Matrix metalloproteinases and their tissue inhibitors in children with chronic hepatitis B treated with lamivudine

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Abstract

Purpose: To evaluate if measurements of MMP-2, MMP-9, TIMP-1 and TIMP-2 have clinical applicability as markers of liver fibrosis and to assess the effect of long-term lamivudine treatment on liver fibrosis in children with chronic hepatitis B (chB).

Material and methods: The observation was carried out on 41 children with biopsy proven chB (HBe/+/, HBVDNA/+/) who were nonresponders to previous IFNα therapy. Lamivudine was administered in the group of 29 children (3 mg/kg/day, maximum 100 mg/daily). The serum concentration of examined markers was measured with ELISA before and after 24 months of therapy. ROC analysis was used to calculate the power of the assays to detect advanced liver fibrosis (score >2 according to Batts&Ludwig).

Results: Serum TIMP-1 and TIMP-2 levels were significantly higher and MMP-9 lower in children with chB compared to controls. There was a significant positive correlation between serum MMP-2 and negative correlation between MMP-9 level and the stage of liver fibrosis. The ability of serum MMP-9 to differentiate children with mild fibrosis from those with advanced fibrosis was significant (AUC=0.75; p=0.03). Other serum markers did not allow a useful prediction. 2-year lamivudine treatment did not improve histological fibrosis but it caused significant decrease of serum TIMP-2 (p=0.01) and increase of MMP-9 level (p=0.00005).

Conclusions: MMP-9 is a better serum fibrosis marker than MMP-2, TIMP-1 and TIMP-2 to diagnose children with advanced liver fibrosis. The significant decrease of TIMP-2 and increase of MMP-9 level during therapy suggest antifibrotic effect of lamivudine in children with chB.

Key words: liver fibrosis, HBV, lamivudine, MMP, TIMP.

Introduction

HBV is still considered one of the most important epidemiological problems because nearly 400 million people all over the world have been chronically infected with this hepatotropic virus [1]. This issue is also important for about 40 millions of Polish citizens, although there was a significant drop from 13 296 HBV incidents in 1993 to 1 473 in 2004 [2]. Due to possible development of liver cirrhosis or primary hepatic carcinoma, chronic hepatitis B is also regarded a significant clinical problem.

During progression of chronic liver disease an imbalance occurs between synthesis and breakdown of extracellular matrix (ECM) components. Matrix metalloproteinases (MMPs) are involved in degrading ECM while tissue inhibitors of matrix metalloproteinases (TIMPs) prevent their fibrolytic action. Hepatic stellate cells (HSC) play a central role in the pathogenesis of liver fibrosis and their activation leads to release of both MMPs and TIMPs [3]. However, MMP-9 are mainly derived from Kupffer cells [4]. These components, which can be measured in serum, are thought to play an essential role in liver injury associated with tissue remodeling but it is still unclear whether their circulating levels are liver-specific [5].

So far the treatment of chronic hepatitis B in children has been based on interferon alpha. The use of this drug allows to expect the inhibition of HBV replication in approximately 30--50% patients [6]. This is certainly not sufficient and therefore, new attempts are made to introduce new antiviral agents. Presently, lamivudine, a nucleoside anologue is considered the most promising drug in the treatment of chronic hepatitis B in

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Received 06.11.2006 Accepted 30.01.2007

children. One of the main goals of lamivudine therapy, even in the absence of virus supression is improvement of liver histology, especially of liver fibrosis, which is a predictor of progression to cirrhosis [7].

To our knowledge, serum fibrosis markers predicting liver fibrosis have not been assessed before in children with chronic hepatits B during long-term lamivudine treatment. Therefore, the aim of the study was to evaluate if measurements of MMP-2, MMP-9, TIMP-1 and TIMP-2 have clinical applicability as markers of liver fibrosis and to examine the effect of lamivudine treatment on liver fibrosis by direct assessment of histological scores and by indirect assessment of serum levels of selected fibrosis biomarkers (MMP-2 and 9 and TIMP-1 and 2) in children with chronic hepatitis B.

Material and methods

Patients

The study was carried out with 41 Caucasian children (mean age 10.5 years, range 6-17, 29 boys and 12 girls) with serologically and biopsy-verified chronic hepatitis B, who were nonresponders to previous intrerferon alpha therapy (3 MU tiw for 20 weeks). The children were still positive for HBs, HBe antigens and serum HBVDNA. Patients with HCV coinfection and with liver cirrhosis were excluded from the study. None of the children was treated with antiviral and immunomodulating drugs during the 12-month period before inclusion into the study. Informed consent was obtained from all patients' parents and the protocol was approved by the local ethical committee of the Medical University of Białystok, Poland. Serum samples were evaluated twice: at the beginning and after 24 months of lamivudine treatment. Serum samples were stored at -70ºC until use. Standard liver tests were measured by validated automated methods and included total bilirubin, albumin, ALT, AST, GGT and ALP. HBsAg and HBeAg were determined by MEIA (Microparticle Enzyme Immunoassay) and HBV DNA were determined by PCR (qualitative) method.

Lamivudine treatment and definition of response

Lamivudine was applied in the group of 29 children at the dose of 3 mg/kg/day up to 100 mg daily for 24 months. HBeAg/antiHBe seroconversion with concomitant clearance of serum HBVDNA after 2 years of treatment was considered the criterion of treatment response.

Measurement of serum fibrosis markers

The concentration of MMP-2, MMP-9, TIMP-1 and TIMP-2 were measured with EIA technique in serum using R&D Systems commercial kits. Nine children (mean age 10 years) were included as control group without anamnestical, clinical or laboratory signs of liver diseases or other chronic diseases.

Histological analysis

Percutaneous liver biopsies were obtained in all patients before treatment and in 13 children after 24-months of lamivudine treatment. The liver specimens were fixed in buffered formalin and embedded in paraffin. Histological sections were stained using hematoxylin-eosin, Masson-Goldner, Masson's trichrome and reticulin stains. Fibrosis stage and inflammation grade were assessed in a blinded fashion by a single pathologist without knowledge of the patients' laboratory or clinical data. In order to determine specificity and sensitivity of the assay we arbitrarily defined advanced liver fibrosis as a score >2 and advanced inflammation as a score >1 according to Batts and Ludwig [8].

Statistics

Results are expressed as means ± SD. Statistical analysis was performed with the Mann-Whitney U test for independent samples and Wilcoxon signed rank test for paired samples. The relationship between non-invasive markers and liver histology scores were analysed by the Spearman rank-correlation test for non-parametric data and by the Pearson method for parametric data. The tests were considered statistically significant at $p<0.05$.

Receiver operating characteristics (ROC) analysis (AccuROC, Montreal, Canada) was used to calculate the power of the assays to detect advanced liver fibrosis [9]. Sensitivity of the assays was plotted against the false positivity (1-specificity). Comparison of the area under the curve (AUC) was performed using a two-tailed p test, which compares the AUC to the diagonal line of no information (AUC 0.5).

Results

Serum concentration of biomarkers in children with chronic hepatitis B

Serum concentration of TIMP-1 and TIMP-2 levels were significantly higher and MMP-9 was lower in children with chronic hepatitis B /n=41/ compared to controls /n=9/ (142.0±32.0 vs 123.2±17.5 ng/ml, p=0.043; 86.7±11.0 vs 72.6±13.8 ng/ml, p=0.008; 185.2±135.7 vs 261.6±107.0 ng/ml; p=0.016, respectively).

There were no significant correlations of the examined biomarkers with age, ALT, AST, GGT, ALP, bilirubin and albumin or histological inflammation according to Batts and Ludwig. However, there was a significant positive correlation between serum MMP-2 and negative correlation between MMP-9 and the stage of fibrosis (r=0.31, p=0.046; r=-0.32, p=0.041, respectively).

Diagnostic value of serum fibrosis markers for identification of patients with advanced liver fibrosis and inflammation

All children had liver fibrosis. Thirty-three children (80.5%) had mild liver fibrosis: 25 of them – score 2 and 8 – score 1, and 8 children (19.5%) had advanced fibrosis (score = 3 according to Batts and Ludwig).

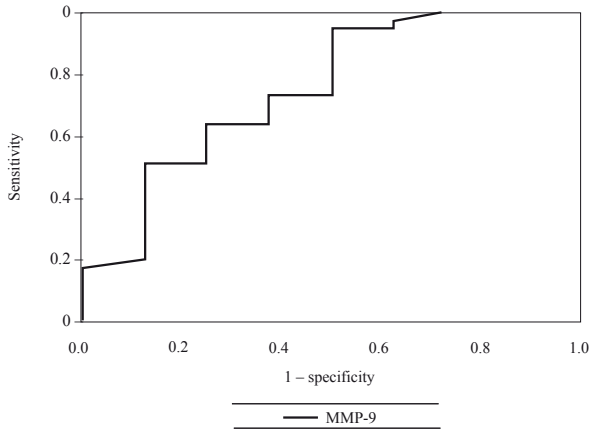
The patients with advanced liver fibrosis did not have significantly higher serum level of liver enzymes, bilirubin, albumin and histological inflammation than patients with mild liver fibrosis. The level of examined biomarkers was not different in children with mild liver fibrosis and with advanced fibrosis except MMP-9 (p=0.03) (*Tab. 1*).

Table 1. **Comparative characteristics of children with mild and advanced liver fibrosis**

Data of the patients	Mild fibrosis (n=33) mean ± SD	Advanced fibrosis (n=8) mean ± SD	p-value
Age (years)	10.4±3.2	11.5±3.5	0.43
ALT (IU/l)	64±35	97±57	0.11
AST (IU/l)	56±23	65±30	0.47
GGT (IU/l)	12±5	12±3	0.99
ALP (IU/l)	277±80	261±116	0.57
Bilirubin (μmol/l)	9.06±4.1	9.75±2.05	0.60
Albumin (%)	63.2±2.8	63.1±3.5	0.79
MMP-2 (ng/ml)	261.7±48.3	283.8±42.6	0.29
MMP-9 (ng/ml)	200.8±145.2	120.6±54.3	0.03*
TIMP-1 (ng/ml)	144.5±27.9	131.9±46.3	0.47
TIMP-2 (ng/ml)	85.4±10.2	92.1±12.2	0.28
Grading	1.36±0.49	1.62±0.74	0.45

* p < 0.05; Normal ranges: AST – 10-40 IU/l, ALT – 10-40 IU/l, GGT – 9-35 IU/l, ALP – 110-350 IU/l, bilirubin – 1.71-18.81 μmol/l, albumin – 58.8-69.6%

Figure 1. **Receiver Operating Characteristic Analysis**



ROC curve of ability of serum MMP-9 to detect mild liver fibrosis according to Batts and Ludwig in children with chronic hepatitis B

Table 2. **The examined serum biomarkers AUC value to detect advanced liver fibrosis (for MMP-9 – mild liver fibrosis) and advanced inflammation according to Batts & Ludwig in children with chronic hepatitis B**

Biomarker	Fibrosis	Inflammation
MMP-2	AUC=0.625±0.1147 p=0.2776	AUC=0.5538±0.097 p=0.5655
MMP-9	AUC=0.75±0.1089 p=0.03*	AUC=0.4013±0.098 p=0.291
TIMP-1	AUC=0.4148±0.1234 p=0.4591	AUC=0.6088±0.0907 p=0.245
TIMP-2	AUC=0.6269±0.1070 p=0.2703	AUC=0.6525±0.0985 p=0.1029

* p< 0.05

The ability of these markers to differentiate the children with mild liver fibrosis from those with advanced fibrosis was not significant except MMP-9 (AUC=0.75±0.1089, p=0.03) (*Fig. 1*, *Tab. 2*). A serum MMP-9 level above 94.6 ng/ml had a sensitivity of 93.9% and specificity of 50%; PPV= 88% and NPV= 66.6%.

None of these markers was a good predictor of histological inflammation (*Tab. 2*)

Effect of lamivudine on serum MMP-2, MMP-9, TIMP-1 and TIMP-2 level in children with chronic hepatitis B

Children with chronic hepatitis B who were treated with lamivudine were analysed according to the type of response. There were 4 responders (14%) and 25 nonresponders (86%).

There were no significant differences between the groups regarding age, the levels of bilirubin and albumin, the activity of AST, ALP, GGT and grade of inflammation as well as the stage of fibrosis. However, the responders displayed a significantly higher activity of ALT (p=0.012). The level of examined serum fibrosis markers before lamivudine therapy was not significantly different in responders and nonresponders either (*Tab. 3*).

During 24 months of lamivudine treatment there were significant changes of examined serum fibrosis markers: TIMP-2 significantly decreased (p=0.01) and MMP-9 level significantly increased (p=0.00005). No significant changes of TIMP-1 and MMP-2 were found at the end of 24 months of lamivudine treatment (*Tab. 4*).

Effect of lamivudine on liver histology in children with chronic hepatitis B

There were no significant changes in liver fibrosis as well as in liver inflammation after 24 months of lamivudine therapy (1.77±0.6 vs 1.77±0.44 and 1.38±0.51 vs 1.69±0.48, respectively).

Discussion

The advanced liver fibrosis and even cirrhosis had been regarded irreversible until quite lately. However, experimental and clinical studies confirmed possibility of stopping or even decreasing the stage of liver fibrosis through causal factor

***Table 3*. The baseline characteristics of the groups of examined children with chronic hepatitis B treated with lamivudine**

Data of the patients	Responders (n=4) mean± SD	Nonresponders (n=25) mean± SD	p-value
Age (years)	12± 3.5	10.2±2.9	0.37
ALT (IU/l)	122±35	63±37	0.008*
AST (IU/l)	69±21	56±26	0.14
GGT (IU/l)	12±3	13±6	0.77
ALP (IU/l)	272±99	279±91	0.83
Bilirubin (μmol/l)	8.38±3.08	8.72±3.08	0.76
Albumin (%)	62.0±3.6	63.6±2.4	0.35
Staging	2.5±0.6	1.96±0.61	0.11
Grading	1.75±0.5	1.48±0.59	0.41
MMP-2 (ng/ml)	278.3±40.8	267.5±45.9	0.47
MMP-9 (ng/ml)	126.6±56.8	164.9±99.8	0.49
TIMP-1 (ng/ml)	160.5±23.8	145.8±34.8	0.23
TIMP-2 (ng/ml)	93.8±5.5	87.0±12.6	0.11

* $p < 0.05$

***Table 4*. Effect of 24-month lamivudine treatment on serum fibrosis markers in children with chronic hepatitis B (n=29)**

Marker	Before treatment	After treatment	p-value
MMP-2 (ng/ml)	269.0±44.7	261.7±59.0	0.24
MMP-9 (ng/ml)	159.6±95.2	359.1±229.0	0.000046*
TIMP-1 (ng/ml)	147.8±33.6	142.8±39.3	0.28
TIMP-2 (ng/ml)	87.9±12.0	82.4±12.2	0.014*

* $p < 0.05$

elimination or application of pharmacological preparation of potential antifibrotic activity, e.g., interferon alfa with ribavirin or lamivudine [10,11]. Therefore, reliable diagnostics is necessary for monitoring hepatic fibrogenesis in patients with chronic hepatitis. Although, the morphological examination of liver biopsy is still regarded a standard method in assessment of the stage of liver fibrosis, it is an invasive procedure and has several limitation such as sampling error and it only provides static information about the amount of fibrotic tissue [12,13]. Recently, several biochemical blood tests for liver fibrosis have been evaluated in adults which give possibility of long-term monitoring of the course of disease and possible changes caused by treatment. The noninvasive markers of fibrosis include extracellular matrix (ECM) components (e.g. hyaluronan, laminin, collagens, MMPs, TIMPs) as well as non-ECM biochemical panels (FibroTest, Forns index, APRI) [14,15].

To our best knowledge non-invasive biomarkers that predict liver fibrosis due to chronic hepatitis B in children are lacking except our previous studies. We found that the combination of serum hyaluronan and laminin-2 as well as APRI can accurately predict significant liver fibrosis [16,17]. The results of our next studies showed that the ability of serum TGF beta 1 or cystatin C to differentiate children with advanced liver fibrosis from those with mild fibrosis was not significant [18,19].

In this study we found significantly higher level of TIMP-1 and TIMP-2 and lower level of MMP-9 in the group of children with chronic hepatitis B than in controls. Our results are in agreement with data presented by Flisiak et al. [20], Mitsuda et al. [21] and Murawaki et al. [22] who also confirmed higher TIMP-1 level and with Leroy et al. [23] and Walsh et al. [24] who showed higher level of TIMP-1 and -2 in patients with chronic hepatitis than in controls. Reif et al. [25] and Walsh et al. [24] found that patients and control group had similar serum level of MMP-2. This finding is not with agreement with data presented by Chen et al. [26] who showed higher level of MMP-2 in patients with hepatitis but they also confirmed, like in our study, that MMP-9 level in patients with chronic liver disease were lower than in controls. The study of Reif et al. [25] is not consistent with ours and Chen et al. [26] findings because they demonstrated that serum activity of MMP-9 was increased in patients with hepatitis C compared to controls. Such discrepancies could be explained by analytic methods used (ELISA or zymography), subject sampling or severity of the disease in the examined group of patients with chronic viral hepatitis.

Although we confirmed significant correlation of MMP-2 and -9, which are the major MMPs in circulation, with the stage of liver fibrosis, using ROC analysis we found that only the ability of MMP-9 to differentiate the children with mild liver fibrosis from those with advanced fibrosis was significant.

In adults with chronic viral hepatitis, mainly caused by HCV, the role of MMPs and TIMPs as markers of liver fibrosis was discrepant. Our findings are in agreement with Leroy et al. [23] who also demostrated a correlation of serum level of MMP-2 and -9 with fibrosis stage. However, in contrast, Reif et al. [25] found that MMP-2 and -9 are markers of inflammation but not of the degree of fibrosis. Morever, Murawaki et al. [27] showed that TIMP-1 correlated both with histological inflammation and fibrosis. Walsh et al. [24] demonstrated TIMP-1 correlation with liver inflammation and TIMP-2 with histological fibrosis, however, MMP-2 was related neither to fibrosis nor histological activity index. Using ROC analysis both TIMP-1 and TIMP-2 (but not MMP-2) had significant diagnostic ability

in detecting advanced liver disease. The usefulness of TIMP-1, which was better than MMP-2, in detecting severe liver fibrosis was also confirmed by Leroy et al. [23] and Boeker et al. [28].

These discrepencies in the estimation of the role of MMPs and TIMPs in liver fibrosis might be related to the use of a different fibrosis scoring systems as well as multiple versus single pathologists scoring the biopsies. Moreover, non-invasive markers will not have complete concordance with histological staging because histological scoring systems are not sensitive enough to detect small changes in fibrosis stage and biomarkers may even be more accurate than biopsy in staging disease [15].

The main aim of this study was to examine the effect of lamivudine treatment on liver fibrosis by direct assessment of histological scores and by indirect assessment of serum levels of fibrosis markers in children with chronic hepatitis B who were nonresponders to previous interferon alpha therapy. Data regarded the effect of lamivudine treatment on liver histology in children are scarce. Only Ozgenc et al. [29] evaluted retrospectively histological response in 29 children who received first combination therapy (interferon alpha and lamivudine) and then continued with prolonged treatment with lamivudine and they found significant decrease of inflammation and fibrosis scores. In our prospective study, neither significant changes in histological evolution of fibrosis nor in inflammation after 2-year lamivudine therapy were found. All 13 children with repeated liver biopsy did not seroconvert to antiHBe. Therefore, probably for that reason we did not observe statistically significant improvement in liver histology, especially in fibrosis.

Only few studies analysed the effect of lamivudine treatment on the level of serum fibrosis markers in adults with chronic hepatitis B. Flisiak et al. [20] showed that lamivudine treatment resulted in a significant decrease of TIMP-1 and TGF beta 1 and increase of MMP-1 level during treatment. Although in our study we examined different kind of MMPs and TIMPs, we found similar changes induced by lamivudine treatment; we demonstrated the significant decrease of TIMP-2 and increase of MMP-9. Other authors analysed other serum fibrosis markers. Poynard et al. [30] found that in patients with chronic hepatitis B a 24-month course of lamivudine treatment lead to significant decrease in fibrosis assessed by FibroTest. Grzeszczuk et al. [31] demonstrated that hyaluronan level decreased during lamivudine treatment both in patients with HBeAg seroconversion and without it. Maxwell et al. [32] showed that amino-terminal propeptide of type I procollagen (PINP)/carboxy-terminal telopeptide of type I collagen (ITCP) ratio was sensitive and specific in detecting responders to 48-week lamivudine treatment.

We conclude that MMP-9 is a better serum fibrosis marker than MMP-2, TIMP-1 and TIMP-2 to diagnose children with advanced liver fibrosis. The significant decrease of TIMP-2 and increase of MMP-9 level during therapy suggest antifibrotic effect of lamivudine in children with chronic hepatitis B but this finding needs to be confirmed in larger studies.

Acknowledgments

This study has been supported by a grant from the Medical University of Białystok, Poland, No 3-43645.

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