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VIRAL HEPATITIS

Matrix-derived serum markers in monitoring liver fibrosis in children with chronic hepatitis B treated with interferon alpha

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Abstract

AIM: To evaluate prospectively 4 selected serum fibrosis markers (tenascin, hyaluronan, collagen VI, TIMP-1) before, during and 12 mo after IFN treatment of children with chronic hepatitis B.

METHODS: Forty-seven consecutive patients with chronic hepatitis B (range 4-16 years, mean 8 years) underwent IFN treatment (3 MU tiw for 20 wk). Fibrosis stage and inflammation grade were assessed in a blinded fashion before and 12 mo after end of treatment. Serum fibrosis markers were determined using automated assays.

RESULTS: IFN treatment improved histological inflammation but did not change fibrosis in the whole group or in subgroups. Only hyaluronan correlated significantly with histological fibrosis(r = 0.3383, P = 0.021). Basal fibrosis markers did not differ between responders (42.5%) and nonresponders(57.5%). During IFN treatment only serum tenascin decreased significantly in the whole group and in nonresponders. When pretreatment values were compared to values 12 mo after therapy, TIMP-1 increased in all patients and in nonresponders, and hyaluronan decreased in all patients and in responders.

CONCLUSION: Tenascin reflects hepatic fibrogenesis and inflammation which decreases during IFN treatment of children with chronic hepatitis B. TIMP-1 correlates with nonresponse and hyaluronan with histological fibrosis.

Key words: Biopsy; Collagen VI; Fibrogenesis; Fibrosis; Hepatitis B virus; Hyaluronan; Serum marker; Tenascin; TIMP-1

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INTRODUCTION

The potential of interferon α (IFNα) to normalize aminotransferase activity, eliminate serum HBV DNA and HBeAg and reduce liver necroinflammation in patients with chronic hepatitis B is widely acknowledged[1-3]. In children with chronic hepatitis B treatment with IFN leads to long-term serological and biochemical remission in less than 50%[4-6]. Nonetheless, several reports suggested that IFN treatment for hepatitic C can halt or even reverse liver fibrosis[7-10], while its antifibrogenic potential in chronic hepatitis B needs to be confirmed[11]. Histological staging of liver fibrosis plays a central role in the liver pathological assessment since progressive fibrosis may lead to cirrhosis, the most important predictor of decompensated liver diseases and death[3, 12].

Liver biopsy is the standard method to assess fibrosis stage which allows to semiquantify the extent of fibrosis, yielding a static view[13]. However, biopsy has significant disadvantages for the assessment of fibrosis and fibrosis progression. Thus despite minor histological fibrosis, progression might be fast, since the accumulated extracellular matrix is the result of a dynamic process characterized by changes in matrix synthesis (fibrogenesis) and removal (fibrolysis)[14]. Furthermore, liver biopsy is invasive and histological scoring systems are not sensitive enough to detect small changes in fibrosis stage. Finally, bioptical sampling error can reach 25%-33% for a difference in one stage, when using the METAVIR system which ranges from 0 (no fibrosis) to 4 (cirrhosis)[15,16]. Therefore, noninvasive markers that may reflect overall hepatic fibrogenesis and fibrolysis in chronic hepatitis would be of great clinical benefit,

allowing repeated assessment of progression or therapeutic interventions, especially in children with chronic hepatitis B or C who are treated with IFN or other antiviral or potential antifibrotic agents[17-19].

The aim of this study was to investigate the clinical usefulness of selected matrix-derived serum markers (tenascin, hyaluronan, collagen VI, tissue inhibitor of metalloproteinase 1 or TIMP-1) in a long-term follow-up of children with chronic hepatitis B treated with IFN α.

MATERIALS AND METHODS

Patients

The study was carried out prospectively in 47 children (mean age 8 years, range 4-16, 31 boys and 16 girls) with serologically and biopsy-verified chronic hepatitis B. The children were positive for HBs and HBe antigens and had increased serum activity of HBV DNA polymerase for at least 1 year. Patients with autoimmune hepatitis or HCV coinfection 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 Bialystok. Serum samples were evaluated at three time points: at the start and the end (5 mo) of IFN α treatment, and 12 mo after end of treatment. Serum samples were stored at -70 ℃ until use. Standard liver tests were measured by validated automated methods and included total bilirubin, albumin, alanine transaminase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (gamma-GT). HBsAg and HBeAg were determined by MEIA (IMx, Abbott).

IFN treatment and definition of response

IFN (IFN α 2a: 30 children or IFN α 2b: 17 children) was applied at the dose of 3 MU tiw subcutaneously for 20 wk according to the schedule approved by the Polish Interferon Study Group[20]. HBeAg/antiHBe seroconversion and lack of HBV DNA polymerase activity 1 year after end of treatment was considered as the criterion of treatment response.

Measurement of serum fibrosis markers

Monoclonal antibodies were used to detect tenascin, collagen VI and TIMP-1 in sandwich immunoassays performed in an automated analyzer employing fluoresceine-labelled capture antibodies and alkaline phosphatase labeled detection antibodies. Hyaluronan was determined using biotinylated cartilage link protein. The immune complexes were separated from serum using magnetic particles covered with monoclonal anti-fluoresceine (anti-biotin in case of hyaluronan). The assays were developed for the BAYER IMMUNO 1 immunoassay system and validated in several cohorts of liver patients and healthy individuals[21-23].

Histological analysis

Percutaneous liver biopsies were obtained before treatment and 12 mo after IFN α discontinuation. 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 according to the method of Batts and Ludwig[24].

Table 1 Baseline characteristics of the group of children with chronic hepatitis B

Data of the patients	Mean	SD	Minimum	Maximum
Age (yr)	8	3.51	4	16
HBV infection (mo)	44	30	10	144
ALT (nkat/L)	1600	1567	350	9902
AST (nkat/L)	1367	1117	567	7718
GGT (nkat/L)	250	150	50	1150
Bilirubin (μmol/L)	8.5	4.3	3.4	23.9
Albumin (g/L)	65.6	4.2	57.2	74.3
Hyaluronan (μg/L)	34.3	21.5	14.6	113.5
Tenascin (μg/L)	748.8	265.9	295.6	1480.2
TIMP-1(μg/L)	558.1	121.2	342.2	852.3
Collagen VI (μg/L)	5.8	2.2	2.3	14.2
Staging	2.0	0.6	1	3
Grading	1.6	0.7	1	3

Normal ranges: AST: 167-667 nkat/L; ALT: 167-667 nkat/L; GGT: 150-583 nkat/L; Bilirubin: 1.7-18.8 μmol/L; Albumin: 58.8-69.6 g/L.

Statistical analysis

Results were expressed as means ± SD. Statistical analysis was performed with the Wilcoxon rank-sum test for independent samples and Wilcoxon signed rank test for paired samples. The relationship between the serum fibrosis markers and liver histology scores was analysed by the Spearman rank-correlation test for nonparametric data and by the Pearson method for parametric data. Tests were considered statistically significant at $P < 0.05$.

RESULTS

Patient characteristics

The baseline characteristics of the 47 children are presented in Table 1. They were classified into responders (n = 20; 42.5%) and nonresponders (n = 27; 57.5%). There were no significant differences between the groups regarding age, gender, body mass, duration of HBV infection, the levels of bilirubin and albumin, the activity of GGT, levels of baseline liver fibrosis markers (tenascin, hyaluronan, collagen VI, TIMP-1) and grade of inflammation. However, treatment responders displayed a significantly higher activity of ALT (2184 ± 2217 *vs* 1150 ± 550 nkat/L) and AST (1717 ± 1567 *vs* 1100 ± 500 nkat/L) (P = 0.0453 for both) and a higher fibrosis score according to Batts and Ludwig (2.3 ± 0.5 *vs* 1.8 ± 0.6; P = 0.004).

Basal levels of serum fibrosis markers

We arbitrarily defined mild/moderate fibrosis as stage ⩽ 2 (n = 39) and advanced fibrosis as stage = 3 (n = 8) accor-

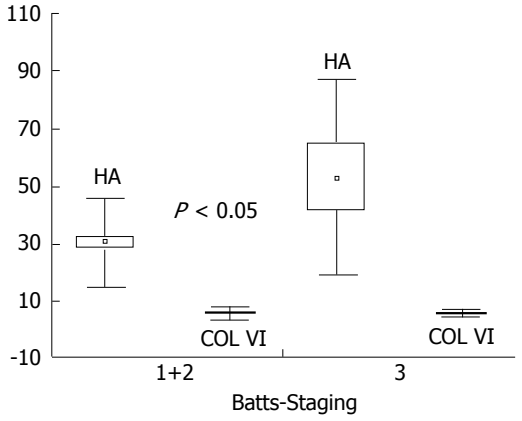

Figure 1 Correlation of serum fibrosis markers with histological staging (mean ± SD).

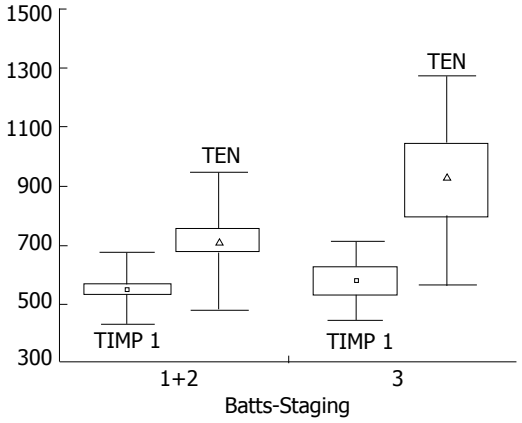

Figure 2 Correlation of serum fibrosis markers with histological staging (mean ± SD).

Table 2 Effect of IFN alpha on serum fibrosis markers in children with chronic hepatitis B (mean ± SD)

Patients	Marker (μg/L)	Before IFN (1)	After IFN (2)	12 mo after IFN (3)	*P* 1 *vs* 2	*P* 1 *vs* 3	*P* 2 *vs* 3
All	TIMP-1	558.1 ± 121.2	569.2 ± 134.8	636.9 ± 125.8	NS	b	NS
n = 47	CollagenVI	5.8 ± 2.2	6.2 ± 1.8	Nd	NS	-	-
	Tenascin	748.8 ± 266.0	641.5 ± 216.8	764.0 ± 250.0	b	NS	b
	Hyaluronan	34.3 ± 21.5	40.0 ± 30.9	28.7 ± 19.3	NS	b	b
Responders	TIMP-1	544.1 ± 120.3	555.4 ± 109.3	608.0 ± 134.9	NS	NS	NS
n = 20	CollagenVI	5.6 ± 1.85	6.7 ± 1.8	Nd	NS	-	-
	Tenascin	766.2 ± 289.9	686.2 ± 225.8	765.5 ± 249.9	NS	NS	NS
	Hyaluronan	40.3 ± 28.1	36.8 ± 18.0	30.7 ± 17.2	NS	a	a
Non	TIMP-1	567.9 ± 123.1	581.2 ± 155.0	657.2 ± 118.3	NS	b	NS
Responders	CollagenVI	6.0 ± 2.4	5.7 ± 1.7	Nd	NS	-	-
n = 27	Tenascin	736.6 ± 252.7	602.6 ± 205.6	762.9 ± 254.9	b	NS	b
	Hyaluronan	29.6 ± 13.6	42.7 ± 39.1	27.2 ± 20.9	NS	NS	b

Nd: Not determined; NS: Not significant; $^{a}P < 0.05$, $^{b}P < 0.01$.

ding to Batts and Ludwig. There were no significant differences in mean serum levels of tenascin, collagen VI and TIMP-1 between children with mild and advanced liver fibrosis, while levels of hyaluronan were higher in the latter group (30.3±15.8 *vs* 53.1±34.3 μg/L; $P = 0.0266$). There was a trend for increased tenascin and TIMP-1 in children with advanced fibrosis (Figure 1, Figure 2). We also arbitrarily defined mild inflammation as grade 1 ($n = 20$) and severe inflammation as grade ⩾2 ($n = 27$) according to Batts and Ludwig. There were no significant differences in mean concentrations of all serum fibrosis markers between children with mild and severe hepatic inflammation.

Effect of IFN on serum fibrosis markers

At end of treatment there were no significant changes in serum fibrosis markers in responders, while in nonresponders only tenascin decreased significantly ($P = 0.0074$). Twelve months after end of treatment serum hyaluronan was significantly lower than before treatment ($P = 0.0076$), while serum TIMP-1 was increased ($P = 0.0072$). In responders only hyaluronan decreased significantly ($P = 0.0304$), while in nonresponders the level of TIMP-1 increased ($P = 0.0064$, Table 2). Tenascin reached pretreatment levels in both responders and nonresponders.

Effect of IFN on liver histology

There were no significant changes in fibrosis stage after IFN therapy in the whole cohort, 2.0 ± 0.6 *vs* 2.1 ± 0.6, according to Batts and Ludwig and in subgroups, responders: 2.3 ± 0.5 *vs* 2.2 ± 0.7; nonresponders: 1.8 ± 0.6 *vs* 2.0 ± 0.6. Histological inflammation improved significantly in the whole group, 1.6 ± 0.7 *vs* 1.2 ± 0.7, $P = 0.0373$.

Correlation between serum fibrosis markers, histology and biochemical parameters

There were no significant correlations between baseline levels of the 4 serum fibrosis markers with liver fibrosis or inflammation according to Batts and Ludwig, or with AST, ALT, GGT, albumin or bilirubin. Only hyaluronan correlated significantly with histological fibrosis ($r = 0.3383$, $P = 0.021$).

DISCUSSION

Liver biopsy has been considered the gold standard for the assessment of hepatic fibrosis. Current recommen-

dations suggest that this procedure precede antiviral treatment in most patients with chronic hepatitits B or C[25]. However, liver biopsy is invasive with the potential for complications, such as bleeding which occurrence ranges from 0.3% to 0.5%[26,27], and mortality up to 0.1%[26,28]. In addition, since the biopsy core only represents 1/20 000 to 1/50 000 of the liver, biopsy is prone to sampling error, and variations in fibrosis staging may be high among different pathologists[15,16,21,29]. For these reasons, especially in children, non-invasive detection of histological liver damage, paticularly of fibrosis, is needed. Ideally serum markers of fibrosis should be applicable to patients with chronic hepatitis to either diagnose the stage of liver fibrosis, potentially replacing liver biopsy for this purpose, or to monitor progression of fibrosis or fibrogenesis, particulary during treatment [17,30]. Markers of the dynamics of fibrogenesis and fibrolysis are urgently needed, e.g. for short-term assessment of antifibrotic drug effects, but difficult to validate.

In this study we evaluated the changes of 4 serum fibrosis markers derived from the extracellular matrix (tenascin, hyaluronan, collagen VI and TIMP-1) before, at the end of and 12 mo after treatment of children with chronic hepatitis B with IFN. Our results showed a significant decrease of hyaluronan in responders and increased TIMP-1 in nonresponders, when levels before and 12 mo after interferon α treatment were compared. While falling during treatment, serum tenascin reached pretreatment levels in both responders and nonresponders. There were no significant changes in histological liver fibrosis 12 mo after the 5-mo course of IFN in all patients or in the subgroups of responders and nonresponders. This was expected, since the rate of fibrosis progression or regression in patients with chronic hepatitis B or C was usually slow. Assuming that IFN has at least some antifibrotic activity, as suggested before in large retrospective analyses of patients with chronic hepatitis C[31, 32], the histological scoring systems are obviously not sensitive enough to detect small changes in liver fibrosis and (modest) antifibrotic treatment effects. Nonetheless, the course of serum fibrosis (fibrogenesis) markers in our small but well defined group of children suggests that IFN indeed has antifibrogenic activity, especially in responders. This antifibrotic effect seems to be transient, as exemplified by serum tenascin which was depressed only during IFN treatment.

Prior to our study there had been no longitudinal, prospective studies of serum fibrosis markers in children with chronic hepatitis B, and only few studies analysed the effect of IFN therapy on the stage of liver fibrosis in children. Our findings are consistent with our previous study[33] and with those of others, who did not observe improvement of liver fibrosis by antiviral treatment in children, when biopsy was performed before and immediately after[34] or 9-12 mo after end of treatment[35, 36], while Gregorio *et al*[37] found significant improvement in staging in responders. However, these reports included small numbers of patients (≤24). It has been demonstrated that fibrosis stage changes more slowly than inflammation grade[7, 38]. This explains why we did not observe a significant improvement in fibrosis, while inflammation was clearly suppressed by IFN treatment.

We found that hyaluronan was the best serum marker to predict advanced liver fibrosis, since its level correlated significantly with histological fibrosis and was significantly higher in children with advanced *vs* mild/moderate liver fibrosis. These data are in keeping with previous results in patients with chronic viral hepatitis[39-43]. Thus the ability of this test to differentiate patients with extensive liver fibrosis from those with mild liver fibrosis was stronger than that of other markers, i.e., PIIINP, collagen IV, MMP-1, MMP-2 and TIMP-1[44] and laminin, collagen IV, PIIINP and TGF β1[45].

In children, up to now serum hyaluronan had only been studied in biliary atresia and cystic fibrosis[46-48], and there had been no data on collagen VI in chronic viral hepatitis or on TIMP-1 or tenascin in childhood liver diseases in general. Previous studies indicated that most serum fibrosis markers are influenced by body growth, especially PIIINP[49,50]. Thus healthy children have higher PIIINP levels than adults, excluding its use as reliable fibrosis marker for pediatric patients. Hyaluronan appears to be a useful marker of fibrosis stage also in children due to its short biological half life of only a few minutes and a prominent uptake by sinusoidal endothelial cells[51]. Similarly, tenascin and TIMP-1 are applicable to children with chronic hepatitis B as markers of fibrogenesis/inflammation and of fibrogenesis, respectively.

Our data suggest that serum hyaluronan, tenascin and TIMP-1 could be useful fibrosis markers in future studies of children with chronic viral hepatitis.

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