IDENTIFICATION OF PRE-CORE AND BASAL CORE PROMOTER MUTANTS IN PATIENTS WITH CHRONIC HEPATITIS B IN THE REPUBLIC OF MACEDONIA

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Abstract: Recent development of molecular techniques has improved our understanding of the role of various mutations of the HBV genome. Most common are mutations in the precore (PC) and basal core promoter (BCP) region, responsible for more serious course of chronic hepatitis.
The purpose of this study was to evaluate the prevalence of PC and BCP mutants in patients with chronic hepatitis B in the Republic of Macedonia.
Serum samples from 69 patients with chronic hepatitis B (47 males and 22 females, average age 49±20y.) were collected in the period from 2002-2012. All serum samples were tested for HBV, HCV and HDV infection and immediately frozen at -70°C. According to the HBeAg status, these patients were divided in two groups: HBeAg positive (15/69 pts or 21, 74%), and HBeAg-negative (54/69 pts or 78,26%).
Molecular examination including extraction and amplification of HBV DNA was performed. To establish if HBeAg-negative status is related to sero-conversion, or as a consequence of viral mutations, we have used INNO-Lipa hybridization assay from Innogenetics to identify the presence of mutations in precore and BCP region of HBV DNA. Molecular analysis was done in 38/54 HBeAg-negative patients (28 males and 10 females).
The prevalence of PC mutants in 84,21% (p=0,0000) and BCP mutants in 68,42% (P=0,0033) were extremely high in 38 examined HBeAg-negative patients. Combination of PC and BCP mutants was detected in HBV DNA of 25/38 HBeAg-negative patients (65,78%).
As a conclusion, HBeAg-negative stage was predominant in our patients with chronic hepatitis B and was related to mutations in PC and BCP region.
Keywords: HBV, HBeAg, HBV DNA, RNA, PC, BCP, HCC, WHO, nt

1. BACKGROUND

Hepatitis B virus is most common cause of chronic hepatitis infection in the world. It is estimated that annually about 1 million dead occurred as a consequence of cirrhosis and hepatocelular carcinoma- HCC [1]. According to World Health Organization (WHO), about 80% of all cases with HCC globally appear as a result of chronic HBV infection [2]. Although HBV does not possess direct citopatogenic effect, the actual cause of liver damage is related to an activation of host immune system, especially in chronic HBV infection. To survive, HBV must evade the immune activity of the host and mutate. There are numerous evidences that natural or induced mutations of the virus influence the severity, clinical outcome of the disease and response to therapy with interferon or nucleoside analogues. HBeAg negativity in HBV chronic infection means e-antigen seroconversion low viral replication and inactive disease. But, so-called HBeAg-negative chronic hepatitis B is characterized by persistent viral replication, active hepatitis and progression to more severe form of liver disease. Molecular analysis of the structure and pathogenesis of HBV revealed that HBeAg- negative form of chronic hepatitis B is caused by viral mutations of "C" gene which leads to progressive disease and bad prognosis HBeAg-negative chronic hepatitis B is most prevalent in Mediterranean Region with 33% and Asia- Pacific with 15% [3]. Male gender affected with chronic HBV infection is more often in HBeAg negative stage than females [4].
Use of molecular techniques showed that substitution of adenine with guanine at nucleoside 1896 in precore region of "C" gene create TAG stop codon which leads to inability of producing of HBe- antigen, while replication of HBV DNA is not compromised, but instead it is increased [5-7]. Due to increase of viral DNA replication, the clinical condition of the patient worsens and therapy with interferon is compromised.
Because of base pairing of nt1896 with nt1858, TAG mutation occurs in HBV DNA with thymine at position 1858 in the stem of the pregenomic RNA loop. If cytosine is present at nt1858, this mutation doesn't occur because of stability of the pregenomic RNA loop. Thus, PC mutation is common in genotype B, D, but it is rare in genotype A or C, which have cytosine at nt1858 [8].
The other common mutation in "C" gene is in basal core promoter region (BCP), which regulates the expression of HBeAg. It's mutation lowers the expression of HBeAg. Most common is double substitution at position 1762 where

adenine (A) is substituted with thymine (T) and at position 1764 where guanine (G) is substituted with adenine. This double substitution results with reduced expression of HBeAg and increased replication of HBV [8]. Because BCP region partly overlap with X-region of the HBV genome, mutations in the nucleotide sequences at position 130 and 131 of this region (K130M and V131) are prognostic markers for development of HCC [9-13]. Development of these two mutations is connected with spontaneously or given α- interferon therapies, which induce HBeAg sero-conversion [14].

There is connection in occurrence of PC and BCP mutations with male gender and certain HBV genotypes. Thus in genotype C there is higher level of HBV replication, which can be as a result of deregulation of precore RNA or pregenomic RNA [15].

There is also geographic distribution of these two mutations, so they are most prevalent in Asia and Mediterranean Region, where genotypes B and C are predominant, while they are not found in North America and North Europe where genotype A is predominant [16-17].

Republic of Macedonia belongs to a region of moderate prevalence of HBV infection, with prevalence of total AntiHBc antibodies in normal population with 21% [18].

So, the purpose of this study was to evaluate the prevalence and the role of mutations in the precore (PC) and basal core promoter (BCP) region of HBV in patients with HBeAg negative chronic HBV infection in Republic of Macedonia.

2. MATERIALS AND METHODS

Serum samples:

As a material we have used serum samples from 69 patients with chronic HBV infection (47 males and 22 females with av. age 49+/- 20y.) collected during the period from 2002y. to 2012y. Patients were diagnosed and treated from HBV infection at the University Clinic of Gastroenterohepatology - Medical Faculty, Skopje. All samples were immediately frozen and stored at -70°C.

Serological analysis:

Serological tests in all 69 serum samples were performed immediately at the time of collection. Detection of 6 HBV markers (HBsAg, AntiHBs, HBeAg, AntiHBe, AntiHBc, AntiHBcIgM) and AntiHCV antibodies was performed with Micro particle enzyme immunoassay (MEIA) using immunoanalyzer Axsym- Abbott.

Serological tests for HDV Ag, AntiHDV IgM, AntiHDV were performed with microelisa by Eiagen Adaltis.

DNA analysis:

Viral DNA was isolated in all 69 serum samples using standard phenol chlorophorm extraction and precipitation with ethanol, and with two commercial kits: Viral Gene-spin^{tm} Viral DNA/RNA (Intron Biotechnology) and Ribo-Spin vRD ^{tm} (Geneall Biotechnology, according to the manufacturer recommendations [19].

DNA amplification:

Amplification of HBV DNA in all 69 isolated samples was performed by nested PCR using HBV test Genekam Biotechnology AG, Germany according to the manufacturer recommendation.

Detection of pre- core (PC) and basal core promoter (BCP) mutants: was performed with INNO-Lipa HBV PreCore v8 line probe hybridization assay from Innogenetics, according to the manufacturer recommendations. The principle of this assay is nested PCR with biotinilated primers and hybridization of amplification product with specific oligonucleotide probes immobilized on membrane strips.

Statistical evaluation: because the obtained results were nonparametric, we have used a nonparametric two-tailed chi square (χ^2) statistical method for comparison and explanation of the obtained results.

3. RESULTS

Serological tests of HBV, AntiHCV and HDV markers were performed upon receipt at the University Clinic of Clinical Biochemistry - Skopje.

DNA extraction, HBV DNA amplification and detection of pre-core (PC) and basal core promoter (BCP) mutants were performed at the Department of Molecular Biology and Genetics at the Faculty of Natural Sciences and Mathematics, University "Ss. Cyril and Methodius"-Skopje.

HBV DNA was successfully isolated and analyzed in 69 patients (22 females and 47 males). Average age of all patients was 49+/-20 years.

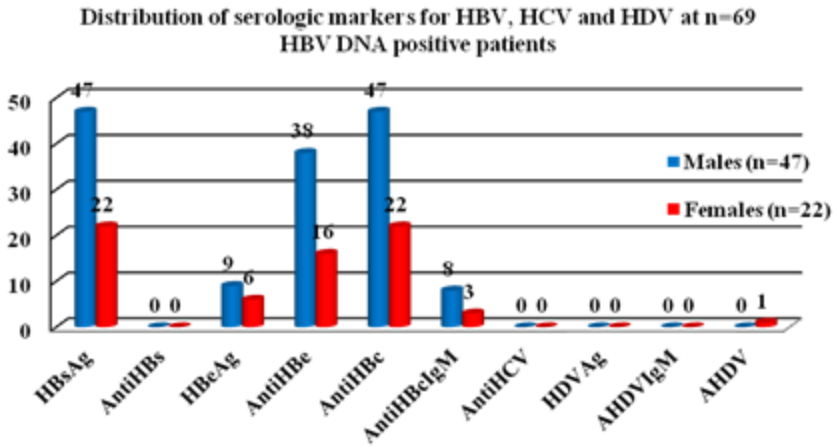
Results of serologic tests in serum samples of 69 patients with positive HBV DNA:

All 69 patients were positive for HBsAg and AntiHBc (100%).

HBeAg positive were 15 of 69 patients or 21,74%, while AntiHBe positive were 54 of 69 patients or 78,26%. AntiHBcIgM antibodies were detected in 11 of 69 patients or 15,9%. All patients were AntiHCV negative 0% as well as HDVAg and AHDVIgM (0%), while only one patient had AHDV antibodies (1/69 or 1,45%).

Chronic HBV infection was more prevalent in male gender in 69 examined patients with 47 of 69 or 68,11%, while 22 of 69 examined patients were female or 31,89%. Gender distribution of obtained results of serologic markers is shown at Chart 1.

Chart 1.



According to the HBeAg status 69 examined HBV DNA positive patients were divided in two groups:

- Group of 15 HBeAg positive patients which belonged to n=9 males and n=6 females patients.
- Group of 54 HBeAg negative patients composed 38 males and 16 females.

Chi square test of number of HBeAg negative patients showed statistic significance of the prevalence of HBeAg negative stage at males patients p=0,000 (Table 1).

Table 1	Males	Females	Total
HBeAg positive	9	6	15
Percent	13,043%	8,696%	21,739%
HBeAg negative	38	16	54
Percent	55,072%	23,188%	78,261%
Total	47	22	69
P= 0,0000			

Results of hybridization assay for detection of precore (PC) and basal core promoter (BCP) mutants:

Due to the objective reason (finances), detection of presence of PC and BCP mutants in HBeAg negative patients was performed in HBV DNA samples from 38 of 54 patients with HBeAg negative status (28 males and 10 females).

Mutations in the Pre-core (PC) region were detected in 32 of 38 HBeAg negative patients with chronic HBV infection or 84,21%. Only 6 of 38 patients have wild type PC region (1896G- 15,78%). Mutation nt1896G-A was detected in 21 of 38 patients or 55,26%, while 11 of 38 patients had mixture of wild type and variant (A) (28,94%). (Table 2).

Chi square test (χ^2) of distribution of PC mutants in relation with gender has been shown at Table 3.

Table 2. Total number of tested HBeAg negative patients (n=38)

Region Pre- core (PC) and variants			
		Positive patients	**%**
Total number of mutants	32/38	84,21%	
Wild type (G)	6/38	15,79%	
Variant (A)	21/38	55,26%	
Combination of wild type + variant (A)	11/38	28,94%	

Table 3
χ^2 test of distribution of PC mutants at n=38 HBeAg negative patients

	Males 28/38 (73,68%)	**Females 10/38 (26,31%)**	**Total**	**P<0,05**
WT	3	3	6	
Percentage	7,89%	7,89%	15,79%	**P=0,0001**
PC	17	4	21	
Percentage	44,73%	10,52%	55,26%	**P=0,037**
WT/PC	8	3	11	
Percentage	21,05%	7,89%	28,94%	**P=0,0009**
Total number of mutants	25	7	32	
Percentage	65,79%	18,42%	84,21%	**P=0,0001**

Mutations in the basal core promoter region (BCP) were detected at 26 of 38 HBeAg negative patients with chronic HBV infection (68,42%) **(Table 4).**
Wild type of BCP was 12/38 HBeAg negative patients (31,57%).
Most prevalent was mutation T1762/A1764 at 19 of 38 patients or 50%.
Mutation A1762/T1764 was not detected in any of n=38 tested samples (0%).

Chi square test (χ^2) of distribution of BCP variants in relation with gender has been shown at **Table 4**.

Table 4.
χ^2 test of distribution of BCP mutants at n=38 HBeAg negative patients

	Males 28/38 (73,68%)	**Females 10/38 (26,31%)**	**Total**	**P<0,05**
Wild type (AG)	7	5	12	
Percentage	18,42%	13,16%	31,58%	**P=0,0033**
Variant (AA)	1	0	1	
Percentage	2,63%	0%	2,63%	**P=0,0000**
Variant (TA)	15	4	19	
Percentage	39,47%	10,52%	50%	**P=0,05**

Combination of wild type +AA	2	0	2	
Percentage	5,26%	0,0%	5,26%	**P=0,0000**
Combination of wild type + TA	3	0	3	
Percentage	7,89%	0,0%	7,89%	**P=0,0000**
Combination of (AA+TA)	0	1	1	
Percentage	0,0%	2,86%	2,86%	**P=0,0000**
Total number of mutants	21	5	26	
Percentage	55,263%	13,158%	68,42%	**P=0,0033**

Combination of mutations in the PC and BCP regions:

Combination of both mutations was detected in 25 of 38 examined HBV DNA positive samples from HBeAg negative patients (65,78%). Of them, 21 were males (55,26%) and 4 were females (10,52%). Chi square test (χ^2) of distribution of mutants in both regions in relation with gender was not significant (p=0,071).

4. DISCUSSION

HBeAg negative form of chronic hepatitis B represents serious health and socioeconomic problem. Literature data have shown that sero-conversion from HBeAg to AntiHBe allows selection of mutants in the precore and basal core promoter region which leads to impaired production of HBeAg, while HBV DNA level is not compromised. At this way HBV can escape host immune system or therapy response. Clinical consequences of such long-term viral infection are cirrhosis and hepatocellular carcinoma. For end-stage liver disease the only therapeutically option is liver transplantation.

Severity of chronic HBV infection is seriously affected by co or super infection with HDVwhich is commonly prevalent in Mediterranean and South America, Africa and Middle East [20]. In the case of co-infection with HDV in previously chronic HBV infection, there is 60-80% risk of cirrhosis and HCC [21].

In this study we have examined 69 patients with chronic HBV infection (22 females and 47 males) with average age of 49+/-20 years. Male gender prevailed in all of 69 examined patients 68,11%.

Serologic tests for detection of for 6 HBV markers, AntiHCV antibodies and 3 HDV markers were performed immediately upon receipt in serum samples from all 69 investigated patients with chronic HBV infection.

All 69 serum samples were AntiHCV negative (0%). Only one patient had AHDV antibodies (1,44%). Co or super infection with HCV and HDV didn't show significant influence on the course of chronic HBV infection in all 69 examined patients with chronic HBV infection.

HBV DNA was successfully extracted and amplified from serum samples of 69 investigated patients collected during the 2002y. and 2012y.

The prevalence of HBeAg negative stage in isolated samples from patients with chronic HBV infection was predominant, and male gender was more affected or 38 of 69 or 55,07% patients (P=0,0000). Our results also correspond with literature data about prevalence of HBeAg negative stage of chronic HBV infection in Mediterranean Basin of 33% [3-4].

INNO-Lipa hybridization assay was used to investigate the prevalence and the role of PC and BCP mutations in the course of the HBeAg negative stage of chronic HBV infection.

Total number of examined patients for detection of the presence of these mutants was n=38 (28 males and 10 females).

Prevalence of PC mutations in 38 HBeAg negative patients was extremely high 84,21%. This mutation prevail among male gender with 65,79%, while 18,42% in female (p=0,0001).

Only 6/38 belonged to the wild type (15,79%). PC mutation was detected in 55,26%, while 28,94% had mixture of wild type and PC mutation. These results correspond with literature data, about the prevalence of PC mutants in HBeAg negative phase of chronic HBV infection in Mediterranean and Southeast Europe [3, 5].

Prevalence of BCP mutants in HBV DNA from n=38 patients with HBeAg negative phase was also high, but lower than prevalence of PC mutation 26 of 38 or 68,42%. As in the case of PC mutants, these BCP mutants prevailed in male gender also 57,89%/ 13,158% (P=0,0033).

Most prevalent was T1762/A1764 variation in 19 of 38 patients or 50%.

The percent of wild type BCP was higher than PC mutants and 12 of 38 patients have no mutations in this region or 31,58%.

Double mutation in the PC and BCP region was found among 25 of 38 examined HBV DNA positive samples from HBeAg negative patients (65,78%). From them, 21 were males (55,26%) and 4 were females (10,52%). Chi square test (χ^2) of distribution of mutants in both regions in relation with gender was not significant (p=0,071). This high percent implies pure prognosis and increased risk of progression to HCC.

All this results for prevalence of PC and BCP mutations in HBeAg negative patients with chronic HBV infection corresponds with literature data about role of these mutants in HBeAg negative stage of the disease and their prevalence in Mediterranean and Balkan region [5].

5. CONCLUSION

HDV and HCV doesn`t have influence on the course of chronic HBV infection in our group of patients.

Male gender is more affected by the chronic HBV infection and HBeAg negative stage of the disease.

HBeAg negative stage of chronic HBV infection in our group doesn't represent normal beneficial phase of chronic HBV infection, but rather it has been caused by mutations in precore (PC) and basal core promoter (BCP) region.

All above conclusions imply need of routine use of sophisticated molecular methods for detection and characterization of HBV in order to define preciously the form of liver disease, as well as to monitor the course and evolution of HBV and the effects of therapy.

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