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Immunohistochemical Study of TNFα, CD41 and PDGFR-β in Chronic Hepatitis C Patients Demonstrating the Relationship of Intrahepatic Platelet Accumulation to Thrombocytopenia and Hepatic Fibrosis

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ABSTRACT

Background: Chronic liver diseases (CLDs) are characterized by persistent liver injury and fibrosis, which are basic features that lead to liver cirrhosis and liver cell failure. Thrombocytopenia is a frequent complication of CLDs. The mechanisms of thrombocytopenia in CLD and cirrhosis are still unclear. **Objective:** The aim of the present work was to study intrahepatic expression of tumor necrosis factor alpha (TNFα), cluster of differentiation antigen 41 (CD41) and platelet-derived growth factor receptor beta (PDGFR-β) in patients with chronic hepatitis C (CHC) and their relation to the grade and stage of CHC. Also, we want to investigate the relationship between intrahepatic platelets (PLTs) and thrombocytopenia in CHC. **Patients and Methods:** Analytical cross-sectional study was conducted on fifty patients with proven CHC who were admitted to Tropical Medicine and Gastroenterology Department, Sohag University Hospital. All patients were clinically examined and fully investigated. Liver biopsy was obtained for histopathological examination and immunohistochemical study using monoclonal antibodies against TNFα, CD41 and PDGFR-β. **Results:** Fifty patients were included, their age ranged from 20-59 years with a mean of 44.42±11.17 years. Forty patients (80%) were males and 10 (20%) were females. The mean average weighted scores (AWS) of imunohistochemical expression of TNFα, CD41 and PDGFR-β were significantly higher in grades 3&4 compared to lower grades (P<0.0001; 0.009; 0.05 respectively), in patients with fibrosis stage 3 versus earlier stages (P<0.0001; 0.001; <0.04 respectively), and in thrombocytopenic group compared to non-thrombocytopenic group (P<0.001 for each). There was a significant negative correlation between peripheral PLT count and the AWS of TNFα, CD41, and PDGFR-β expression in CHC patients (r= -0.37; P= 0.008 & r= -0.41; P= 0.007 & r= -0.34, P= 0.045 respectively). **Conclusion:** Expression of TNFα, CD41 and PDGFR-β markers was associated with progression of disease activity and fibrosis. Intrahepatic PLT accumulation in CHC may be involved in thrombocytopenia and liver fibrosis.

Key words: Chronic hepatitis C, TNFα, CD41, PDGFR-β, histopathological grading and staging, and thrombocytopenia.

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INTRODUCTION

Viral hepatitis is a worldwide health problem affecting hundred millions of people and is the main cause of liver cirrhosis and hepatocellular carcinoma (HCC)[1]. Egypt hasthe highest hepatitis C virus (HCV) prevalencein the world[2].

Liver fibrosis results from progressive damage of liver parenchyma along with accumulation of extracellular matrix (ECM) proteins[3]. Liver fibrosis was previouslythought to be a passive and irreversible process however; it is now considered a form of wound-healing response to chronic liver injury[4].

Liver sinusoidal cells and cells of inflammatory infiltrates are the most common sources of the cytokines. In special circumstances, endothelial cells, hepatic stellate cells (HSCs), bile duct epithelium, and hepatocytes can synthesize TNF and interleukin 1 (IL-1)[5]. In CHC, reports on cytokine expression in different inflammatory grades and fibrosis stages are divergent[6].

TNFα is a pleiotropic cytokine that can be produced by many immune cells including macrophages/monocytes[7]. TNFα is a well-known mediator of hepatocyte death[8]. On the other hand, TNFα is needed for normal

hepatocyte proliferation and it has anti-apoptotic activity[9].

In addition to the haemostatic properties of PLTs, it has features of inflammatory cells when activated where it releases many active compounds as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and transforming growth factor-beta (TGF-β)[10]. The multipotential properties of PLTs, such as angiogenesis[11], wound healing[12], and metastasis in cancer[13]were previously described. After viral infection, PLTs accumulate in the liver and increase the immunopathological processes in liver[14].

PDGF plays an important role in hepatic fibrosis[15]. PDGFRs have important role in activating HSCs and enhancing liver fibrosis and cirrhosis. Whether PDGFR-α and PDGFR-β have different roles in fibrogenesis is not known[16].

Thrombocytopenia is a frequent complication of CLD and cirrhosis, it occurs in about 60-70% of patients with cirrhosis and⁄or fibrosis[17]. Thrombocytopenia has a bad effect on the health of patients especially in advanced stages when the PLT count falls below $50x10^3/\mu L$[18]. Thrombocytopenia frequently prevents patients from receiving crucial interventions such as invasive diagnostic or therapeutic procedures[19].

The mechanisms of thrombocytopenia in patients with HCV-related CLD are not clearly defined. Many pathogenetic mechanisms are involved such as direct bone marrow suppression, hypersplenism, and autoimmune destruction of PLTs[20]. The overexpression of PDGF and PDGFR in livers of an experimental injury model was previously reported[21]. PDGF is a potent mitogen that stimulates proliferation and migration of mesenchymal cells[15].

The aim of the present work was tostudy intrahepatic expression of TNFα, CD41, and PDGFR- β in patients with CHC and their relation to the grade and stage ofCHC. Also, we want to investigate the relationship between intrahepatic PLTs and thrombocytopenia in CHC.

PATIENTS AND METHODS

The study protocol was approved by the Ethical Committee of Sohag Faculty of Medicine. Written informed consents were obtained from all participants before inclusion in the study. An analytical cross-sectional study was conducted on fifty patients with proven CHC infection admitted to Tropical Medicine and Gastroenterology Department, Sohag University Hospital.

The inclusion criteria were provenCHC patients by positive hepatitis C virus antibodies (HCV Abs) and hepatitis C virus ribonucleic acid (HCV RNA) by polymerase chain reaction (PCR) for more than 6 months, and compensated liver disease. The exclusion criteria were patients who received or are receiving any anti-HCV treatment, patients with liver cirrhosis or coagulopathy (patients whose PLT count was less than $100x10^3$ /μL and/or prothrombin time was more than three seconds over the control), co-infection with hepatitis B virus (HBV), autoimmune liver disease, alcoholic liver disease, drug induced liver disease, Wilson's disease, haemochromatosis, HCC, and patients with chronic medical problems as chronic renal failure.

All patients were subjected to full history taking, complete clinical examination, complete blood count, and liver function tests (alanine transaminase, aspartate transaminase, serum albumin, bilirubin, and prothrombin time and concentration). Abdominal ultrasound examination was done for evaluation of the liver, portal vein and spleen. Liver biopsy was obtained from all patients forhistopathological and immunohistochemicalstudy. All specimens were stained with hematoxylin and eosin and examined under a light microscope for grading and staging of CHC. Immunohistochemical study using monoclonal antibodies against TNFα, CD41 (as a marker for PLTs) and PDGFR-β (as a marker for fibrosis) was done using staining kit (Catalogue # AEX080-IFU, ScyTek Laboratories). The intensity of expression of

each marker was presented by calculation of the mean AWS on a 0-12 scale.

Liver damage was evaluated for inflammation and fibrosis. The inflammatory activity (grade of disease activity) was classified according to Scheuer and coworkers[22] into:- Grade 0: no inflammation, Grade 1: minimal inflammation, Grade 2: mild inflammation, Grade 3: moderate inflammation and Grade 4: marked inflammation. The severity of fibrosis was classified into:- Stage 0: no fibrosis, Stage 1: mild portal fibrosis, Stage 2: moderate periportal fibrosis, Stage 3: marked fibrosis (bridging fibrosis with lobular distortion), and Stage 4: cirrhosis. Patients were categorized according to the grade of inflammatory activity into patients with minimal and mild inflammation (Grade 1+Grade 2= 32 patients) and those with moderate and marked inflammation (Grade 3+Grade 4= 18 patient). Also, they were categorized according to the stage of fibrosis into patients with no, mild, and moderate fibrosis (stages 0, 1 and 2 = 40 patients) and those with marked fibrosis (stage 3 = 10 patients). In addition, the patients were recategorized into thrombocytopenic group: 10 patients with PLT count less than $150x10^3/\mu L$ and non-thrombocytopenic group: 40 patients with normal PLT count ($150x10^3$-$450x10^3/\mu L$).

Statistical analysis:

Data were analyzed using Statistical Package for Social Sciences (IBM SPSS) software package version 20. Data were expressed as mean ± standard deviation (SD) for continuous variables. Comparison of mean in 2 groupswas carried out by Student's t- test. Categorical variables were expressed as frequency and percentage and the comparison between these variables was carried out using Chi-square (x^2) test. Pearson's correlation was used to study the correlation. $P<0.05$ was considered statistically significant.

RESULTS

Fifty patients were included, their age ranged from 20-59 years with a mean of 44.42±11.17 years. Forty patients (80%) were males and 10 (20%) were females. Most patients (60%) had mild grade of inflammation while 48% had moderate fibrosis (Table 1).

The mean AWS of immunohistochemical expression of TNFα, CD41 and PDGFR-β were significantly higher in grades 3 & 4 compared to lower grades, in patients with fibrosis stage 3 versus earlier stages (Table 2), and in thrombocytopenic group compared to non-thrombocytopenic group (Table 3). The mean peripheral PLT count was significantly lower in patients with stage 3 fibrosis than those with earlier stages (Table 2).

In our study, the thrombocytopenic group included 10 patients; their mean PLT countwas 126±15.8. The non-thrombocytopenic group included 40 patients; their mean PLTcount was 308±88.64. There was statistically insignificant difference between thrombo- cytopenic and non-thrombocytopenic groups regarding age, gender, residence and prothrombin time. The mean alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, AWS of TNFα, CD41 and PDGFR-β were significantly higher in thrombocytopenic group compared to non-thrombocytopenic group. On the other hand, mean serum albumin was significantly lower in thrombocytopenic group compared to non-thrombocytopenic group (Table 3 & Figures 1-3).

Table (1): Grading and staging of CHC patients according to Scheuer and coworkers[22]

Grades of inflammatory activity:	Number (%). Total number=50
Minimal inflammation (Grade 1)	2(4%)
Mild inflammation (Grade 2)	30(60%)
Moderate inflammation (Grade 3)	8(16%)
Marked inflammation (Grade 4)	10(20%)
Stages of liver fibrosis:	
No fibrosis (stage 0)	2(4%)
Mild portal fibrosis (Stage 1)	14(28%)
Moderate periportal fibrosis (Stage 2)	24(48%)
Marked fibrosis (bridging fibrosis with lobular distortion; Stage 3)	10(20%)

Table (2): Comparison between different grades of necroinflammation, and between different stages of fibrosis

	Grades 1&2 N=32	Grades 3&4 N=18	P-value	Stages 0,1&2 N=40	Stage 3 N=10	P-value
TNFα	1.66±1.58	6.28±3.64	**<0.0001**	2.25±2.31	7.6±3.53	**<0.0001**
CD41	5.19±3.52	7.94±3.28	**0.009**	5.88±3.24	7.26±3.53	**<0.001**
PDGFR-β	6.44±3.72	8.44±2.96	**0.05**	6.7±3.6	9.2±2.7	**0.04**
PLT count	290.44±91.57	243.83±124.74	0.13	280.78±100.88	237.2±133.83	**0.02**

TNFα: Tumor necrosis factor alpha, CD41: Cluster of differentiation antigen 41, PDGFR-β: Platelet-derived growth factor receptor beta, PLT count: Platelet count. Significant P-values are in bold.

Table (3): Comparison between thrombocytopenic and non-thrombocytopenic groups

	Thrombocytopenic group N=10	Non-thrombocytopenic group N=40	P-value
Age (years)	48.1±5.7	34.5±12.02	0.248
Gender (Male/Female)	3/7	7/33	0.314
Residence (Rural/Urban)	7/3	30/10	0.515
Alanine aminotransferase (IU/L)	95.1±55.76	48.48±29.86	**<0.001**
Aspartate aminotransferase (IU/L)	91.93±47.9	56.7±34.2	**0.01**
Prothrombin time (Second)	13.2±1.06	12.57±0.94	0.072
Total bilirubin (mg/dl)	1.25±0.37	1.06±0.34	**0.045**
Albumin (g/dl)	3.6±0.71	4.49±0.7	**<0.001**
TNFα	5.6±2.86	2.7±3.03	**<0.001**
CD41	5.8±2.86	3.43±3.03	**<0.001**
PDGFR-β	6.9±3.6	5.27±3.92	**<0.001**

TNFα: Tumor necrosis factor alpha, CD41: Cluster of differentiation antigen 41, PDGFR-β: Platelet-derived growth factor receptor beta. Significant P-values are in bold.

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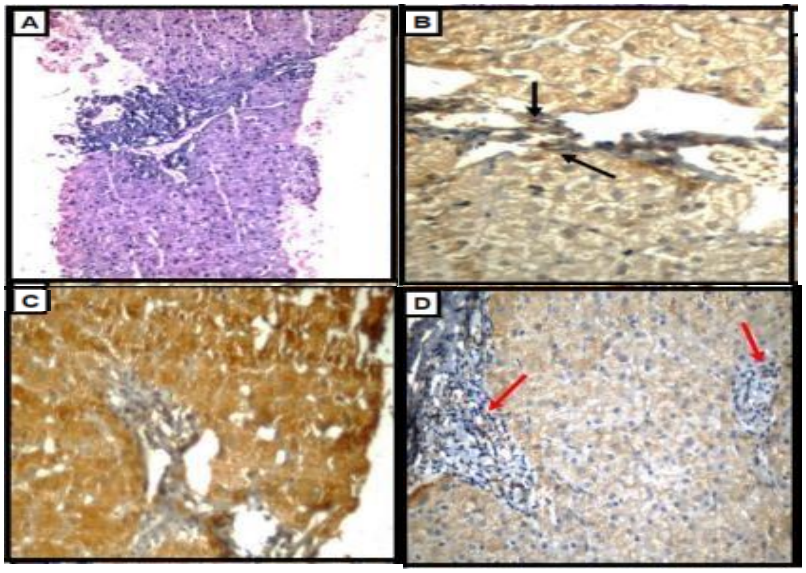

Figure (1): A case of chronic hepatitis (Grade II, Stage II) associated with thrombocytopenia showing: **A:** Mild piecemeal necrosis and fibrous septa extending from portal areas (H&E staining, X200). **B:** Moderate cytoplasmic staining for TNFα in many infiltrating mononuclear cells (black arrows) in portal areas (X400). **C:** Strong cytoplasmic staining for CD41 in hepatocytes (X400). **D:** Moderate staining for PDGFR-β in fibroblasts (red arrows) of the fibrous septa extending from the portal areas (X400).

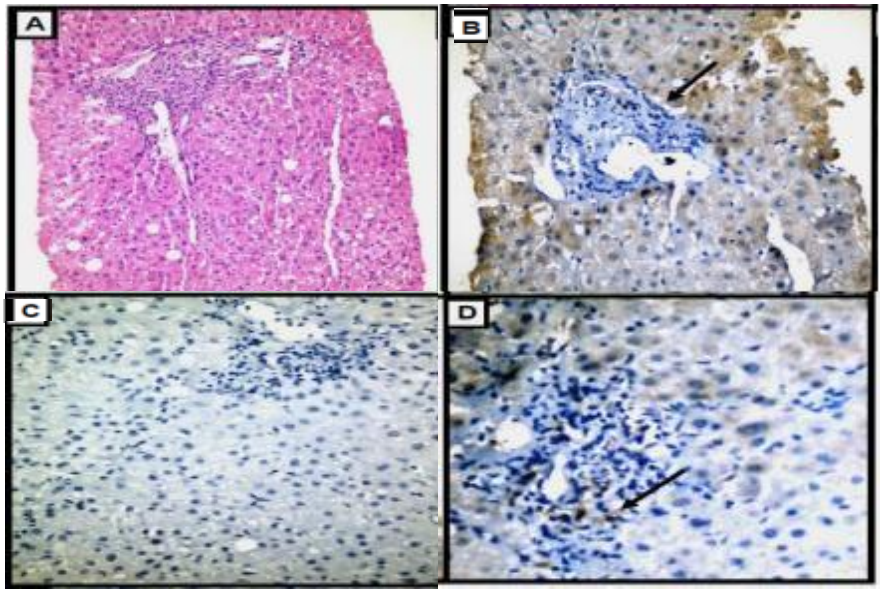

Figure (2): A case of chronic hepatitis (Grade II, Stage II) with normal platelet count showing: **A:** Mild piecemeal necrosis and fibrous septa extending from portal areas (H&E staining, X200). **B:** Negative staining for TNFα in infiltrating mononuclear cells (black arrow) in portal areas (X200). **C:** Negative staining for CD41 in hepatocytes (X400). **D:** Mild staining for PDGFR-β in fibroblasts (black arrow) of the fibrous septa (X400).

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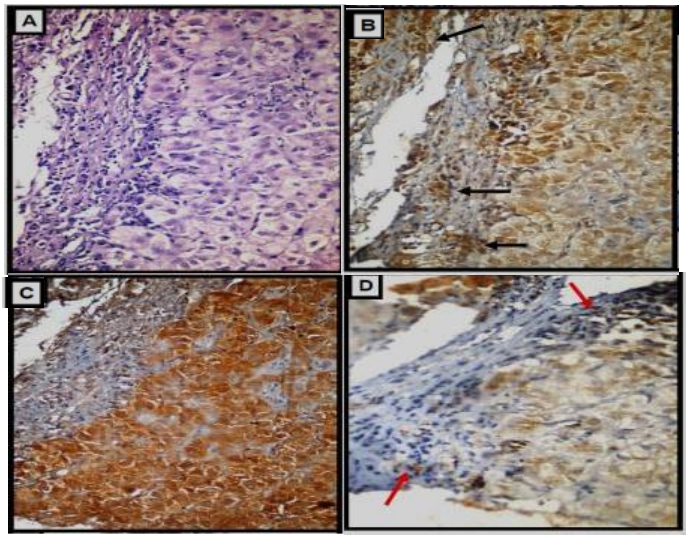

Figure (3): A case of chronic hepatitis (Grade IV, Stage III) associated with thrombocytopenia showing: **A:** Severe piecemeal necrosis and bridging fibrosis (H&E staining, X400). **B:** Strong cytoplasmic staining for TNFα in many infiltrating mononuclear cells (black arrows) in portal areas (X400). **C:** Strong cytoplasmic staining for CD41 in hepatocytes (X400). **D:** Moderate staining for PDGFR-β in fibroblasts (red arrows) of the fibrous septa (X400).

There was a significant negative correlation between peripheral PLT count and the AWS of TNFα$_2$, CD41, and PDGFR-β expression in CHC patients (r= -0.37; P= 0.008 & r= -0.41; P= 0.007 & r= -0.34, P= 0.045 respectively) (Figure 4).

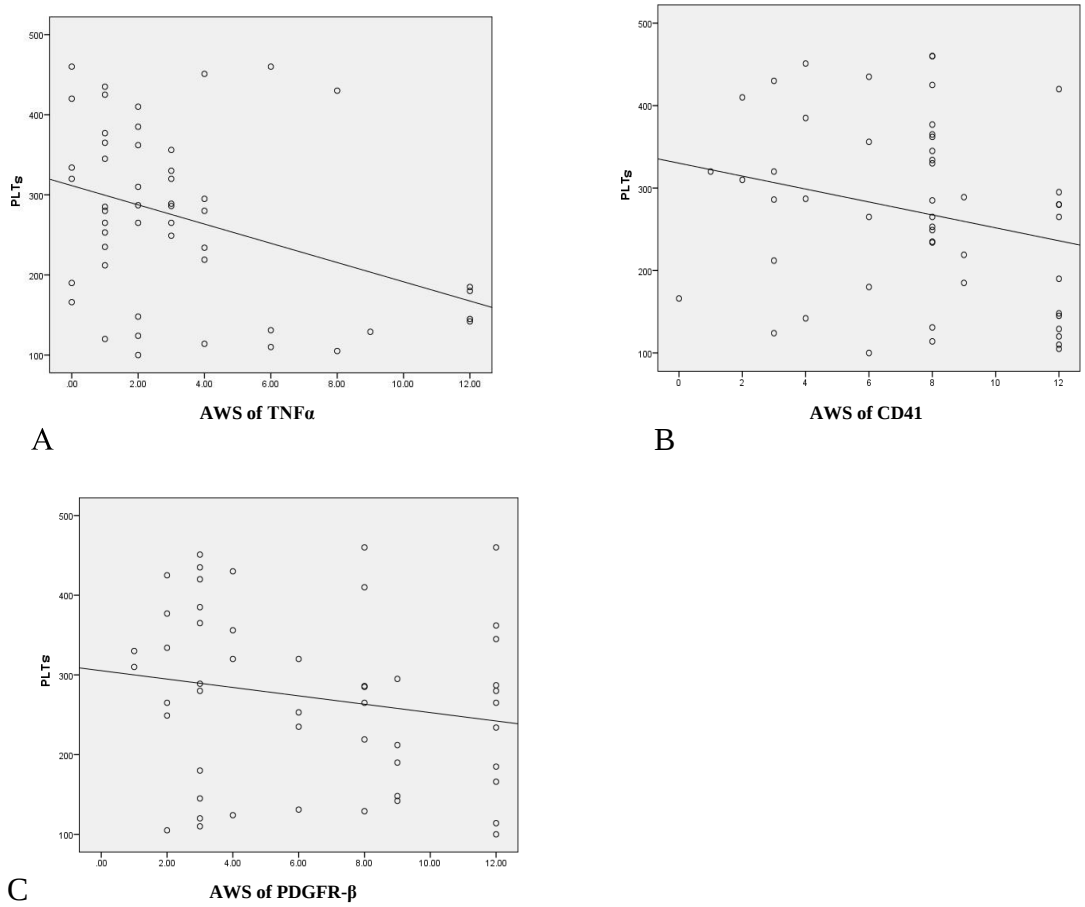

Figure (4): There is a significant negative correlation between peripheral PLT count and the AWS of TNFα$_2$ (A), CD41 (B), and PDGFR-β (C).

DISCUSSION

Liver fibrosis is a pathological condition that badly affects hepatic function but currently few therapeutic options areavailable for it. HSCs play an important role inhepatic fibrogenesis secondary to all types of liver injury[23].

There is a complex interaction between PLTs and liver fibrosis. Our finding of a significantly higher immunohistochemical expression of TNFα, CD41 and PDGFR-β in grades 3& 4 compared to lower grades is consistent with others[24] who highlighted the role of TNFα and PDGFR-β in hepatic inflammation and fibrosis and found thatTNFα induces sinusoidal alteration that leads to adherence of PLTs to sinusoidal endothelium in the same way as that occurs in blood vessels. On response to TNF and IL-1, the endothelium synthesizes and releases platelet-activating factor (PAF). Activated PLTs release PDGF which plays an important role in hepatic fibrosis.

We found a significantly increasedmean AWS of imunohistochemical expression of TNFα in infiltrating mononuclear cells (MNCs) in portal area in higher grades of inflammatory activity and advanced stage of fibrosis. TNFα and its receptors (TNFRs) were detected in hepatocytes and infiltrating MNCs in chronic hepatitis B (CHB) patients and the expression of the receptors was correlated with liver histology[25]. Also, some authors found a positive correlation between expression of TNFα and staging in CHC[26].

We found a significantly increased expression of PDGFR-β in liver tissues of patients with advanced stage of fibrosis than those with earlier stages and this is in agreement with many authors who found many cells expressing PDGFR-β in the periportal area as myofibroblast-like cells and that thenumber of these cells correlated with the grade of inflammation and the stage of fibrosis in patients with CLD[27]. These cells are thought to be arising from Ito cells and proliferate in response to PDGF[28].

We found that patients in stage 3 fibrosis had significantly lower mean peripheral PLT count than those with earlier stages of fibrosis. Ikeda *et al.*[29] found similar result.

We found a significantly increased immunohistochemical expression of CD41 in liver tissue of CHC patients with thrombocytopenia than those with normal PLT count and this is in agreement with Kondo *et al.*[30] who found that PLTs accumulated in the liver tissue in patients who underwent hepatic resection for HCC secondary to chronic HCV. They found increased expression of CD41 in the non-cancerous areas in patients with chronic hepatitis or cirrhosis along with an increase in histological liver damage, although the peripheral PLT count was significantly decreased. They reported that PLTs were mainly present in the sinusoidal spaces of periportal area with inflammation. Similarly, Sata *et al.*[31] used ^{111}Indium-Labelled PLTs to clarify the mechanism of thrombocytopenia observed in patients with CHB. They found intrahepatic PLT accumulation in 50% of the studied patients. Yan *et al.*[32] found intrahepatic PLT accumulation in patients with CHB using monoclonal Ab against CD61 PLT surface antigen.

Starlinger and Assinger[33] found PLT accumulation within the liver and a decrease of PLT count in the hepatic veins when compared to the portal vein, indicating that PLTs adhere in the hepatic vasculature and remain in the liver. PLT accumulation in liver tissues may enhance liver fibrosis through activation of HSCs[21].

Based on these findings, it would be reasonable to consider intrahepatic PLT accumulation as an important factor for hepatic fibrosis and as a possible mechanismof thrombocytopenia in CHC patients.

CONCLUSION

Our result indicate that the expression of TNFα, CD41 and PDGFR-β markers plays a central role in the pathogenesis of CHC and was associated with progression of disease activity and fibrosis.

We have demonstrated that PLT accumulation in the liver of patients with CHC may enhance hepatic fibrosis through the action of PDGF on HSCs and could be a possible mechanism of thrombocytopenia in

these patients. Further studies about the biological characteristics and function of different cells in the hepatic tissue and their cytokines may help in identifying new treatment for liver fibrosis and thrombocytopenia in CHC patients.

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Conflict of interest: Nil.

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