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ORIGINAL ARTICLE

Ascitic Hepatocyte Growth Factor as a marker for Diagnosis of Spontaneous Bacterial Peritonitis in Cirrhotic Patients

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ABSTRACT

Background: *Spontaneous bacterial peritonitis (SBP) is a common and potentially lethal complication of decompensated cirrhosis. Hepatocyte growth factor (HGF) is a protein produced by mesenchymal cells, and regulated by different physiological and pathological conditions e.g pregnancy, aging and infections.* ***Objective:*** *This study evaluated ascitic HGF as a local marker in diagnosis of SBP.* ***Methodology:*** *Eighty cirrhotic ascitic patients were divided equally into 40 patients with SBP and 40 without. All involved patients were subjected to assay of ascitic HGF using ELISA technique.* ***Results:*** *Ascitic HGF was significantly related to SBP (P=0.000). The sensitivity and specificity of HGF for differentiating SBP were 80 and 82.5%, respectively, at a cut-off value of 2981.34 pg/ml. There was a significant positive correlation between ascitic HGF and TLC (P=0.018), and transaminases; AST and ALT (P =0.007 and P=0.023 respectively).* ***Conclusion:*** *Ascitic HGF is a reliable minimally invasive biomarker for diagnosis of SBP in cirrhotic patients.*

INTRODUCTION

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

Spontaneous Bacterial Peritonitis (SBP) is an infection of the previously sterile ascitic fluid not preceded by a history of intra-abdominal septic focus or visceral perforation[2]. 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 [3].

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 induces SBP [4,5]. The microorganisms more commonly isolated from cases of SBP are *E.coli* (around 70%), *Klebsiella* species (around 10%), *Proteus* species, *Enterococcus faecalis* (around 4% each), *Pseudomonas* species (around 2%) and others (around 6%) [6].

Hepatocyte growth factor (HGF)/scatter factor was primarily recognized in 1984 and molecularly cloned as a potent mitogen of primary cultured hepatocytes [7]. 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 HG [8]. Interaction of the active HGF with its specific receptor prompts an intracellular signal pathways ensuing in regeneration and repair of damaged tissue [9].

High HGF has been detected systemically during infectious injuries[10], such as sepsis [11], pneumonia [12], gastroenteritis, skin and soft tissue infections and pyelonephritis. Similarly, in cerebrospinal fluid during meningitis [11].

Despite performing diagnostic paracentesis; ascitic fluid culture is negative in at least 40% of cases with an elevated polymorphs neuclear leucocytes (PMN) count [13]. Thus, detection of high amounts of cytokines during inflammatory diseases is not a distinctive finding. However, determination of HGF in plasma/ serum and urine proved to be a sensitive method that could detect specific clinical problems [14].

The aim of the present work is to evaluate the role of ascitic HGF in the diagnosis of SBP in cirrhotic patients via a) correlating the level of HGF in ascitic fluid of cirrhotic patients with the laboratory features of SBP, b) identifying the cutoff value of HGF in the ascitic 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 METHODOLOGY

The present study was conducted from September 2016 to January 2017 in the Medical Microbiology and Immunology Department Laboratories, Cairo University. Eighty cirrhotic ascitic patients were recruited from Tropical Medicine Department, Faculty

of Medicine, Cairo University (Kasr AlAiny Hospital) and classified into 2 equal groups: forty patients with SBP, and 40 patients without SBP who served as control group.

The approval of Tropical Medicine Department as well as informed consents 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:

a. 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)**.**
b. Laboratory criteria: i. Worsening of liver function, ii. Absolute neutrophil count of ≥250 cells/ mm^3 in the ascitic fluid in the absence of an intra-abdominal source of infection [4] regardless the ascitic fluid culture.

Exclusion criteria:

- Non cirrhotic ascites e.g heart failure, renal failure, …etc.
- Cirrhotic patients without ascites.
- Secondary peritonitis diagnosed by absolute neutrophil count of ≥250 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:* [A volume of 5 ml ascetic fluid was collected in a sterile container. Ascitic fluid was centrifuged in the laboratory for 3 min at 5000 rpm.by (NÜVE NF 815 CENTRIFUGAL (8x15 mL), Turkey). A direct Gram stained smear of the specimen was examined then processed as follows: Direct inoculation onto two sets of each of three agar plates (chocolate agar, blood agar, Mac Conkey agar). The first set was incubated aerobically at 37°C for 24-48 hours. The second set was incubated anaerobically in gaspak system for 4-5 days and were examined daily for growth. Indirect inoculation in thioglycolate broth and examined daily for turbidity. Then subcultures from the broth were done on three agar plates as mentioned before and incubated both aerobically and anaerobically. Further processing as was determined by Gram staining and colony morphology then, necessary biochemical tests were performed. In case *staph auruas* (Coagulase +ve and yellow colonies on MSA) was recovered then subculture on Oxacillin Resistant Screening Agar Base (ORSAB) to detect if it is methicillin resistant *Staph auruas* (MRSA) by the growth of blue colonies[15]**.**
   c. **Detection of hepatocyte growth factor level using quantitative ELISA**. (Quantikine ELISA Catalog Number DHG00; R&D Systems Inc., Minneapolis, Minnesota, USA). One ml volume of ascitic fluid was centrifuged at 3000 rpm for 5 minutes and supernatant was stored at -70^0c till processing. Quantitative detection of HGF was determined in both groups using a sandwich ELISA kit 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.
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 ascetic 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, Cairo University. Eighty cirrhotic ascitic inpatients were equally divided into 2 groups; forty patients with SBP which constituted 25 males (62.5%) and 15 females (37.5%) with mean age 53± 10 years, and 40 patients without SBP (control group) which constituted 16 males (40%) and 24 females (60%) with mean age 44± 12 years. Comparison between SBP and control groups in terms of the main laboratory tests including the ascitic HGF is shown in table (1).

Table 1: The main laboratory tests of the studied patients

Laboratory test			SBP	Control	*P* value
Complete blood count		Hb	10.2 ± 1.6	9.14± 1.5	0.48
		WBC (x 10^3)	9.82 ± 4.18	7.59 ± 3.5	0.29
Liver biochemical profile	Transaminases	AST	42.25 ± 3.2	60.57 ± 6.4	0.198
		ALT	71.72 ± 5.1	40.4 ± 3.7	0.095
	International normalized ratio	INR	1.23 ± 0.07	1.6 ± 0.09	0.667
C-reactive protein (CRP) (mg/l)			17.12	10.20	0.108
Ascitic fluid analysis		TLC	2440.75 ± 479	90.35 ± 11.5	**0.001**
		HGF (pg/ml)	8346. 4354± 805.18	2417.6454 ±157.25	**0.000**

SBP= Spontaneous bacterial peritonitis, Hb=Haemoglobin, WBC= White blood cells, AST=Aspartate aminotransferase, ALT=Alanine aminotransferase, INR=International normalized ratio, TLC=Total leucocytic count, HGF=Hepatocyte growth factor

Receiver Operator Characteristic (ROC) curve was done to determine the best cut off limit of HGF level to diagnose SBP as illustrated in fig. 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.

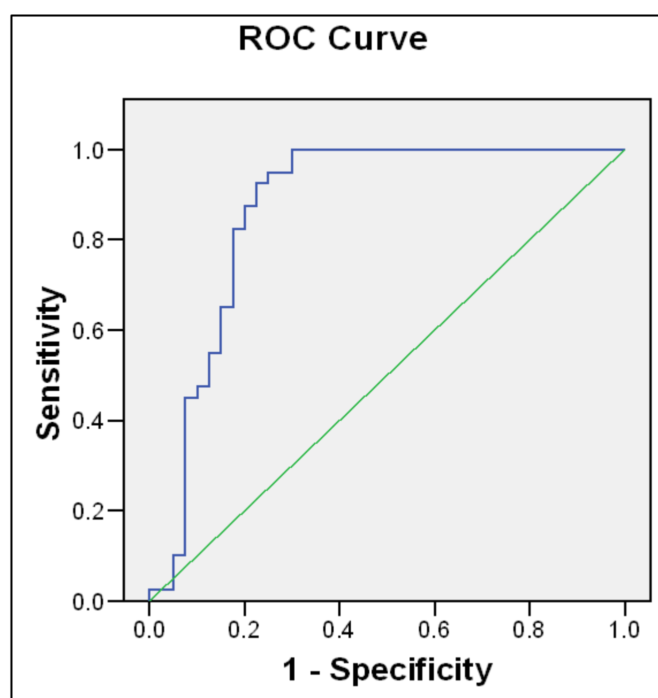

Fig. 1: ROC analysis of HGF level in diagnosis of SBP

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).

Table 2: Ascitic fluid microbiologic cultures of SBP patients

Microbiologic cultures of SBP number (percent)		
Culture		**Organisms detected**
Negative 32 (80)		
Positive 8 (20)	Gram negative 6 (75)	*Pseudomonas* 3 (37.5/ 50) *Klebsiella* 2 (25/ 33.3) *Proteus* 1 (12.5/ 16.7)
	Gram positive 2 (25)	*Strept. pneumonia* 1 (12.5/ 50) *MRSA* 1 (12.5/ 50)

SBP=Spontaneous bacterial peritonitis, MRSA=Methicillin resistant *Staph. 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.

Table (3): Correlation between ascitic HGF and the main laboratory findings

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

HGF=Hepatocyte growth factor, r= Spearman correlation coefficient

DISCUSSION

Cirrhotic patients with ascites are predominantly vulnerable to SBP due to altered gut permeability, suppression of the reticuloendothelial system and bacterial overgrowth [16].

Hepatocyte growth factor (HGF) is a pleiotropic cytokine. High levels of HGF are produced both locally and systemically in case of injuries induced by infections [17], moreover, it plays as a prognostic factor in inflammatory diseases [18].

In the present work, the 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 including the tuberculous meningitis [19], and in faeces in infectious diarrhea (*P* <0.0001) [20].

In this study, CRP in SBP was not significantly different from those without SBP. In agreement with our results Viallon et al. [21] who found that CRP was less sensitive (62%) to diagnose SBP in cirrhotic patients, and Pieria et al. [22] found CRP has a weak predictive index for infection and prognosis in patients with decompensated/advanced cirrhosis. On the contrary, Papp et al. [23] 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%), and Preto-Zamperlini et al. [24] who found that CRP was significantly higher in patient with SBP than in non-infected ascites (p<0.001). 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 [22].

In this study, ascitic HGF was not associated with CRP. That may be explained by Guillén et al. [25] who showed that hepatocyte synthesis of CRP is independent from HGF during acute-phase reactions. 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 [12], in acute Brucellosis in which both of them were significantly correlated and significantly declined after treatment [26].

In this study, ascitic HGF had a significant positive correlation with both transaminases in SBP. That partially agreed with previous studies e.g a significant positive correlation between serum HGF and transaminases [27], a positive correlation with AST but not ALT 26, or not at all [28].

In our study, a significant positive correlation was found between ascitic HGF and ascitic TLC 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., found a positive correlation between CSF-HGF and CSF white blood cell counts [29], while Özden et al. [19] did not find this.

In this study, only 1/5 of the SBP ascitic cultures were positive. This figure was close to the range reported before i.e 25% [30] and 30% [31] (25%, 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 [13]. However, others documented higher prevalence e.g 40% [32] and 50% [33].

The microbiological spectrum of SBP patients in our study was mainly (75%) gram negative bacteria (GNB). Similarly, Li et al found GNB the most common causing SBP were community-acquired 34, but with less prevalence in different studies [35,36]. In this study, *Pseudomonas spp* (50%) was the commonest, followed by *Klebsiella* spp. (33.3%) and *Proteus* spp (16.7%). This was different from many other studies that found E. coli the most common pathogen e.g Khalil et al. *E. coli* (58.3%) followed by *Enterobacer* spp. (25%), *Pseudomonas* spp (8.3%) and *Klebsiella* spp. (8.3%)[37], Haider et al., reported E.coli 30%, *Klebsiella* spp 14%, and each of *Enterobacter* spp and *Pseudomonas.* spp was 4% [35], and Li et al,; *E. coli* (24.2%), *Klebsiella pneumoniae* (18.9%) [34].

On the other hand, quarter of the SBP positive cultures (25%) were Gram positive organisms. This was consistent with other studies 24% and 27.8% [38,26] respectively. On the contrary, Gou et al., reported the predominant gram-positive pathogens in ascites of SBP patients 36. Similarly, 57.1%, 55%, and 47.8% [37, 31, 39] respectively. The last one had that figure versus 44.9% GNB due to high fungal SBP (7.2%) [39].

In our 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%)[37], Methicillin resistant Staph. aureus (MRSA) was the most common isolate (77%)[40], and *Streptococcus* spp was the most prevalent organisms[31].

It is worth noting that marked changes in the causative pathogenic organisms of SBP and risky changes in their antibiotic resistance profiles took place. This may be explained by increasing antibiotic prophylaxis, numerous invasive techniques and contact with the hospital environment [31].

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.

Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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