

CLINICAL STUDY

Alpha-2-macroglobulin and hyaluronic acid as fibromarkers in patients with chronic hepatitis C

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ABSTRACT

BACKGROUND: Liver fibrosis is the final common pathway of chronic liver diseases of various etiology. From the practical standpoint, it would be ideal to have a noninvasive fibromarker. The aim of our study was to investigate the levels of alpha-2-macroglobulin, potential fibromarker, in correlation to histological staging and another potential fibromarker, hyaluronic acid, in patients with chronic hepatitis C.

METHODS: Population groups in this study consisted of 51 healthy volunteers and 54 patients with chronic hepatitis C. Liver biopsies were obtained under ultrasound guidance. Alpha-2-macroglobulin was determined by electroimmunodiffusion and hyaluronic acid with enzyme-linked binding protein assay.

RESULTS: Both potential fibromarkers, alpha-2-macroglobulin and hyaluronic acid, were increased in patients with chronic hepatitis C. The alpha-2-macroglobulin levels were not significantly increased in the groups F0–F1. In the groups F2–F4, alpha-2-macroglobulin levels were significantly higher than in the control group. The changes of hyaluronic acid were similar to changes of alpha-2-macroglobulin. Regression analysis showed a significant correlation between hyaluronic acid and alpha-2-macroglobulin levels.

CONCLUSIONS: According to the results of our study, it can be concluded that alpha-2-macroglobulin and hyaluronic acid might be useful markers of liver fibrosis (*Tab. 2, Ref. 15*). Text in PDF www.elis.sk.

KEY WORDS: chronic hepatitis C, fibromarkers, liver fibrosis, alpha-2-macroglobulin, hyaluronic acid.

Introduction

Liver fibrosis is the final common pathway of chronic liver diseases of various etiology (e.g. viral infections, alcohol abuse, autoimmune conditions or metabolic diseases). It is a complex dynamic process that includes an increase in extracellular matrix components, activation of cells producing matrix material, cytokine release and tissue remodelling. Progression to an advanced fibrosis can be rapid or slow, depending on disease state and the degree of activity of liver injury. The assessment of liver fibrosis is important for disease prognosis and for determination of the urgency of treatment as well as the response to therapy.

From the practical standpoint, it would be ideal to have a noninvasive fibromarker that can (a) stage the degree of liver fibrosis, (b) reflect the rate of matrix deposition or its removal, (c) monitor the impact of therapy. It should be (d) readily available, (e) reproducible and (f) inexpensive. Unfortunately, so far, none of the known and clinically used fibromarkers can meet all these requirements.

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Despite of its invasive character, liver biopsy remains to be the “gold standard“ in the diagnosis and staging of liver fibrosis. For both, the physician and the patient, the decision to proceed with liver biopsy is not trivial. Significant complications occur in 1–5 % of patients with a reported mortality rate of between 1 : 1000 and 1 : 10000 (1). Due to the limitations of liver biopsy mentioned above, much effort has been invested to find a reliable serum liver fibromarker that would be a useful adjunct to clinical assessment and would reduce the need of repeated liver biopsy. Several biochemical candidates have been proposed as markers of fibrosis, either isolated or combined together in a scoring systems, such as PGA index, Fibro Test, Fibrometer test, Hepa Score or ELF score (2).

Liver fibrosis is a complex process involving production and deposition of different components that constitute the extracellular matrix (ECM). To these components belong proteins such as: collagens, noncollagenous glycoproteins (fibronectin, laminin, undulin, vitronectin, tenascin, osteonectin, elastin), proteoglycans and polysaccharide (hyaluronan)(3). Due to an increased production of ECM components in liver fibrosis, also the interest in determination of substances related to ECM as serum fibromarkers increases. One of the most useful fibromarkers is hyaluronan (hyaluronic acid, HA) (4–6). Hyaluronic acid is a chief component of ECM and plays an important structural role in the formation of ECM. Hyaluronic acid is a linear, anionic, nonsulfated glycosaminoglycan with unique characteristics. HA molecules are polymers of disaccharides (up to 25000 disaccharide repeat units in length)

Tab. 1. Standard laboratory tests in patients with chronic hepatitis C and healthy controls.

	ALT ($\mu\text{kat.l}^{-1}$)	AST ($\mu\text{kat.l}^{-1}$)	Cholinesterase ($\mu\text{kat.l}^{-1}$)	Albumin (g.l^{-1})
Healthy controls	0.32±0.07	0.35±0.05	66.02±13.33	39.3±3.5
Fibrosis grade 0	1.11±0.42	0.62±0.25	77.16±15.83	37.8±4.2
Fibrosis grade F1	1.22±0.53	0.70±0.33	74.52±13.92	37.9±4.1
Fibrosis grade F2	1.42±0.55	0.78±0.32	62.01±13.25	36.5±6.9
Fibrosis grade F3	0.78±0.48	1.15±0.53	59.53±12.58	29.9±7.0
Fibrosis grade F4	1.05±0.47	1.18±0.48	29.88±5.83	25.6±5.9

Results are given as the mean ± SD (standard deviation)

composed of D-glucuronic acid and N-acetyl-D-glucosamine, linked via alternating $\beta(1\rightarrow4)$ and $\beta(1\rightarrow3)$ glycosidic bonds (7).

Another potential serum fibromarker is alpha-2-macroglobulin (AMG). AMG is a large plasma protein with molecular weight approximately 720 kDa. It is a versatile protease inhibitor with a different mechanism than serpins. AMG has four identical subunits with molecular weight of 180 kDa, occurring as a pair of dimers. The dimers are disulfide-linked, whereas the pairs are held together by noncovalent bonds (8,9). The dimer is the active unit, one molecule of AMG binds up to two protease molecules. AMG is synthesized primarily by hepatocytes and has a half-life of several days. After binding of the protease molecule, AMG-protease complex is cleared from circulation primarily by receptors on hepatocytes (10). The half-life of complexes is only a few minutes. The major function of AMG is the inhibition of proteases. AMG also binds to a variety of other proteins. AMG has been measured as one component of some multiparameter indices of hepatic fibrosis (11).

The aim of our study was to investigate the levels of alpha-2-macroglobulin, potential fibromarker, in correlation to histological staging and to another potential serum fibromarker – hyaluronic acid – in patients with chronic hepatitis C.

Patients and methods

Study population

Population groups under study consisted of 51 healthy volunteers – blood donors and 54 patients with chronic hepatitis C (37 men and 14 women, mean age 37.6 years). All patients had chronic hepatitis C virus (HCV) infection as confirmed by HCV-RNA polymerase chain reaction analysis in serum. Coexisting liver diseases attributable to alcohol, hepatitis B, autoimmune hepatitis, primary biliary cirrhosis or haemochromatosis were reasonably excluded by history and standard clinical, laboratory, imaging and histologic studies. Human immunodeficiency virus coinfection and post-transplant patients were also excluded. Five ml of peripheral blood was taken from each patient in fasting state, one hour before performing liver biopsy. Liver biopsies were performed as part of routine clinical care in the staging and grading of liver disease. All patients and controls gave an informed consent for use of data and serum for research purposes. Local Ethics Committee approved the study.

Liver biopsy

Liver biopsies were obtained under an ultrasound guidance and were deemed adequate on the base of either specimen size or the

Tab. 2. Levels of alpha-2-macroglobulin and hyaluronic acid in patients with chronic hepatitis C and healthy controls.

	Alpha-2-macroglobulin (mg.l^{-1})	Hyaluronic acid ($\mu\text{g.l}^{-1}$)
Healthy controls	1308±220	15.52±9.37
Fibrosis grade F0	1375±210	20.74±12.93
Fibrosis grade F1	1620±340	25.75±11.5
Fibrosis grade F2	1820±310**	34.53±18.0*
Fibrosis grade F3	2810±565***	81.85±14.25***
Fibrosis grade F4	3475±1115***	361.72±158.8***

Results are given as the mean ± SD, statistical significance * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

number of portal tracts. Biopsies were fixed, paraffin-embedded and stained with at least hematoxylin-eosin-safran and Masson's trichrome or picrosirius red for collagen. Biopsies were interpreted according to the scoring schema developed by METAVIR group (12). Fibrosis was scored on a 5-point scale: F0 – no fibrosis, F1 – portal fibrosis alone, F2 – portal fibrosis with rare septae, F3 – portal fibrosis with many septae, F4 – cirrhosis.

Laboratory methods

Standard laboratory assessments including a complete blood cell count, routine biochemical parameters – ALT, AST, cholinesterase, albumin, bilirubin, were performed by a licensed clinical laboratory in fresh serum within 36 hours of collection. Other analyses were performed on sera stored frozen at -80°C . Alpha-2-macroglobulin was determined immunochemically by electroimmunodiffusion according to Laurell (13), using monospecific antisera (Q-SwAHu/A2M, Sevapharma a.s., Praha, Czech republic). Hyaluronic acid was determined with an enzyme-linked binding protein assay that uses a capture molecule known as hyaluronic acid binding protein (Hyaluronic acid test kit, Corgenix, Inc., CO, USA).

Statistical analysis

Results are given as the mean ± SD (standard deviation). Means were compared using the non-parametric Wilcoxon test. We have considered p values of less than 0.05 to be statistically significant.

Results

The results of standard biochemical tests are presented in the Table 1. Albumin and cholinesterase, parameters reflecting the liver function, are in the groups F0 and F1 not significantly different from healthy controls. In the patients with fibrosis grade F2, these parameters are slightly decreased. Albumin level and

cholinesterase activity were significantly decreased in the group F4 ($p < 0.001$).

Both potential fibromarkers – hyaluronic acid and alpha-2-macroglobulin – were increased in patients with HCV infection (Tab. 2). The level of alpha-2-macroglobulin in the group F0 was practically the same as in healthy controls. The level of alpha-2-macroglobulin was slightly increased in the group F1 compared to healthy controls, but this difference was statistically not significant. In the groups F2–F4, AMG levels were significantly higher than in the control group. The differences between F2 – F3 ($1820 \pm 310 \text{ mg.l}^{-1}$ vs $2810 \pm 565 \text{ mg.l}^{-1}$) and F3 – F4 ($2810 \pm 565 \text{ mg.l}^{-1}$ vs $3475 \pm 1115 \text{ mg.l}^{-1}$) were also statistically significant.

The results of hyaluronan determination showed that there was no difference between the group F0 and healthy controls ($20.74 \pm 12.93 \text{ } \mu\text{g.l}^{-1}$ vs $15.52 \pm 9.37 \text{ } \mu\text{g.l}^{-1}$). The levels of hyaluronan were slightly increased in the group F1 and F2 compared to the control group ($25.75 \pm 11.5 \text{ } \mu\text{g.l}^{-1}$, resp. $34.53 \pm 18 \text{ } \mu\text{g.l}^{-1}$ vs $15.52 \pm 9.37 \text{ } \mu\text{g.l}^{-1}$), but the difference between the group F1 and F2 was not significant. The levels of HA in the group F3 ($81.85 \pm 14.25 \text{ } \mu\text{g.l}^{-1}$ vs $15.52 \pm 9.37 \text{ } \mu\text{g.l}^{-1}$) and the group F4 ($361.72 \pm 159.8 \text{ } \mu\text{g.l}^{-1}$ vs $15.52 \pm 9.37 \text{ } \mu\text{g.l}^{-1}$) were significantly increased in comparison with the control group. The levels of HA in the groups F3 and F4 were also significantly higher than in the group F2.

Regression analysis of the relationship of hyaluronic acid and alpha-2-macroglobulin levels showed a statistically significant correlation between these two parameters in sera of patients with chronic hepatitis C ($r = 0.43$, $p < 0.001$).

Discussion

Chronic hepatitis C is a progressive disease. Because the prognosis of patients with this disease depends mainly on the development of liver fibrosis, this process needs to be regularly evaluated. Despite the fact that histological examination is the „gold standard“ for assessing liver fibrosis, liver biopsy is potentially associated with severe complications and for ethical reasons cannot be repeated to monitor liver status. As a consequence of the difficulties linked to liver histological examination, serum fibromarkers are now used for monitoring of the liver fibrosis in patients with HCV infection. Developing an efficient and non-invasive method for liver fibrosis is important for prognosis and treatment of patients with chronic hepatitis type C. A noninvasive diagnosis of liver fibrosis could also enhance the development of antifibrotic therapies.

There are several types of non-invasive markers utilized for evaluation of severity of fibrosis in patients with chronic hepatopathy. Indirect markers utilize routine laboratory parameters such as: transaminases, albumin, prothrombin time or other parameters, which relate to the liver disease stage. Direct serum markers of fibrosis are laboratory parameters, which reflect the ECM turnover and can be used for staging of fibrosis, and also for monitoring the progression or regression of liver fibrosis. These markers include several glycoproteins, collagens, collagenases and their inhibitors. These have been studied individually as well as in combinations in different scoring systems.

In the present study, we have evaluated and compared alpha-2-macroglobulin and hyaluronan serum levels as markers of liver fibrosis. Hyaluronan is the most studied direct serum marker. It is a glycosaminoglycan that is synthesized in extracellular space by hepatic stellate cells and is degraded by sinusoidal endothelial cells (14). The results of our study showed that the levels of HA in the group of patients with chronic hepatitis C were increased, when compared to the healthy controls. This increase correlates well with the severity of liver fibrosis. Patients with mild fibrosis (fibrosis grade \leq F2) had only slightly increased levels of HA, but this increase was not significantly different from reference values. Significantly increased levels of HA were found in the patients with an extensive liver fibrosis (fibrosis grade F3 and F4). The results indicated that the hyaluronic acid level used as the fibromarker is a more reliable diagnostic tool in patients with more severe fibrosis than in patients with moderate form of fibrosis. The explanation of increased levels of HA in patients with liver fibrosis could be the activation of hepatic stellate cells, which is characteristic for liver fibrosis, and/or the endothelial dysfunction that occurs as fibrosis progresses. Our results of investigation of hyaluronic acid levels in patients with chronic HCV infection are in good agreement with results presented by Guechot et al (4). These authors presented cut off values for diagnosing extensive liver fibrosis in patients with chronic hepatitis C as $85 \text{ } \mu\text{g.l}^{-1}$. The mean value of HA in the group F3 in our patients is practically the same – $82 \text{ } \mu\text{g.l}^{-1}$.

The second investigated parameter, alpha-2-macroglobulin, was also increased in our group of patients with chronic hepatitis C. The changes of AMG levels were similar to changes of hyaluronic acid. In the group of patients with F0 fibrosis, there were no changes compared to the control group. In the patients with a slight grade of fibrosis (F1–F2), the levels of AMG were moderately increased, but they were still in the reference range. The significant increase in AMG levels was found only in patients with severe grade of fibrosis (F3–F4). One of the possible explanations of increased AMG levels in the patients with liver fibrosis is associated with the function of this protein. AMG is one of the major antiproteinases, present in plasma of vertebrates. Another important property of AMG is its ability to bind a variety of other proteins. AMG binds several cytokines and growth factors. The biological activity of some cytokines is inhibited when bound to AMG. During liver fibrosis, TGF β (transforming growth factor beta) stimulates the production of matrix. It has been shown earlier that AMG inhibits the activity of TGF β . Tiggelman et al (15) observed that TGF β -induced collagen formation by human liver myofibroblasts is reduced in vitro by AMG. It is possible, that AMG may have an anti-fibrogenic effect on TGF β -induced activation of stellate cells and the increase of AMG levels in patients with liver fibrosis is a compensation mechanism decreasing the production of extracellular matrix. Another mechanism for the increase of AMG levels in patients with liver fibrosis is a decreased clearance of AMG-protein complexes. These complexes are cleared from circulation primarily by receptors on hepatocytes (10). Damage of hepatocytes in chronic hepatopathies and disorders of the structure of hepatic sinusoids, as a consequence

of accumulation of extracellular matrix in perisinusoidal space, may lead to a decreased uptake of alpha-2-macroglobulin-protein complexes by hepatocytes leading to an increase of plasma alpha-2-macroglobulin levels.

The similarity of changes of alpha-2-macroglobulin and hyaluronic acid levels was confirmed also by the regression analysis, which showed the statistically significant correlation between these two parameters ($p < 0.001$).

According to the results of our study, we can conclude that the determination of alpha-2-macroglobulin and hyaluronic acid plasma levels may be a useful diagnostic tool in the verification of liver fibrosis and in the monitoring of the activity of hepatic fibrogenesis. Our results showed that these parameters are more reliable in patients with more severe grade of fibrosis. In regard to an interindividual variability of these parameters, their utilization is more favorable for monitoring the fibrogenic activity and effects of therapy than for the determination of the grade of liver fibrosis.

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