Original Article

# Serum prolidase activity may be an index of liver fibrosis in chronic viral hepatitis

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**Abstract.** It has been shown that an increase in prolidase enzyme activity is correlated with increased rates of collagen turnover. In previous studies, it has been investigated that whether prolidase activity may be an index of liver fibrosis, but the results are controversial. Therefore, the aim of this study was to investigate serum prolidase activity in patients with biopsy-proven chronic viral hepatitis (CVH) and to find out whether prolidase enzyme activity is correlated with histopathological findings in those CVH patients. Fifty-four patients with CVH and 44 healthy controls were enrolled. Serum prolidase enzyme activity was measured spectrophotometrically. Serum prolidase activity was significantly higher in CVH than controls (p<0.001). A significant correlation was observed between serum prolidase activity and fibrosis score in patients with CVH (r=0.525, p<0.05). Our findings indicated that prolidase activity seems to be correlated with the level of fibrosis. Thus, serum prolidase activity may be an adjunctive tool in predicting the degree and stage of liver histopathological findings.

Key words: Chronic viral hepatitis, prolidase activity, liver biopsy, fibrosis

## 1. Introduction

Chronic liver diseases represent a major cause of morbidity and mortality worldwide. Chronic hepatitis B (CHB) and chronic hepatitis C (CHC) are the two most important causes of chronic hepatitis (CH) and hepatocellular carcinoma (HCC) (1). It has been shown that viral and host factors such as viral hepatitis persistent inflammatory reaction, alcohol or other toxic damages may be responsible for the progression of the chronic liver injury in chronic viral hepatitis (CVH). Chronic liver injury leads to an inflammatory response including the infiltration and activation of immune cells and to the proliferation and transdifferentiation activation of mesenchymal cells within the liver, especially of hepatic stellate cells. Hepatic stellate cells produce an excess of extracellular matrix (ECM) proteins that are deposited in the liver. On the other hand, hepatic fibrosis may progress to liver cirrhosis and liver failure (2-4).

Clinical investigators have been searching for noninvasive many different serum markers of fibrosis including single markers of ECM, such as hyaluronic acid, and combinations of ECM markers and markers of hepatic function (5), and these have been reviewed (6). These tests have to be reliable, accurate, reproducible, and easy to perform. In addition, these markers must reflect and they must total mass of liver collagen and ECM and be able to reflect both fibrogenesis and fibrosis regression. Further, the ideal marker test would be able to accurately stage disease and also be sensitive to changes in fibrosis induced by therapy or the natural history of disease progression.

Collagen is the best known ECM component and is responsible for the maintenance of the architecture and the integrity of all connective tissue components (7). One of the enzymes involved in collagen biosynthesis is prolidase [E.C.3.4.13.9]. Prolidase activity is easy to measure in plasma. Prolidase is a manganese-requiring homodimeric iminodipeptidase, which releases carboxy-terminal proline or hydroxyproline from oligopeptides and was first identified in 1937 (8). Prolidase activity has been documented in erythrocytes, leukocytes, plasma, dermal fibroblasts, kidney, brain, heart, thymus, and uterus (9). It has been shown that prolidase enzyme activity was correlated with collagen turnover rate (10). Also, it has been suggested

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that prolidase enzyme activity was increased in patients with nonalcoholic steatohepatitis (NASH) (11, 12). In a few experiments, serum prolidase activity has been investigated that whether prolidase enzyme activity may be an index of liver fibrosis but the results are controversial (13,14). On the other hand, this is because the knowledge related to the relationship of serum prolidase enzyme activity with the degree of liver damage in patients with CVH is limited (15). Recently, a study showed that prolidase activity increased in patients with CHC (16).

Therefore, in this study, we aimed to investigate serum prolidase enzyme activity in patients with biopsy-proven CVH and to find out whether prolidase enzyme activity is correlated with histopathological findings in those CVH patients. Further, our main clinical goal was to find out whether the measurement of serum prolidase enzyme activity would be useful as an index of liver fibrosis in CVH.

## 2. Materials and methods

### 2. 1. Subjects

A total of 54 consecutive patients with biopsy-proven CVH and 44 healthy controls were enrolled in this study. The aetiology of CVH was chronic hepatitis B (CHB) (n=29, 53.7%) and chronic hepatitis C (CHC) (n=25, 46.3%) infection. The diagnosis of CVH was made by the histopathological evaluation based on the modified Knodell score (17). The study protocol was carried out in accordance with the Helsinki Declaration as revised in 2000 and approved by the local human institutional review committee. All subjects were informed about the study and written consent was obtained.

### 2. 2. Exclusion criteria

Exclusion criteria included the use of supplemental vitamins, history of diabetes mellitus, coronary artery disease, renal disease, rheumatoid arthritis, cancer, systemic or local infection, the existence of alcohol intake, smoking habit, pregnancy, non-alcoholic steatohepatitis and cirrhosis.

### 2. 3. Blood sample collection

Blood samples were obtained following an overnight fasting state. Samples were withdrawn from an antecubital vein into blood tubes and immediately stored on ice at $4^{o}$C. The serum was then separated from the cells by centrifugation at 3000 g for 10 min and serum prolidase enzyme activity were analysed.

### 2. 4. Determination of prolidase activity

Serum was diluted 40 fold with 2.5 mmol/l Mn21, 40 mmol/l trizma HCl buffer (pH: 8.0) and preincubated at $37^{o}$C for 2 hr. The reaction mixture containing 30 mmol/l gly-pro, 40 mmol/l trizma HCl buffer (pH: 8.0), and 100 ml of preincubation serum in 1ml was incubated at $37^{o}$C for 30 min. A total of 0.5 ml of 20% trichloroacetic acid solution was then added to stop the incubation reaction. The supernatant was used for measurement of proline by the method proposed by Myara et al. (15, 18), which is a modification of Chinard's method (19). Intra-assay coefficient of variation of the assay was 3.8%.

### 2. 5. Other Parameters

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were determined via an autoanalyzer (Aerosets, Wiesbaden, Germany) using commercially available assay kits (Abbotts, Abbott® Park, IL).

### 2. 6. Statistical analysis

Data were expressed as mean±standard deviation (SD). Fibrosis scores and necroinflammatory grades of CVH were analysed using Student's t-test. Qualitative variables were assessed by $X^2$ test. Continuous variables were compared using Student t-test. Pearson correlation analysis was used to find out the correlations of prolidase enzyme activity with ALT level. Spearman correlation analysis was used to find out the correlations of prolidase enzyme activity with liver biopsy specimens' histological findings. P value of less than 0.05 was regarded as significant.

## 3. Results

The demographic and clinical data of study population are shown in Table 1. There were no statistically significant differences between two groups in regard to age, gender, and BMI (all p>0.05).

Serum prolidase enzyme activity was significantly higher in patients with CVH than controls (p<0.001). Spearman correlation analysis revealed a significant correlation between serum prolidase enzyme activity and fibrosis score in patients with CVH (r=0.525, p<0.05).

However, serum prolidase enzyme activity, fibrosis score and necroinflammatory grades were not correlated with AST, ALT levels and AST/ALT ratio in patients with CVH (all p>0.05).

Table 1. Demographic characteristics and prolidase activity of CVH patients

| Parameters | CVH  (n=54) | Controls (n=44) | p |
|---|---|---|---|
| Age (years) | 42.1±7.4 | 44.4±5.9 | ns |
| Sex (female/ male) | 26/28 | 20/24 | ns |
| Body mass index (kg/m$^2$) | 22.3±1.4 | 21.5±2.1 | ns |
| AST (U/L) | 32.1±14.2 | 20.5±4.2 | 0.001 |
| ALT (U/L) | 68.1±12.2 | 23.7±6.3 | 0.001 |
| Prolidase (U/L) | 144.8±23.5 | 113.1±11.1 | 0.001 |

Values are mean ± SD; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CVH, chronic hepatitis; SD, standard deviation; ns = non significant

When patients with CVH were divided according to aetiologic factors, no differences were observed between CHB and CHC in respect to prolidase enzyme activity, and ALT levels and fibrosis scores and necroinflammatory grades (all p > 0.05).

## 4. Discussion

Normal liver contains type I and type III collagens as well as small amounts of several basement-membrane collagens. The ratio of type I to type III has been determined by different authors in both normal and cirrhotic liver, but with marked discrepancies (20-22). The development of hepatic fibrosis in CVH is due to increased synthesis, deposition, and possibly reduced degradation of hepatic extracellular matrix components, especially collagens, such as interstitial type I and III, basement membrane type IV, microfibrillar type VI, and pericellular type V, non-collagenous proteins, such as laminin, fibronectin, undulin, etc., and various types of proteoglycans, such as hyaluronan, etc (23).

The management and prognosis of CVH greatly depend on the extent and progression of liver fibrosis. The progression is subtle. The value of laboratory tests to diagnose liver fibrosis is limited. Also, there is no ideal reference for the assessment of liver histology. On the other hand, liver biopsy is still the gold standard method in assessing the severity of liver fibrosis and cirrhosis. The liver biopsy provides a static picture of the changes that have already taken place in the liver. The common approach for diagnosing or assessing the activity of connective tissue in this organ is the histological examination of a biopsy, if one is performed by a specialist physician (24). However, liver biopsy is an invasive procedure and may cause potential complications including bleeding, pneumothorax, and perforation of colon or gallbladder. It is also prone to sampling errors (25). Thus, it is difficult to perform liver biopsy for all patients who need

to be assessed repeatedly. In addition, biopsy samples are usually too small to diagnose the disease accurately and diagnostic opinions often differ among pathologists (26). As a result of these limitations, there is a need for simple, inexpensive, and reliable noninvasive monitoring methods for evaluating the severity of hepatic fibrosis. The measurement of certain enzymes of connective tissue proteins in serum or plasma may the reflect activity of liver fibrogenesis. They offer the potential for diagnosis and therapeutic control.

Some are directly linked to the modifications in ECM turnover occurring during fibrogenesis, the so-called "direct markers", while others reflect alterations in hepatic function but do not directly reflect ECM metabolism, the so-called "indirect markers" (27,28). These markers have a pathophysiologic rationale since they may be an expression of either deposition or removal of ECM, thus giving information on its metabolism. They may potentially be used not only to stage liver fibrosis, but also to assess the speed of liver fibrogenesis with the most relevant prognostic value, and also to estimate and monitor the efficacy of and the response to antifibrotic drugs. A limitation to the clinical use of direct markers of liver fibrosis is that they are not routinely available in all hospital settings. The indirect markers of liver fibrosis are biochemical parameters that are measurable in the peripheral blood. They are an indirect expression of liver damage and have a statistical association with liver fibrosis stage. While direct markers of liver fibrosis reflect the process of fibrogenesis, indirect markers satisfy the request for a simple and easy-to-perform marker. Both direct and indirect markers for liver fibrosis may be single or a combination of parameters. In recent years, many noninvasive tests, e.g., AST/ALT ratio, AST/platelet ratio index, hyaluronic acid, YKL-40, N-terminal propeptide of type III collagen, FibroTest, SteatoTest, and NashTest (29-36), have been evaluated in clinical practice to replace

liver biopsy for the assessment of the degree of fibrosis in patients with chronic liver disease due either to chronic viral hepatitis or NASH. Besides these markers, transient elastography, 13C-caffeine breath test, and DNA sequencedbased serum protein glycomics have also been proposed for the evaluation of liver fibrosis in chronic liver diseases (37-39). However, many of these tests are not widely available, and nonspecifically elevated in the presence of various circumstances. Thus, the recent trend is to use a combination of these tests in order to improve the accuracy of fibrosis prediction (40).

Prolidase plays an important role in the recycling of proline for collagen synthesis and cell growth (10). Also, prolidase enzyme activity increases in important quantities during collagen biosynthesis (41). In addition, many workers have measured, in liver and blood samples from human and animal, prolidase enzymes activity that are involved in collagen formation (13,14,15, 42-46). In these studies, it has been investigated that whether serum prolidase activity seems to be correlated with the level of liver fibrosis during chronic liver diseases. However, there are conflicting reports concerning the association of serum prolidase activity and liver fibrosis (13-15). Myara et al. (15) investigated serum prolidase enzyme activity in patients with chronic hepatitis and cirrhosis. All the patients with high prolidase activity had chronic liver diseases in the study of Myara et al. (15). However, of 27 patients with biopsy-proven cirrhosis, they found increased prolidase activity only in 5 patients. Thus, they speculated that plasma prolidase activity might be high in the early stage of fibrosis and might subsequently drop in advanced fibrosis. Also, Myara et al. (15) reported that plasma prolidase activity might be useful in evaluating fibrotic processes in chronic liver disease in the human. Recently, Duygu et al. (16) investigated serum prolidase activity in in patients with CHC, and they demonstrated significantly increased serum prolidase activity in patients with CHC. Furthermore, Brosset et al. (47) investigated prolidase enzyme activity in patients with alcoholic liver disease and they observed that cirrhotic patients with alcoholic hepatitis had significantly higher enzyme activity in comparison with those without alcoholic hepatitis. On the other hand, Zuyderhoudt et al. (13) investigated whether plasma prolidase activity showed a correlation with the extent of liver fibrosis in the rat. They have conclude that plasma prolidase activity, at least in the rat, is very much influenced by liver cell leakage and does not seem to reflect active stages of liver

fibrosis (13). In contrast, Abraham et al. (14) showed a correlation with the extent of liver fibrosis in the rat. After Cd4 administration to rats, liver collagenolytic activity rose in early stages of fibrosis and declined in advanced fibrosis (47). Although collagen synthesis has been widely explored in relation to the pathogenesis of hepatic fibrosis, only a few experiments have been devoted to collagen catabolism. In human liver, collagenase activity was shown to be high in cirrhosis, but low in alcoholic hepatitis (48).

Besides chronic liver diseases, previous clinical studies have shown increased serum prolidase activity in Helicobacter pylori gastritis (49), breast cancer (50), coronary artery disease (51) and hypertension (52). Conversely deficient/reduced serum prolidase activity was reported to be associated with lupus-like syndrome characterized by non-healing skin ulcers owing to defective collagen turnover (53). Reduced serum prolidase activity was also reported in renal insufficiency (54) and uremia (55).

Chronic liver diseases are slow, progressive diseases characterised by advancing hepatocellular necrosis, inflammation and fibrosis. An increase in the amount of collagen in the liver is a bad sign indicator for chronic liver diseases. It is very important to note that circulating biochemical markers of fibrogenesis, fibrolysis or both may not reflect hepatic fibrosis or cirrhosis, since they are not liver-specific. Thus, the best diagnostic approach would be the identification and measurement in serum of the driving force of fibrogenic process. Also, some more efficient laboratory tests are required to evaluate liver hepatocellular necrosis, inflammation and fibrosis. In this study, in an attempt to search for a suitable marker for the prediction of liver fibrosis severity in patients with CVH, we measured the serum prolidase activity in these patients. In accordance with the previous studies, especially study of Myara et al. (15), in the present study, we observed that serum prolidase enzyme activity is significantly higher in patients with CVH than healthy controls, and significantly associated with liver biopsy specimens' histopathological findings in those patients with CVH. However, when patients with CVH were divided according to aetiologic factors, no differences were observed between CHB and CHC in respect to prolidase enzyme activity, and ALT levels and fibrosis scores and necroinflammatory grades. Since serum prolidase enzyme activity is a noninvasive marker that significantly correlates with the liver collagen

content in hepatic fibrosis (14, 56, 57), it would be helpful to assess the fibrosis process in CVH. The measurement of serum prolidase enzyme activity is a simple, reliable, fast, inexpensive and readily automated method that is compatible with random access analysis in barcoded primary tubes and can be performed in most automated analysers used for standard biochemical liver fibrosis. Therefore, biochemical parameters, particularly serum prolidase anzyme activity, may be considered to be more accurate than liver biopsy in reflecting the fibrogenesis in hepatocytes. Thus, it can be used as a noninvasive marker in the follow up of liver fibrosis severity in patients with CVH. Thus, we considered that serum prolidase anzyme activity could be a useful noninvasive diagnostic marker in routine clinical practice for determining the patients with CVH.

The findings of the present study confirm that serum prolidase activity seems to be correlated with the level of fibrosis. Also, this study demonstrated that monitoring serum prolidase activity may be a useful adjunctive tool in predicting the degree and stage of liver histopathological findings, especially in the absence of advanced fibrosis and other conditions, which may affect the interpretation of prolidase activity in CH.

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