monitoring is useful for the diagnosis of OHC. If the patient is available for liver biopsy, the biopsy should be performed for HCV RNA testing. If the patient is not available for liver biopsy, it is

necessary to investigate HCV RNA in serum after ultracentrifugation and in PBMCs. When at least one of the diagnostic test results is positive, OHC can be diagnosed. If the results of both tests are negative, it is suggested to exclude the diagnosis of OHC and perform the tests every three to four months for a period of one year¹². Similar methodological approaches could be useful to assess pathological, biochemical, and clinical results of OHC in patients receiving hemodialysis.

Antiviral therapy provides HCV RNA clearance, with biochemical and histological improvement in CHC patients. Although OHC is a mild disease, it can cause histological liver damage, including cirrhosis^{7,31}. Detection of low levels of viral replication in hepatocytes, may indicate ongoing liver damage, and oncogenic transformation³². For this reason it is important to identify those patients in terms of treatment³⁰. The presence of low amounts of HCV RNA may remain detectable lifelong and could be important for transmission through hemodialysis units.

CONCLUSION

In this study prevalence of OHC infection (3.6 %) determined by the presence of HCV RNA in PBMCs among hemodialysis patients. As a result of this study when a liver biopsy is not available for HCV RNA detection, the diagnosis can be made by testing with a highly sensitive real time PCR technique, for the presence of viral RNA in PBMCs. This study have some important limitations. We did not able to demonstrate genotype of HCV strains and follow up viremia periodically. There is still a significant proportion of patients remained undiagnosed so further investigations in larger patient groups are needed.

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