The Study of VHB Genotypes in a Population – a Pilot Study

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Identifying the hepatitis B virus (HBV) genotype is crucial for hepatocellular carcinoma (HCC) surveillance related to HBV infection. The aim of this pilot study was to evaluate the prevalence of HBV genotypes at the patients with chronic hepatitis in the N-E region of the country. Material and Method: 20 patients with chronic hepatitis B virus were included in this study during July–October 2014 period and were tested for serological markers (chemiluminiscence assay, viral load (Real Time PCR assay) and genotyping method. We detected the D genotype of HBV in 19 (95%) of the patients; one had only genotype A (5%) like unique infection, and in other case was diagnosed two associated genotipes, A and D(5%) We found statistical significant difference among the mean of viremia (DNA/HBV) and the genotype of HBV (p=0.004) , the presence / absence of HBeAg and antiHBe (p=0.001), the severity of fibrosis(p=0.006), and the presence / absence of interferon therapy (p=0.001). The D genotype of HBV is the most frequent in our area, alone or associated with A genotype. Genotyping of HBV should be routinely introduced in our hospital, as our data suggest that testing patients for serological markers and viral load in association with the HBV type is very useful for monitorization of chronic B hepatitits patients.

Keywords: HBV, genotype, INNOLiPA, HBeAg negativ, antiviral therapy

GLOBOCAN mentions Romania as ranking second in Europe in the incidence of hepatocellular carcinoma (HCC), with a mortality of 10.5 / 100,000 for men and 4 / 100,000 for women, [1] infection caused by HBV being associated with an increased risk for this cancer. WHO (World Health Organization) specifies that world wide there are more than 240 million individuals chronically infected with HBV [2]. Romania is part of the intermediate endemicity area, porting chronic HBsAg prevalence between 2-7%. According with HBsAg prevalence in general population, Romania belong to intermediate endemicity area. Although HBV vaccine, introduced in Romania since 1995 in new-born, significantly reduced the morbidity and mortality through HPV infection, chronic hepatitis, cirrhosis and liver cancer remain a public health problem as in many parts of the world; complications of chronic HBV infection remain the 7th leading cause of death from infectious diseases worldwide [3]. A ECDC (European Centre of Diseases Control) report states that, in 2012, 14,745 cases of hepatitis HBV have been reported by the 27 EU countries, Romania being cited with 486 cases of acute hepatitis HBV. Trends over time are difficult to interpret due to changes in case definitions and practices reported in some countries. It is important to distinguish between acute and chronic infection with HBV [4]. According to EASL (European Association for the Study of the Liver) clinical practice guidelines, pre-treatment assessment of liver disease involves assessing biochemical markers (AST, ALT), detection of DNA / HBV, particularly by Real Time PCR quantification (known method for sensitivity, specificity, accuracy and range limitations results), testing for other causes of chronic hepatitis (HDV co-infections, HCV and / or HIV), liver biopsy (recommended for determining the degree of necrosis and fibrosis) [5].For the treatment of CBH the FDA (Food and Drugs Administration) has approved two different antivirals: conventional interferon or pegylat alfa and nucleoside analogues (NA) (lamivudine, telbivudine, emtricitabine, entecavir) and nucleotide (adefovir and tenofovir).

As predictors of response to interferon therapy, international criteria were established, based on the amount of DNA / HBV (<2 x 10 UI/ mL) determined by RT PCR, useful for initiation and continuation of viral therapy, increased transaminases and HBV genotypes: genotypes A and B proved to be associated with a good response in the appearance of anti-HBe Ab, secondary sero-conversion and loss of HBsAg compared with genotypes D and C [6,7].The 10 types of VHB have different geographical distributions and population shifts occur due to intravenous drug use, sexual behavior and migration situation. For making therapeutic decisions based on clinical evidence, studies have been conducted, which compared the effectiveness of antiviral medication - NA *versus* interferon. Clinical decisions, individualized for each patient should be taken immediately based on response rate to therapy and on the basis of adverse reactions.[8,9] Management of antiviral therapy should be based on virological monitoring to facilitate early evaluation of partial response to treatment, and treatment failure.[10]

The aim of this study is to assess the HBV genotype prevalence in a pilot study from North-eastern population of our country, and second, we aimed to correlate the type of HBV genotypes with the response to antiviral therapy.

Experimental part

Material and methods

A prospective cohort study of 20 chronic B hepatitis patients was conducted at the Iaºi *Sf. Parascheva*

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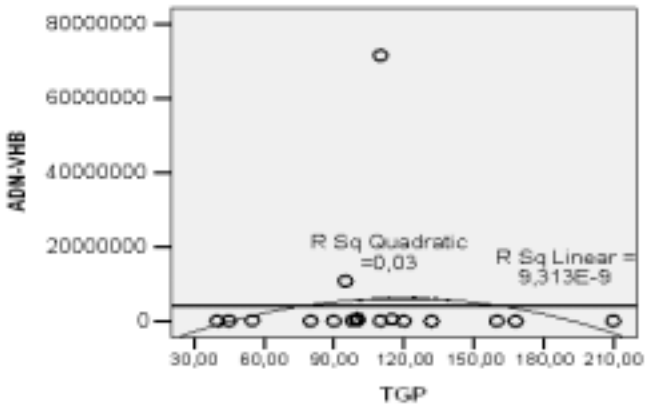

Fig. 1. Mathematical model DNA/HBV – ALAT values for prediction of D genotype of HBV

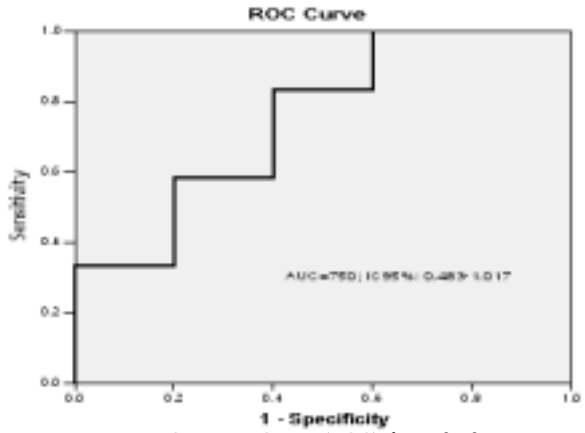

Fig. 2. Receiver Operator Curve (ROC) for calculating the DNA/HBV level differentiating HbeAg negative

University Hospital for Infectious Diseases in the interval July – October 2014. The serological markers (HBsAg, HBeAg and anti-HBe Ab) were tested with the *ARCHITECT i1000 SR, Immunoassay (Abbott DiagnosticsAnalyzer)* and the DNA / HBV were detected using *COBAS TaqMan 48 Real-Time PCR System* for Quantitation of Hepatitis B Virus *(Roche Diagnostics).* Blood samples collected in EDTA tubes were then sent to the Microbiology Laboratory of the Iasi *Gr. T. Popa* University of Medicine and Pharmacy for HBV genotyping

The HBV genotyping: DNA/HBV purification: DNA/*HBV* was purified using the kit Instruction for the Invisorb® Spin Virus DNA Mini Kit, STRATEC Molecular GmbH, D-The purity and concentration of DNA were analyzed with *Nano Drop Pearl* nan photometer. **INNO LiPA PCR amplification** purposed two steps:(*outer and nested amplification)*each of them using dedicated primers. For both amplifications (outer and nested) we used Gene *Amp PCR System* 9700, *Applied Biosystem thermocycler.* Test procedure for LiPA involved the next steps: denaturing, hybridizing the samples, washing the strips, developing the color using twincubator hybridization bath.

Statistical analysis: Datawere analyzed using SPSS version 20.0 (SPSS, Chicago, IL, USA). The level of statistical significance (p-value, the probability of maximal error) was considered 0.05 (5%), a probability (confidence interval) of 95% showing that the decision was fair. Thus, statistical significance was defined as $p < 0.05$ (95% CI).

Research ethics: Written informed consent was obtained from all patients enrolled in the study or a legal guardian, after receiving the approval from the Research Ethics Committee of the Ia°i *Gr T. Popa* University of Medicine and Pharmacy. The study conforms to international recommendations on human studies and respects the ethical standards for laboratory tests performed on pathological products from patients as specified in the Declaration of Helsinki. The HBV genotyping was a blind testing, without knowing clinical and microbiological data for the patients.

We genotyped the DNA/HBV from 20 patients aged 20 – 66 years old (mean 37.75), 8 (40%) women, 10 (50%) being from urban areas. 13 cases(65%) were positive for HbeAg, and 14 (70%) presented anti HbeAb. The mean of ALAT values was 107.9 (40 – 210 IU/mL). Fibrosis grade 1 and 2 was detected each for 7 (35%) of patients, F3 for 5 (25%) and F3 – F4 for 1 (5%) patient. 15(75%) of the patients are under Pegasys / Pegintron therapy. The mean of DNA / HBV was 36477105,3000 IU/mL (2500,00 - 717250000,00 IU/mL). The D genotype of HBV was detected for 18 (90%) of the patients. One had only genotype A (5%) and another one had double co-infection with both D and A genotypes (5%).Overestimation of incidence of hepatitis B virus mixed-genotype infections by use of the new line probe INNO LiPA geno-typing assay.Qutub MO et al, 2006 [11], developed a standardized, single-round INNO-LiPA PCR amplification protocols and automated sample processing by the MagNA Pure LC instrument were evaluated, with improved efficiency and suitability for routine laboratory use. One alternative of INNO LiPA genotyping method is TRUGENE™ HBV mgenotyping method, which was found to have better sensitivity, by Basaras M et al. 2013 [12].

Mercier Met al. in 2011found that INNO-LiPAover-estimates mixed infections as a result of erroneous genotype H detection, in comparison with sequencing of PCR-amplified DNA /HBV samples [13]. The high

Author/ year	Country	VHB genotype	Prevalence
Bokharaei-Salim Fet al. 2014	Azerbaijan	D	95.2%
		A	5.8%
		A+ D	0.97%
Sayan Met al. 2014	Turkey	E	First case
Constantinescu I et al.. 2014	Romania	D	60.5%,
		A+ D	31.4%,
Chamni Net al. 2014	Thailand	C	81.3%
Vutien Pet al. 2014	California	B	67.5%
		C	24.2%
Basu DM et al. 2013	SUA	A	43.7%
		C	4.34%
		D	4.34%
		E	4.34%
Baha Wet al. 2013	Maroc	D	90.45%
		A	5.9%
Scotto Get al. 2010	South Italy	E	45.15%
		D	18.1%
		B	15.3%
		C	13.2%
		A	4.9%

Table 1
GENOTYPES VHB FRECQUENCY DETECTED BY DIFFERENT AUTHORS INNO LiPA- METHOD

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Min	Max	P F_{ANOVA} test
					Lower Bound	Upper Bound			
Genotip									
A	2	6450	778	550	-538	13438	5900	7000	**0.004**
D	19	40528717	16890616	3981156	-4346634	12452377	2500	7E+008	
Complex a									
HBeAg (+) anti HBe (-)	5	147280	298052	133293	-222800	517360	2500	680000	**0.001**
HBeAg (+) antiHBe (+)	2	5500	4243	3000	-32619	43619	2500	8500	
HBeAg (-) antiHBe (+)	12	60731284	206772694	59690135	-70645818	192108386	5000	7E+008	
HBeAg (-) antiHBe (-)	1	7000	.	.	.	.	7000	7000	
HBeAg									
Absent	13	56060185	19868488	5510527	-6400389	17612426	5000	7E+008	**0.001**
Present	7	106771	253007	95628	-127221	340763	2500	680000	
anti HBe									
Absent	6	123900	272668	111316	-162247	410047	2500	680000	**0.002**
Present	14	52036172	19147726	5117443	-58499509	16261185	2500	7E+008	
Fibrosis									
F1	7	131129	246071	93006	-96450	358707	2500	680000	**0.006**
F2	7	104015243	27044083	10221026	-14610080	35413129	3000	7E+008	
F3	5	100041	201858	90274	-150599	350681	5600	461000	
F3-F4	1	5000					5000	5000	
PEG tratament									
Absent	5	14360340	3206782	1434116	-25457119	54177799	8500	7E+008	**0.001**
Present	15	813540	276272	71333	-716404	2343485	2500	10774400	

Table 2
DESCRIPTIVE STATISTICAL INDICATORS OF DNA/HBV (COPIES/mL)

prevalence of D genotype of HBV in our tested patients explain the lack of response to interferon therapy, as none of the patients had <2 x 10 copies /ml DNA / HBV. The only one patient positive for A genotype / HBV had positive HBe antibodies and a low value of viral load of DNA/HBV (5900 IU/mL), which is in accord with the known favorable response in case of this genotype from the literature. We have used F test ANOVA to test for differences among the mean of viremia (DNA/HBV) and the genotype of HBV, the presence / absence of HBeAg and antiHBe,Ab, the severity of fibrosis, and the presence / absence of interferon therapy. There was a statistically significant difference between groups as determined by one-way ANOVA (p = 0.004 for HBV genotype, p = 0.001 for HbeAg status, p = 0.002 for anti Hbe Ab, p = 0.006 for the degree of fibrosis and p = 0.001 for interferon therapy) We also have tested the differences between ALAT (alaninaminotransferase) values and the same previous analysed parameters, but we did not found any statistical significant difference.. The mathematical models showed that for our treated patients with interferon, the high level of viremia associated with a high level of TGP (16.1%) are predictors for D genotype of HBV, but these results cannot be extrapolated to general population (p > 0.05) (fig. 1).

We have used ROC curve for calculating the HBV DNA level differentiating HBeAg-negative and the cut-off value was set at 2.9×10^4 copies/mL with 75% sensitivity and 80% specificity (fig. 2). 55% (11/20) of our patients were infected with Mediterranean HBV strains, being HbeAg negative, anti-HBe Ab positive with high levels of DNA/HBV. The D genotype of HBV detected in 90% is known to be associated with precore mutation and non-A HBV genotypes. The most important strength of our study is that we are assessing for the first time the HBV genotype prevalence in patients from Northeastern Romania. The weakness of our study is that the number of tested patients is low, but our results are correlating with other studies performed in our country, which stated that the most prevalent genotype is D. In the close future we intend to extend our HBV genotyping in more patients and also to assess the antiviral therapy efficiency according with the HBV genotypes.

Conclusions

In this pilot study regarding the genotypes circulated in Northeast region of Romania, the most predominant genotype detected was D (90 %). It would be useful for our chronic B hepatitis patients to adapt antiviral therapy according with the HBV genotype, in order to obtain like endpoints HBeAg seroconversion, HBsAg loss, DNA/HBV undetectability.As risk factors with statistic significancefor the unfavourable evolution were identified: high viral load, the type genotype, HBeAg, the severity of fibrosis and IFNtherapy.

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