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Original Article

The diagnostic value of neutrophil gelatinase-associated lipocalin and hepcidin in bacteria translocation of liver cirrhosis

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Abstract: Objective: Bacterial translocation (BT) or bacterial DNA (bactDNA) translocation is a critical pathogenesis mechanism of spontaneous bacterial peritonitis. Studies of BT or bactDNA translocation are limited in humans. Neutrophil gelatinase associated lipocalin (NGAL) can efficiently distinguish bacterial and nonbacterial ascites in ascitic patients. Hepcidin is a useful marker of bacterial infection in the late-onset sepsis. However, the relationship between NGAL, hepcidin and BT was still unclear. In present study, the levels of NGAL, hepcidin and their relationship with BT or bactDNA translocation were investigated. Material and methods: Weekly doses of carbon tetrachloride (CCl4) were given to induce liver cirrhosis in Sprague-Dawley rats. Trypticase (blood) soy agars were used to culture bacteria. BactDNA was sequenced by ABIPRISM 310 automated sequencer. The levels of NGAL and hepcidin were assessed by ELISA. Receiver operating characteristic (ROC) curve was used to determine the cut-off values and compare the diagnostic performance of NGAL and hepcidin. Results: 56 cirrhotic and 10 normal rats were included in this study. The levels of both two biomarkers were significantly higher in BT or bactDNA translocation group compared to non-translocation group. The area under ROC curve for the diagnosis of BT was 0.910 for serum NGAL, 0.858 for serum hepcidin and 0.940 for their combination, whereas that for the diagnosis of bactDNA translocation was 0.906 for NGAL, 0.779 for hepcidin and 0.950 for their combination, respectively. The combination of NGAL and hepcidin improved the ability to detect BT or bactDNA presence in MLNs and ascites. Conclusion: BT and the presence of bactDNA in MLNs were observed in a rat cirrhotic model. Serum NGAL and hepcidin can serve as sensitive and specific tests for diagnosis of BT or bactDNA translocation. NGAL in combination with hepcidin can improve the accuracy of diagnosis.

Keywords: Bacterial translocation, bacterial DNA translocation, liver cirrhosis, neutrophil gelatinase associated lipocalin, hepcidin, ROC curve

Introduction

Bacterial infections are prevalent complications in liver cirrhotic patients, with an incidence of about 34% during hospitalization [1]. Spontaneous bacterial peritonitis (SBP), urinary tract infections and pneumonia are the most common infections in cirrhotic patients, and 80% of them are caused by Gram-negative bacilli, especially Escherichia coli. These data indicate that majority of the infection in patients with cirrhosis are enteric origin (reviewed in 2). SBP affects 10-30% of hospitalized patients with cirrhosis and ascites [3]. Bacterial translocation (BT), which is a critical development mechanism of SBP, is defined as viable bacteria from the intestinal lumen passing through the intestinal wall to mesenteric lymph nodes (MLNs) and other organs [4]. BT occurs in 25-30% of patients with cirrhosis and liver dysfunction and about 58% of rats with ascitic cirrhosis [5, 6]. However, in humans, studies of BT are limited because of the requirement for surgery and the removal of MLN at improper conditions. There have been several indicators such as lipopolysaccharide, lipopolysaccharide bind-

ing protein to diagnose BT. However, both of them have some drawbacks to be applied as perfect biomarkers. Recent years, detection of bactDNA in biologic fluid has been thought to be a surrogate biomarker of BT in patients with advanced liver cirrhosis [2]. The presence of bactDNA in culture-negative MLNs in an animal model of cirrhosis was considered as bactDNA translocation [7]. However, detection of bactDNA does not mean the presence of invaded viable bacteria. For the translocation of viable bacteria in cirrhotic patients was associated with the pathogenesis of portal hypertension and hepatorenal syndrome, whereas presence of bactDNA is associated with aggravation of peripheral vasodilation and worsening of intrahepatic endothelial dysfunction [7, 8]. Therefore the clinical consequences of bactDNA presence may be different compared with the presence of viable bacteria. Detection of bactDNA or viable bacteria in MLNs may have some clinical significance.

Neutrophil gelatinase associated lipocalin (NGAL) is a novel 24 KDA glycoprotein released by human neutrophil granules and is able to bind iron-laden siderophores of different invading bacteria [9]. Animal studies have demonstrated that NGAL-deficient mice exhibit increased susceptibility to Escherichia coli infection because of deficiency of NGAL-sequestered iron [10]. Serum and urinary level of NGAL have been measured in human studies, and its level is a useful biomarker to distinguish acute bacterial from viral infection or controls [11, 12]. Furthermore, a recent report has shown that NGAL in peritoneal fluids can efficiently identify bacterial ascites in ascitic patients [13]. Urinay and cerebrospinal fluid NGAL also can recognize patients with acute bacterial infection [14, 15]. Since BT includes the process of bacteria invasion from intestinal lumen to blood or lymphocyte, NGAL may be upregulated after encountering invasion bacteria. Therefore, it would be a good biomarker for BT in patients with advanced liver cirrhosis. Trude H. Flo et al demonstrated that in vivo, LPS induces a 200-fold increase in NGAL messenger RNA transcription and a 20-fold increase in protein concentration in a TLR4-dependent manner, whereas bacterial CpG-DNA induced only a 34.9 fold increase in NGAL mRNA of mouse macrophages [16, 17]. Therefore, NGAL may distinguish the differences between the presence of bacteria or bactDNA and absence of them.

Hepcidin, a 25-amino acid peptide produced by hepatocytes and macrophages which can bind the iron exchange molecule ferroportin and result in its degradation, is released into the circulation after generation [13]. The main role of hepcidin is to maintain iron homeostasis by decreasing iron absorption from the duodenum and iron release from liver or macrophages. Hepcidin concentration, which is increased during infections and inflammatory situations, can decrease the amount of iron available in the peripheral circulation. This mechanism aims to limit the overgrowth of iron-requiring potential pathogens such as bacteria in acute infections through the decrease of circulating iron [18]. Serum hepcidin has been identified as a useful marker of infection in the evaluation of late-onset sepsis in very low birth weight infants and its concentration was much higher in children with infection than that of healthy control [19, 20]. In humans injected with LPS, hepcidin was demonstrated to dramatically secret into urine [18]. A recent report also pointed out that hepcidin is present in serum and ascitic fluids [21]. Macrophages treated with Pam3CSK4 and LPS, ligands respective to TLR2 and TLR4, significantly increased hepcidin expression [22]. Therefore, after BT, hepcidin in biological fluid would be up regulated and be detectable while encountering invaded bacteria or bactDNA. It would be another effective biomarker for the diagnosis of BT or bactDNA translocation and the level of hepcidin would be different between the presence of bacteria or bactDNA and absence of them.

Both NGAL and hepcidin influence the iron metabolism of bacteria through different mechanism. Therefore, combination use these two indicators may be a more powerful diagnosis tool for BT or bactDNA translocation. In present study, we investigated the level of NGAL, hepcidin and their relationship with BT or bactDNA translocation in a liver cirrhosis rat model. Whether NGAL and hepcidin could identify BT or bactDNA translocation was further examined. We still determined whether ascitic NGAL and hepcidin could distinguish bacterial peritonitis from ascites without bacterial infection. The relationship between the level of NGAL, hepcidin and bactDNA presence in ascites was also investigated.

Material and methods

Animal

Male Sprague-Dawley rats were obtained from *experimental animal center of Wuhan University* (*Wuhan*, China). Rats were raised in cages under a constant room temperature of 21°C and exposed to a reversed 12 h light-dark cycle from 7 am to 7 pm.

Induction of cirrhosis

Male Sprague-Dawley rats weighing from 100 to 120 g were fed with 1.5 m mol/L phenobarbital in tap water for about 10-14 days [23]. All of them were raised with standard rodent chow until their weight increased to 200 g [23]. Weekly doses of CCl4 were intragastrically injected using a sterile pyrogen free syringe with an attached stainless steel lavage needle without anesthesia [23]. The first dose of CCl4 was 20 μl, and subsequent doses were regulated based on changes in weight 48 hours after the last dose [23]. After ascites appeared, the dose was cut down to 20 ul per week and it was increased if ascites resolved. After 12-16 weeks of induction, the cirrhosis model was successfully established [23].

Laparotomy

Rats with induced liver cirrhosis were killed when ascites was obvious. Laparotomy was carried out under anesthesia with 10 mg/kg xylazine and 50 mg/kg ketamine in completely sterile conditions. Diagnostic centesis were performed when ascites became massive enough for drainage. After abdominal wall depilatory preparation and iodine sterilization, paracentesis were performed with sterile technique. Then, the abdomen was incised widely and the remaining ascites was emptied. All detectable mesenteric lymph nodes (MLNs), especially those from the ileo-cecal area, sterilely separated, removed, and then liquidized in aseptic saline for bacterial culture. An aliquot of liquidized MLNs was stored into aseptic Eppendorf pipes for bactDNA study. Blood was gathered from the cava vein and injected in pyrogen-free centrifugal tubes. Rats were then killed with intravenous injection of sodium thiopental. Control rats were killed following the same protocol. This study was approved by the Animal Research Committee of Huazhong University of Science and Technology. Animals received care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals.

MLNs isolation and culture

Rats were killed and placed in the anaerobic glove box. The middle MLNs were excised under sterile conditions and transferred to grinding tubes which contained trypticase soy broth in the anaerobic glove box. The MLNs were liquefied with Teflon grinders and incubated at 37°C for at least 3 days. After incubation, the MLNs homogenates were stained with Gram stain, and 0.1 ml of them was spread with sterile glass L-rods onto enriched trypticase soy agar plates which were able to support the growth of a wide variety of strictly anaerobic bacteria. These plates were also incubated in the anaerobic glove box at 37°C for at least 3 days.

Rats were killed and their MLNs were transferred to grinding tubes containing sterile culture medium to detect aerobic bacteria. The MLNs were homogenized, and these tubes were incubated aerobically in 10% carbon dioxide at 37°C at least 3 days. After incubation, 0.1 ml of the MLNs homogenate was spread onto blood agar plates to detect aerobic bacteria. The organ homogenates also were stained with Gram stain to confirm bacteria present in the homogenates grew on the agar plates.

Detection and identification of bactDNA in rat models and liver cirrhotic patients

DNA isolation: 10 ml cirrhotic patients' ascites were centrifuged and the pellet was resuspended in 200 μl of supernatant. These 200 μl supernatants and serum, ascites and MLNs homogenate from rats were used for DNA extraction with the Bacteria Genomic DNA Preparation Kit according to the manufacturer's instructions (TIANGEN, Beijing, China). Samples were centrifuged and DNA was finally eluted with 50 μl water. The yield and purity of DNA were examined by reading A260 and A260/A280 in a DR 2800 Spectrophotometer.

DNA amplification and electrophoresis: A polymerase chain reaction (PCR) for the universal amplification of a region of the 16S rRNA gene was employed. PCR was performed using broad-range primers (5'-AGAGTTTGATCATGGCTCAG-3' and 5'-ACCGCGACTGCTGCTGGCAC-3') [24]. A 35-cycle PCR was run in a S1000 Thermal Cycler (Bio-Rad, USA) using the following program: 94°C 30 seconds, 55°C 30 sec-

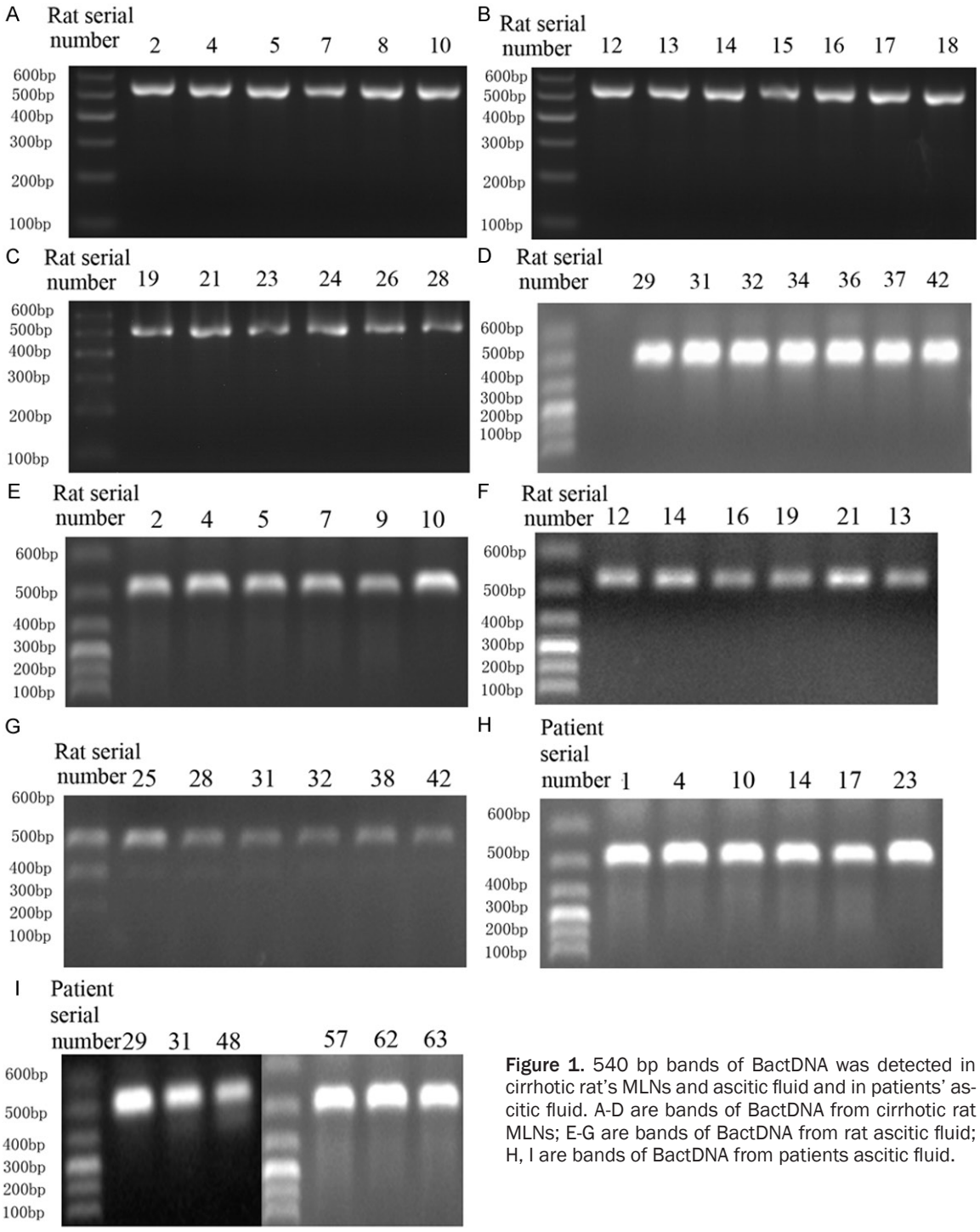

Figure 1. 540 bp bands of BactDNA was detected in cirrhotic rat's MLNs and ascitic fluid and in patients' ascitic fluid. A-D are bands of BactDNA from cirrhotic rat MLNs; E-G are bands of BactDNA from rat ascitic fluid; H, I are bands of BactDNA from patients ascitic fluid.

onds, and 72°C 60 seconds. 5 μl products were examined by 2% agarose gel electrophoresis and UV visualization. A 540 bp band would be obtained from different bacterial cultures as expected.

DNA sequencing: Nucleotide sequences of PCR products were determined by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and ABIPRISM 310 automated sequencer according to the manufacturer's instructions. The PCR products were sequenced with primer 5'-ACCGCGACTGCTGCTGGCAC-3' [25]. The sequences identification was carried out by BLAST at www.ncbi.nlm.nih.gov. Then the nucleotide sequences of bacteria in MLN

Rat serial number	Sequence of bacteria in BT'MLNs	Sequence of bacteria in cultured BT's MLNs	Sequence of bacteria in BT's ascitic fluid	Bacterial type
2	……cagatgtgaa atccccgggc tcaacctggg aactgcatct ……	……cagatgtgaa atccccgggc tcaacctggg aactgcatct ……	……cagatgtgaa atccccgggc tcaacctggg aactgcatct ……	Escherichia coli
4	……gctgatgtgc gaaagcgtgg ggatcaaaca ggattagata……	……gctgatgtgc gaaagcgtgg ggatcaaaca ggattagata……	……gctgatgtgc gaaagcgtgg ggatcaaaca ggattagata……	Staphylococcus aureus
5	……agacacggcc cagactccta cgggaggcag cagtagggaa……	……agacacggcc cagactccta cgggaggcag cagtagggaa……	……agacacggcc cagactccta cgggaggcag cagtagggaa……	Enterococcus faecalis
7	……gacgaagact gacgctcagg tgcgaaagcg tggggagcaa……	……gacgaagact gacgctcagg tgcgaaagcg tggggagcaa……	……gacgaagact gacgctcagg tgcgaaagcg tggggagcaa……	Escherichia coli
10	……gttttgattt gaaaggcgct ttcgggtgtc gctgatggat……	……gttttgattt gaaaggcgct ttcgggtgtc gctgatggat……	……gttttgattt gaaaggcgct ttcgggtgtc gctgatggat……	Enterococcus faecium
14	……ataccctggt agtccacgcc gtaaacgatg tcgacttgga……	……ataccctggt agtccacgcc gtaaacgatg tcgacttgga……	……ataccctggt agtccacgcc gtaaacgatg tcgacttgga……	Escherichia coli
16	……gatgcgtagc cgacctgaga gggtgatcgg ccacactgga……	……gatgcgtagc cgacctgaga gggtgatcgg ccacactgga……	……gatgcgtagc cgacctgaga gggtgatcgg ccacactgga……	Staphylococcus epidermidis
21	……ggcgacgatc cctagctggt ctgagaggat gaccagccac……	……ggcgacgatc cctagctggt ctgagaggat gaccagccac……	……ggcgacgatc cctagctggt ctgagaggat gaccagccac……	Escherichia coli
23	……tgcgaaagcg tggggatcaa acaggattag ataccctggt……	……tgcgaaagcg tggggatcaa acaggattag ataccctggt……	……tgcgaaagcg tggggatcaa acaggattag ataccctggt……	Staphylococcus aureus
28	……caaggcaacg atgcatagcc gacctgagag ggtgatcggc……	……caaggcaacg atgcatagcc gacctgagag ggtgatcggc……	……caaggcaacg atgcatagcc gacctgagag ggtgatcggc……	Staphylococcus aureus
32	……tactgggcgt aaagcgcacg caggcggcaa ttaagtcaga……	……tactgggcgt aaagcgcacg caggcggcaa ttaagtcaga……	……tactgggcgt aaagcgcacg caggcggcaa ttaagtcaga……	Proteus vulgaris
42	……gtcccgcaac gagcgcaacc cttattgtta gttgccatca……	……gtcccgcaac gagcgcaacc cttattgtta gttgccatca……	……gtcccgcaac gagcgcaacc cttattgtta gttgccatca……	Enterococcus faecium

Figure 2. Results of bactDNA sequence detection and bacterial type identification through Nucleotide Sequencing.

Table 1. Concentrations of neutrophil gelatinase-associated lipocalin (NGAL) and hepcidin in serum of liver cirrhotic animal model and controls

	Control	Non-BT group	BT group	BactDNA negative group	BactDNA translocation group
Serum NGAL	77.6±39.4	101.0±45.8	205.5±81.5*	87.2±43.0	170.5±65.5*
Serum hepcidin	10.2±3.1	15.9±8.1	31.4±12.3*	15.1±9.3	24.2±10.7*

*P < 0.05 BT or bactDNA translocation group vs. control or non-BT or bactDNA negative group.

and ascites were compared to determine whether they were the same bacterium.

Detection of NGAL and hepcidin in blood and ascites of rat models or ascites of patients

Rats' blood and ascitic samples were obtained for determination of NGAL, hepcidin concentration. Patient's paracentesis was performed and samples of the ascites were obtained for determination of hepcidin and NGAL concentrations. Quantitative measurements of ascitic fluid hepcidin and NGAL concentrations were determined using a polyclonal antibody-based enzyme-linked immunosorbent assay specific for rat and human hepcidin (Eiaab Science Co.

Table 2. Diagnostic performance of serum NGAL, hepcidin or their combination in liver cirrhotic animal model

		Area under curve	*P*-value	Sensitivity	Specificity
Viable bacteria	Serum NGAL (> 158 ng/ml)	0.910 (95% CI, 0.792-1.000)	0.34Δ	0.833 (95% CI, 0.365-0.991)	0.926 (95% CI, 0.742-0.987)
Presence in rat MLNs	Serum hepcidin (> 15.7 ng/ml)	0.858 (95% CI, 0.703-1.000)	0.18Δ	0.833 (95% CI, 0.365-0.991)	0.667 (95% CI, 0.460-0.828)
	Combination	0.941 (95% CI, 0.853-1.000)			
	Either test positive			1.000 (95% CI, 0.517-1.000)	0.667 (95% CI, 0.460-0.828)
	both tests positive			0.667 (95% CI, 0.241-0.940)	0.963 (95% CI, 0.791-0.998)
		Area under curve	*P*-value	Sensitivity	Specificity
BactDNA	Serum NGAL (> 97 ng/ml)	0.906 (95% CI, 0.802-1.000)	0.25Δ	1.000 (95% CI, 0.717-1.000)	0.700 (95% CI, 0.457-0.872)
Presence in rat MLNs	Serum hepcidin (> 14.3 ng/ml)	0.779 (95% CI, 0.621-0.937)	0.01Δ	1.000 (95% CI, 0.717-1.000)	0.600 (95% CI, 0.364-0.800)
	Combination	0.950 (95% CI, 0.870-1.000)			
	Either test positive			1.000 (95% CI, 0.717-1.000)	0.4 (95% CI, 0.200-0.636)
	both tests positive			1.000 (95% CI, 0.717-1.000)	0.9 (95% CI, 0.669-0.982)

The *p* value means the difference between NGAL or hepcidin alone and the combination of them.

Table 4. Diagnostic performance of neutrophil gelatinase-associated lipocalin (NGAL), hepcidin or their combination in ascites of liver cirrhotic animal model

		Area under curve	*P*-value	Sensitivity	Specificity
Bacteria	Ascitic NGAL (> 116.5 ng/ml)	0.879 (95% CI, 0.750-1.000)	0.08Δ	1.000 (0.517-1.000)	0.727 (0.496-0.884)
Presence in rat	Ascitic hepcid in (> 19.3 ng/ml)	0.883 (95% CI, 0.759-1.000)	0.09Δ	1.000 (0.517-1.000)	0.773 (0.542-0.913)
Actites	Combination	0.977 (95% CI, 0.824-1.000)			
	Either test positive			1.000 (0.517-1.000)	0.545 (0.367-0.749)
	both tests positive			1.000 (0.517-1.000)	0.955 (0.751-0.998)
		Area under curve	*P*-value	Sensitivity	Specificity
BactDNA	Ascitic NGAL (> 97 ng/ml)	0.795 (95% CI, 0.692-0.962)	0.03Δ	0.889 (95% CI, 0.567-0.994)	0.737 (95% CI, 0.486-0.899)
Presence in rat	Ascitic hepcidin (> 14.30 ng/ml)	0.804 (95% CI, 0.644-0.964)	0.02Δ	1.000 (95% CI, 0.629-1.000)	0.684 (95% CI, 0.435-0.864)
Ascites	Combination	0.956 (95% CI, 0.881-1.000)			
	Either test positive			1.000 (95% CI, 0.629-1.000)	0.474 (95% CI, 0.252-0.705)
	both tests positive			0.889 (95% CI, 0.507-0.994)	0.947 (95% CI, 0.719-0.997)

ΔThe *p* value means the difference between NGAL or hepcidin alone and the combination of them.

Table 3. Concentrations of NGAL and hepcidin in ascites of liver cirrhotic animal model

	Bacteria negative group	Bacteria positive group	BactDNA negative group	BactDNA positive group
Ascites NGAL in rats	103.9±43.4	172.2±35.6Δ	103.2±46.6	150.9±43.2Δ
Ascites hepcidin in rats	15.3±5.7	25.0±7.6Δ	13.1±5.2	22.4±6.7Δ
Ascites NGAL in patients			100.32±68.7	219.2±123.9Δ
Ascites hepcidin in patients			21.9±6.7	48.8±39.9Δ

ΔP < 0.05 bacteria or bactDNA positive group vs. bacteria or bactDNA negative group.

Ltd, china) and NGAL (BioPorto Diagnostics A/S, Denmark). The present study was approved by the Ethics Committees of Huazhong University of Science and Technology.

Statistical analysis

Quantitative variables were analyzed by Student's t-test and Mann-Whitney test and qualitative variables were compared by Chi-squared test and Fisher test. Results were expressed as mean ± standard deviation or frequencies. An ROC curve analysis was performed to determine the optimal cutoff value of NGAL and hepcidin. Multiple logistic regression was used to evaluate the performance of these biomarkers in combination as one predictor. *P*-values < 0.05 were considered statistically significant. SPSS15.0 was used to perform all analyses.

Results

BT and bactDNA translocation in rat model

120 rats were included in this study. 54 rats died during the induction period of cirrhosis, and 56 other animals were successfully induced to cirrhosis with ascite. 10 rats were fed with normal food and used as controls. BT was observed in 12 of 56 cirrhotic rats with ascites (21.4%) but in none of control ones. Gram-positive cocci were detected in 6 of 12 culture-positive MLNs (50%), while gram-negative bacteria were found in the remaining 6 samples (50%). BactDNA was detected in all MLNs with positive cultures, and there was a 100% match in sequence of the bacteria type identified in MLN and its culture (**Figures 1**, **2**).

BactDNA was identified in the MLNs of 26 rats, 12 being from culture-positive MLNs and the remaining 14 from culture-negative MLNs. BactDNA was detected in 14 of 44 rats with culture-negative MLNs (31.8%). Similarly as the above result, BactDNA was also identified in 18 ascites samples of rat model, 12 being from BT rats and the remaining 6 from rats with culture-negative MLNs. In all these cases, bactDNA detected in ascitic fluids was the same as those identified in either culture-positive or negative MLNs.

Levels of NGAL and hepcidin in serum in different groups according to translocation status and their diagnostic value for diagnosing BT and bactDNA translocation

Different groups were divided according to the status of bacteria or bactDNA presence. BT group was defined as those who had culture-positive MLNs, non-BT group was defined as those who had culture-negative MLNs. BactDNA translocation group was defined as those who had bacterial DNA in MLNs, bactDNA negative group was defined as those who did not have bactDNA in MLNs. **Table 1** shows levels of NGAL and hepcidin measured in serum and ascites in groups described previously. Marked difference of the levels of NGAL and hepcidin in serum and ascites were observed between BT group and non-BT group (P < 0.05). Similar to the above results, the levels of NGAL and hepcidin in serum and ascites also had significant difference between bactDNA translocation and bactDNA negative group (P < 0.05). To further determine whether NGAL and hepcidin in serum can used as a diagnosis tool to discriminate BT group from non-BT group, the optimal cutoff value of NGAL and hepcidin in serum was determined by means of the ROC curve (**Table 2**). At a cutoff value of 158 ng/ml, NGAL had a sensitivity of 83.3% and a specificity of 92.6%. At a cutoff value of 15.7 ng/ml, hepcidin had a sensitivity of 83.3% and a specificity of 66.7%. The combination of these two indicators (both tests positive) had a sensitivity of 66.7% and a specificity of 96.3%. The area under the curve (AUC) was 0.910 for NGAL, 0.858 for hepcidin and 0.941 for their combination (both tests positive). Although the addi-

tion of NGAL to hepcidin could improve the ability to distinguish BT from non-BT, no significant difference was observed (P > 0.05). The cutoff value of NGAL and hepcidin was also determined for the diagnosis of bactDNA translocation. With a cut off value of 97 ng/ml, sensitivity and specificity were 100% and 70% for NGAL. With a cut off value of 14.3 ng/ml, sensitivity and specificity were 100% and 60% for hepcidin. The combination of these two indicators (both tests positive) had a sensitivity of 100% and a specificity of 90%. The AUC was 0.906 for NGAL, 0.779 for hepcidin and 0.950 for their combination (both tests positive). The combination of NGAL and hepcidin was more accurate than hepcidin alone (P < 0.05).

The diagnostic value of NGAL and hepcidin in bacteria or bactDNA presence in ascites of rats

The 56 rats with asites were divided into following four subgroups: viable bacteria positive group (SBP) and bacteria negative group; bactDNA positive group and bactDNA negative group. Significant differences of the levels of NGAL and hepcidin in ascites were observed between bacteria positive (SBP) and bacteria negative group, or between bactDNA positive and bactDNA negative group (as show in **Table 3**). To further determine whether ascitic NGAL and hepcidin could be used as a diagnosis tool to discriminate SBP from bacteria absence in ascites, the optimal cutoff value of NGAL and hepcidin in ascites was determined by means of the ROC curve (**Table 4**). At a cutoff value of 116.5 ng/ml, NGAL has a sensitivity of 100% and a specificity of 72.7%. At a cutoff value of 19.3 ng/ml, hepcidin has a sensitivity of 100% and a specificity of 77.3%. The combination of these two indicators (both tests positive) has a sensitivity of 100% and a specificity of 95.5%. The area under the curve (AUC) was 0.879 for NGAL, 0.833 for hepcidin and 0.977 for their combination (both tests positive). Although the combination of NGAL and hepcidin could improve the ability to discriminate SBP from bacteria absence, no significant difference was observed (P > 0.05).

Whether NGAL and hepcidin in ascites could be used as a diagnosis tool to discriminate ascites bactDNA of non-bactDNA presence was further examined. The optimal cutoff value of NGAL and hepcidin in ascites was determined by means of the ROC curve (**Table 4**). At a cutoff value of 97 ng/ml, NGAL has a sensitivity of 88.9% and a specificity of 73.7%. At a cutoff value of 14.3 ng/ml, hepcidin has a sensitivity of 100% and a specificity of 68.4%. The combination of these two indicators (both tests positive) has a sensitivity of 88.9% and a specificity of 94.7%. The area under the curve (AUC) was 0.795 for NGAL, 0.804 for hepcidin and 0.956 for their combination (both tests positive). The addition of NGAL to hepcidin could significantly improve the ability to distinguish bactDNA from non-bactDNA presence (P < 0.05).

The diagnostic value of NGAL and hepcidin in bactDNA in ascites of cirrhosis patients

Since it was difficult to obtain the human MLNs, we decided to determine the relationship between NGAL, hepcidin and bacteria in ascites of cirrhotic patients. Ascitic fluid samples were obtained from patients of liver cirrhosis and were analyzed for bacterial culture and bactDNA. All the samples were culture negative. BactDNA was detected in these ascites and bactDNA positive or negative group were divided according to DNA status. Similarly as the results in rats, we found that bactDNA was detected in 12 of 68 ascites of liver cirrhotic patients and the level of NGAL and hepcidin were significantly different between these two group (**Figure 1**, **Table 3**). To further determine whether NGAL and hepcidin in ascites can used as a diagnosis tool to discriminate ascites bactDNA of non-bactDNA presence, the optimal cutoff value of NGAL and hepcidin in ascites was determined by means of the ROC curve (**Table 4**). At a cutoff value of 99.5 ng/ml, NGAL has a sensitivity of 85.7% and a specificity of 77.8%. At a cutoff value of 45.4 ng/ml, hepcidin has a sensitivity of 85.7% and a specificity of 88.9%. The combination of these two indicators (both tests positive) has a sensitivity of 85.7% and a specificity of 100%. The area under the curve (AUC) was 0.786 for NGAL, 0.829 for hepcidin and 0.929 for their combination (both tests positive). The combination of NGAL and hepcidin was more accurate than NGAL alone (P < 0.05).

Discussion

SBP is a severe complication in patients with cirrhosis and ascites and in-hospital mortality rates may range from 20 to 30% [26]. BT is the

major mechanism of SPB and bacteremia in these patients. BactDNA translocation is connected with a marked inflammatory response in cirrhotic animal models [27]. However, the diagnostic methods for BT and bactDNA translocation are also limited because of the need for removing MLN in patients with liver cirrhosis and ascites under appropriate conditions. Therefore, it is important to develop new diagnostic tools to detect BT or bactDNA translocation in the future. Since NGAL is closely related with SBP and can be used as diagnostic tools for SBP, hepcidin is also correlated with bacteria-mediated sepsis which is caused by BT [18-20], it would be useful to use them as tools to diagnose BT in patients with cirrhosis.

Ascitic fluid NGAL and serum hepcidin have been identified as biomarkers of SBP or bacterial sepsis whereas BT is the important development mechanism of them [2, 19, 28]. We hypothesized that in cirrhotic animal model, after BT or bactDNA translocation, the viable bacteria or bactDNA in MLNs can effectively activate the expression or secretion of NGAL and hepcidin from hepatocytes, macrophages and other immune cells, therefore NGAL and hepcidin could be used as biomarkers for BT or bactDNA translocation. In our study, BT was detected in a rat liver cirrhosis model and the sequences of bacteria in MLNs before and after culture were highly consistent with each other. Marked differences of the levels of NGAL and hepcidin in serum were observed between BT group and non-BT group or between bactDNA translocation and bactDNA negative group. Serum NGAL and hepcidin could discriminate BT or bactDNA translocation from non-translocation rats. Furthermore, NGAL and hepcidin in ascites of rats and patients could also identify bactDNA presence in ascites. Similarly as the results exhibited in other study, the bactDNA sequence detected in MLNs of cirrhotic rats was the same as that presented in the ascites [27]. A combination use of these two biomarkers exhibited a better performance than that of NGAL or hepcidin alone in distinguishing ascitic bactDNA presence from bactDNA absence.

Recent reports pointed out that hepcidin was described as a liver expressed antimicrobial peptide, therefore the expression of hepcidin in serum of controls should be higher than that in liver cirrhotic animal model [21]. However, there was no significantly difference in serum hepcidin between controls and bacteria negative liver cirrhotic rats in our study. The reason for this may be that the baseline expression of hepcidin is major produced by hepatocytes, whereas mouse macrophages and neutrophils also produce hepcidin [22]. Layoun A et al indicated that macrophages treated with Pam3CSK4 and LPS, ligands respective to TLR2 and TLR4, significantly increased hepcidin expression, while TLR stimulated by bacterial nucleic acids which are TLR9 ligands had no effect on hepcidin expression using primary macrophages, murine peritoneal macrophages [22]. Our data show that bactDNA translocation also stimulate the secretion of hepcidin. This is a little inconsistent with their observations. This inconsistency may be due to the presence of dead bacterial cell walls in our bactDNA translocation group. Further study is needed to clarify this inconsistency. The combination of ascitic NGAL and hepcidin was more accurate than NGAL or hepcidin alone in identifying bactDNA presence in ascites of rats ($P < 0.05$), whereas the combination of NGAL and hepcidin was only more accurate than NGAL alone in that of liver cirrhotic patients. We don't know the exact reason for this, additional research may be needed to resolve this discrepancy. All the samples obtained from liver cirrhotic patients were culture negative, this may due to the majority patients included in this study were already subjected to antibiotic therapy. This is consistent with the European Association for the Study of the Liver clinical practice guidelines which declared that immediate empirical antibiotic treatment is needed once after the clinical diagnosis of bacterial peritonitis.

Presence of bactDNA is associated with aggravation of peripheral vasodilation and with worsening of intrahepatic endothelial dysfunction [7]. The presence of bactDNA in ascites of patients with cirrhosis is an indicator of poor prognosis and cirrhotic patients with bactDNA in ascites have a higher risk of hepatorenal syndrome, SBP and mortality [28-30]. Hence, NGAL and hepcidin can be good indicators to prognoses cirrhotic patients with ascites. This is the first time that we reported that hepcidin in ascites can predicate the presence of bactDNA. More attentions should put on the ascitic patients with higher level of NGAL and hepcidin in ascites. Since bactDNA in ascites was considered as the molecular evidence of BT [31],

ascitic NGAL and hepcidin should be further verified in large scale for their diagnostic value of bactDNA presence in ascites of cirrhotic patients in the future.

In conclusion, BT or bactDNA translocation was observed in a rat cirrhotic model and cirrhotic patients. After BT or bactDNA translocation, serum NGAL and hepcidin increased significantly in the rat cirrhotic models. Elevated level of NGAL and hepcidin can discriminate BT or bactDNA translocation from non-translocation. NGAL and hepcidin in the ascites can also identify bactDNA presence in the ascites of rats and patients. Further studies are needed to evaluate the relationship between NGAL, hepcidin and BT in cirrhotic patients.

Disclosure of conflict of interest

None.

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