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Research Article

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Ascitic Fluid Hepatocyte Growth Factor: New Insights for Diagnosis of Spontaneous Bacterial Peritonitis in Cirrhotic Patients, an Egyptian Pilot Study

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Abstract

Background: Spontaneous bacterial peritonitis (SBP) is a common and potentially fatal complication of patients with decompensated cirrhosis and ascites. Hepatocyte growth factor (HGF) is a protein produced by mesenchymal cells including hepatocytes. It is regulated under different physiological and pathological conditions, for instance, during pregnancy, aging and infectious disease. This study focuses on evaluation of HGF as a local acute phase response marker in ascitic fluid of cirrhotic patient that could be used for early laboratory diagnosis of SBP.

Methodology: Forty cirrhotic patients with SBP and 40 cirrhotic patients without SBP were included in this study. All involved patients were subjected to estimation of HGF level in the ascitic fluid using ELISA technique, in addition to conventional culture and biochemical reactions for identification of the bacteria involved of SBP.

Results: There was a highly significant statistical difference in HGF levels in ascitic fluid between cirrhotic patients with SBP and those without SBP (P=0.000). The sensitivity and specificity of HGF for selective detection of the SBP group over the non-SBP group were 80 and 82.5%, respectively, at a cut-off value of 2981.34 pg/ml. There was a considerable positive correlation of HGF levels with TLC in ascitic fluid (r=0.372, p=0.018). There was a remarkable positive correlation between HGF levels and transaminases; AST and ALT (r=0.423, p=0.007 and r=0.359, P=0.023 respectively).

Conclusion: HGF could be used as minimally invasive biomarker for early detection of SBP in cirrhotic patients.

Keywords: Hepatocyte growth factor (HGF); Spontaneous bacterial peritonitis (SBP); Ascites; Cirrhosis

Introduction

Cirrhotic patients are in a state of immune umbalance known as cirrhosis associated immune dysfunction syndrome, characterized by excessive activation of pro-inflammatory cytokines, in which patients are predisposed to infections [1,2].

Spontaneous Bacterial Peritonitis (SBP) is an infection of the previously sterile ascitic fluid in absence of a visceral perforation or an intra-abdominal septic focus [3]. It is a very frequent bacterial infection in cirrhotic ascitic patients accounting for 10% in hospitalized patients and 1.5%-3.5% in non-hospitalized patients [4,5].

In advanced liver disease, increased intestinal permeability together with pathological bacterial translocation in which intestinal bacteria cross the enteral wall and colonize intestinal and/or mesenteric lymph nodes favouring *SBP* via a diversity of mechanisms [6-8]. Microorganisms isolated from ascitic fluid in patients with SBP comprise Gram-negative bacilli (GNB) mainly *E.coli*, *Klebsiella*, *Enterobacter*, and *Pseudomonas* followed by Gram positive cocci mainly *Streptococcus spp.*, Staphylococcus ssp. and Enterococcus spp. Less frequent causative organisms of SBP are anaerobes [9,10].

Hepatocyte growth factor (HGF)/scatter factor was primarily recognized in 1984 and molecularly cloned as a potent mitogen of primary cultured hepatocytes [11]. It is a growth factor manufactured by mesenchymal cells during injuries in several organs. HGF is translated as a single-chain precursor, proteolytic cleavage at the site of injury activate it into double-chained active HGF [12]. Interaction of the active HGF with its specific receptor (c-Met) prompts an intracellular signal pathways ensuing in regeneration and repair of damaged tissue [13,14].

High HGF has been detected systemically during infectious injuries [15], such as sepsis [16,17], pneumonia, gastroenteritis, skin and soft tissue infections and pyelonephritis. Similarly, in cerebrospinal fluid during meningitis [16].

Despite performing diagnostic paracentesis; ascitic fluid culture is negative in at least 40% of cases with an elevated polymorphs neuclear leucocytes (PMN) count [18].

It is crucial although often difficult to make a rapid diagnosis of bacterial infections in cirrhosis due to nonspecific manifestations, which are indistinguishable from other non-infectious causes of systemic inflammatory response syndrome (SIRS) and the symptoms of liver deterioration. Therefore, serum biomarkers that are sensitive, reliable and inexpensive are being followed in order to enhance the diagnosis of bacterial infection in the setting of cirrhosis. General inflammatory markers, such as C-reactive protein (CRP, synthesized by the liver), ferritin (synthesized by the liver) or white blood cells (WBC) count, lack specificity for bacterial infections [19].

Detection of high amounts of cytokines during inflammatory diseases is not a distinctive finding. However, determination of HGF in plasma/ serum and urine has been found to be a sensitive method that could detect specific clinical problems [20].

Aim of the Work

To evaluate the role of ascitic HGF in the diagnosis of SBP in cirrhotic patients *via* a) correlating the level of HGF in ascetic fluid of cirrhotic patients with clinical and laboratory features of SBP, b)

identifying the cutoff value of HGF in the ascetic fluid required for the diagnosis of SBP in cirrhotic patients and c) identifying the bacterial spectrum involved in SBP. Sub-objective: To compare the sensitivity of HGF and CRP in diagnosis of SBP.

Patients and Methods

The present study was conducted from September 2016 to January 2017 in the Medical Microbiology and Immunology Department Laboratories, Cairo University. Eighty cirrhotic patients recruited from the in-patient section of the department of Tropical Medicine, Kasr Al Ainy Hospital Cairo University were included in this study. They were classified into 2 groups:

Group 1: This group included 40 Cirrhotic ascitic patients with SBP.

Group 2: This group included 40 Cirrhotic ascitic patients without SBP who served as a control group.

The approval of Tropical Medicine Department as well as an informed consent from patients were taken.

Inclusion criteria: Cirrhotic patients with ascites (cirrhosis being diagnosed clinically, biochemically, radiologically, and histopathologically in some cases) and ascites was diagnosed (clinically and radiologically).

Inclusion criteria for group1and exclusion criteria for group 2: Evidence of SBP according to at least one of the following criteria:

Clinical criteria: i. Local abdominal symptoms (abdominal pain, abdominal tenderness, vomiting, diarrhea, deteriorated hepatic condition e.g encephalopathy, and /or ileus) and/ or ii. Systemic manifestations (hypotension, hyper or hypothermia, chills, tachycardia, and/or tachypnea).

Laboratory criteria: i. Worsening of liver function, ii. An absolute neutrophil count of250 cells/ mm3 in the ascitic fluid in the absence of an intra-abdominal source of infection [4] regardless the ascitic fluid culture.

Exclusion criteria of the study:

Non-cirrhotic ascites e.g heart failure, renal failure, …etc.

Cirrhotic patients without ascites.

Secondary peritonitis diagnosed by absolute neutrophil count of250 cells/ mm^3 in the ascitic fluid in the presence of an intra-abdominal source of infection.

Bacterascitis in which cultures of ascitic fluid are positive but there is normal ascitic neutrophil count (<250/mm^3).

All subjects of both groups were subjected to 1] Full history taking, 2] Thorough clinical examination including general, abdominal, cardiopulmonary, and neurological examination, 3] Comprehensive Laboratory investigations were done including complete blood count (CBC), liver biochemical profile (bilirubin, transmainases, albumin, international normalized ratio (INR), C-reactive protein level (CRP), 4] Ascitic fluid analysis: ascitic fluid sample was obtained by diagnostic paracentesis under strict sterile conditions and after application of local anesthesia. a. Ascitic fluid neutrophil count was done using a manual counting chamber (Fuchs-Rosenthal bright blue, Superior Marienfeld, Germany) which was proven to be more reliable than automated cell counting. b. Ascitic fluid culture: i. A volume of 5 ml ascetic fluid was collected in a sterile container then centrifuged in the laboratory for 3 min at 5000 rpm. (NÜVE NF 815 CENTRIFUGAL (8 * 15 mL), Turkey. ii. Direct Gram stained smear of the ascetic fluid was examined then processed as follows:

Direct inoculation: Several drops were inoculated directly onto two sets of each of three agar plates (chocolate agar, blood agar, Mac Conkay agar). The first set was incubated aerobically at 37°C for 24-48 h. The second set was incubated anaerobically in gaspak system for 4-5 days and were examined daily for growth.

Indirect inoculation: A volume of 0.5 ml of deposit was placed in 10 ml thioglycolate broth and examined daily for turbidity. Subcultures from the broth were done on three agar plates (chocolate agar, blood agar, Mac Conkay agar) and incubated both aerobically and anaerobically.

Further processing was done according to the nature of the isolate, as was determined by Gram staining and colony morphology then, necessary biochemical tests were performed.

Gram negative organisms were identified using oxidase test, citrate utilization, urea hydrolysis, triple sugar iron (TSI), lysin decarboxylation, indole production.

Staphylococcus spp. was identified on the basis of positive catalase test, coagulase enzyme and growth on mannitol salt agar.

Streptococcus spp. was identified by negative catalase test and esculin hydrolysis.

Yeasts were identified by Gram's staining and germ tube test (GTT) [21].

Detection of hepatocyte growth factor level in ascitic fluid was done using quantitative ELISA. i. One ml volume of ascetic fluid was transported to laboratory of the Medical Microbiology and Immunology to be centrifuged at 3000 rpm for 5 minutes and supernatant was stored at -70°C till processing. ii. Quantitative detection of HGF level in ascetic fluid was determined in both groups using a sandwich ELISA kit (Quantikine Human HGF Immunoassay, catalog number DHG00).

A 96-well plate precoated with monoclonal antibody specific for human HGF was used.

Standards and samples were pipetted into the wells and any HGF present is bound by the immobilized antibody.

After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human HGF was added to the wells.

Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of HGF bound in the initial step. The optical density was measured at 450 nm using a microplate reader.

A stock solution of 80,000 pg/mL lyophilized recombinant human pro-HGF in a buffered protein base was used to produce a dilution series of seven standards. The 8000 pg/mL standard served as the highest standard while the 125 pg/mL standard served as the lowest standard.

A standard curve was generated by plotting the average optical density (O.D.) (450 nm) obtained for each of the standard concentrations on the Horizontal (X) axis versus the corresponding HGF concentration (pg/ml) on the vertical (Y) axis. The sample concentrations were then interpolated from the standard curve.

5] Statistical analyses were performed using IBM SPSS advanced statistics version 20 (SPSS Inc., Chicago, IL). Mean and standard deviation were used to describe HGF concentrations and other numerical data like laboratory values. Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. The associations between baseline clinical or laboratory characteristics and ascitic HGF levels were analyzed with Mann–Whitney test for categorical variables and Spearman's correlation coefficient for continuous variables. Comparison between 2 groups was done using Kruskal-Wallis test (non-parametric ANOVA) then post-Hoc "Schefe test" was used for pair-wise comparison based on Kruskal-wallis distribution. A P-value<0.05 was considered significant.

Results

Eighty cirrhotic inpatients were recruited from the Tropical Medicine Department, Faculty of Medicine (Kasr Al Ainy Hospita), Cairo University. They were equally divided into 2 groups; Group 1 (cirrhotic ascitic patients with SBP): This group included 40 patients; 25 (62.5%) males and 15 (37.5%) females with mean age 53 ± 10 years, and Group 2 (control cirrhotic ascitic patients without SBP): This group constituted 16 (40%) males and 24 (60%) females with mean age 44 ± 12 years.

Comparison between group 1 and group 2 in terms of the main laboratory tests including the ascitic HGF is shown in table 1.

Laboratory test		Group 1	Group 2	P value
Complete blood count	Hb	10.2 ± 1.6	9.14 ± 1.5	0.48
	WBC (x 103)	9.82 ± 4.18	7.59 ± 3.5	0.29
	AST	42.25 ± 3.2	60.57 ± 6.4	0.198
	ALT	71.72 ± 5.1	40.4 ± 3.7	0.095
	INR	1.23 ± 0.07	1.6 ± 0.09	0.667
C-reactive protein (CRP) (mg/l)		17.12	10.2	0.108
Ascitic fluid analysis	TLC	2440.75 ± 479	90.35 ± 11.5	0.001
	HGF (pg/ml)	8346.435	2417.645	0
		± 805.18	± 157.25	

Table 1: The main laboratory tests of the studied patients.

Hb:Haemoglobin; WBC:White blood cells; AST:Aspartate aminotransferase; ALT:Alanine aminotransferase; INR:International normalized ratio; TLC:Total leucocytic count; HGF:Hepatocyte growth factor

ROC (Receiver Operator Characteristic) curve was done to determine the best cut off limit of HGF level to diagnose SBP as illustrated in Figure 1. Analysis showed that the best cut off limit of HGF in diagnosing SBP was 2981.3424 pg/ml which achieves sensitivity of 80% and specificity of 82.5%. The area under the curve was 0.870.

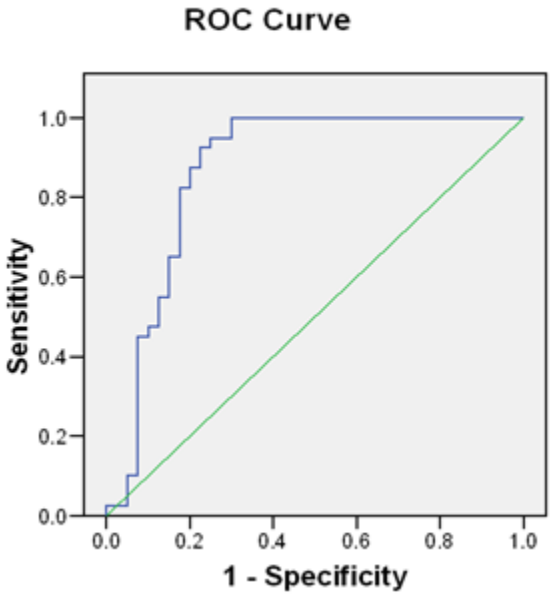

Figure 1: ROC analysis of the cut off value of HGF level for diagnosis of SBP.

Ascitic fluid cultures are shown in Table 2. Only 8 cultures of the SBP patients (20%) were positive. All of them were monomicrobial, and there was a significant association between ascitic fluid TLC values and ascitic fluid cultures in SBP group (r=0.239, P=0.033).

In addition, the mean HGF was not significantly different (8837.21pg/ml in negative cultures versus 6383.32 pg/ml in positive cultures, P=0.101).

Culture			Group 1
			Number (Percent)
Negative			32 (80)
Positive	Total		8 (20)
	Gram negative	Pseudomonas	3 (37.5/ 50)
	6 (75)	Klebsiella	2 (25/ 33.3)
		Proteus	1 (12.5/ 16.7)
	Gram positive	Strept.pneumonia	1 (12.5/ 50)
	2 (25)	MRSA*	1 (12.5/ 50)

*MRSA:Methicillin resistant Staphyloccus aureus

Correlation between ascitic HGF and some of the main laboratory results are shown in Table 3. It was positively correlated with the leucocytic count, whether in the blood or in the ascitic fluid, and transaminases.

Laboratory results			Ascitic HGF	
			R	P
Complete blood count		Haemoglobin	0.145	0.373
		White blood cells	0.416	0.008
Liver biochemical profile	Transaminases	AST	0.423	0.007
		ALT	0.359	0.023
	International normalized ratio (INR)		0.076	0.641
C- reactive protein (CRP)			0.145	0.373
Ascitic fluid analysis		Total leucocytic count	0.372	0.018
		Culture	0.185	0.101

Table 3: Correlation between ascitic HGF and some of the main laboratory results.

AST:Aspartate aminotransferase; ALT:Alanine aminotransferase

Discussion

Cirrhotic patient with ascites are predominantly vulnerable to SBP due to altered gut permeability, suppression of the reticuloendothelial system and bacterial overgrowth [22]. Once infection advances, an unwarranted response of pro-inflammatory cytokines on a pre-existing hemodynamic dysfunction in the setting of cirrhosis further trigger the development of serious complications such as shock, renal failure, acute-on-chronic liver failure, and bacteremia. These complications are important prognostic landmarks in the natural history of cirrhosis. Remarkably, the incidence of infections from resistant bacteria has increased significantly in healthcare-associated settings [2].

HGF is a pleiotropic cytokine. High levels of HGF are produced both locally and systemically in case of injuries induced by infections [23], moreover, it plays as a prognostic factor in inflammatory diseases [24].

The aim of this work is to assess the role of ascitic HGF in diagnosing SBP including identifying sensitivity, specificity, and its ideal cut off value. This is a pilot study i.e was not studied before, whether in the disease or the sample, including its significant associated laboratory tests.

Ascitic HGF in cirrhotic patients was significantly higher in SBP than those without (P=0.000) which may suggest that HGF is locally produced in ascitic fluid in cirrhotic patients confirming its role in infections. To our knowledge, data of HGF values in ascitic fluid in cirrhotic patients had not been published before. But there were many researches about the significant high HGF value in many other infections e.g in cerebrospinal fluid in acute bacterial meningitis [25,26] (P<0.001 and <0.05 respectively) including the tuberculous meningitis [26], in exhaled breath in pneumonia (P=0.0005) [27], and in faeces in infectious diarrhea (P<0.0001) [28].

In this study, CRP in SBP was not significantly different from those without (P=0.108). In agreement with our results Viallon et al. found that CRP was less sensitive (62%) to diagnose SBP in cirrhotic patients [29], and Pieria et al. found CRP has a weak predictive power for infection and prognosis in patients with decompensated/advanced cirrhosis especially in the intensive care setting [30]. On the contrary, Papp et al. stated that the best marker for infection in cirrhotic patients was CRP with the best accuracy detected at 9.2 mg/L (sensitivity 88.1% and specificity 87.8%) [31], and Papp et al. who found that CRP was significantly higher in patient with SBP than in non-infected ascites (p<0.001) [32]. In cirrhotic patients, the basal level of CRP is elevated due to chronic hepatic inflammation, however, the analytical power of CRP for infection and prognosis is weak in patients with decompensated/advanced cirrhosis. Nevertheless, persistently elevated CRP levels can help recognize patients with a higher short term risk of mortality [30].

In this study, ascitic HGF was not associated with CRP. That may be explained by Guille´n et al. who showed that HGF had no effect on C-reactive protein synthesis when they studied its role as regulator of synthesis of acute-phase proteins in human hepatocytes [33]. However, this was contrary to many studies which found a significant positive correlation between them in many infections e.g in pneumonia including being a therapeutic predictor [16], in acute Brucellosis in which both of them were significantly correlated and significantly declined after treatment (r: 0.922; p<0.001)[34], in acute graft versus host disease (P=0.0056)[35].

In this study, ascitic HGF had a significant positive correlation with both transaminases; ALT levels (r=0.359, P=0.023) and AST (r=0.423, p=0.007) in SBP. That particular point was not studied before for the previous studies evaluated the serum HGF and in the setting of liver cirrhosis per sue e.g a significant positive correlation between serum HGF and transaminases i.e AST and ALT levels (r=0.653, P=0.000 and r=0.458, P=0.000 respectively [36], a positive correlation with AST (P=0.0057) but not ALT [34], or not at all [37].

In this study, a significant positive correlation was found between ascitic HGF and ascitic (r=0.372, P<0.018) which supports our hypothesis that HGF significantly increases in bacterial infections. However, conflicting results were found in other studies e.g in acute meningitis, Nayeri et al., 2000 found a positive correlation between CSF-HGF and CSF white blood cell counts [25], while Ozden et al. [26] did not find this.

In this study, only 1/5 of the SBP ascitic cultures were positive. This figure was close to many previous studies which reported low rate of culture positivity [38-41] (25%, 25.6%, 28.5% and 30% respectively). Reduced rate of culture positivity might be attributed to the copious

use of prophylactic antibiotics and to the progressively earlier diagnosis of SBP, as well as to the low bacteria population (1-2 bacteria/mL) in ascitic fluid [18]. However, others documented higher prevalence [42-44] (39%, 40% and 50% respectively).

The microbiological spectrum of SBP patients in this study was mainly (6/8; 75%). Other studies reported the same finding, where most common Gram-negative bacteria causing SBP were community-acquired [45,46], but with less prevalence i.e 58.2% [46], and 60% [47]. In this study, Pseudomonas spp (3/6; 50%) was the commonest, followed by Klebsiella spp.(2/6; 33.3%) and Proteus spp (1/6; 16.7%). Li et al.; *E. coli* (24.2%), *Klebsiella pneumoniae* (18.9%)[46], similarly Haider et al. reported different prevalence of bacteria i.e *E.coli* 30%, Klebsiella spp 14%, and each of Enterobacter spp and Pseudomonas. spp was 4% [47] and *E. coli* (58.3%) was the commonest bacteria amongst the GNB followed by Enterobacer spp. (25%), Pseudomonas spp (8.3%) and Klebsiella spp. (8.3%) [39].

On the other hand, quarter of the SBP positive cultures (2/8; 25%) were Gram positive organisms. This was consistent with other studies 24% [47], and 27.8% [46]. On the contrary, Gou et al., reported the predominant Gram-positive pathogens in ascites of SBP patients [48,49]. Similarly, 57.1%, 57%, 55.7%, 55%, and 47.8% [39,50,51,41,52] respectively. The last one had that figure versus 44.9% Gram-negative bacteria due to high fungal SBP (7.2%) [52].

In this study, the Gram positive cultures were equally Strept. pneumoniae and MRSA (50%). That was different from other studies e.g Enterococcus spp. (56.2%) was the commonest followed by S. aureus (43.8%)[39], coagulase-negative Staphylococcus 27%, Enterococcus 24%) [49], MRSA was the most common isolate (77%) [50], and Streptococcus spp was the most prevalent organisms [41].

It is worth noting that marked changes in the causative bacteria of SBP and risky changes in their antibiotic resistance profiles took place. In particular, the potential emergence of Enterococci, methicillin-resistant *S. aureus* (MRSA), or fluoroquinolone-resistant bacteria [53]. This may be explained by increasing antibiotic prophylaxis, numerous invasive techniques and contact with the hospital environment [41].

Conclusion

HGF could be used as minimally invasive biomarker for early detection of SBP in cirrhotic patients. The sensitivity and specificity of HGF for SBP were 80% and 82.5%, respectively at a cut-off value of 2981.34 pg/ml.

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