Factors associated with outcome of liver surgery and hepatocellular carcinoma

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CHAPTER 7

LEVELS OF ANGIOGENIC PROTEINS IN PLASMA AND PLATELETS ARE NOT DIFFERENT BETWEEN PATIENTS WITH HEPATITIS B/C - RELATED CIRRHOSIS AND PATIENTS WITH CIRRHOSIS AND HEPATOCELLULAR CARCINOMA

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Abstract

Introduction: Increasing evidence suggests that levels of angiogenic proteins within blood platelets change at the earliest stages of cancer development and may thus provide a promising diagnostic and prognostic tool. Patients with cirrhosis have increased risk of developing hepatocellular carcinoma (HCC).

Aims: We aimed to study whether development of HCC in hepatitis related cirrhosis results in changes in platelet levels of angiogenic proteins.

Materials and methods: We studied the intraplatelet levels of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), endostatin, platelet factor 4 (PF4) and thrombospondin type 1 (TSP-1) in 38 consecutive patients with hepatitis B- or C-related liver cirrhosis with or without HCC in addition to plasma levels of the same proteins. Twenty healthy volunteers were included to establish reference values for the various tests.

Results: Intraplatelet levels of VEGF, bFGF, HGF and endostatin were significantly higher in patients compared to controls. Intraplatelet levels of PDGF, PF4 and TSP-1 were comparable between patients and controls. Plasma levels of VEGF, bFGF and endostatin were comparable between patients and controls. Plasma levels of PDGF, PF4 and TSP-1 were decreased in patients, but this difference disappeared when levels were corrected for platelet count. Intraplatelet and plasma levels of all proteins assessed were comparable between patients with and without HCC.

Conclusion: The intraplatelet levels of some angiogenic proteins are elevated in cirrhosis, but do not discriminate between patients with and without HCC. Thus, intraplatelet levels of angiogenic proteins do not seem useful as diagnostic or prognostic biomarker of HCC in cirrhotic patients.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide.[1] Although numerous treatment modalities are available for HCC,[2] only 30%–40% of patients are eligible for curative interventions, and in these patients, the five-year survival is reported between 66% and 90%.[3-5] Therefore, early detection of tumor growth and recurrence could save many lives.

In general, tumor growth beyond one to two millimeters in size is dependent on angiogenesis.[6] Angiogenesis is regulated by a continuous interplay of stimulatory and inhibitory proteins.[7] Since HCC is a hypervascularized cancer,[8,9] the levels of angiogenic proteins may represent a desirable diagnostic and prognostic tool.[10,11] Therefore, several studies suggested that serum or plasma levels of angiogenesis regulatory proteins are of prognostic value.[12-14] However, the plasma and serum levels of these proteins may not be accurate predictors since the biovariability of these proteins may vary widely over time.[15-18]
It has been well established that platelets play a key role in tumor growth and metastasis.[19-24] Furthermore, it has been demonstrated that platelets actively sequester angiogenic proteins from the blood circulation.[25,26] In mice, platelet levels of angiogenic proteins were already elevated when tumors were smaller than one millimeter, without an increase of levels of these proteins in plasma.[26] Based on these results, it has been postulated that the concentration of angiogenic proteins within platelets is a sensitive and early marker for the presence of tumor, and that platelet levels are superior predictors of progression or recurrence of cancer compared to levels measured in plasma or serum. Indeed, one study in humans showed that some, but not all, angiogenic proteins were elevated in platelets from patients with colorectal cancer,[27] and platelet (but not plasma) levels of these proteins were independent predictors of the presence of tumor.

To our knowledge, no study has yet been performed investigating platelet levels of angiogenic proteins in patients with HCC. Since cirrhosis, especially in combination with Hepatitis B or C virus (HBV or HCV) infection, is a major risk factor for developing HCC, we hypothesized that the intraplatelet levels of angiogenic proteins might be predictors of the presence of HCC in patients with hepatitis-related cirrhosis. Therefore, we studied platelet and plasma levels of various angiogenic proteins in patients with hepatitis-related cirrhosis in presence and absence of HCC.

METHOD

STUDY POPULATION

From February to April 2013, a total of 38 consecutive patients with cirrhosis who visited the outpatient clinic or were admitted to the Hepatology ward of the Department of Gastroenterology of University Medical Center of Padua, Padua, Italy, were enrolled into this

study. We included all patients who had established cirrhosis due to HBV or HCV infection, with or without HCC. Excluded were those who used platelet inhibitory drugs such as acetylsalicylic acid or P2Y12 inhibitors, patients who were undergoing eradication treatment for HBV or HCV infection, patients with an active HCC who had a treatment-free interval less than two months at the time of inclusion and patients who were on dialysis. The diagnosis of HCC was obtained by using the European Association for the Study of the Liver criteria, i.e. two imaging procedures (spiral CT, MRI with paramagnetic contrast injection or ultrasound with second generation intravenous contrast (Sonovue, Bracco, Italy)) confirming the presence of a lesion.

We included 20 adult employees of the University Medical Center Groningen, The Netherlands, to establish reference values for the tests performed. Exclusion criteria for the control group were platelet-inhibitory drugs usage such as acetylsalicylic acid or P2Y12 inhibitors documented history of congenital coagulation disorders, documented history of hepatic disease and history of viral infection (52 weeks). This study was performed in accordance with the declaration of Helsinki and was approved by the local ethical committee. Written informed consent was obtained from each individual.

Study Variables

Patient characteristics and variables were obtained from patient charts. These included age, gender, the severity of liver cirrhosis (according to the Child-Pugh classification) and platelet count. We calculated the Model for End-Stage Liver Disease (MELD) score based on the last laboratory measurements prior to the blood draw. For patients with HCC, we also obtained the number of tumor lesions, the size of the tumors and the total mass of HCC, the presence of vasoinvasion and if the patients were previously treated for the HCC. When necessary, computer-stored hospital files were reviewed for other relevant clinical parameters.

Platelet Count, Isolation of Blood Platelets and Preparation of Plasma

Blood was drawn from the antecubital vein through 20-gauge needles into vacuum 3.2% sodium citrate (9:1, v/v) tubes. Blood platelet count was determined using a Beckman Coulter LH755 Analyser (Miami, FL). Platelets were isolated by differential centrifugation. First, samples were spun at 200 g for 15 min at ambient temperature to obtain platelet-rich plasma (PRP). PRP was transferred to a clean tube and recentrifuged (500 g for 15 min at ambient temperature) in the presence of iloprost (2 ng/ml) purchased from Santa Cruz Biotechnology (Dallas, TX). The supernatant, platelet-poor plasma, was then collected in 2ml tubes, snap-frozen and stored at -80°C until use. The platelet pellet was resuspended in Hepes-Tyrode buffer (10mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 137mM NaCl, 2.68mM KCl, 0.42mM NaH2PO4, 1.7mM MgCl2, 5mM d-glucose, pH 7.4) and recentrifuged (500 g for 15 min, ambient temperature) in the presence of iloprost (2 ng/ml). Then, the supernatant was discarded, and the platelet pellet was resuspended in

Hepes-Tyrode buffer. The platelets count in this suspension was determined and platelets were lysed by repeated free–thaw cycles and stored at -80°C until use.

Tabel 1: Participant characteristics

Demographic characteristics	Controls (n = 20)	Cirrhosis without HCC (n = 16)	Cirrhosis with HCC (n = 22)	*P*
Participant variables				
Gender, Male	11 (55%)	10 (63%)	20 (95%)	0.01
Age, mean (SD)	29 (±5)	55 (±14)	70 (±10)	< 0.01
Platelet count, (G/L), median (IQR)	-	107 (48-159)	102 (68-152)	0.69
INR, median (IQR)	-	1.3 (1.2-1.6)	1.2 (1.1-1.2)	0.03
MELD, median (IQR)	-	12 (9-16)	10 (8-11)	0.09
Etiology of liver Cirrhosis	-			0.22
HBV		3 (19%)	7 (33%)	
HCV		13 (81%)	12 (57%)	
HBV + HCV		0	2 (10%)	
Child Pugh, n (%)	-			0.02
A		4 (25%)	11 (52%)	
B		9 (56%)	6 (29%)	
C		3 (19%)	2 (10%)	
AFP, median (IQR)	-	6 (5-23)	14 (3-24)	0.55
Tumor characteristics	-	-		
Treated (TACE/RFA), yes		-	13 (59%)	
Number of lesions, median (IQR)		-	2 (1-3)	
Total size, mm (IQR)		-	40 (25-71)	

AFP indicates Alpha fetoprotein; HBV, hepatitis B virus, HCC; Hepatocellular carcinoma, HCV, hepatitis C virus; INR, International Normalised Ratio; IQR, interquartile range; MELD: Model of End stage Liver Disease; RFA, radiofrequency ablation; SD, standard deviation, TACE, transarterial chemoembolization.

Levels of Angiogenic Proteins in Plasma and within Platelets

Plasma levels of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), endostatin, platelet factor 4 (PF4) and thrombospondin type 1 (TSP-1) were assessed using commercially available enzyme-linked immunosorbent assays (Duoset, R&D systems, Abingdon, UK) as previously described.[18] Levels of these proteins in platelet lysates were determined with the same assay. Platelet residues in the lysates were spun down prior to the assay. Levels of the angiogenic proteins in the platelet lysates were corrected for the actin content of the lysates as described previously [18] using a commercially available monoclonal antibody (MAB1501R; Milipore, Amsterdam, The Netherlands). For detection, this antibody was biotinylated using a commercially available biotinylation kit (Pierce, Rockford, IL).

All assays were validated by spiking experiments in which a known concentration of the protein was added to plasma or platelet lysate from a single healthy donor after which the recovery of the protein was determined by ELISA. Recoveries for the various tests ranged

from 83 to 94% in plasma and from 65 to 96% in platelet lysate. Accuracy of the measurements was further ensured by control samples on each ELISA plate. Between plate coefficients of variation varied between 3 and 19% for the various tests.

Statistical analysis

Statistical analyses were performed using the statistical software package SPSS 20 (IBM SPSS, Chicago, IL). Categorical variables are shown as numbers and percentages and groups were compared using Pearson's chi-squared test. Continuous variables are presented as means with standard deviation or as medians with interquartile range (IQR) based on their distribution. Continuous variables were compared using a standard t-test or the Mann–Whitney U-test, as appropriate. A $P < 0.05$ was considered statistically significant.

Results

Study Ppopulation

Patient characteristics are summarized in Table 1. Included were 38 patients with hepatitis-related cirrhosis of whom 22 (58%) also had HCC. One patient with HCC was excluded due to the loss of samples during pre-analytical handling. The patients that had HCC were more frequently male than patients that did not have HCC (95% vs. 63%, respectively). There was no significant difference between the patients with or without HCC regarding the type of hepatitis as underling cause of liver cirrhosis (HBV or HCV, 33% and 57% in the patients with HCC vs. 19% and 81% in the patients without HCC). Concomitant HBV and HCV infection was present in two (10%) patients who had HCC.

Thirteen (59%) of twenty two patients that had an active HCC due to a recurrence or a residual tumor at the time of inclusion had previously been treated, ten patients with trans-arterial chemoembolization and one patient with radiofrequency ablation. The patients were classified according to the Child-Pugh classification.[28] The patients who had HCC appeared to have milder liver disease compared to those who did not have HCC. However, the difference between the groups was not statistically significant.

Furthermore, the patients with HCC were significantly older, had a lower INR and MELD score, but had similar platelet counts compared to the patients without HCC. The patients with HCC had a median of two lesions (IQR 1–3), a cumulative tumor size of 40mm (IQR: 30–70) and a serum alpha fetoprotein (AFP) of 12.5 ng/ml (IQR: 3.8–23.5). One patient had an AFP level of 2.750 ng/ml and a cumulative tumor size of 85 mm. No patient had portal vein thrombosis, extrahepatic manifestation of HCC or vasoinvasion.

Levels of Proangiogenic Proteins in Plasma and Platelets from Patients with Hepatitis-related Cirrhosis with and without HCC

Figure 1 shows levels of proteins involved in stimulation of angiogenesis in plasma and platelets from patients and controls. Plasma levels of VEGF were below the detection limit in the majority of patients and controls. Plasma levels of bFGF were not different between patients and controls. Plasma PDGF levels were lower in patients compared to controls, and plasma HGF was higher in patients as compared to the controls. No differences in plasma levels of any of the proangiogenic factors were present between patients with HCC compared to the patients who did not have HCC.

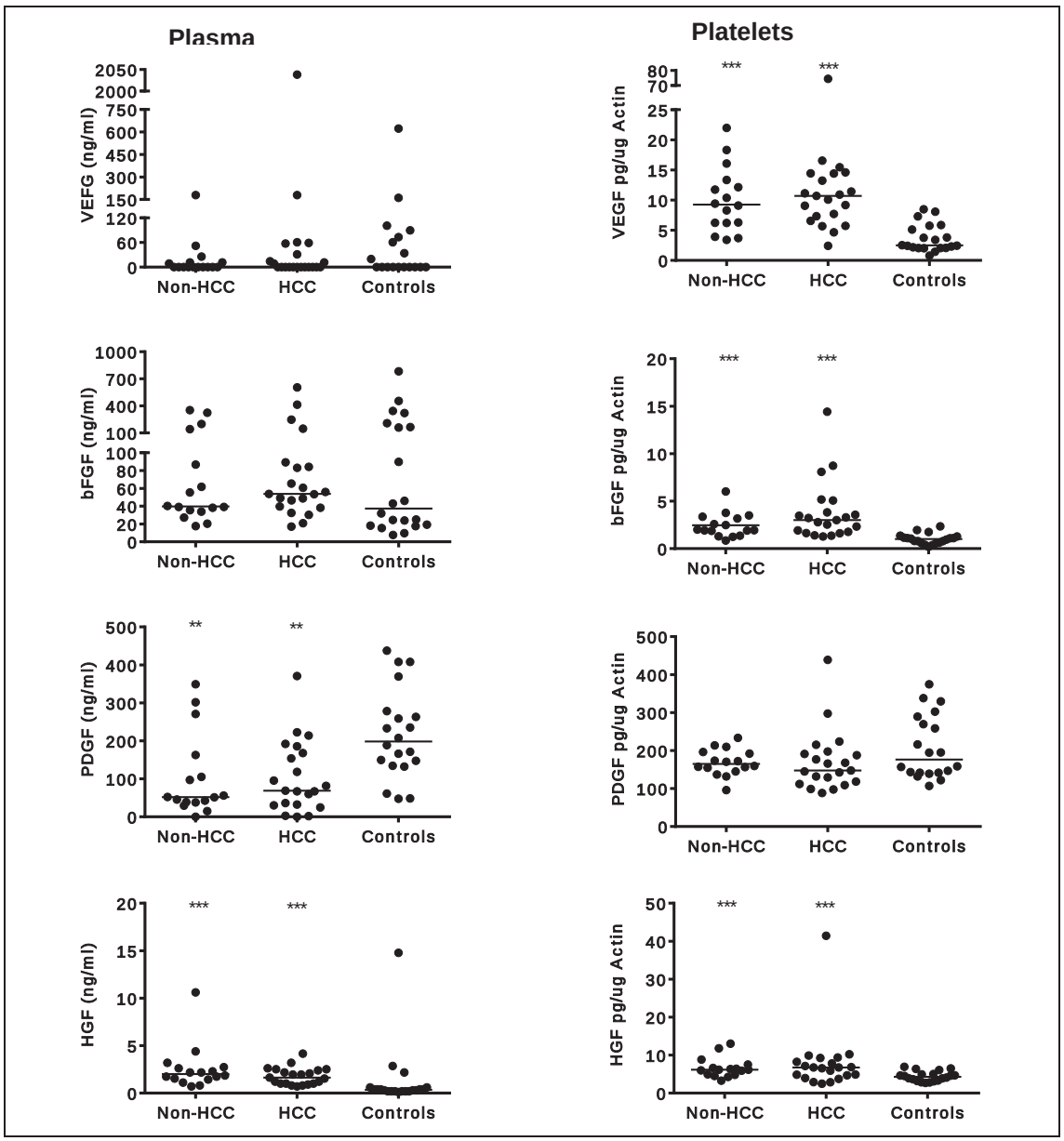

Figure 1: Plasma and intraplatelet levels of angiogenesis inhibitory proteins in patients with or without HCC and control subjects. Horizontal bars indicate median. ** $P < 0.01$, *** $P < 0.001$, all v.s. control

Platelet levels of VEGF, bFGF and HGF were all higher in patients compared to controls, but no differences were detected between patients with and without HCC. Platelet levels of PDGF were not different between patients and controls. Plasma and platelet levels of all proteins were not different between HCC patients who were treatment-naıëve and patients who had been treated for their HCC prior to inclusion in the study (all with $P>0.2$).

Levels of Antiangiogenic Proteins in Plasma and Platelets from Patients with Hepatitis-Related Cirrhosis with and without HCC

Figure 2 shows levels of antiangiogenic proteins in plasma and platelets from patients and controls. Patients had decreased plasma levels of PF4 and TSP-1 compared to controls, and plasma levels of endostatin were similar between patients and controls. No differences were present in plasma levels of antiangiogenic proteins between patients with and without HCC. Platelet levels of PF4 and TSP-1 were similar between patients and controls, and platelet endostatin levels were slightly, but significantly, increased in patients patients compared to controls. No differences in platelet antiangiogenic proteins were detected between patients who did and those who did not have HCC. Plasma and platelet levels of all proteins were not different between HCC patients who were treatment-naıëve and patients who had been treated for their HCC prior to inclusion in the study (all with $P>0.2$).

Discussion

In this study, we found no differences in platelet and plasma levels of seven angiogenic proteins between patients that had hepatitis-related cirrhosis and patients that had hepatitis-related cirrhosis and HCC. We did, however, detect differences in platelet or plasma levels of several of the proteins studied between patients and healthy controls. We hypothesized to find increased intraplatelet levels of the angiogenic markers based on studies showing increased intraplatelet levels of these proteins in patients with colorectal cancer[27] and on studies showing that intraplatelet levels of these proteins increase already at very small tumor sizes in mice.[25,26] The clear absence of a difference between intraplatelet levels of angiogenic proteins in patients with HCC and those without indicates that intraplatelet levels of angiogenic proteins cannot be used as a biomarker for the presence of cancer in all types of cancer. Nevertheless, the small cohort size, heterogeneity of the patient population and the fact that the majority of HCC patients were not ‘‘treatment-naıëve’’ does not allow the firm conclusion that no differences between patients with cirrhosis and those with cirrhosis and HCC exist.

The apparent absence of a difference between angiogenic proteins in patients with and without HCC may be explained by the angiogenic response occurring in patients with cirrhosis who have not (yet) developed HCC. It has been well established that the progression of fibrosis to cirrhosis is associated with a profound angiogenic response

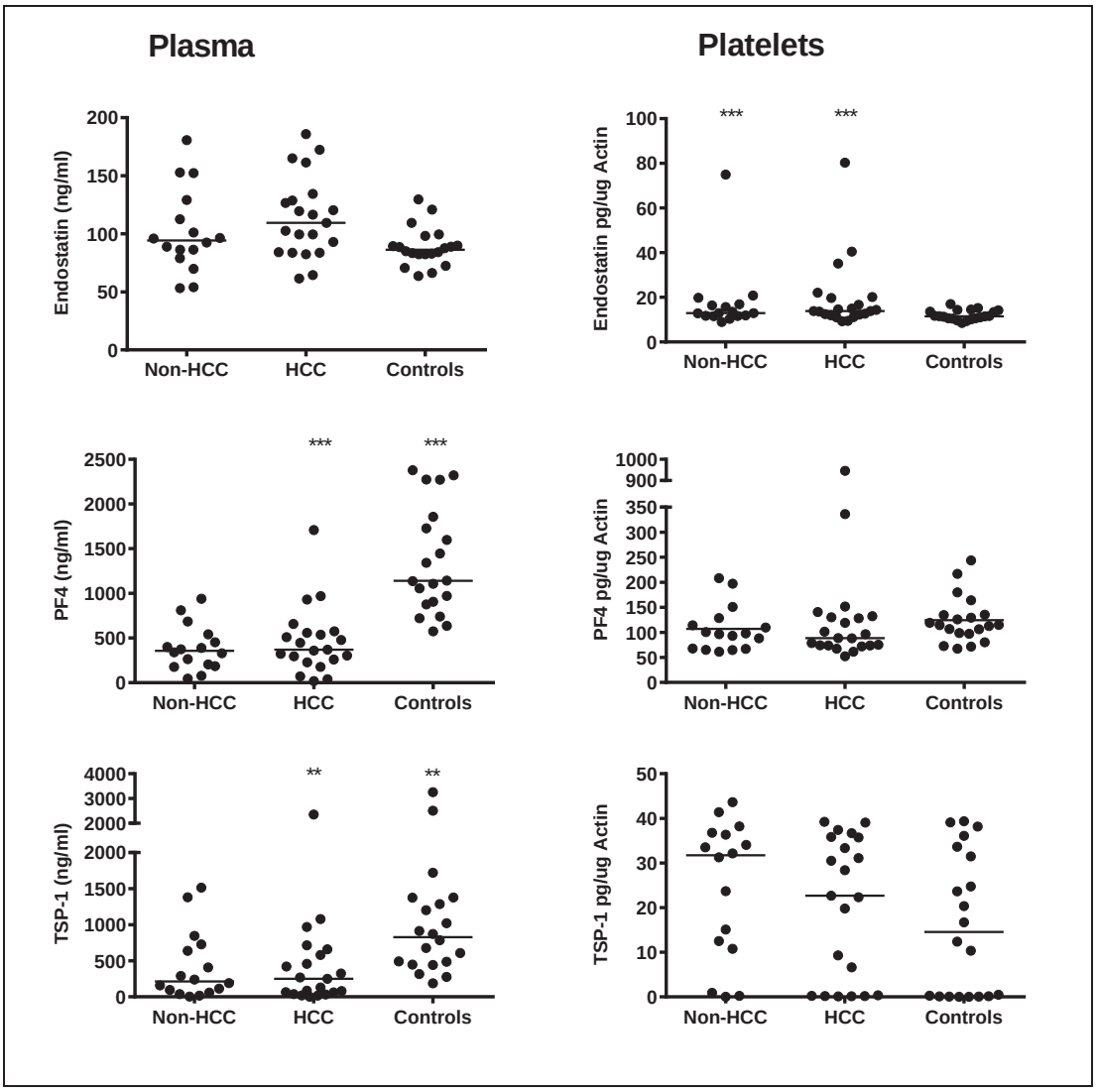

Figure 2: Plasma and intraplatelet levels of angiogenesis inhibitory proteins in patients with or without HCC and control subjects. Horizontal bars indicate median. ** P < 0.01, *** P < 0.001, all v.s. control.

initiated by multiple mechanisms including changes in blood flow, hypoxia and inflammation.[29,30] Importantly, since our data show that plasma levels of the angiogenic proteins are not elevated, it is likely that the angiogenic proteins that are induced in cirrhosis are taken up by platelets, similar to the uptake of these proteins in patients with cancer. Although it has not been established whether platelets play a role in the cirrhosis associated angiogenic response, our data suggest that intraplatelet angiogenic proteins are elevated in cirrhosis. Given the role of platelets in inflammation and their multiple roles in liver diseases,[31] it may be that platelets are active players in disease progression by delivery of angiogenic proteins within the liver.

The additional angiogenic response that occurs when patients with cirrhosis develop HCC may thus simply be too small to result in additional increases in intraplatelet levels of

angiogenic proteins. Our study population consisted of patients with cirrhosis who were followed up on regular basis with active HCC surveillance programs. The median cumulative tumor size in our cohort was 40 mm, and none of the patients had extrahepatic manifestations or macroscopic vasoinvasion suggesting that the tumors were detected early due to the regular follow up. The levels of angiogenic factors produced by these relatively small tumors may also have been too small to result in detectable increases in intraplatelet levels of these proteins. Our results are, however, at variance with previously published data showing serum levels of HGF to be elevated in patients with cirrhosis and HCC compared to patients with cirrhosis alone.[32,33]

Plasma levels of the angiogenic proteins were not different between patients and controls, except for levels of PDGF, PF4 and TSP-1, which were all decreased in patients compared to controls. All three proteins are synthesized by megakaryocytes, and the decreased plasma levels in patients with cirrhosis may simply be a reflection of the decreased circulating platelet count in cirrhosis. Indeed, when plasma levels of PDGF, PF4 and TSP-1 were normalized for the platelet count, the differences between patients and controls fully disappeared (data not shown).

In conclusion, intraplatelet levels of some angiogenic proteins are elevated in cirrhosis, but do not distinguish between patients with cirrhosis who do and who do not have HCC. Intraplatelet levels of angiogenic proteins thus cannot be used as biomarkers of HCC in patients with cirrhosis. Whether platelet levels of angiogenic proteins are prognostic in patients with HCC in the absence of cirrhosis requires further study.

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