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

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BACKGROUND-AIM

Background: According to World Health Organization (WHO), about 80% of all cases with HCC globally appear as a result of chronic HBV infection [1].This stage of chronic HBV infection is most prevalent in Mediterranean Region with 33% and Asia- Pacific with 15% [2]. Male gender affected with chronic HBV infection is more often in HBeAg negative stage than females [3].

There are two forms of chronic hepatitis due to infection with B virus: HBeAg positive and HBeAg negative chronic hepatitis B, with different clinical features and therapeutically outcome.

Recent development of molecular techniques has improved our understanding of natural history of HBV infection as well as the role of various mutations of the HBV genome.

HBV replicates through RNA intermediate using reverse transcriptase which lacks proof reading ability thus allowing mutations to occur and accumulate in the viral genome. Thus mutations can be induced naturally by host immune system or by treatment with nucleoside analogues.

Most common are mutations in the precore (PC) and basal core promoter (BCP) region, leading to inability of producing HBeAg, increased HBV replication and high viral load.

Both mutations are responsible for acquiring HBeAg negative state of chronic HBV infection.

BCP mutation has been associated with increased risk of HCC.
Republic of Macedonia belongs to a region of moderate prevalence of HBV infection, with prevalence of total AntiHBc antibodies in normal population with 21% [4].

Aim of the study was to evaluate the prevalence and the role of PC and BCP mutants in patients with chronic hepatitis B in the Republic of Macedonia.

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		

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

Region and variants of Pre- core (nt 1896)		
	Positive patients	%
Total number of mutants 7	32/38	84,21%
Wild type	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

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

MATERIAL AND METHODS

MATERIALS:

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.

METHODS:

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.

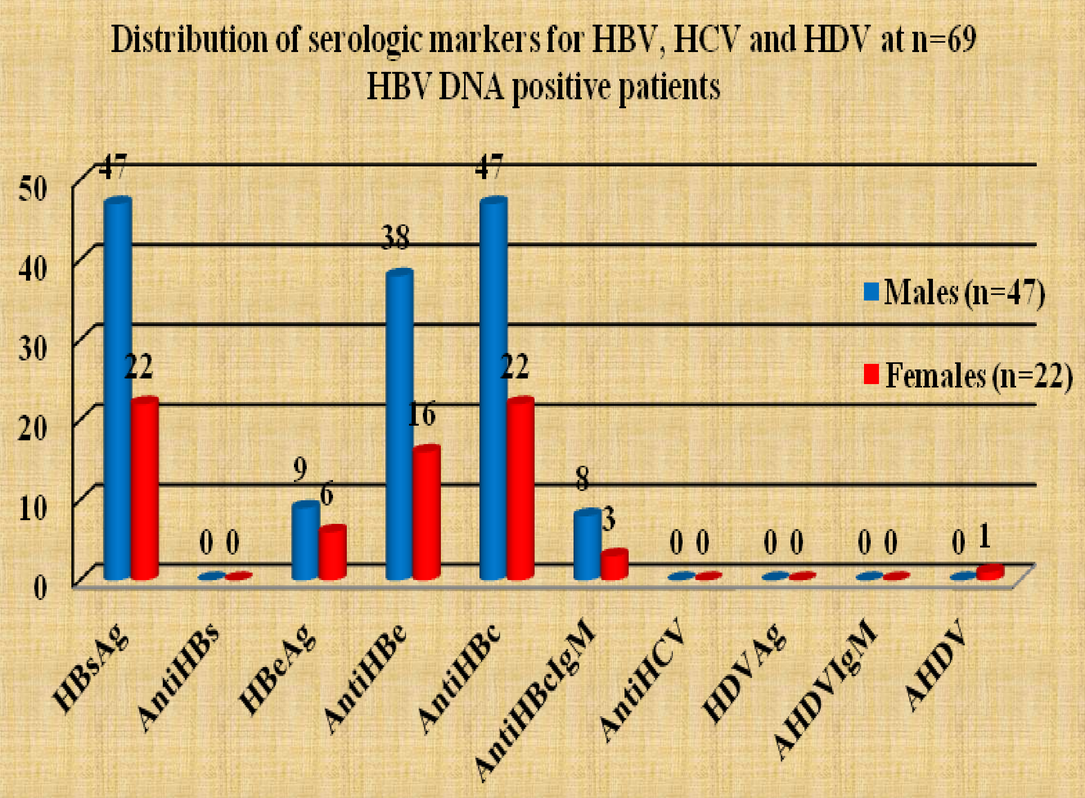
Detection of pre- core (PC) and basal core promoter (BCP) mutants: To establish if HBeAg-negative status is related to sero-conversion, or as a consequence of viral mutations, we have used INNO-Lipa hybridization assay from Innogenetics to identify the presence of mutations in precore and BCP region of HBV DNA. Molecular analysis in 38/54 HBeAg-negative patients (28 males and 10 females) 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.

RESULTS AND DISCUSSION

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.

Chart1



•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% [2-3].

• The prevalence of PC mutants in 38 HBeAg negative patients was extremely high 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%).

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

All mutations prevailed in male patients.

CONCLUSIONS:

• Hepatitis B virus is very stable virus, we have succeeded isolated HBV DNA in srum samples collected since 2002y.

• 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.

• Our 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].

• 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.

Key words: HBV, HBeAg, HBV DNA, RNA, PC, BCP, HCC, WHO, nt.

REFFERENCES:

1. **Rosental KS.** Hepatitis Viruses. Medical Microbiology, eds: Murray PR et al. 1994; 702-718.
2. **Funk ML,Rosenberg DM, Lok ASF.** World-wide epidemiology of HBeAg-negative chronic hepatitis B and associated precore and corepromoter variants. Journal of Viral Hepatitis 2002; 9:52-61.
3. **Чалоска-Иванова В, Јоксимовиќ Н.** Хроничен вирусен хепатит- дијагноза и лекување 2012, Скопје Р. Македонија.
4. **Колоска В.** Ензим- имунолошки методи за детекција на Хепатитис Б и Ц Вирус и маркери за црнодробно оштетување. Маг. Труд 2000.
5. **Sanchez-Tapias JM, Costa J, Mas A, Bruguera M, Rodes J.** Influence of hepatitis B virus genotype on the long-term outcome of chronic hepatitis B in western patients. Gastroenterology. 2002;123(6):1848-56.