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Original Contribution

POSITIVE HBV DNA IN LIVER SAMPLES IN PATIENTS WITH CHRONIC HCV INFECTION

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ABSTRACT

PURPOSE: To determine prevalence of positive HBV DNA in liver tissue samples in patients with chronic HCV infection (CHCV) and to investigate whether there is an association between it and underlying clinical condition. **METHODE:** For the period 2004 – 2010 in the Gastroenterology Clinic at the Military Medical Academy – Sofia, 96 patients CHCV (77% males, 23% females, mean age 50, 5 ± 10, 02 years) – chronic hepatitis (n-42), cirrhosis (n-35) and HCC (n-19) were included in the study. All of them were HBs Ag – negative. Liver samples were tested for the presence on of tree gene regions of HBV: S-, Core- and X- antigen coding regions. Serum levels of HBV DNA were also investigated. **RESULTS:** Positive liver HBV DNA was found in 40,6% of patients with CHCV. The presence of S-gen coding region was detected in 82% (n-32), Core-gen coding region - in 87% (n-34) and X -gen coding region - in 46,1% (n-18) of cases. Occult HBV infection was significantly associated with the presence of cirrhosis and HCC (P<0,0001) and with positive serum markers for past HBV infection (P=0,001). The prevalence of positive serum HBV DNA (20,8%) was significantly lower than the prevalence of positive liver HBV DNA (P<0,0001). **CONCLUSION:** Positive liver HBV DNA was found in 40,6% of cases with CHCV, which is significantly higher than the presence of positive serum HBV DNA (20,8%) in the same patients. Occult HBV infection in patients with CHCV was associated with more severe liver damage in comparison with the patients monoinfected with HCV.

Key words: Nested PCR, liver HBV DNA, S-, Core- and X-antigen coding regions

INTRODUCTION

The development of highly sensitive methods for detection of small amounts of HBV DNA forms the basis for identification of occult HBV infection. The occult HBV infection is defined as presence of HBV DNA in the liver with or without presence of HBV DNA in the serum in individuals who are HBs Ag – negative as confirmed by currently available serological analyses (1). The occult hepatitis B virus infection is due to long-term persistence of the virus genome in the hepatocytes, thus the most precise method for detection of the occult infection is the analysis of DNA, extracted from liver tissue (1-3). Liver samples, though, are scarcely available and, besides that, currently there are no standardized and validated tests for detection of HBV DNA in liver tissues.

The importance of occult HBV infection in patients with HCV infection is quite contradictory. The number of scientific publications presenting studies on the effect of occult HBV infection in patients with hepatitis C infection is constantly increasing. Certain papers identify occult HBV infection as a significant risk factor for more severe liver damage and quicker progress of liver fibrosis, thus for earlier development of liver cirrhosis and hepatocellular carcinoma, respectively. It has been confirmed that the persistence of HBV genome in liver cells, itself, without development of liver cirrhosis, increases the

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risk for hepatocellular carcinoma in patients with HCV infection. In fact, there are also scientific papers that do not prove a relationship between presence of occult HBV infection and the clinical course of HCV infection. There are contradicting data for almost each issue concerning epidemiological, clinical, biochemical, virusological and histological aspects of occult HBV infection in patients with concomitant HCV infection.

PURPOSE

The aim of the present survey is to establish the rate of positive HBV DNA in liver tissue of patients with chronic HCV infection and a probable relationship between it and the relevant clinical finding.

METHODS

The survey covered 96 patients with chronic HCV infection – chronic hepatitis (n = 42), liver cirrhosis (n = 35) and HCC (n = 19) who have been treated at the Gastroenterology Clinic, Military Medical Academy – Sofia, in the period March 2004 – December 2010. Seventy seven per cent of them (n = 74) were men and 23% (n = 22) – women. The patients were 19 to 82 years old, mean age 50.5 ± 10.02 yrs. Eighty four (87,5%) of the patients were infected with HCV genotype 1 and 12,5% with HCV genotype 3 (n = 12). All involved patients were HBsAg – negative and 54,2% (n = 52) showed evidence for past HBV infection. All 96 patients were subjected to liver biopsy. It was performed after Menghini's method, needle 1,2 – 1,4 mm, Hepafix ® Braun Melsungen AG Germany. The assessment of the histological changes in the liver was made implementing METAVIR system (4). The biopsy material has also been used for detection of HBV DNA. The "nested PCR" methodology was used, as it enabled detection and amplification of very small DNA amounts. The extraction of DNA from the deparaffinized liver tissue was made using trade kit QIAamp® DNA Mini Kit, (QIAGEN Ltd, Crawley, UK) for extraction of DNA from tissues according to the manufacturer's requirements. The liver samples were tested for presence of nucleotide sequences in three gene regions of HBV: S-, Core- and X-antigen coding regions (5,6). The following primers were used for detection of S-antigen coding region of HBV: for the first PCR reaction: Primer 1: 5′- CCTGCTGGTGGCTCGAGTTC - 3′ (nt 58–77) and Primer 2: 5′ - CAAACGGGCAACATACCTTG - 3′ (nt 486–467) and for the second PCR reaction: Primer 3: 5′- ACATCAGGATTCCTAGGACC- 3′ (nt 169–188) and Primer 4: 5′- CGCAGACACATCCAGCGATA- 3′ (nt 389–370) (5). For detection of Core – antigen coding region the following primers were implemented for the first PCR reaction: Primer 5: 5′- GGAGTGGGATTCGCACTCC - 3′ (nt 2269–2288) Primer 6: 5′ - ATACTAACATTGAGATTCCC - 3′ (nt 2457–2438) and for the second PCR reaction: Primer 7: 5′- AGACCACCAAATGCCCCTAT - 3′ (nt 2299–2318) and Primer 8: 5′- GATCTTCTGCGACGCGGCGA - 3′ (nt 2429–2410) (5). The detection of X-antigen coding region in the liver tissue involved the following primers: for the first PCR reaction: Primer MD24: 5′- TGCCAACTGGATCCTTCGCGGGACGTCC TT-3′, (nt 1392-1421) and Primer MD26: 5′- GTTCACGGTGGTATAAATG-3′, (nt 1625-1607); primers for the second PCR reaction: HBx1: 5′-GTCCCCTTCTTCATCTGCCGT-3′ (nt 1487-1507) Primer HBx2: 5′- ACGTGCAGAGGTGAAGCGAAG-3′ (nt 1604-1584) (6). The visualization of the products from the nested PCR, size of the expected amplicones: ~221 bp for S-gene, ~ 131-135 bp for Core-gene and ~117 bp for X-gene was made on 2% agaros gel with added ethidium bromide, final concentration 0,5μg/ ml. The sensitivity of the method was tested with cloned HBV DNA with preset concentration (108 copies/ml), with falling ten-fold dilutions performed (from 10^{-1} to 10^{-8}). The sera of all patients with chronic HCV infection were analyzed for presence of HBV DNA by real -time PCR with trade kit for diagnostics: Cobas® TaqMan® HBV Test (Roche Molecular Systems, Inc.), limit of detection 6 IU/ml, (35 copies/ml). The quantitative measurement of HCV RNA in the serum was performed by real-time PCR with trade kit for diagnostics Cobas® TaqMan® HCV Test v 2.0 (Roche Molecular Systems, Inc.), limit of detection 25 IU/ml.

RESULTS

The liver biopsy samples of 96 patients with chronic HCV infection were tested for presence of HBV DNA. It was established that 40,6% (n = 39) of the HCV–positive patients whose liver samples were tested had positive HBV DNA in the liver tissue. The sera of all 96 patients with tested liver samples were analyzed also for HBV DNA and it was found in 20,8% (n = 20) of them. The detected rate of positive hepatic HBV DNA (40,6%) was significantly higher than the rate of positive HBV DNA in sera samples (20,8%) of patients with chronic HCV infection (P<0,0001).

The analysis of the patients with positive HBV DNA in the liver samples (n = 39) revealed that 82% (n = 32) of them had positive gene sequence of S – gene; 87% (n = 34) of them had positive gene sequence of Core–gene and 46,1% (n = 18) of them – for X-gene. Twenty one patients (54%) were simultaneously positive for S–and Core–gene, 5 patients (13%) were positive for both S– and X–gene and 7 patients (18%) were positive for S–and Core–gene at the same time. Six patients (15%) had all three gene sequences – of S-, Core-, and X–gene - positive. **(Table 1)**

Table 1*. S -, Core - and X –gene sequences in patients with positive HBV DNA in liver samples (n-39)*

Simultaneously positive gene sequences	**Positive HBV DNA (n-39), (IU/ml)**	
	Number of patients	**Percent**
S gene и Core gene	21	54%
S gene и X gene	5	13%
Core gene и X gene	7	18%
S gene, Core gene и X gene	6	15%

No difference was established in the distribution of the particular HCV genotypes of the patients with positive and negative hepatic HBV DNA (P=0,802).

No difference was established in the serum level of HCV RNA of the patients with and without positive HBV DNA in the liver samples (P=0,292). The mean values of HCV RNA of the patients with and without occult HCV infection were: 1 247 860 ± 120 574 IU/ml (median – 774 000, 00) and 1 162 771 ± 82 453 IU/ml (median – 786 500,00) respectively.

The values of liver enzymes of patients with positive hepatic HBV DNA (AST - 104 ± 48,07 IU/ml) did not differ significantly from those of patients with negative hepatic HBV DNA (AST - 90,41 ± 56,55 IU/ml) (P=0,325).

Positive hepatic HBV DNA was found in 34 patients of those, positive for anti HBc Ab (34/52, 65,4%) and in 5 patients negative for anti HBc Ab (5/44, 11,4%). Statistically significant relationship was revealed between the presence of positive hepatic HBV DNA and presence of anti HBc Ab (P=0,001). Positive hepatic HBV DNA was found in 20 patients with positive anti HBs Ab (20/36, 56%) and in 19 patients, negative for anti HBs Ab (19/60, 32%). Significant relationship was found between the presence of positive hepatic HBV DNA and anti HBs Ab (P=0,050).

The rate of positive hepatic HBV DNA in the particular clinical groups of HCV-positive patients – chronic hepatitis, liver cirrhosis and HCC was different. The highest rate of positive hepatic HBV DNA was found for the patients with HCC - 58% (11/19), followed by that for the patients with liver cirrhosis - 45,7% (16/35), and the rate among the patients with chronic hepatitis was 26,8% (12/42). The differences in the rates of positive hepatic HBV DNA and of negative hepatic HBV DNA in the individual clinical groups was statistically significant (P=0,001). **(Table 2)**

Table 2. *Positive liver HBV DNA in different clinical groups in patients with chronic HCV infection*

Group of patients with chronic HCV infection n-96	**Positive liver HBV DNA (n-39), (IU/ml)**	
	Number of patients	**Percent**
Chronic hepatitis, n-42	12	28,6%
Cirrhosis, n-35	16	45,7%
HCC, n-19	11	57,9%
Fisher (P)	P<0,0001	

The group of patients with HCV-positive HCC showed the presence of positive hepatic HBV DNA in 58% (11/19) of the patients. Six of the patients with positive hepatic HBV DNA had concomitant cirrhosis (2/11, 54,5% of the patients with HCC and cirrhosis) and 5 of the patients with positive hepatic HBV DNA did not manifest cirrhotic parenchymal rearrangement (5/11, 45,4% of the patients with HCC without cirrhosis). It was established that patients with HCC and positive hepatic HBV DNA were younger than those

with HCC and negative hepatic HBV DNA (mean age 41±9,89 yrs. and 66±2,12 yrs. respectively) (P=0.0018).

In the group of patients with chronic hepatitis (n = 42) it was established that the positive hepatic HBV DNA was associated with more severely expressed stage of necroinflammatory activity in patients with chronic hepatitis C and that this association was statistically significant (P=0,032). Positive hepatic HBV DNA was established in 11% (1/9) of the A1 stage patients; 28% (5/18) of the A2 stage patients and in 43% (6/14) of the A3 stage patients, and was not revealed in patients with A0 stage of necroinflammatory activity. Statistically significant difference was established in the distribution of positive hepatic HBV DNA in the different fibrosis stages of patients with chronic hepatitis C (P<0,0001). Positive hepatic HBV DNA was found in 23,5% (4/17) of the patients with F2 fibrosis and in 36,4% (8/22) of the patients with F3 fibrosis, and was not established in patients with F0 and F1 fibrosis.

DISCUSSION

Occult HBV infection has been the focus of multiple clinical studies of patients with chronic HCV infection. The results of those clinical studies are quite contradictory referring to the rate and to the clinical importance of occult HBV infection as well. In fact, almost all surveys have shown that the rate of occult HBV infection was greater among patients with chronic HCV infection – between 22% and 35 % as an average, than among HCV-negative healthy individuals where it varied between 0 and 17% depending on the geographic region (1-3, 7).

The rate of occult HBV infection established when testing serum samples of patients with chronic HCV infection – 20,8% within the frames of this survey corresponded to rates of occult HBV infection found in sera of HCV-positive patients in other studies: Marusawa et al. (8) – 24,1%, Japan; Kazemi-Shiraz et al. (25) – 22%, Austria; Mrani et al. (10) – 23%, France; Georgiadou et al. (11) – 26,2%, Greece, Zhelev - 19%, Bulgaria (12).

An important factor, substantially affecting the rate of established positive HBV DNA was the tested biological matter – serum, liver samples or peripheral mononuclear cells. The majority of studies have found that the rate of positive HBV DNA in liver samples was greater than that in serum samples. The rate of positive HBV DNA in HBsAg-negative patients with HCV infection, found at testing liver samples was as an average between 50%-56%, reaching even up to 87,6% (13) – 91,9% (14) in individual surveys, while the analysis of serum samples provided a rate of positive HBV DNA between 22-35% (1, 2, 7, 8, 10, 11, 14).

The rate of positive hepatic HBV DNA in patients with chronic HCV infection, as found by this survey, was 40,6% and was significantly greater than the established rate of positive HBV DNA in the sera of the same patients - 20,8% (P<0,0001). The established rate of positive hepatic HBV DNA in HCV-positive patients was similar to the results obtained by other authors too: Shetty et al. (39) found a rate of occult HBV infection, based on positive serum HBV DNA of 28%, and on the basis of positive intrahepatic HBV DNA - 50%; Abdelmalek et al. (32) evidenced presence of positive HBV DNA in 29 % of the liver samples and in 6% of the serum samples; Cacciola et al. (7) had observed positive hepatic HBV DNA in 30% of the tested patients and positive serum HBV DNA in only 18% of them. The higher rate of detection of positive HBV DNA in the liver than in the serum could be explained from biological point of view. At this stage it is recognized that the molecular basis of occult HBV infection is due to the intrahepatic persistence of viral cccDNA, associated with strongly suppressed replication and gene expression (1). The testing of liver samples for presence of HBV DNA is the most precise methodological approach of detection of occult HBV infection but its implementation in clinical practice is difficult.

The researchers of occult HBV infection have implemented various test methods with different limit of detection as well as various “definitions” for presence of occult HBV infection. In this particular study the detection method for HBV DNA in liver samples was the nested PCR technology with amplification of the sequences of S-, Core- and X-gene coding regions of the viral HBV DNA. An occult HBV infection is considered as relevant when at least two positive gene sequences are detected in the liver sample. It was established that 82% of the patients with positive HBV DNA in the liver samples had positive gene sequence for S–gene; 87% - had positive gene sequence for Core–gene and 46,1% - had positive gene sequence for X–gene. Simultaneous positive S– and Core–gene were found in 54% of the patients, in 13% were positive at the same time for S– and X– gene and 18% of the patients were positive for both S– and Core–gene. All three gene sequences

were positive in 15% of the patients - S-, Core- and X-gene. Similar results with prevailing detection of S– and Core– gene and combinations have been obtained also by: Cacciola et al. (7) – 88% of the patients with positive HBV DNA has positive S- and Core-gene at the same time; Fabris et al. (17) – reported positive S–gene in 40% of the patients, positive Core–gene in 80% of the patients and only 20% of the patients were positive for both genes. Villa et al. (18) have established that the S-gene was most often positivized in 42 of the patients, X–gene – in 15,8% of the patients and Core–gene – in only 5,3%. In the presence of two positive genes the association is always with S–gene between Core– and/or X-gene (19).

No relationship has been established between the presence of occult HBV infection and the demographic indicators – patient's sex and age, HCV genotype, serum level of HCV RNA and the biochemical activity of the liver disease at the test, and liver samples. The obtained results were confirmed by other studies as well (1, 9, 11, 20-24). The number of studies that have established an association between the presence of occult HBV infection and the genotype of HCV virus is small (25, 26), serum level of HCV RNA (9, 13, 15) or transaminase activity (25, 27).

One factor that is significantly associated with occult HBV infection is the serological status of past HBV infection. The rate of positive HBV DNA in anti HBc Ab positive patients varies between 32% and 80% (1-3, 7, 8, 11, 14, 15, 26, 28). Our study confirmed the results of the above cited surveys and established that the occult HBV infection was significantly related to the presence of anti HBc Ab (P=0,001) and the presence of anti HBs Ab (P=0,050) as well. The rate of serologically negative occult HBV in patients with HCV infection has been found within the interval 10% - 18% (14, 17), and this survey showed a rate of seronegative occult HBV infection at testing liver samples of 11%.

The presented study established significant relationship between the presence of occult HBV infection and the gravity of clinical presentation of HCV infection – more often in patients with HCC and cirrhosis and, to a smaller extent in patients with chronic hepatitis. Positive hepatic HBV DNA was detected in: 28,6% of the patients with chronic hepatitis, 45,7% of the patients with cirrhosis and 58% of HCC patients (P<0,0001). HCV-positive patients without occult HBV infection do not show such relationship. The results of this study showed that in the cases when the individual clinical states were well distinguished, as chronic hepatitis, cirrhosis and HCC, the test of serum samples could provide reliable information about the presence and effect of occult HBV infection in patients with chronic HCV infection. In the cases with chronic hepatitis C the positive hepatic HBV DNA was significantly related to more severe necroinflammatory changes (P=0,032) and more serious fibrosis (P<0,0001). The obtained results for the clinical significance of occult HBV infection in patients with chronic HCV infection confirmed with the results of other authors (1- 3, 7- 9, 13, 14, 21-23, 25, 26, 30, 31). Other researchers, though, supported that occult HBV infection in patients with chronic HCV infection was only a concomitant state without any effect on the clinical course of liver damage (9, 11, 17, 29, 32).

CONCLUSION

Positive liver HBV DNA was found in 40,6% of cases with chronic HCV infection, which is significantly higher than the presence of positive serum HBV DNA (20,8%) in the same patients. Occult HBV infection in patients with chronic HCV infection was associated with more severe liver damage in comparison with the patients mono-infected with HCV, which requires more strict control and aggressive treatment of HCV infection.

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