Evaluation of Plasma Urokinase-Type Plasminogen Activator Receptor (UPAR) in Patients With Chronic Hepatitis B, C and Non-Alcoholic Fatty Liver Disease (NAFLD) as Serological Fibrosis Marker

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Background/aims: Progressive hepatic fibrosis is the main predictor of outcome and prognosis in chronic liver diseases. The importance of the coagulation cascade has been defined in liver fibrosis; however, the role of the fibrinolytic pathway has not been clear yet. We aimed to evaluate the association between the plasma levels of soluble urokinase Plasminogen Activator Receptor (uPAR) and the severity of liver fibrosis in chronic hepatitis B, C and Non-Alcoholic Fatty Liver Disease (NAFLD). **Methods:** 96 chronic hepatitis B, 22 chronic hepatitis C and 11 NAFLD patients together with 47 healthy controls were enrolled in the study. uPAR plasma levels were detected by Enzyme-Linked Immunosorbent Assay (ELISA) method. **Results:** The plasma levels of uPAR in patients with chronic hepatitis B and C significantly exceeded those of healthy controls ($P < 0.001$) while mean uPAR levels in patients with NAFLD were not different from healthy controls. Mean uPAR levels in chronic viral hepatitis patients with F1–F3 fibrosis and F4–F6 fibrosis were higher than those of control group ($P < 0.001$). Mean uPAR level in patients with F4–F6 fibrosis was significantly higher than that of patients with F1–F3 fibrosis ($P < 0.001$). **Conclusion:** This is the first study that investigated uPAR as a fibrosis marker in NAFLD and chronic hepatitis B patients. It is suggested that plasma levels of uPAR are closely related to the fibrosis stage in chronic hepatitis B and C and that uPAR might be a noninvasive marker of liver fibrosis. (J CLIN EXP HEPATOL 2019;9:29–33)

Chronic hepatitis can lead liver cirrhosis and hepatocellular carcinoma that have high morbidity and mortality, and the most common causes of chronic hepatitis are chronic hepatitis B, C and Non-Alcoholic Fatty Liver Disease (NAFLD).^{1–3} Hepatic stellate

cells produce extracellular matrix proteins as a response to chronic injuries to hepatocytes and cause liver fibrosis and cirrhosis as the common consequence of all chronic liver injuries.^{4–6} The mechanisms of fibrinogenesis pathway are the point of interest in many studies and researches for preventive and therapeutical interventions to produce antifibrotic drugs to be used in chronic liver diseases are increasing every day.

Plasminogen and the plasminogen activators are components of extracellular proteolytic steps in humans. Tissue-type Plasminogen Activator (tPA) or urokinase-type Plasminogen Activator (uPA) can activate plasminogen and generate plasmin that degrades Extracellular Matrix (ECM) components such as fibrin.⁷

In recent years, several studies have demonstrated the role of uPA and urokinase Plasminogen Activator Receptor (uPAR) in cancer pathogenesis,^{8–11} certain inflammatory conditions like pancreatitis,^{12,13} sepsis,^{14,15} rheumatoid arthritis^{16,17} and acute myocardial infarction.¹⁸

We aimed to evaluate plasma uPAR levels in patients with chronic liver diseases due to hepatitis B, C, and NAFLD, and the relationship between uPAR levels and histological staging of liver fibrosis in this study.

Keywords: non-alcoholic fatty liver disease, hepatitis B, hepatitis C, urokinase-type plasminogen activator receptor

Received: 27 February 2017; **Accepted:** 8 February 2018; **Available online:** 16 February 2018

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Abbreviations: ALT: Alanine Aminotransferase; ECM: Extracellular Matrix; ELISA: Enzyme-Linked Immunosorbent Assay; HRP: Horseradish Peroxidase; HSCs: Hepatic Stellate Cells; MMPs: Matrix Metalloproteinases; NAFLD: Non-Alcoholic Fatty Liver Disease; NIA: Necro-Inflammatory Activity; OD: Optical Densities; PAIs: Plasminogen Activator Inhibitors; TGF-beta: Transforming Growth Factor-beta; TIMPs: Tissue Inhibitors of Metalloproteinases; tPA: tissue-type Plasminogen Activator; uPAR: urokinase Plasminogen Activator Receptor; uPA: urokinase-type Plasminogen Activator

<https://doi.org/10.1016/j.jceh.2018.02.001>

MATERIALS AND METHODS

Subjects

96 chronic hepatitis B, 22 chronic hepatitis C and 11 NAFLD patients that followed by gastroenterology clinic of Gazi University Hospital were enrolled in this work. 47 healthy individuals that suitable for age and gender were recruited as control group at the same time. The patients who participated in the study were between 18 and 65 years old and no one had a history of alcohol use. Exclusion criteria included the presence of chronic serious disease and pregnant or breast-feeding patients.

Persistence of HBsAg in serum for at least 6 months was descriptive for chronic HBV infection, and chronic HCV infection was diagnosed by detection of positive anti-HCV and HCV RNA in serum for at least 6 months. We used 3 criteria for the diagnosis of NAFLD: histologic picture of steatohepatitis, evidence of minimal or no alcohol consumption (<20 g/day), absence of serological, virological or immunological markers for other chronic hepatitis etiologies.

Percutaneous liver biopsies were performed in all patients who had participated in the study. The length of the core liver biopsy specimens is ranged between 1 cm and 2.2 cm each including minimum 6 well defined portal tracts. Masson's trichrome stain was used in order to assess liver fibrosis, histochemically. Ishak scoring system was used to evaluate liver biopsy specimens in order to determine the grade of Necro-Inflammatory Activity (NIA) (between 0 and 18) and the stage of fibrosis (between 0 and 6).

Methods

Ten milliliters (10 cc) of peripheral venous blood sample was collected from patients and healthy control volunteers and filled into a sodium citrate containing tube for measurement of plasma uPAR level and routine liver function test analysis. All blood samples were centrifuged at $1600 \times g$ for 15 min and isolated plasma samples were stored in aliquots at -80°C until the day of uPAR analysis. Liver function tests from blood samples were performed daily in routine biochemistry laboratory. Plasma uPAR levels were evaluated by using a sandwich Enzyme-Linked Immunosorbent Assay (ELISA) kit for Plasminogen Activator Receptor (uPAR), (available trademark: for Homo sapiens (Human); USCN Life Science Inc., Wuhan, China) according to manufacturer's instructions. The microplate in the kit was covered by an antibody specific to uPAR. The second antibody was a biotin-conjugated antibody specific to uPAR. Avidin conjugated to Horseradish Peroxidase (HRP) was added for augmentation. The concentrations of uPAR in the samples were determined by comparing the optical densities (OD) of samples to standards with known concentrations by means of

regression-correlation analysis with computerized statistical program called Microsta. Cut-off limit for the kit was 0.113 ng/ml.

Ethics Statement

All patients and healthy control volunteers confirmed written informed consent before enrolment and ethics approval for conducting this study was obtained from the Ethical Committee of Gazi University. All procedures were appropriate for the ethical criterions of the committee on human experimentation of our institution and for the Declaration of Helsinki.

Statistical Method

All statistical analysis was performed using a computer-based statistics software program (SPSS, version 11.5; Inc., Chicago, USA). Plasma uPAR levels of different disease groups and of different stages of fibrosis were compared with variance analysis. Homogeneity of variances was controlled with Shapiro-Wilk test and Levene's test. The mean uPAR levels of different groups were evaluated statistically with One-Way Anova test, and the mean uPAR levels of patient groups and control group were compared with Kruskal-Wallis test. The results were accepted as significant for P value <0.05 .

RESULTS

There were 4 groups in our study; 96 chronic hepatitis B (B Group), 22 chronic hepatitis C (C Group) and 11 NAFLD patients as well as 47 healthy controls (Control) were recruited.

The demographic features of patients and control subjects are given in [Table 1](#).

The mean ALT levels in chronic hepatitis B group and NAFLD groups were statistically higher than that of control group; besides the mean ALT level in NAFLD group was statistically higher than those of chronic hepatitis B and C groups ($P < 0.001$) ([Table 2](#)).

The mean uPAR plasma levels were found to be higher in chronic hepatitis B and C groups compared to the control group while there was no statistically significant difference between NAFLD and control groups in this respect ([Table 2](#)).

We classified the chronic hepatitis patients according to the Necro-Inflammatory Activity (NIA) based on liver biopsies as mild-moderate (NIA 0–9) and severe.^{10–18} When we compared mild-moderate, severe and control groups, the mean uPAR plasma levels in both the mild-moderate and the severe groups were found to be higher than that of the control group ($P < 0.001$). In addition, the mean uPAR plasma level in the severe group was found to be higher than that of the mild-moderate group ($P < 0.001$) ([Figure 1](#)).

Table 1 Demographic Features of Control and Patient Groups.

	Control	B Group	C Group	NAFLD	P-value
Age	46.2 ± 14.6	45.6 ± 14.2	53.8 ± 8.8	47.2 ± 11.3	0.088
Gender					0.006
Male	25 (53.2%)	67 (69.8%)	8 (36.4%)	4 (36.4%)	
Female	22 (46.8%)	29 (30.2%)	14 (63.6%)	7 (63.6%)	

Control: healthy control subjects.

B Group: chronic hepatitis B patients.

C group: chronic hepatitis C patients.

NAFLD: Non-Alcoholic Fibrotic Liver Disease Patients.

Table 2 ALT and uPAR Levels of Control and Patient Groups.

	Control	B Group	C Group	NAFLD	P-value
ALT (IU/ml)	20.0 (10.0) ^{a,b}	27.0 (20.5) ^{a,c}	23.0 (15.7) ^d	44.0 (37.0) ^{b,e,d}	<0.001
uPAR (ng/ml)	2.1 (2.0) ^{a,e}	4.6 (5.5) ^a	3.8 (8.0) ^e	2.8 (2.3)	<0.001

^aStatistically significant difference between chronic hepatitis B patient (B) group and the control group ($P < 0.001$).

^bStatistically significant difference between NAFLD group and the control group ($P < 0.001$).

^cStatistically significant difference between B group and NAFLD group ($P < 0.001$).

^dStatistically significant difference between chronic hepatitis C (C) group and NAFLD group ($P < 0.001$).

^eStatistically significant difference between C group and the control group ($P < 0.001$).

NAFLD: Non-Alcoholic Fibrotic Liver Disease patients.

ALT: Alanine Aminotransferase.

uPAR: urokinase-type Plasminogen Activator Receptor.

Figure 1 The mean uPAR levels in patient groups—classified according to NIA (mild-moderate, severe)—and in control group. *Horizontal line in each bar graphic represents the mean value (50th percentile) while the lower end of the bar represents 25th and the upper end represents the 75th percentile. The upper and lower end-points of the vertical lines represent the maximum and the minimum values respectively. uPAR: urokinase-type plasminogen activator receptor; NIA: necro-inflammatory activity.

We evaluated the association of uPAR levels with the histologic fibrosis stage in chronic hepatitis B and hepatitis C patients. We first classified the patients based on biopsy findings as mild-moderate fibrosis (F1–3) and

severe fibrosis (F4–6). We determined that uPAR plasma levels of both mild-moderate fibrosis and the severe fibrosis groups were statistically higher than those of control group ($P < 0.001$). Besides, uPAR plasma levels of severe fibrosis group were higher compared to those of mild-moderate fibrosis group ($P < 0.001$) (Figure 2).

DISCUSSION

Liver cirrhosis is a common result of the long process of chronic liver diseases. Chronic hepatitis, especially viral hepatitis and non-alcoholic steatohepatitis—related to obesity and metabolic syndrome—have a very high potential risk for liver fibrosis. They are the most important causes of end-stage liver disease due to their high prevalence in population.^{1–3} ECM construction and destruction is a dynamic process; hence, continuous inflammation and imbalance between formation and degradation of extracellular matrix are the pathophysiological basis of hepatic fibrosis.^{4–6}

This condition resembles the processes happening after a thrombotic event and after activation of coagulation cascades to cause increased fibrin deposition and inadequate fibrinolysis. Many studies on uPA and uPAR have been limited to fibrinolysis/coagulation functions, and very few have addressed their roles associated with fibrosis in chronic viral hepatitis.^{19–21}

In this study, we have revealed the correlation between uPAR plasma levels and stages of fibrosis in chronic

Figure 2 The mean uPAR levels in patient groups—according to fibrosis stage (mild-moderate, severe)—and in control group. *Horizontal line in each bar graphic represents the mean value (50th percentile) while the lower end of the bar represents 25th and the upper end represents the 75th percentile. The upper and lower end-points of the vertical lines represent the maximum and the minimum values respectively. uPAR: urokinase-type plasminogen activator receptor; FS: fibrosis stage.

hepatitis B and C patients. Our results showed that uPAR levels were significantly higher in severe fibrosis (F4-6) compared to those in mild-moderate fibrosis cases (F1-3) and healthy controls.

The activation of plasminogen by plasminogen activators (tPA and uPA) is an important extracellular step in mammalian proteolysis.⁷ The uPA, which is secreted as a proenzyme, should be activated with proteolysis after binding to uPAR on the cell surface.⁸ The uPAR distribution is quite broad, including reticuloendothelial system cells like monocytes and macrophages, connective tissue cells and cancer cells, they can all express uPAR as a glycosylphosphoinositol-anchored protein.^{9,10} uPAR also can be found as a soluble molecule (suPAR) in serum, produced by proteolytic cleavage from the plasma membrane.¹⁰

Organogenesis, tissue remodeling, wound healing, inflammation, tumor invasion and metastasis are examples of processes that plasmin involved in degradation of ECM.⁸⁻¹⁰ In matrix turnover, plasmin has the two important roles; proteolysis and activation of Matrix Metalloproteinases (MMPs).²² In addition, plasmin can activate Transforming Growth Factor-beta (TGF-beta) which is a fibrogenic cytokine that main component of liver fibrosis.²³ The plasminogen-plasmin system is under the control of specific Plasminogen Activator Inhibitors (PAIs).²⁴ Which is the main cell type in liver fibrosis called Hepatic Stellate Cells (HSCs), transforms into myofibroblast-like cell (activated stellate cell) and produces large amounts of connective tissue in response to liver damage.^{25,26}

Activated HSCs seem to produce uPA and MMPs that lead to matrix degradation. Besides, PAI-1 and tissue inhibitors of metalloproteinases (TIMPs) (which inhibit

MMP functions) secreted by HSCs cause accumulation of collagen fibrils as well.^{27,28}

However, there has been no consensus about the role of plasminogen-plasmin system and activated HSCs in the liver fibrosis yet.

uPAR facilitates connection of plasminogen on the cell membrane and increases the efficiency of proteolysis which is an important step for plasmin transformation²⁹ also, PAI-1 inhibits this step by affecting activity of uPA and tPA.¹³ It seems that amount of uPAR can affect ECM proteins degradation by plasmin very vigorously,³⁰ and the rise of uPAR may enhance degradation of extracellular proteins in the initial stages of liver fibrosis.²⁷ Activated HSCs produce excessive amounts of ECM proteins, regardless of degradation capability of uPA, and insignificant amounts of ECM can be seen in early stages of fibrosis.²⁵ As fibrosis progresses, increased expression of both PAI-1, uPA, uPAR and tPA may be associated with decreased degradation of the matrix.³¹ According to this, the findings in the advanced stage of cirrhosis, degradation system of ECM may be downregulated while activated HSCs produce greater amount of ECM leading to progressive fibrosis.

Consistent with these data, in some animal researches, it was found that plasminogen deficiency in mice could cause a redundant collection of ECM after induction of liver fibrosis with carbon tetrachloride. The enhanced fibrotic activity in these mice was due to the additional effects of decreased proteolysis and increased matrix formation.³² Another animal model study showed that systemic application of anti-uPAR monoclonal antibodies might cause hepatic fibrin accumulation in mice whose tPA insufficient.³³

For this reason, the fibrinolytic process appears to be a substantial molecular pathway in the pathophysiology of hepatic fibrosis.

Thus, suPAR level may be a valuable diagnostic marker for estimation of severity of liver fibrosis.²⁰ However, the measurement of elevated plasma uPAR levels alone as a marker of advanced hepatic fibrosis may not be reliable since elevated levels of uPAR can be seen in the many other conditions like inflammatory diseases.¹⁴

In the literature, our study is the first one to assess uPAR as a biomarker of fibrosis in NAFLD and chronic hepatitis B patients. This is also the first study that included three different chronic hepatitis patient groups in the same trial.

The most important limitation of our study is the small number of patients; especially in the NAFLD group. The mean uPAR level of the NAFLD cases was similar to the control group. The low number of cases of NAFLD may have affected the average of the uPAR in this group.

In conclusion, the mean level of uPAR in patients with chronic viral hepatitis was significantly higher than the control group, but the NAFLD cases did not reach this

result. Also plasma uPAR levels were significantly higher in the advanced fibrosis (F4–F6) than mild-moderate fibrosis (F1–F3) group.

As uPAR is related with fibrinolysis fundamentally, our study revealed the relationship of plasminogen activator system and liver fibrosis. Furthermore, our data may constitute the basis for future studies evaluating the uPAR as a novel biomarker for liver fibrosis in chronic viral hepatitis B and C. The correlation between uPAR plasma levels and stages of fibrosis should be investigated for other hepatitis causes such as alcoholic hepatitis or autoimmune hepatitis; similar in chronic hepatitis B and C patients.

CONFLICTS OF INTEREST

The authors have none to declare.

ACKNOWLEDGEMENT

This study was supported by internal medicine specialists association funds, partially.

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