Levels of HBV-DNA, sFas and sFasL among healthy HBsAg carriers in period of three years

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Abstract

Purpose: The object of the study was the usefulness of sFas and sFasL concentration in the prognosis of disease development in healthy HBsAg carriers.

Patients: 34 healthy HBsAg carriers were examined over a three-years period.

Material and methods: HBV-DNA was extracted using the Gene Elute Mammalian Genomic DNA Miniprep Kit (Sigma, USA). HBV-DNA concentration and YMDD mutations were measured by RT-PCR based on TaqMan Universal Master Mix (Applied Biosystems, USA). HBeAg and anti-HBe in serum were detected by MEIA method (ABBOTT, Germany). The concentration of sFas and sFasL in serum was estimated by ELISA method (Bender MedSystems, Austria).

Results: Within three year observation period the number of carriers with absent HBV-DNA increased from 19% to 33%. HBV-DNA above 105 copies/ml, which was detected in 63% of carriers, decreased to 11% ($p<0.05$). After 3 years, a reduction of HBV-DNA levels was observed in 89% of carriers ($p<0.05$).

The occurrence of sFasL decreased from 56% to 48%. sFasL correlated with HBV-DNA ($p<0.05$). The concentration of sFas decreased ($p<0.01$). Chronic hepatitis B developed in 11% of men carriers, and 11% eliminated HBeAg, anti-HBe and HBV-DNA. YMDD mutant was not detected in any of the HBsAg carriers.

Conclusions: High concentration of sFasL in serum may suggest the development of chronic hepatitis and it seems that sFasL detection is never a good prognostic factor.

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Introduction

According to National Institute of Health [1], HBV-DNA above 105/mL in serum of chronically HBV infected persons is the basic condition of the diagnosis of chronic hepatitis B. Such diagnosis does not demand increased ALT activity, or other abnormal biochemical indicators of liver damage, to be discovered. Among persons infected with YMDD mutant HBV, lower HBV viral load in comparison to the one discovered in persons infected with the "wild" virus is responsible for the development of chronic hepatites [2]. Limited studies determining the correlation of HBV viraemia with histological changes in liver have shown a possible relation between those two factors. However, besides the level of viral load, immunity is also of significant importance in the development of changes produced by HBV infection.

The death of hepatocytes in HBV infection is the consequence of inflammatory necrotic changes or processes of programmed cell death. In HBV infection, programmed cell death is initiated mainly by the activation of Fas "death domain" present on hepatocytes. It seems that the processes of programmed cell death play a significant role in the development of chronic hepatitis [3,4].

In our previous studies [5] performed in healthy HBsAg carriers, HBV replication of above 105/mL was detected in 65% while the concentration of sFas was lower in relation to its concentration in patients with chronic hepatitis B. Current studies performed 3 years after the original analysis are the continuation of the undertaken issue and their main aim is to determine the importance of HBV viral load and the concentration of programmed cell death indicators in HBsAg carriers' state. Moreover, the rate of YMDD mutant presence in HBsAg carriers was also determined in the current studies.

Material and methods

The study included 34 HBsAg healthy carriers, 15 women (aged 20-43 yrs) and 19 men (aged 21-38 yrs) in 2002 year. The examinations were re-performed in 27 persons, 12 women (aged 23-43 yrs) and 15 men (aged 24-39 yrs) after 3 years.

Inclusion and exclusion criteria of healthy HbsAg carriers were: age 18 and over, HbsAg presence in serum for at least 1 year, normal range of ALT, AST, bilirubin, albumin and prothrombin within 1 year, unremarkable changes in USG liver examination, lack of past or present history of drug or alcohol abuse, autoimmune disorders, HCV infection, immunomodulation treatment.

Informed consent was obtained from each patient and the Bioethics Committee at the Medical Academy of Białystok approved the study protocol.

Methods

HBsAg, HBeAg and anti HBe in serum were detected by MEIA test (ABBOTT, USA).

Extraction of HBV-DNA from patients' sera

HBV-DNA was extracted from 200 ml of patients' serum using kits for DNA isolation: the Gene Elute Mammalian Genomic DNA Miniprep Kit (Sigma, USA). The part of HVB-DNA were amplificated by PCR system with primer complementary to conservative part of genome (sense 5'-AG GGG AGG AGA TTA GGT TAA-3' antisense 5'-AGG AGT GCG AAT CCA CAC TC-3') in 20 ml reaction mixture: 200 mM dNTPs, 0.4 mM all primers, 1.5 mM $MgCL_2$, 1.0 U Taq polymerase (Sigma) and 4 ml DNA solution. 40 cycles amplification was performed (in 96°C for 30 s, in 57°C for 60 s and in 72°C for 60 s). The products of amplification were appointed in 2% agar gel by electrophoresis, and next stained with ethidine bromide. Electrophoregrams were visualized in system of record and computer analyses UVI-KS400i/ /Image PC (Syngen Biotech, USA). Part DNA solution was kept in temperature -20°C for further stages of work.

HBV-DNA concentration in sera was evaluated by real-time detection PCR based on TaqMan chemistry. Amplification was performed in 25-ml reaction mixture containing 2 x TaqMan Universal Master Mix (Applied Biosystems, USA) with uracil N'-glycosylase, 30 pmol of forward primer, 30 pmol of reverse primer, 30 pmol TaqMan probe (5'-FAM) and 5 ml of isolated DNA. After incubation for 2 minutes at 50°C, which enables uracil N'-glycosylase to inactivate possible contaminating amplicons, incubation for 10 min at 95°C allowed AmpliTaq Gold polymerase to activate and inactivate the uracil N'-glycosylase. Next cycles PCR were moved. Number of copies was counted by interpolation from definite standard curve. The detection limit of this system was as few as 10 HBV-DNA copies/ml of serum. A linear standard curve was obtained between 10 and 108 DNA template copies/reaction.

YMDD mutation

Mutation of gene of virus's polymerase was performed with use of reagents "Blood Mini" (A&A Biotechnology, Poland). DNA solutions about positive results of amplification were investigated mutatory analysis with use of direct sequenced technique. Before of sequention reaction, the part of gene of virus's polymerase with rt180 and rt204 code became duplicated in single PCR or nested-PCR reaction in dependence from concentration HBV-DNA in studied sample. Amplification were performed in 20 μl mixture contain 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM every from dNTPs, 0.5 mM every from primers, 1 U DNA polymerase and 5 ml of solution of DNA, what responded of 5 ml patients serum. Amplification on road single PCR were realized with primer 840 (5'-ACCCCATCTTT TTGTTTTGTTAGG-3') and 377 primer (5'-GGATGTGTCTGCGGCGTTT-3'). In nested-PCR reaction external primer 12F (5'-AGACTCGTGGTGGACTTCTCT-3') and 5RC (5'CAAAAGAAAATTGGTAACAGCGGTA-3'), as well as internal primers 840 and 377 were used.

Amplification in thermocycler GeneAmp PCR System 2400 (Applied Biosystems, USA) having accord to following thermal profile: 5 min in 94°C, 40 cycles for 30 s in 94°C, 30 s in 55°C and 60 s in 72°C and 5 min in 72°C were executed. Identification of PCR products was executed in agar elektroforesis and dying brom etydyne.

Cleaning of PCR products before of seuquentione reaction were prepared with reagents Clean-Up (A&A Biotechnology Poland).

Reaction of sequence were executed cyclic method, in 10 μl of reactionary mixture from primers sensible 377, using finished kid of BigDye Apprentice Termister Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA). Products of sequenced were cleaned by Ex Terminator Kit (A&A Biotechnology, Poland).

The sequencing was performed ABI PRISM 377 analisator (Applied Biosystems, USA) and use of software Sequencing Analysis 3.4.1.

sFas and sFasL

The sFas and sFasL concentration in the serum was measured twice by immunology – enzymatic assay test (ELISA, Bender MedSystems, Austria) [6].

Statistical analyses

Statistical analysis was performed by use of non-parametric Mann–Whitney U and Spearman tests. Values of $p<0.05$ were considered to be significant.

Results

HBV-DNA was not detected in 5/34 (19%) persons in the year 2002 and in 9/27 (33%) in the year 2006. The number of persons with HBV-DNA above 105 copies/ml, decreased from 17/27 (63%) in the year 2002 to 3/27 (11%) in the year 2006. A significant reduction of HBV viraemia after 3 years of observation was discovered in 89% of carriers, which is comparable to the reference data (*Fig. 1*).

In the year 2002, sFasL presence in serum was detected in 19/34 (56%), after 3 years of observation in 13/27 (48%) HBsAg carriers (the protein was not detected in the group of healthy persons). The rate of sFasL presence (studies from the years 2002 and 2006) correlated with high HBV viraemia ($r=0.349$, $p<0.05$). No relation between the detection of sFasL in serum

Figure 1. **YMDD mutant infection was not discovered in any of the HBsAg carriers**

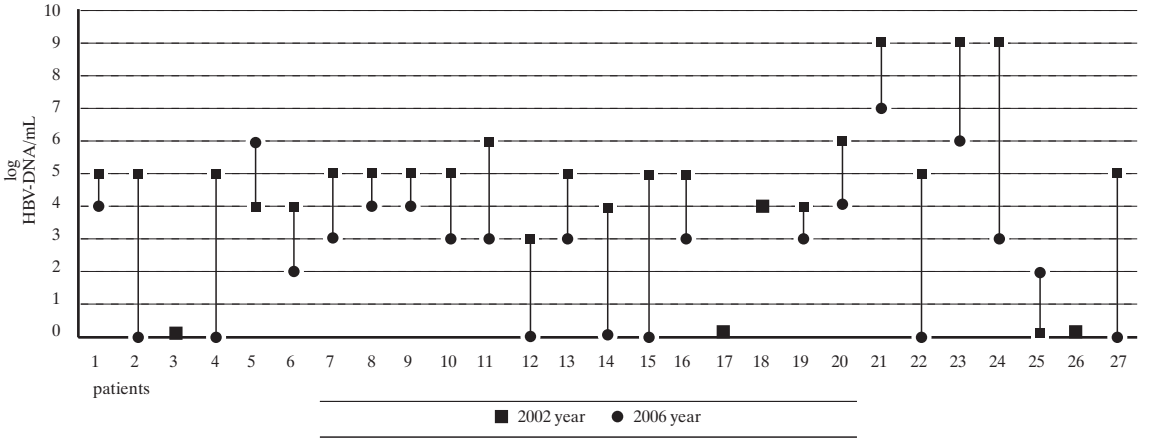
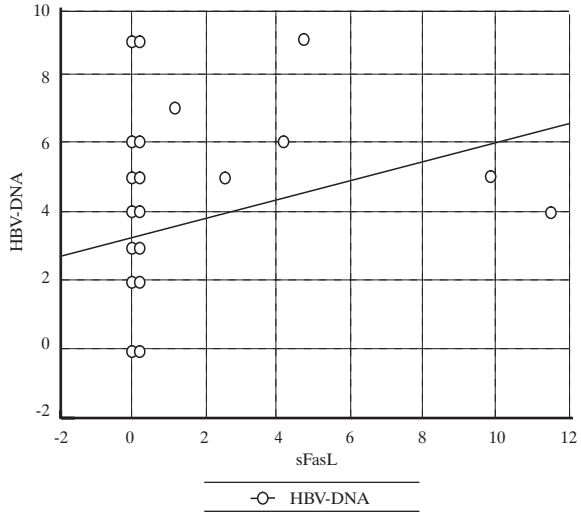


Table 1. **HBV-DNA and sFas concentration as well as percentage of sFasL in haealthy HBsAg carriers**

	n – patients (%)		**sFas concentration** (pg/ml)		**percentage of patients with sFasL** (%)	
year	2002	2006	2002	2006	2002	2006
healthy (n=12)				18.4	Absent	
healthy HBsAg carriers	34 (100)	27 (100)	15.4	12.6	19/34 (56)	13/27 (48)
HBV-DNA >105/mL	22 (65)	3 (11)	16.3	9.3	12 (35)	3 (11)
HBV-DNA <105/mL	5 (15)	15 (56)	12.9	12.9	4 (12)	8 (29)
HBV-DNA =0	7 (20)	9 (33)	14.1	13.1	3 (9)	2 (7)
HBeAg positive patients	10 (29)	2 (7)	15.8	10.0	4 (12)	2 (7)
HBV-DNA >105/mL	8 (23)	2 (7)	16.0	10.0	4 (12)	2 (7)
HBV-DNA <105/mL	0	0	0	0	0	0
HBV-DNA =0	2 (6)	0	14.8	0	0	0
anti-HBe positive patients	24 (71)	22 (81)	15.1	13.0	15 (44)	10 (37)
HBV-DNA >105/mL	14 (41)	1 (4)	16.5	8.0	8 (23)	1 (4)
HBV-DNA <105/mL	5 (15)	15 (55)	12.9	12.9	4 (12)	8 (29)
HBV-DNA =0	5 (15)	6 (22)	13.9	14.4	3 (9)	1 (4)

Figure 2. **The concentration of sFas after 3 years of observation underwent a significant reduction (Spearman test, p<0.01). No correlation between the concentration of sFas and HBV-DNA viraemia was observed despite the fact that both the HBV viral load and the concentration of sFas underwent a reduction**



and the presence of HBeAg or the anti-HBe antibody was discovered (*Tab. 1*, *Fig. 2*).

Chronic hepatitis B developed in 3/27 (11%) men carriers. After 3 years of observation, the increase in ALT activity was discovered in 3/27 (11%) men carriers. In the mentioned persons, after a morphological examination of liver tissue, clinical chronic hepatitis B was diagnosed (*Tab. 2*).

Discussion

The reduction of HBV viral load in most carriers within the period of 3 years confirms the importance of natural immunological processes in HBV infections [7]. The presence of sFasL in sera of HBsAg carriers indicates HBV stimulated expression of the domains present on lymphocytes responsible for the recognition of infected cells. In own studies, the correlation between HBV-DNA and the level of sFasL presence was detected, which indicates the importance of the processes of programmed cell death in the elimination of HBV infection. A high concentration of sFasL together with high HBV viral load seems to be a risk prognostic factor of a chronic inflammatory process. The study of Han at al. [8] proved that in comparison to patients with

Figure 3. **Within the 3-year period of observation 8/27 (30%) of carriers eliminated the HBeAg and 2/27 (7.4%) eliminated HBV-DNA**

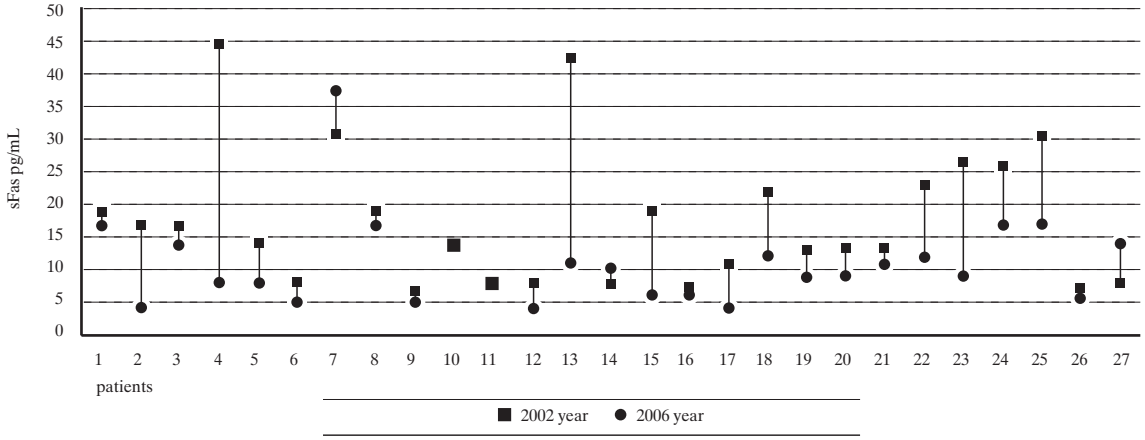


Table 2. A) three healthy HBsAg carriers which eliminated HBV-DNA, HBe and anti-HBe; B) three HBsAg carriers which followed chronic hepatitis B

A)

Person	Aged – years	HBV-DNA/mL		HBeAg		anti HBe		sFas (pg/mL)		sFasL (pg/mL)	
		2002	2006	2002	2006	2002	2006	2002	2006	2002	2006
C-M	26	1.33 x 105	0	+	-	-	-	19	6	0	0.14
K-H	31	1.01 x 1059	0	+	-	-	-	23	12	0	0
L-A	28	1.10 x 105	0	-	-	+	-	7.5	14	0	0

B)

Person	Aged – years	HBV-DNA/mL		HBeAg		anti HBe		SFas (pg/mL)		SFasL (pg/mL)	
		2002	2006	2002	2006	2002	2006	2002	2006	2002	2006
M-P	24	9.09 x 104	6.12 x 106	-	-	+	+	14.0	8.0	11.5	4.16
B-S	27	1.93 x 109	1.14 x 107	+	+		-	13,0	11.0	4.73	1.15
M-O	24	1.39 x 105	5.34 x 103	-	-	+	+	43.0	11.0	9.89	0.09

chronic hepatitis B and those with liver cirrhosis the concentration of soluble TRAIL (TNF-related apoptosis inducing ligand) molecules in HBsAg carriers is lower. The results of the studies, similarly as in the studies being presented, indicate the existence of a relation between high concentration of the indicators of programmed cell death, such as sTRAIL, and the exacerbation of liver damage. This is confirmed by the observed development of chronic hepatitis in carriers with such type of parameters. This is important because in most carriers with HBV viral load above 105 copies/ml and low concentration of sFasL (or the absence of the protein in serum), after 3 years of observation no traits of the disease development were detected and the HBV viral load underwent a reduction.

The concentration of sFas, similarly to viral load, decreased within the period of a few years' observation. Such result can indicate a reduction of programmed cell death activity in the states of hypostimulation of these processes by viruses. A reduction of viral load and the concentration of sFas is a very good indicator, indicating HBV infection being reduced. The studies of Xin at al. [9] confirm the relations between the concentration of Fas in serum and liver tissue and the activity of apoptosis whereas the studies of Hayashi and Mint [10] confirm the coexistence of the elimination of infected hepatocytes and cytotoxic activity.

It was not proved in own studies that seroconversion in HBe system influences the elimination of HBV, however, the virus replication was observed. At present, it is hard to determine whether the elimination of HBV together with the appearance of anti-HBe is a prognostically good factor. The studies of Chu at al. [11] confirm this suggestion.

Antiviral treatment of HBsAg carriers with viral load above 105 copies/ml is justifiable, which is also confirmed by own studies. YMDD mutant (514C-A, 523C-A, 562T-A or 667C-A) was not discovered in any HBsAG carrier. Peng and all. [2], examining HBV infected patients with the presence of anti-HBe antibodies and with viral load from 10 to 105 copies/ml showed the presence of precore mutant 1 869G-A in 86.9%, and mutant 1 762A-T/1 764G-A in 32.8% persons. The results of these studies indicate frequent mutations in the case of HBV infections and as it is widely known the mutants of this virus can cause significant liver damages even in the case of low viral load. It is expedient thus to use such prognostic factors that will enable to make a decision of treatment of HBV infected patients despite detecting low viral load. It seems that the examination of the presence and concentration of sFasL simultaneous with the examination of viral load can facilitate the initiation of antivirus treatment.

Conclusions

The concentration of sFas and the rate of sFasL presence (the indicators of programmed cell death) correlate with HBV-DNA among HBsAg carriers. High HBV-DNA viral load together with high concentration of sFasL in serum may suggest the development of chronic hepatitis. The absence of sFasL in serum seems to be the factor of the elimination of HBV-DNA.

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