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New Markers for Evaluation of the Efficacy of Interferon-alpha Therapy in Hepatitis C Patients

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Abstract: Background: hepatic fibrosis is the most important factor for estimating clinical outcome and determining therapeutic strategy, especially interferon therapy, in patients with hepatitis C virus (HCV)-associated liver diseases. Aim of the work: to evaluate the clinical utility of serum fibrosis markers including matrix metalloproteinase (MMP-2) and N-terminal peptide of type III procollagen (PIIINP) in patients with HCV-associated liver disease and response to interferon (IFN) therapy. Subjects and Methods: seventy patients with HCV-associated liver disease and twenty apparently healthy individuals of matched age and sex were enrolled in the study, PIIINP and MMP-2 were measured and we evaluate the changes of their levels before and after IFN therapy for patients. Results: increase in serum levels of fibrotic markers was correlated with the progression of the disease and their levels were significantly decreased after IFN therapy. Conclusion: This study demonstrates that serum PIIINP and MMP-2 reflect fibrogenesis in patients with hepatitis C virus associated liver disease and that they may be serological markers used as a non-invasive markers for evaluating the efficacy of interferon therapy in these patients.

Key words: HCV- liver fibrosis- MMP-2 – PIIINP - IFN-alpha.

INTRODUCTION

Hepatic fibrosis represented a net increase in scar tissue rather than collapse of existing stroma. Simply stated, fibrosis represents a wound healing response and is the common end point of a variety of different insults to the liver as: hepatitis viruses, alcohol & drug toxicity, inherited metabolic disorders and cholestatic & immune diseases[1]. Chronic hepatitis C virus (HCV) infection is a silent disease in the majority of patients, until significant hepatic fibrosis has developed[2]. The prognosis is determined mainly by the progression of fibrosis and the ultimate development of cirrhosis[3].

Hepatic fibrosis is the result of an imbalance between enhanced matrix synthesis and diminished breakdown of connective tissue proteins, the net result of which is increased deposition of extra cellular matrix [4].

Several attempts have been made to find accurate noninvasive markers of disease activity and fibrosis, several laboratory markers, such as platelet counts, ALT/AST ratio[5]. N-terminal peptide of type III procollagen (PIIINP)[6]. matrix metalloproteinases (MMPs)[4]. or type IV collagen[7]. have been proposed to represent hepatic fibrosis. Some have combined several biochemical and clinical markers with scoring system to predict the presence or absence of fibrosis, PIIINP levels have been well studied in patients with chronic liver disease[8]. and revealed a correlation with histological inflammation more than fibrosis. MMP-2 has been also studied and revealed positive relationships with the degree of periportal necrosis, fibrosis and total score of histological activity index[9].

Hence, there is a need to develop simple, accurate and reliable noninvasive markers for evaluating the severity of fibrosis, so as to avoid the hazards and complications of liver biopsy. The present study was carried out to investigate the effects of alpha interferon therapy on the levels of MMP-2, PIIINP and ALT in sera of patients with HCV to intense light on the possible effect of therapy on such patients.

Subjects and Methods: Seventy patients with chronic liver disease were studied and all patients had detectable serum HCV-RNA. Liver biopsies were done for Fifty-one patients for histological examination of the liver under the guide of ultrasound with Menghini needle. Tissue sections from the patients were stained with hematoxylin and eosin and evaluated for the stage of liver fibrosis and the grade of liver activity. Twenty-nine patients, whose diagnosis was based on clinical, biochemical and imaging findings, were classified as having liver cirrhosis, because they had much risk for the biopsies. None of the patients had other causes of chronic liver injury (a history of alcohol consumption or hepatocellular carcinoma).

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Twenty healthy individuals were also included in the study of matched age and sex. All tissues and serum samples were obtained with informed consent.

The patients were sub-classified into five groups based on the stage of liver fibrosis according to the Metavir scoring system[10]. Fibrosis was staged on a scale of F0-F4 as shown in table 1.

The grade of liver activity was classified into four groups according to Metavir classification: A0 (no activity), A1 (mild activity), A2 (moderate activity) and A3 (severe activity).

Table 1: Metavir Fibrosis.

Category	Histological characteristics
F0	Normal
F1	Periportal fibrosis
F2	Incomplete septae
F3	Complete septae
F4	Cirrhosis

Laboratory Tests: Ten milliliters of fasted venous blood (6 Hours of fasting) were taken from each subject participating in the study, about 1.8ml of blood was added into 0.2 ml citrate for determination of and prothrombin concentration and the rest of blood was left to clot and the serum was separated by centrifugation and stored at -20°c for analysis of:

Liver Function Tests: Total bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (γ-GT), alkaline phosphatase (ALP), total proteins and albumin.

Markers of Hepatitis Virus: Hepatitis B surface antigen (HBsAg), hepatitis B core antibody (HBcAb), hepatitis C virus antibody (HCV-Ab), and hepatitis C virus RNA (HCV-RNA).

Related Liver Fibrosis Markers: matrix metalloproteinase-2 (MMP-2) and N-terminal peptide of type III procollagen (PIIINP).

ALT, MMP-2 and PIIINP were measured six months later after treatment with alpha interferon (three million units three times a week) and compared to normal subjects who were known to be free from any diseases especially liver diseases.

Liver function tests were performed using a Beckman Auto-analyzer (Synchron CX4, USA). A diazotization method determined serum total bilirubin [11]. Activities of ALT,AST, ALP and γ-GT were determined by the enzyme rate method [12,13,14].

Serum total proteins were measured by a buiret method[15]. and albumin was determined[16]. Prothrombin concentration was determined using standard thromboplastin method[17].

HCV antibodies were detected using a third generation enzyme-linked immunosorbent assay (Sorin Biomedica Diagnostics, Italy),[18]. Serological assay for HBV markers (HBsAg and anti-HBc) were performed by a direct non-competitive sandwich assay (DiaSorin, Italy) based on ELISA technique[19]. Serum samples were assayed for HCV-RNA by reverse transcriptase polymerase chain reaction (RT-PCR) technique. RNA was extracted with guanidinium thiocyanate[20]. and primers from 5' untranslated region of the HCV genome were used[21].

The determination of PIIINP by radioimmunoassay (Orion Diagnostica, P.O.Box 83, 02101 Espoo, Finland) [22].

The determination of MMP-2 by sandwich enzyme immunoassay technique (R&D systems, Inc. 614 McKinley Place, Minneapolis, MN 55413, USA)[23].

Ultrasound examination was done for all patients to exclude liver cirrhosis and to determine hepatic size.

Statistical Analysis: Results were expressed as mean ± SD. Data was statistically analyzed using SPSS package for windows, version 7.5 (F-test and r-coefficient).

RESULTS AND DISCUSSION

Table 2, represents the characteristics of the patients and controls included in the study. 71% of the patients were males. The median age at biopsy was 42±13 and the median duration of infection was 17±8 years.

Patients with fibrosis of F2 or greater are considered as candidates of antiviral therapy.

There was a highly significant increase in the mean levels of total serum bilirubin, AST,ALT, ALP and γ-GT in patients compared with the control group. Also table 2, shows that the serum levels of PIIINP and MMP-2 were highly significantly increased compared to the control group. On the other hand there was a significant decrease in the mean levels of serum albumin ,total protein and prothrombin concentration in patients compared to control group.

Table 3, shows serum levels of ALT, PIIINP and MMP-2 for patients before and after 6 months of therapy with interferon, there was a highly significant reduction of serum levels of ALT, PIIINP and MMP-2 after therapy $P<0.001$.

Table 4, shows the correlation between PIIINP, MMP-2 and liver function tests. There was a positive significant correlation between PIIINP and total bilirubin, AST, ALT, ALP, γ-GT, total protein and MMP-2 and a negative significant correlation between PIIINP and albumin & prothrombin concentration.

Positive correlation was found between MMP-2 and total bilirubin, AST, ALT, ALP, γ-GT and total protein while negative correlation were found between MMP-2, albumin and prothrombin concentration.

Table 2: characteristics of the studied patients and controls

Characteristics	Patients	Controls	
Demographics			
Median age at biopsy	42±13(years)		
Median duration of infection	17±8		
Male sex	50(71%)	10(50%)	
Fibrosis stage			
F0	8(15.7%)		
F1	17(33.3%)		
F2	13(25.5%)		
F3	7(13.7%)		
F4	6(11.8%)		
Necroinflammatory activity grade			
None (N0)	11(21.6%)		
Mild (A1)	18(35.3%)		
Moderate (A2)	13(25.5%)		
Severe (A3)	9(17.6%)		
Significant histologic lesions			
(activity≥A2,fibrosis≥F2	24(47%)		
Laboratory tests			P*
Total bilirubin (mg/dl)	1.18±0.19	0.55±0.26	<0.05
AST (U/l)	79.8±6.70	25.0±4.3	<0.05
ALT (U/l)	87.4±9.31	19.4±9.3	<0.01
ALP (U/l)	90.4±7.8	68.20±4.7	<0.05
γ-GT(U/l)	133.1±10.2	18.2±3.4	<0.01
Albumin (g/dl)	3.65±0.35	4.4±0.6	<0.05
Total protein (g/dl)	7.21±0.07	7.36±0.07	<0.05
Prothrombin concentration (%)	83.29±13.06	91.82±7.2	<0.01
HBsAg	Negative	Negative	
HBcAb	Negative	Negative	
HCV Ab	Positive	Negative	
HCV-RNA	Positive	Negative	
MMP-2 (ng/ml)	832.0±201.4	305.0±95.7	<0.001
PIIINP (µg/l)	16.74±4.9	4.8±1.2	<0.001

P*<0.05: Significant. P*<0.01 and <0.001: Highly significant.

Table 3: parameters before and after therapy

ALT	PIIINP	MMP-2 (µg/l)	(U/l) (ng/ml)
Before treatment	87.4±9.31	16.7±4.9	832.0±201.4
P<0.05	P<0.001	P<0.001	
After treatment	24.2±6.3	7.8±4.1	610.0±98.1
	P<0.001	P<0.001	P<0.001
Control	19.4±9.3	4.8±1.2	305.0±95.7

Table 4: Correlation between PIIINP, MMP-2 and parameters of liver function

Parameters	PIIINP		MMP-2	
	r	p	r	p
AST	0.36	<0.05	0.33	<0.05
ALT	0.94	<0.01	0.91	<0.01
ALP	0.98	<0.01	0.95	<0.01
γ-GT	0.56	<0.01	0.53	<0.01
Albumin	-0.89	<0.01	-0.81	<0.01
Total protein	0.74	<0.01	0.70	<0.01
Prothrombin concentration	-.82	<0.01	-0.84	<0.01
Total bilirubin	0.40	<0.05	0.49	<0.05
MMP-2	0.94	<0.01	----	----
PIIINP	--	--	0.98	<0.01

Abbreviations:
AST: aspartate aminotransferase. ALT: alanine aminotransferase. γ-GT :γ-glutamyl transferase.. MMP-2: matrix metalloproteinase type 2. PIIINP: N-terminal peptide of type III procollagen.

Discussion: Chronic hepatitis C virus (HCV) infection results in the development of liver fibrosis and cirrhosis in 20 to 25% of patients. The main task of the physician when examining a patient with a verified HCV infection is to identify the activity of inflammatory and necrotic processes in the liver, as well as the stage of fibrosis, and the reversibility of detected changes. Along with other clinical and laboratory parameters, this plays a major role in forecasting the course of hepatitis, as well as determining the therapeutic approach in each specific case[24]. Liver biopsy remains the best way to assess the severity of chronic hepatitis C. The risk of developing cirrhosis depends on the stage (degree of fibrosis) and the grade (degree of inflammation and necrosis) observed in the initial liver biopsy[25]. Non-invasive diagnostic approaches attempt to evaluate the serum markers of fibrogenesis. Biochemical markers of fibrosis scoring include thrombocyte counts, the prothrombin concentration, ratio of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, the level of γ-glutamyl transferase and the quantity of blood serum albumin. Another set of markers is based on the detection of molecular junctions that activate fibrosis, or participate in the generation of the liver extra cellular matrix. The most applicable include hyaluronic acid (HA), type IV collagen (IV-C), N-terminal propeptide of type III procollagen (PIIINP), metalloproteinases (MMP), inhibitors of metalloproteinases (TIMP), and growth-transforming factor beta (GTF beta).[26].

In this study we measured PIIINP, MMP-2, ALT at the start of the study and six months later after therapy with interferon-alpha. There was a highly significant increase in serum markers at baseline with P<0.001 compared to the control group.

Metalloproteinases and their tissue inhibitors have been shown by several groups to correlate with the development of cirrhosis, the extent of toxic damage to the liver in alcoholic liver disease and the inflammatory activity in patients with chronic viral hepatitis[3,4,7]. Other studies measured PIIINP, type IV collagen and hyaluronic acid. PIIINP is a component of extra cellular matrix deposited in the space of tissues. It is produced from type III procollagen in hepatic stellate cells and released into the circulation. Some authors confirmed that PIIINP correlates better with inflammation and is thought to reflect primary active hepatic fibrogenesis in chronic liver disease[6,8,27]. and in monitoring the progression of fibrosis and in assessing the therapeutic effect of anti-fibrogenic drugs [27,28].

Based on our data, there was a strong correlation between biochemical responses to interferon therapy (normalization of ALT) and serological response as confirmed by highly significant reduction of MMP-2 and PIIINP after six months of therapy. Several studies were in agreement with us[27,28,29,30].

Abdelaziz[31]. reported that as high serum levels of PIIINP were shown in many pathological conditions including liver fibrosis, liver cirrhosis and malignant disease, one can expect liver damage after viral

infection due to accumulation of connective tissue components including collagen type III[32]. which may provide a relatively non-invasive mean following the disease progression[33].

Conclusion: This study demonstrates that serum PIIINP and MMP-2 reflect fibrogenesis in patients with hepatitis C virus associated liver disease and that they may be a serological marker used as a non-invasive marker for evaluating the efficacy of interferon therapy in these patients.

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