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CASE STUDY

A RETROSPECTIVE INVESTIGATION OF BLOOD COAGULATIVE PARAMETERS PT, KPTT, PROTEIN C, AND ANTITHROMBIN III IN 95 PATIENTS WITH LIVER DISEASE

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Abstract

Background: The liver is a crucial synthesis of blood coagulation factors and anticoagulative serine proteases such as protein C(PC) and antithrombin III (ATIII), which exerts a key role in the regulation of hemostatic balance. Activated protein C (APC) and protein S complex inactivate the activated factor Va and VIIIa, thus limiting Xa and thrombin formation. The excess protein S can drive cancer cellular proliferation and cell survival through oncogenic receptor Axl. In presence of heparin binding, antithrombin III (ATIII) and thrombin form an inactive complex in a 1:1 molar ratio. ATIII also inactivate factor IXa, Xa, XIa and XIIa at slow rate. In the setting of liver diseases, this reduced dysregulation can be attributed to decreased synthesis by the liver and increased consumption of coagulative factors and protein C and ATIII.
Methods: In current study, using routine detection for the blood coagulative parameters in 75 patients with liver diseases. Protein C antigen was determined by Laurell rocket electroimmunoassay. The clotting assay was used for detecting ATIII activity (ATIII:C), ATIII antigen (ATIII:Ag) was measured using immunoassay (EIA).
Results: The results showed that there exist one or three coagulative parameters PT, KPTT, and TT abnormal longer. Moreover, the abnormal intensity of coagulative parameters was associated to the severity of liver diseases. In our detection of 20 liver cirrhosis, the results showed significantly decreased plasma protein C antigen (PC:Ag 0.5501 vs 1.0578 μ/ml) and antithrombin III level (ATIII: Ag 21.8 vs 39.8 mg/dl, ATIII:C 40.25 vs 105.04%), respectively.
Conclusions: The measurement of multidispillary analyses of coagulative and anticoagulative system protein C and ATIII level are helpful to monitoring the liver diseases and might play a predictable marker.
Keyword: Antithrombin III, blood coagulative function, liver disease, Protein C.

INTRODUCTION

Liver disease is a significant threat to human health. The liver is closely related to blood coagulation and bleeding. Most coagulation factors are synthesized in the liver. The main reasons for coagulation disorders in liver diseases include reduced synthesis, increased consumption and increased anticoagulant substances. Thus, patients with serious liver diseases can cause bleeding diathesis, which would monitored for the assessment of those coagulative factors deficiency, and natural anticoagulants PC and ATIII decrease. Anticoagulative protein S, protein C system[1], two vitamin K-dependent coagulation factors, are involved in the regulation of hemostatic balance. Activated protein C inactivate Va and VIIIa and stimulates fibrinolysis[1-3]. In this process, protein S serve as cofactor of activated protein C. Moreover, excess protein S can drive cancer cellular proliferation and cell survival via high activation of oncogenic receptor Axl[4-9].

In addition to Protein S, regarding anticoagulative system and fibrinolysis, many topics focused on the urokinase-type plasminogen activator uPA-uPAR (plasminogen activator receptor) in its oncogenic transformation in tumor cells[10-12]. In an orthotopic lung cancer model[13], uPAR (H249A -D262A) double mutant HD cells that eliminate β1 integrin interaction but maintaining uPA binding showed reduced tumor size in comparison of cells expressing wild-type uPAR. In addition, tumor incidence was also reduced in HD-injected mice, which indicate that expression of uPAR

had its survival advantage to tumor cells *in vivo*. It has been demonstrated that uPAR directly associated with integrin α3β1 and integrin α5β1, initiating epithelial to mesenchymal transition (EMT). Association of uPA-uPAR and β1-integrin is crucial to activate multiple signaling pathways including ERK and Jak-Stat1 or PI3K-Akt-GSK-3β signaling[11-13], and/or WNT/-catenin signaling[14], while uPA-mediated activation of Akt, GSK-3β and Stat1 were all impaired in HD cells[13]. Therefore, uPA-uPAR-β1 integrin signaling can modulate lung tumor progression *in vivo*. High levels of uPAR are expressed at the invasive area of tumors, which are linked to the risk of relapse and metastasis[11]. Additional data has been shown that oncogenic androgen receptor is confirmed to be a risk factor in the role of benign prostatic hyperplasia (BPH), prostatic cancer (Pca) and in recent male hepatocellular carcinoma (male HCC)[4-6,15-26]. AR overexpression confers a highly tumorigenic phenotype[15,16,19-21,23]. The existence of AR splice variants (AR-SVs, AR-V7) through its constitutive transcriptional activity leading to increased AR oncogenic signaling can promote cellular proliferation, cell migration and invasion via EMT, which are responsible for the regulation of some oncogenic processes within PCa[6,23]. This is a novel area and its etiology of hormonally driven cancers even growth factors involved in this process.

In clinics, decreased plasma PC and ATIII levels can reflect a decreased synthesis and/or consumption coagulopathy (heparin-like substances), which often occur the dysregulation of hepatocytes in liver diseases. In current study, we carried out the measurement of multidisciplinary analysis of coagulative system protein C and ATIII detection in several of 95 patients with liver disease.

MATERIALS AND METHODS

Total 95 patients with liver diseases were contained two groups. One group of 74 patients were entered in the measurement of coagulative parameters during early March 1985 to August 1985. The sex ratio of male and female was 65:10 respectively. The criteria of diagnosis of liver diseases are according to the rules where physicians have in common used in clinics. The liver diseases included acute viral hepatitis 9 cases, chronic persistent hepatitis 10, chronic active hepatitis 19, hepatitis gravis 8, hepatitis cirrhosis 5, primary hepatocellular carcinoma 2, cholestatic hepatitis 1, HBV carrier 21 cases. Another group of 20 patients with liver cirrhosis were included during early March 1988 to December 1988. The sex ratio of male: female was 13:7 respectively. The clinical diagnosis of liver cirrhosis were hepatitis B liver cirrhosis 15 cases (including 13 decompensated hepatic cirrhosis), cholestatic cirrhosis 1, alcoholic hepatocirrhosis 1, schistosomiasic hepatocirrhosis 1, hepatic cirrhosis complicated with hepatocellular carcinoma(HCC) 1, hepatitis cirrhosis complicated with diabetes 1 case, respectively. Normal control group included 50 health individuals.

Fibrinogen concentration was measured by Sodium sulfite-dibiuret method; Prothrombin time (PT) was determined with Quick one-stage (Quick AJ, 1971; Mial JB and Lafond DJ, 1969a)[27-29]; Kaolin partial thromboplastin time (KPTT) and thrombin time (TT) were used routinely. VIIIR:Ag: Factor VIII-related antigen (VIII R:Ag) was measured by Laurell rocket electroimmunoassay. Also, factor VIII antigen in plasma is electrophoresed through agarose gel containing factor VIII antiserum. The antigen develops a rocket-like immunoprecipitate, the length of rocket is directly proportional to the concentration of VIII R:Ag. The detail method was described in a previous study[28]. In order to the statistical analyses, the abnormal assays were designed as PT>16s, KPTT >50s, and TT >23s. Correction of prolonged TT time to normal or to over 5s was evaluated as corrected TT time.

Protein C antigen (PC:Ag) assay: Add 60 µl amount of rabbit protein C antiserum into indubiose A37, swirl to mix, then prepared a 100x60x2 mm agarose slide. Just before using, punch holes using template pattern, 3 mm in diameter, approximately 3 mm apart and 2cm from the edge of the plate. Prepare normal plasma samples the following dilutions with Tris barbital buffer: undiluted, 1:2 dilution, 1:4 dilution, and 1:8 dilution. Patient samples were quick-thawed or fresh, tested undiluted and 1:2 in buffer. Add 13 µl of plasma to each well. Run the electrophoresis at 120v, 4-6v, 0.7 mA/plate for 18-20 hours overnight. When electr-ophoresis is finished, put slide in Coomassie Brilliant Blue stain for 10 minutes. Finally, measure the length of rockets from the top of the well to the apex. Construct a reference curve and the linear regression equation using the four normal plasma dilutions. The height of each dilution peak is plotted on log-log paper against the percent of normal. Determination the percent of PC:Ag in each patient sample from the curve. The details has been in previous study[1,2,7].

ATIII: Ag assays: ATIII kits were kindly provided by Shanghai Institute of Biological Products. Antithrombin III antigen (ATIII: Ag) in serum and plasma was electrophoresed through agarose gel containing antithrombin III antiserum. The antigen develops a rocket-like immunoprecipitate, which is directly proportional to the concentration of ATIII antigen. Antithrombin III activity (ATIII:C) was used with clotting assay. All results were statistically analyzed with "t" test.

RESULTS

The coagulative parameters in liver diseases

Among all types of hepatitis, except for HBV carriers, there exist one or three mean values of coagulative parameters PT, KPTT, and TT which were abnormal longer than that of normal control group. The abnormal intensity of prolonged prothrombin time (PT) was in turn hepatitis gravis (HG), hepatitis cirrhosis (HC), chronic active hepatitis (CAH), acute viral hepatitis (AVH), and chronic persistent hepatitis (CPH). There was significantly difference in comparison of normal control group (p<0.001). The intensity of prolonged

KPTT and TT were in turn hepatitis gravis, hepatitis cirrhosis, chronic active hepatitis, acute viral hepatitis, and chronic persistent hepatitis. There was a significant statistical difference compared with normal control group (p<0.001). Moreover, prolonged KPTT was negatively correlated with serum albumin level (r=0.5274, p<0.05), which suggest that decreased serum albumin in patients with chronic active hepatitis was associated with prolonged KPTT value. The coagulative parameters of HBV carriers was in the normal range.

Using methylaniline blue correction test in 14 patients with prolonged TT, the prolonged TT in 11 patients (78.6%) was corrected to normal or shortened by over 5 seconds, suggesting an increased heparin-like substance. Notably, 4 patients died of hepatitis gravis or hepatitis cirrhosis. It was found that the mean values of PT, KPTT and TT were significantly prolonger than that of those survival patients. The results were seemed to be drawn that the abnormal intensity of coagulative parameters were associated to the severity of liver diseases. In various of liver diseases, factor VIII-related antigen (VIII R:Ag) was predispose to be an increased tendency. Moreover, increased VIII R:Ag was positively with prolonged KPTT and TT value. The increased VIII R:Ag was in turn hepatitis gravis, primary hepatocellular carcinoma, hepatitis cirrhosis, chronic active hepatitis, chronic persistent hepatitis, acute viral hepatitis, and HBV carriers.

Table 1: Blood coagulative parameters in 75 liver diseases.

Diagnosis	No	Fibrinogen (g%)	PT(s)	KPTT(s)	TT(s)	VIII R: Ag(%)
AVH	9	0.32 ±0.08	13.30±2.22	51.3±8.84	19.1±5.93	205±73.45
CPH	10	0.29±0.09	12.5±2.05	42.3±9.34	18.4±3.58	238.5±81.86
CAH	19	0.42±0.20	15.1±2.72	55.1±19.0	23.4±7.34	280.08±99.09
HG	8	0.36±0.13	16.9±3.98	63.5±16.8	28.7±9.42	476.23±119.78
HC	5	0.40±0.12	15.2±1.71	64.0±25.8	28.7±2.14	346.14±52.04
PHC	2	0.30	12.4	43.70	25.70	423.86±12.64
CH	1		20.8	61.0	21.0	
HBVc	21	0.31±0.07	12.6±0.97	40.3±6.79	17.3±3.42	194.63±80.09
NCG	40	0.20~0.40	12.4±1.16	37.5±4.6	15.8±2.12	113.36±39.38

AVH: acute viral hepatitis; CPH: chronic persistent hepatitis; CAH: chronic active hepatitis; HG: hepatitis gravis; HC: hepatitis cirrhosis; PHC: primary hepatocellular carcinoma; CH: cholestatic hepatitis; HBVc: HBV carrier; NCG: normal control group.

Table 2: Plasma protein C and antithrombin III levels in 20 hepatic cirrhosis.

Liver diseases	No	PC:Ag(μ/ml)	ATIII:Ag(mg/dl)	ATIII:C(%)	ATIII:C/ATIII:Ag
Liver cirrhosis (LC)	20	0.5501±0.2536*	21.8±16.99*	40.25±30.89*	1.85
Hepatitis B LC	14	0.5066±0.2514*	20.84±14.93*	36.04±25.87*	1.73
HBLC with hepato-renal syndrome	2	0.3139, 0.2876	33.19, 1.85	35.83, 10	
HBLC with hepato-encephalopathy	2	0.3139, 0.9291	33.19, 13.5	35.83, 29.06	
NCG	50	1.0578±0.1886	39.8±6.54	105.04±15.81	2.64

Note: * < 0.001 LC:liver cirrhosis HBLC:hepatitis B liver cirrhosis

Protein C and Antithrombin III levels in liver diseases

The mean value of plasma protein C antigen (PC:Ag) in 19 liver cirrhosis was 0.5501 μ/ml (range 0.1436~0.9291 μ/ml), which was significantly lower than that of normal control group(1.0578 μ/ml). Moreover,11 (73.7%) of 15 decompensated liver cirrhosis the PC:Ag was below the lower limit of normal control (range: 0.1436- 0.6264 μ/ml). 6 patients with severe liver cirrhosis had their lowest level of plasma protein C antigen 0.1436, 0.1846, 0.1919, 0.2876, 0.5147 and 0.5438 μ/ml, respectively. The total serum protein was 3.6, 4.15, 4.6, 5.2, 5.1, and 5.8 g/dl, respectively. Plasma PC:Ag level was positively correlated with serum albumin and total protein synthesis (r=0.9680, p<0.05; r=0.8211, p< 0.05). One patient with compensated cirrhosis had a normal range of PC:Ag level (0.7785 μ/ml). Intriguing, plasma PC:Ag level was 1.0883 μ/ml in 1 patient complicated with diabetes mellitus.

Determination of antithrombin III was carried out in 20 patients with liver cirrhosis. The results found an obviously decreased antithrombin III antigen (ATIII: Ag, 21.8 vs 39.8 mg/dl) and antithrombin III activity (ATIII:C, 41.63 vs 105.04%). Both ATIII:Ag and ATIII:C level showed a parallelism decrease (r=0.7959, p<0.001), and the ratio of ATIII:C/ATIII: Ag was normal. There was no statistical correlation between plasma ATIII: Ag and ATIII:C levels and content of serum protein (r=-0.0534, -0.0122, respectively, p> 0.5)

DISCUSSION

The liver is a crucial synthesis of blood coagulation factors I, II, V, VII, VIII, IX, X, XI, and XII. When liver cells are severely damaged such as cirrhosis and hepatitis, there will be a defect in the synthesis of clotting factors. This can give rise to blood clotting disorders, manifested as an increased tendency to bleed. Thus, detecting blood clotting function in cases of liver diseases, including determining the levels of blood coagulation factors. Deutsch[30] found that at least one coagulation test was abnormal in 85% of patients with liver diseases. Among 62 cases of acute and chronic hepatitis reported by Tongji Hospital in Wuhan[31], China, the coagulation changes in severe hepatitis were the most significant. Those with prolonged PT accounted for 64.3%, and those with prolonged KPTT accounted for 71.4%. Similar to the results of this group, those with prolonged KPTT accounted for 66.7%.

Table 3: The detail evaluation of PC:Ag, ATIII:Ag, and ATIII:C levels in 20 liver cirrhosis.

No	Diagnosis	Sex	Ages	SP (g/dl)	Alb (g/dl)	PC:Ag (μ/ml)	ATIII:Ag (mg/dl)	ATIII: C (%)
1	CLC	F	51	7.0	3.2	0.7785	35.65	85.5
2	DLC	M	43	3.6	2.6	0.1436	13.7	36.03
3	DLC	M	46	7.0	4.3	0.6264	29.34	42.6
4	DLC	F	48	6.0	3.6	0.9291	13.5	29.06
5	DLC	M	39	6.67	3.15	0.7442	8.88	14
6	DLC	M	76	4.6	2.1	0.1919	5.81	16
7	DLC	F	30	5.2	2.64	0.2876	1.85	10
8	DLC	F	58	4.15	2.6	0.1846	28.88	19.6
9	DLC	M	72	6.98	2.92	0.4609	22.79	2.74
10	DLC	M	52	6.0	4.6	0.6188	6.51	39.22
11	DLC	F	50	5.1	2.5	0.5438	18.21	21.4
12	DLC	M	38	5.8	3.2	0.5147	59.65	87.76
13	DLC	M	38				21.19	33.57
14	DLC	M	24			0.3139	33.19	35.83
15	DLC	F	49			0.7575	13.52	67.32
16	DLC, DM	M	59	6.6	3.1	1.0883	8.88	33.57
17	DLC, PHC	M	72	7.9	3.4	0.5036	2.75	20.2
18	SHC	M	44			0.5857	19.8	20.03
19	AHC	M	48	6.86	2.47	0.5086	27.98	74.09
20	BHC	F	45			0.6691	63.95	119.37

CLC: compensated liver cirrhosis; DLC: decompensated liver cirrhosis; M: male; F: female; PHC: primary hepatocellular carcinoma; DM: diabetes mellulitus; SHC: schistosomic hepatocirrhosis; AHC: alcoholic hepatocirrhosis; BHC: billiary liver cirrhosis

When their existence severe lesions in liver cells, the synthesis of antiplasmin is reduced and the production of heparinase is insufficient, leading to an increase in heparin-like substances in plasma. Wang *et al.*[32], examined 51 patients with liver diseases, and 26 cases (50.9%) had increased heparin-like substances. Among the 14 cases with prolonged TT in this group, 11 cases (78.6%) were confirmed to have an increase in heparin-like substances. In two patients, the prolonged TT could not be corrected by toluidine blue, and the fibrinogen content was normal. It is speculated that there may be abnormalities in fibrinogen quality. In this group, four patients died. The coagulation indexes PT, KPTT, and TT were significantly prolonged compared with the survival group. To a certain extent, it suggests that the severity of liver disease is related to the severity of coagulation factor defects.

Some authors have observed the PC level in liver diseases. Griffin *et al.*[33], detected the PC:Ag level in 15 patients with liver diseases, and the level decreased in 11 cases. Among 11 cases of malignant diseases without liver diseases, only one case showed a decrease. Mannucci *et al.*[34], detected the amount of PC in 58 cases of liver diseases. The results showed that the PC content in all patients was lower than normal, and the degree of decrease was generally proportional to the severity of the disease and was related to protein synthesis disorders. Boyer *et al.*[35], simultaneously determined the plasma PC level in 15 patients with cirrhosis by EIA and ELISA methods, and the results were 18.4% and 19.0%, respectively. While Mannucci *et al.*[36], measured 19 cases by the same method and the results were 32% and 30%, respectively. In this group, 14 cases of hepatitis cirrhosis were detected, and the mean value of PC:Ag was 0.5066 μ/ml. In 6 cases with significantly lower total serum protein than normal, the amount of PC decreased more significantly (mean value 0.3110 μ/ml), indicating that the plasma PC concentration is related to the amount of liver synthesized protein. This reduction can be attributed to decreased synthesis by the liver and increased consumption of protein C in the setting of liver cirrhosis. In one case of biliary cirrhosis, the PC:Ag was low, but the ATIII level was normal. This is due to secondary vitamin K deficiency caused by bile excretion disorders, and insufficient synthesis of PC leads to a decrease in content. Therefore, like many vitamin K-dependent proteins, PC is produced in the liver, so its level decreases in liver diseases. These findings showed that protein C deficiencies may be demonstrated in the dysregulation of hepatic PC synthesis, and might act an important role as a predictor index in liver diseases.

Antithrombin III is another important anticoagulant protein. Similar to protein C, antithrombin III levels are also affected in liver diseases. In patients with liver cirrhosis, antithrombin III levels are often significantly reduced. Chan[37] used RIA to detect the ATIII levels of 36 healthy men and 21 healthy women. The results were 19.9 mg/dl and 19.1 mg/dl respectively. The ATIII levels of 17 patients with cirrhosis were significantly reduced (9.6±2.4 mg/dl). Yamagachi *et al.*[38], reported that 60% of patients with acute hepatitis, 95% of 83 patients with cirrhosis, 75% of 15 chronic hepatitis, and 100% of 6 primary liver cancer had low plasma ATIII levels. Yashimura and Satake[39] reported 142 cases of various liver and gallbladder diseases. Except for drug-induced intrahepatic cholestasis and primary biliary cirrhosis, the ATIII levels of other liver diseases were all reduced. Therefore, the determination of ATIII is helpful for the differentiation of primary biliary cirrhosis from cirrhosis and chronic active hepatitis, and can be used as one of the indicators for observing and judging the degree and prognosis of chronic liver damage such as chronic hepatitis and cirrhosis. In this group, 20 cases of various types of cirrhosis were detected. The mean values of plasma ATIII:Ag and ATIII:C were obviously reduced, especially in hepatitis cirrhosis. In one case of biliary cirrhosis, PC:Ag was low, but the ATIII level was

within the normal range. This suggests that the simultaneous detection of PC and ATIII can be used as one of the indicators for differentiating biliary cirrhosis and hepatitis cirrhosis.

It is recently noted that the changes in plasma protein C and antithrombin III levels in liver diseases have important clinical implications. Decreased levels of two natural anticoagulant proteins can lead to a hypercoagulative state, increasing the risk of thrombosis. This is particularly relevant in those cases with liver cirrhosis and even hepatocellular carcinoma (HCC), which are already at an increased risk of developing portal vein thrombosis and other thrombotic complications. Monitoring protein C and antithrombin III levels can help clinicians to assess the coagulation status of patients and guide treatment designs. In some cases, supplementation of these proteins may be possibly considered to prevent or treat thrombotic events.

Factor VIII-related antigen (VIIIR:Ag) shows an increase in numerous groups of liver diseases, and the increased degree is positively correlated with the prolongation of KPTT and TT. Possible mechanisms for the increase of VIIIR:Ag: (1). Hepatitis virus or antigen-antibody complexes damage liver vascular endothelial cells, promoting an increase in the release of VIIIR:Ag. (2). In liver diseases, the clearance ability of VIIIR:Ag is reduced. Maisonneuve *et al.*[40], examined 11 cases of acute viral hepatitis and found that VIIIR:Ag was increased in all cases. Our results are consistent with theirs, and it is suggested that the degree of increase in VIIIR:Ag has a certain relationship with the severity of liver diseases and can be used as another monitoring index reflecting the coagulation defects in liver diseases.

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AUTHOR'S CONTRIBUTION

Zhu G: Writing original draft, methodology. **Broekmans AW:** data curation, literature survey. **Bertina RM:** review and editing. All authors reviewed and approved the final version of the article.

DATA AVAILABILITY

The data will be available to anyone upon request from the corresponding author.

CONFLICT OF INTEREST

None to declare.

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