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Original Article

Ascitic Fluid Lactoferrin and Proinflammtory Cytokines (TNF-α and IL-6) for the Diagnosis of Spontaneous Bacterial Peritonitis

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Purpose: Spontaneous bacterial peritonitis (SBP) is an important cause of morbidity and mortality in patients with cirrhosis and ascites. Fecal lactoferrin is a sensitive and specific marker for the differentiation of IBD and IBS and for the evaluation of disease activity of IBD. The aims of this study were to assess the utility of ascitic fluid lactoferrin concentration (AFLAC) for the diagnosis of SBP and evaluate the relationship between proinflammatory cytokines and SBP.

Method: A total of 111 ascitic fluid samples from 66 patients were included in this study. Ascitic fluid samples were obtained from hospitalized patients for determination of cell counts, cultures, and lactoferrin, TNF-α, IL-6, and HsCRP concentrations. Receiver operating characteristic (ROC) curve was used to identify a cut-off level for future development of a rapid bedside test.

Results: The percentage of SBP in ascitic fluid samples was 19.8%. The AFLAC concentration in SBP samples (mean, 51.23±34.34 ng/ml) was significantly higher than in non-SBP samples (mean, 18.44±18.98 ng/ml; P<0.001). Only the area under the curve (AUC) of lactoferrin showed promising utility (0.788, 95% confidence interval, 0.670-0.907). The cut-off point of ascitic lactoferrin was 46.07 ng/mL for distinguishing SBP and non-SBP samples.

Conclusion: AFLAC can help in the diagnosis of SBP in cirrhotic patients. Further studies using larger numbers of samples should be performed to validate the utility of ascitic lactoferrin in diagnosing SBP.

Key words: Lactoferrin, spontaneous bacterial peritonitis, liver cirrhosis, ascites, cytokines

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Introduction

Lactoferrin is a red iron-binding protein that exists mainly in external secretions (ex: breast milk, polymorphonuclear neutrophils). Due to lactoferrin's bacteriostatic properties in an iron-

depleted state, it may play an important role in the defense mechanism of mucosal surfaces.[1] Lactoferrin is released from polymorphonuclear neutrophils on activation of these cells and its presence in body fluids is proportional to the flux of neutrophils.[2, 3, 4, 5] Lactoferrin is synthesized during the transition of neutrophils from promyelocytes to myelocytes and stored in secondary granules.[21] It is believed that lactoferrin protects against enteric pathogens and contributes to the antimicrobial armory of neutrophils.[2,4,6]

Lactoferrin has been shown to be remarkably stable and resistant to degradation at room temperature.[7] Diagnosis of some digestive system diseases is challenging. A previous study has indicated that fecal lactoferrin is a sensitive and specific marker for the differentiation of IBD and IBS.[19] In addition, lactoferrin in pancreatic secretion may be a precipitate protein in stone formation in chronic pancreatitis. Serum anti-lactoferrin might contribute to the clarification of a pathogenetic mechanism of autoimmune pancreatitis and liver diseases, although its diagnostic and prognostic value appears to be limited.[20]

Spontaneous bacterial peritonitis (SBP) is an important cause of morbidity and mortality in patients with cirrhosis and ascites. In clinical practice, there is still no rapid or efficient method for diagnosing SBP. SBP is identified in 10%–30% of patients hospitalized with ascites,[8] and mortality can approach 30%.[9] The diagnosis of SBP is based on a polymorphonuclear leukocyte (PMN) count ≥250/mm.[3,22] The cell count and differential are generally performed manually, although automated cell counts may give comparable results.[11] The diagnosis of SBP may be delayed when hospital laboratory personnel are not available at off hours and in the office setting as specimens must be sent to an offsite laboratory. As PMNs in ascites degrade and lactoferrin is relatively stable, lactoferrin is an attractive marker for diagnosing inflammation based on body fluids.

In 2008, Parsi et al. assessed the utility of ascitic fluid lactoferrin for the diagnosis of SBP in a total of 218 consecutive ascitic fluid samples from 148 patients with cirrhosis.They concluded that qualitative bedside assays for the measurement of ascitic fluid lactoferrin can be easily developed and may serve as a rapid and reliable screening tool for SBP in patients with cirrhosis.[5] Relatively little research has been conducted on lactoferrin in clinical use in Asia, although many patients with liver cirrhosis present with SBP in Asia. We designed a prospective study to determine the value of ascitic fluid lactoferrin concentration (AFLAC) in the rapid diagnosis of SBP in clinical setting.

The aim of this study was to assess the value of diagnosing SBP via AFLAC and proinflammatory cytokine levels. Using receiver operating characteristic (ROC) curve analysis, a cut-off level was identified for future development of a rapid bedside test.

Material and Methods

Study design and definitions

This prospective study was conducted at a medical center, and was approved by the institutional review boards of both involved institutions with verbal consent obtained from all patients or patients' family member. Requirement for written consent was waived by the institutional review boards. Consecutive patients with ascites due to cirrhosis who fulfilled the inclusion criteria were enrolled in the study. The diagnosis of cirrhosis relied on clinical, biological, and morphologic criteria. Patients were admitted either for treatment of ascites or for complications of liver cirrhosis (ex: infection, gastrointestinal bleeding, hepatic encephalopathy, alcoholic hepatitis, acute renal failure). The diagnosis of SBP was based on PMN count equal to or greater than 250/mL in ascitic fluid, with or without positive ascitic fluid culture. Patients with SBP underwent repeat paracentesis 3 days after antibiotic therapy.

Paracentesis

Ascitic fluid was collected from inpatients with liver cirrhosis and ascites between January 1, 2010 and December 31, 2010. Ascitic fluid samples were obtained for determination of cell counts, cultures, and lactoferrin, TNF-α, IL-6, and HsCRP concentrations.[8] Total and differential

Tab 1. ***Epidemiologies of ascitic fluid samples***

	SBP(Yes)	*SBP(No)*	*P value*
Age (year)	57.55±9.24	60.46±11.22	0.213
Male (%)	14(64%)	58(65%)	0.893
Type of hepatitis			0.672
B	7(31.8%)	21(23.6%)	
C	8(36.4%)	36(40.4%)	
B+C	2(9.1%)	1(1.1%)	
nonB; nonC	5(22.7%)	31(34.8%)	
Child classification			0.924
A	0(0%)	1(1.1%)	
B	9(40.9%)	34(38.2%)	
C	13(59.1%)	54(60.7%)	
IL-6(pg/ml)	8782.21±11918.16	3034.28±2817.69	0.035*
TNF(pg/ml)	11.86±5.86	9.16±6.54	0.079
Lactoferrin (ng/ml)	51.23±34.34	18.44±18.98	0.001*
HsCRP (mg/dl)	3.90±6.27	0.98±1.25	0.041*

** Statistical significance*

cell counts were determined using an optical microscope. Bacterial cultures were obtained by bedside inoculation of 10 mL of ascitic fluid into aerobic and anaerobic bottles.[12,13,14] Quantitative measurements of AFLAC were determined using a polyclonal antibody-based enzyme-linked immunosorbent assay specific for human lactoferrin conducted by a laboratory blinded to the patients' clinical information and other laboratory results.

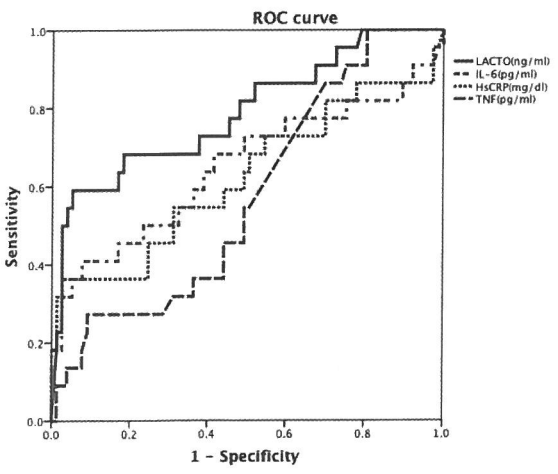

Fig.1. ROC curves for different proinflammatory cytokines and lactoferrin
LACTO: lactoferrin

Patient criteria

The inclusion criteria were hospitalized patients (1) over 18 years of age, (2) with known cirrhosis, and (3) with detectable ascites. The exclusion criteria were patients with (1) abdominal surgery within 3 months of the start of the study period, and (2) other causes of neutrocytic ascites (ex: pancreatitis, tuberculosis, appendicitis, hemorrhagic ascites, peritoneal carcinomatosis). Ascitic fluid was obtained from each patient one to three times based on clinical need to monitor SBP control.

Statistical analysis

Descriptive statistics were computed for all variables. In addition, ROC curve analysis was used to estimate potential cut-off values of AFLAC to predict SBP with optimal sensitivity and specificity. SPSS version 21 software (IBM) was used to perform all analyses.

Results

A total of 111 ascitic fluid samples from

Tab 2. ***Areas under curve for different biomarkers***

	Area under curve	95% confidence interval	
		Lower limit	Upper limit
Lactoferrin (ng/ml)	0.788	0.670	0.907
IL-6 (pg/ml)	0.648	0.495	0.801
HsCRP (mg/dl)	0.623	0.470	0.776
TNF (pg/ml)	0.571	0.441	0.701

66 patients were included in this study. The epidemiologies and results of analyses of ascitic fluid samples are shown in Tab 1. SBP was diagnosed in 22 samples (19.8%). AFLAC concentration in SBP samples (mean, 51.23±34.34 ng/ml) was significantly higher than in non-SBP samples (mean, 18.44±18.98 ng/ml; P<0.001). ROC curve was generated according to the results of lactoferrin, TNF-α, IL-6, and HsCRP. (Fig. 1) The areas under the ROC curve (AUCs) calculated for lactoferrin, TNF-α, IL-6, and HsCRP with 95% confidence interval are shown in Tab 2.

AUC value of AFLAC was the only one that reached 0.788 (95% confidence interval, 0.670-0.907). The cut-off point of ascitic lactoferrin was 46.07 ng/ml, with highest combined sensitivity (59.1%) and specificity (94.8%) for distinguishing SBP samples and non-SBP samples. There were no significant differences in the traditional inflammatory biomarkers TNF-alpha, IL-6, and HsCRP in our study. These biomarkers did not possess clinical value for diagnosing SBP using ROC curve method.

Discussion

Lactoferrin is a major whey protein that is useful for differentiating between IBD and IBS. It can serve as an adjunct to blood parameters for determining IBD with ongoing inflammation. The presence of lactoferrin in body fluids is proportional to the neutrophils in body fluids. It is a major immune protein of neutrophils, stored in secondary granules until cellular activation.[23] The AUC of lactoferrin in our study was 0.788, which indicated acceptable discrimination.

The AUCs of IL-6, HsCRP and TNF were 0.648, 0.623, and 0.571, respectively, which indicated nondiscrimination. Therefore, lactoferrin is an acceptable biomarker for diagnosing SBP in patients with liver cirrhosis. Parsi et al. assessed the utility of ascitic fluid lactoferrin for the diagnosis of SBP in 218 consecutive ascitic fluid samples from 148 patients with cirrhosis. SBP samples had a significantly higher lactoferrin concentration when compared with non-SBP samples. Qualitative bedside assays of ascitic fluid lactoferrin are considered a potential screening tool for SBP in patients with liver cirrhosis.[5] In our study, the cut-off value for lactoferrin for diagnosing SBP was 46.07ng/dL (sensitivity: 59.1%, specificity: 94.8%), which was significantly lower than the cut-off level reported by Parsi et al. (242ng/dL, sensitivity: 95.5%, specificity: 97%). This discrepancy in lactoferrin cut-off level between the two studies may be due to different etiologies of cirrhosis and total patient numbers, although the mean ages were similar in SBP and non-SBP groups in these two studies. In the study by Parsi et al., the etiologies of liver cirrhosis were alcoholism alone in 25% (n = 37), chronic viral hepatitis alone in 33% (n = 49), combination of alcoholism and chronic viral hepatitis in 10% (n = 14) and other factors in 32% (n = 48) (primary sclerosing cholangitis, primary biliary cirrhosis, autoimmune hepatitis, nonalcoholic steatohepatitis, cryptogenic cirrhosis, Wilson's disease, α1-antitrypsin deficiency, or hemochromatosis). In our study, the etiology of liver cirrhosis was mostly related to chronic viral hepatitis (75/111, 67.6%). Among the SBP-positive samples, 17/22 (77.3%) were from patients with underlying chronic viral hepatitis. Whether this etiology led to lower cut-off lactoferrin level in patients with SBP needs further verification via

large-scale studies. In the present study, the longer period from collection of ascitic fluid samples to laboratory lactoferrin check may have led to degradation of lactoferrin level and, thus, lower cut-off level..

The prevalence of SBP in patients with liver cirrhosis was 19.8%, which was twice as high as in the study by Parsi et al.(10%). Based on Child-Pugh classification (Child A: 0, Child B: 40.9% and Child C: 59.1%) cirrhosis was also more severe than in the study by Parsi et al. The higher prevalence of SBP in patients with cirrhosis in our study may be related to the severity of cirrhosis or comorbid diseases during hospital stay. This indicates that the etiology of cirrhosis in Taiwan is different from that in Western countries. Cirrhosis tends to be more severe in Taiwan than in Western countries. Large-scale studies are needed to elucidate and identify efficient cut-off value of lactoferrin for SBP diagnosis in Taiwan.

Fecal lactoferrin is a sensitive and specific marker for the differentiation of IBD and IBS and for the evaluation of the disease activity of IBD. Lactoferrin in pancreatic secretion may be a precipitate protein in stone formation in chronic pancreatitis. Serum anti-Lf might contribute to the clarification of the pathogenetic mechanism of autoimmune pancreatitis and liver diseases, although its diagnostic and prognostic value appears to be limited. Further studies are required for confirmation. Fecal lactoferrin has been evaluated as a means for diagnosing inflammatory diarrhea in a community setting where cell lysis and specimen transport might lead to false-negative results.[4,19] In a large clinical trial of 1041 patients undergoing 2,123 procedures, leukocyte reagent strips with a threshold of 2+ for positivity had a sensitivity of only 45%.[17] Even when the threshold for positivity was lowered, the sensitivity of the test only improved to 79%.[18] Although specificity remained high in most studies and a strong positive result could predict SBP, the varied sensitivities in clinical trials make current leukocyte reagent strips suboptimal for the diagnosis of SBP. According to the findings of Parsi et al and the present study, the variation in the cut-off level of lactoferrin in cirrhotic patients with SBP makes it difficult to determine a definitive value for diagnosing SBP using rapid qualitative test.

For clinicians with busy practices and for in-house staff or hospital staff taking care of large numbers of patients with liver disease, a rapid test is needed to identify patients with SBP.. In our study, traditional inflammatory biomarkers (TNF-alpha, IL-6, HsCRP) in ascitic fluid did not have clinical value for diagnosing SBP using ROC statistical method as there were no significant differences between SBP and non-SBP samples. However, there was significant difference in lactoferrin between SBP and non-SBP samples. Lactoferrin, a product of activated PMNs, is a logical marker. Perhaps, in time, a qualitative assay for lactoferrin will make bedside diagnosis of SBP possible. Further studies that include larger numbers of patients with SBP should be performed to validate the results and to further assess the optimal lactoferrin threshold for identifying elevated ascitic fluid PMN count.

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