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ORIGINAL ARTICLE

Nucleotide-Binding Oligomerization Domain-Containing Protein 2 Variants in Patients with Spontaneous Bacterial Peritonitis

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Abstract
Background The occurrence of spontaneous bacterial peritonitis (SBP) is significantly increased in carriers of nucleotide-binding oligomerization domain-containing protein 2 (NOD2) variants, suggesting that local immune alterations might be implicated in bacterial translocation (BT).
Aims We aimed to assess the role of the NOD2 gene in conferring susceptibility to SBP. We also sought to determine whether levels of serum interleukin-6 (IL-6), lipopolysaccharide-binding protein, and soluble TNF-α receptor, along with the presence of bacterial DNA (bactDNA) in ascitic fluid, are appropriate markers for BT in patients with liver cirrhosis and SBP.
Methods A cohort of 171 patients was divided into two groups: patients with SBP ($n = 82$) and those without SBP ($n = 89$). The presence of the most common NOD2 variants (p.R702W, p.G908R, and c.3020insC) was determined in these patients.
Results We detected the p.G908R variant in four patients (4.9 %) of the SBP group. No significant difference was observed between the SBP and non-SBP groups for NOD2 risk variants. The frequency of bactDNA in ascitic fluid was higher for patients with NOD2 variants than for patients without variants ($p = 0.021$). Serum IL-6 levels in the SBP group were higher than those in the non-SBP group.
Conclusions The frequent detection of bactDNA in ascites of patients with the p.G908R variant suggests there is a strong association between NOD2 risk variants and BT in SBP patients. In addition, increased serum IL-6 levels and bactDNA in ascitic fluid could be considered surrogate markers for BT in patients with cirrhosis.

Keywords NOD2 · Bacterial translocation · Spontaneous bacterial peritonitis · Cirrhosis

Introduction

Spontaneous bacterial peritonitis (SBP) is unique to patients with cirrhosis and occurs in the absence of a contiguous or remote identifiable source of infection. The main mechanism of SBP appears to be translocation of bacteria (BT) and associated products (i.e., bacterial DNA) from the gut lumen to mesenteric lymph nodes, to systemic circulation (bacteremia), and then to existing fluids (ascites and/or hydrothorax) [1, 2].

Intestinal bacterial overgrowth, increased intestinal permeability, and impaired immunity possibly favor BT [3]. The immune systems of patients with cirrhosis are significantly altered and can promote the development of infections and BT. Some of these alterations have a genetic basis; as an example, nucleotide-binding oligomerization domain-containing protein 2 (NOD2) plays an important role by recognizing bacterial molecules (peptidoglycans) and stimulating an immune reaction [4]. The occurrence of SBP is significantly increased in carriers of NOD2 variants,

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suggesting that local immune alterations might be implicated in BT [5]. Additionally, NOD2 variants increase the risk of culture-positive SBP and bacterascites in cirrhosis patients, likely affecting their survival [6].

Intestinal bacterial overgrowth and translocation are common in cirrhosis with ascites. This can lead to the activation of monocytes and lymphocytes, and increased levels of proinflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6). The binding of TNF-α to its transmembrane receptors triggers an inflammatory response. Following activation by TNF-α, transmembrane TNF-α receptors in activated monocytes and other immune cells are cleaved to form a soluble TNF-α receptor (sTNFαR). Thus, the paracrine effects and activation of TNF-α are mirrored in the levels of sTNFαR [7]. Bacterial endotoxins, such as lipopolysaccharide (LPS), promote the synthesis of LPS-binding protein (LBP) in the liver and form an LPS–LBP complex that binds to CD14. Measurement of serum LBP levels has been proposed as a surrogate marker of BT [8]. At present, the most appropriate marker for BT remains to be elucidated.

The aim of the present study was to assess the potential role of the NOD2 gene in conferring susceptibility to SBP. Additionally, we attempted to determine whether serum IL-6, LBP, and sTNFαR levels, along with the presence of bacterial DNA (bactDNA) in ascitic fluid, were markers of BT in patients with liver cirrhosis and SBP.

Materials and Methods

Patients

Patients with cirrhosis and ascites who were admitted to the Department of Hepatology, Turgut Ozal Medical Center (Turkey), between October 2011 and April 2013 were included in our study. Cirrhosis was defined by clinical, laboratory, and radiological findings, or histology if available. Diagnostic paracentesis was performed in all patients with ascites. SBP was diagnosed when the ascites polymorphonuclear neutrophil cell count was $> 250/\mu L$, based on the criteria of the International Ascites Club [9]. Patients with secondary peritonitis, acute pancreatitis, or peritoneal carcinomatosis were excluded. Before commencement of the study, we determined patient numbers for groups based on the frequencies of NOD2 risk variants reported in previous studies. Patients ($n = 171$) were divided into two groups: patients with SBP ($n = 82$) and those without SBP ($n = 89$). All patients gave their informed consent to participate in the study. The ethics committee of Inonu University Medical Faculty approved our study. The severity of the underlying liver disease was assessed according to the Model of End-stage Liver Disease (MELD) and the Child–Pugh score.

Sample Collection

Approximately 25 mL ascitic fluid was taken out for every patient. Total and differential cell counts in ascitic fluid were defined by an automated blood cell count for samples in 2.7 mL of EDTA, with total protein and albumin levels also analyzed. Ascitic fluid (10 mL) was inoculated into blood culture bottles for aerobic and anaerobic cultures. Ascitic fluid (5 mL) was obtained under strictly sterile conditions and used in the analysis of bacterial DNA. Venous blood samples were collected in bottles containing 9 mL of EDTA and used in the analysis of NOD2 risk variants. We also obtained blood and serum samples, with EDTA used as an anticoagulant, for standard hematological, biochemical, and coagulation tests. For cytokine analyses, blood samples were centrifuged ($3500 \times g$ for 10 min) and the supernatants (serum) collected and aliquoted. Samples were subjected to the relevant biochemical analyses or stored at −70 °C until required.

Detection and Identification of BactDNA in Peritoneal Fluid Samples

Total nucleic acid was isolated from peritoneal fluid samples using the QIAsymphony automated extraction system and a DSP Virus/Pathogen Midi Kit V1 (Qiagen, Hilden, Germany).

We used primers p8FPL (5′-AGT TTG ATC CTG GCT CAG-3′) and p806R (5′-GAC TAC CAG GGT ATC TAA T-3′) for polymerase chain reaction (PCR) amplification of an approximately 800-bp fragment of 16S rDNA. The thermal cycling profile involved an initial denaturation step (95 °C for 4 min), 35 amplification cycles (95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min), and a final extension step (72 °C for 7 min). Amplicons were subjected to 2 % (w/v) agarose gel electrophoresis, excised from gels, and purified using a QIAquick PCR Purification Kit (Qiagen). Sequencing of amplicons was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Obtained sequences were submitted to the Basic Local Alignment Search Tool program on the NCBI blast server (http://blast.ncbi.nlm.nih.gov). We selected the MegaBLAST algorithm and nucleotide collection (nr/nt) searches; returned hits were evaluated for query coverage and e-values.

Detection of NOD2 Variants

Genomic DNA was extracted from venous blood samples using a QIAamp DNA Blood Mini Kit (Qiagen), according to the manufacturer's protocol.

The most frequently studied NOD2 variants are p.G908R, p.R702W, and c.3020insC. Therefore, we preferred these NOD2 gene variants. These variants were detected using quantitative real-time PCR assays according to a previously described protocol, with some minor modifications. The oligonucleotide primers and probes (Table 1) we used in the current study were synthesized by PrimerDesign (Primer Design Ltd., UK). The binding targets of primers and probes, and locations of mutations in the NOD2 gene are shown in Figs. 1, 2, and 3. Each reaction contained 0.4 μM primers and 0.2 μM probes in each reaction. We used a QuantiTect Probe PCR Kit (Qiagen) and a Rotor-Gene Q Real-Time PCR instrument (Qiagen) to carry out quantitative PCR assays. Thermal cycling conditions involved an initial denaturation step at 95 °C for 15 min, followed by 35 amplification cycles (94 °C for 15 s, 60 °C for 60 s). Mutations were confirmed by DNA sequencing following the detection of NOD2 variants.

Serum Levels of LBP, IL-6, and sTNFαR

The concentration of LBP, IL-6, and sTNFαR in serum samples was determined using specific enzyme-linked immunosorbent assays from Hangzhou Eastbiopharm (China), Boster Immunoleader (Wuhan, China), and Bender MedSystems (Vienna, Austria), respectively.

Statistical Analysis

We used the Kolmogorov–Smirnov test for statistical evaluation and found that our data were not normally distributed ($p > 0.05$). Fisher's exact, Chi-square, and Mann–Whitney U tests were subsequently used for comparisons, with $p < 0.05$ considered statistically significant.

Results

Patient Characteristics

For the SBP group, 62.6 % of patients were males (62.6 %), and the median age of patients was 54.09 ± 14.15 years. For the non-SBP group, 70.7 % of patients were males, and the median age of patients was 55.24 ± 13.08 years. The etiologies of all patients were as follows: 78 (45.1 %) were positive for hepatitis B; 41 (23.7 %) were cryptogenic; 25 (14.5 %) were positive for hepatitis C; ten (5.8 %) were alcoholic; and 27 (11.9 %) were other diseases. The majority of patients were in the advanced stages of liver disease. In the SBP group, 22 (26.8 %) and 60 patients (73.2 %) had Child–Pugh scores of B and C, respectively. In the non-SBP group, 24 (26.9 %) and 65 patients (73.1 %) had Child–Pugh scores of B and C, respectively. The median MELD score at the time of admission was 20 (range 4–44) for the SBP group and 18 (range 9–39) for the non-SBP group.

NOD2 Risk Variants

We detected the p.G908R NOD2 risk variant in four patients (4.9 %) of the SBP group. Other NOD2 risk variants (c.3020insC and p.R702W) were not seen in this group. We did not detect any NOD2 risk variants in the non-SBP group (Table 2). There was no significant difference between the two groups with respect to the presence of NOD2 risk variants (Table 2).

Ascitic Fluid Culture and BactDNA

We observed a significant association between NOD2 risk variants and culture-positive ascitic fluid ($p = 0.021$). Ascitic fluids were culture-positive for 11 patients, with two of these patients belonging to the non-SBP group. We identified *Escherichia coli* in four patients (36.3 %), *Enterobacter* in one patient (9.1 %), *Acinetobacter* in one patient (9.1 %), *Streptococcus* spp. in two patients (18.2 %), methicillin-resistant *Staphylococcus aureus* in

Table 1 Sequences of oligonucleotide primers and probes used to detect NOD2 risk variants

NOD2 risk variant	Primer name	Primer sequence (5′–3′)	Probe sequence (5′–3′)
p.R702W	MGB_F	CTGAGTGCCAGACATCTGAGAAG	VIC-CCTGCTCTGGCGCC
	MGB_R	GCTGCGGGCCAGACA	Yakima Yellow-CTGCTCCGGCGCC
p.G908R	MGB_F	TGATCACCCAAGGCTTCAGC	VIC-ACTCTGTTGCGCCAGA
	MGB_R	GAACACATATCAGGTACTCACTGACAC	Yakima Yellow-CTGTTGCCCCAGAAT
c.3020insC	MGB_F	CCAGGTTGTCCAATAACTGCATC	VIC-TGCAGGCCCCTTG
	MGB_R	CCTTACCAGACTTCCAGGATGGT	Yakima Yellow-CTGCAGGCCCTTG

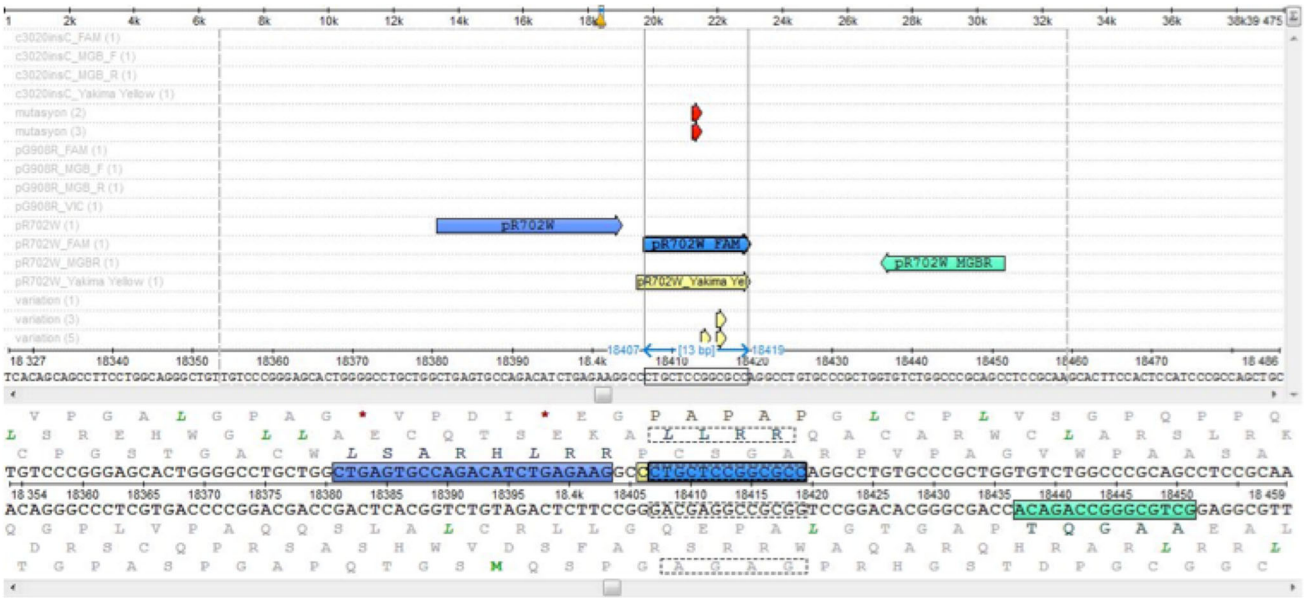

Fig. 1 Binding sites of probes and primers on the NOD2 gene for detection of the p.R.702W mutation. The map of the NOD2 gene was made with UGENE software (http://ugene.unipro.ru)

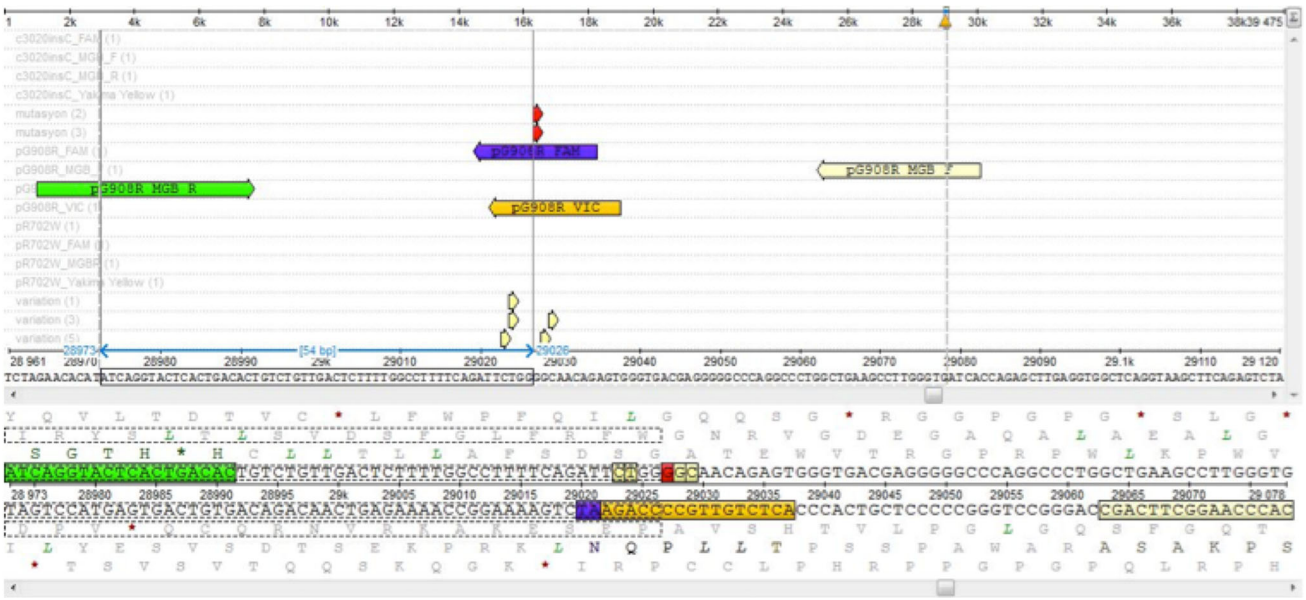

Fig. 2 Binding sites of probes and primers on the NOD2 gene for detection of the p.G908R mutation. The map of the NOD2 gene was made with UGENE software (http://ugene.unipro.ru)

two patients (18.2 %), and *Candida* spp. in one patient (9.1 %). We detected bactDNA in the ascitic fluid of culture-positive patients (Table 3). There was a significant difference in the presence of ascitic fluid bactDNA between SBP and non-SBP groups ($p = 0.027$). The frequency of bactDNA in the ascitic fluid of patients with NOD2 risk variants was higher than of patients lacking NOD2 risk variants ($p = 0.021$).

A comparison of Child–Pugh and MELD scores, along with serum IL-6, sTNFαR, and LBP levels for SBP and non-SBP groups, is given in Table 4. There was no significant difference for Child–Pugh and MELD scores and sTNFαR levels between the SBP and non-SBP groups. However, serum IL-6 and LBP levels ($p < 0.01$ and $p < 0.001$, respectively) were significantly different between the two groups. Serum IL-6 levels in the SBP

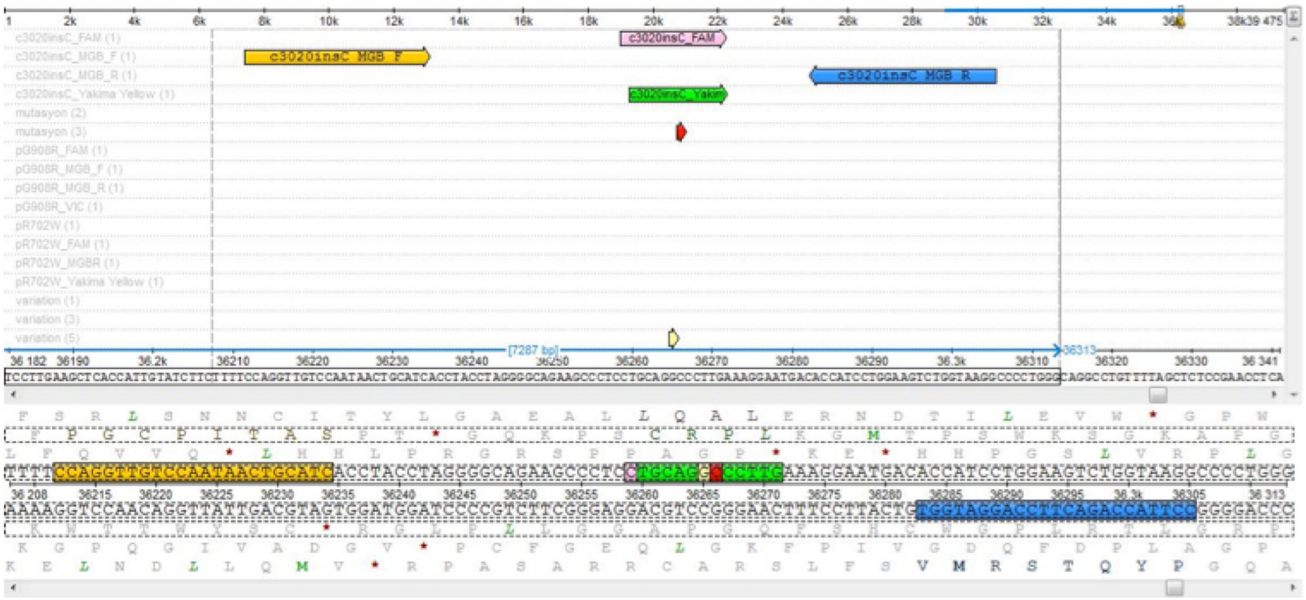

Fig. 3 Binding sites of probes and primers on the NOD2 gene for detection of the c.3020insC mutation. The map of the NOD2 gene was made with UGENE software (http://ugene.unipro.ru)

Table 2 Association between NOD2 risk variants and SBP

Groups	Number lacking NOD2 risk variants (%)	Number of p.G908R variants (%)	Total (%)	*p* value
SBP	78 (95.1)	4 (4.9)	82 (100)	0.051
Non-SBP	89 (100)	0 (0)	89 (100)	

Table 3 Ascitic fluid bactDNA results in patients with NOD2 risk variants and SBP

	Presence of bactDNA in ascitic fluid			
	Negative (%)	Positive (%)	Totals (%)	*p* value
NOD2 risk variants				
Lacking NOD2 risk variants	158 (94.6)	9 (5.4)	167 (100)	0.021
p.G908R	2 (50)	2 (50)	4 (100)	
Patient groups				
SBP	73 (89)	9 (11)	82 (100)	0.027
Non-SBP	87 (97.8)	2 (2.2)	89 (100)	

group were higher than those in the non-SBP group, while serum LBP levels in the SBP group were lower than those in the non-SBP group. We did not observe a significant difference in Child–Pugh and MELD scores, and serum IL-6, sTNFαR, and LBP levels between patients with and without NOD2 risk variants.

Discussion

We were unable to determine a significant association between NOD2 risk variants and SBP patients with cirrhosis. Although NOD2 gene mutations have been widely studied in various inflammatory diseases such as Crohn's disease and Blau syndrome [10], there are few studies regarding NOD2 variants in patients with SBP and cirrhosis of the liver.

Appenrodt et al. [5] reported that patients carrying NOD2 variants developed SBP with greater frequency and had reduced mean survival time compared with those lacking NOD2 variants. They demonstrated that the carrier status for any *NOD2* risk allele was a significant risk factor for death ($p = 0.001$). Although the risk allele frequencies of all *NOD2* variants tended to be higher in patients with SBP, no statistically significant differences were observed for single nucleotide polymorphisms of NOD2. We

Table 4 Comparative Child–Pugh and MELD scores, and serum IL-6, sTNFαR, and LBP levels for SBP and non-SBP patient groups

	Child–Pugh score (range)	MELD score (range)	IL-6 (pg/mL)	sTNFαR (ng/mL)	LBP (ng/mL)
NOD2 risk variants					
Lacking NOD2 risk variants ($n = 167$)	11 (6–15)	19 (7–44)	60.15 ± 7.0	2.33 ± 1.99	8.57 ± 7.38
p.G908R ($n = 4$)	13.5 (9–15)	29.5 (15–40)	55.07 ± 2.7	2.26 ± 0.50	6.75 ± 6.07
Groups					
SBP ($n = 82$)	11 (6–15)	20 (4–44)	65.52 ± 6.43[a]	2.67 ± 2.35	6.33 ± 5.81[b]
Non-SBP ($n = 89$)	11 (7–15)	18 (9–39)	52.32 ± 6.72	2.04 ± 1.53	10.36 ± 8.08

[a] $p < 0.01$ compared with the non-SBP group

[b] $p < 0.001$ compared with the non-SBP group

analyzed three NOD2 risk alleles (c.3020insC, p.G908R, and p.R702W) and detected the p.G908R mutation in four patients (4.9 %) with SBP. We failed to detect the other two mutations in any patients from either group ($p > 0.05$). Although the difference between the two groups was not statistically significant, the presence of the p.G908R mutation in four patients of the SBP group suggests that NOD2 risk variants might play some role in SBP. In addition, our findings also suggest that analysis of several more NOD2 risk alleles would be beneficial in determining any association between NOD2 and SBP.

At least 30 NOD2 risk variants have been found in Caucasians [11]. Hugot et al. [12] investigated the prevalence of NOD2 mutations in healthy Caucasian people and reported allele frequencies of 4.3, 1.2, and 2.3 % for the R702W, G908R, and 1007 fs mutations, respectively. Large geographic fluctuations of the G908R, 1007 fs, and wild-type alleles were also observed. To the best of our knowledge, there are no reports concerning NOD2 frequency in cirrhosis patients of Turkey. However, the frequency of NOD2 gene variants in the Turkish population has been investigated for other diseases, such as ankylosing spondylitis, sepsis, and Crohn's disease [13–15]. G908R mutation frequencies were 5, 5.5, and 1.4 % in ankylosing spondylitis, sepsis, and Crohn's disease, respectively. These mutation frequencies were similar to those observed in our current study, with a 4.9 % frequency of this mutation in SBP patients with liver cirrhosis observed. In our study, there was no significant association of NOD2 variants between groups. The frequency of NOD2 risk alleles appears to differ for every country [16, 17], and therefore, our results could be related to ethnicity.

The NOD-like receptors are a family of pattern recognition receptors that play a significant immunological role in the recognition and clearance of bacterial pathogens. The NOD2 gene encodes a cytosolic protein that recognizes the bacterial cell wall component muramyl dipeptide, and activates the innate pathways of host defense. There is some evidence that alterations in NOD2 might impair neutrophil activation and their migration toward inflammatory stimuli [6, 18]. Bruns et al. [6] investigated three NOD2 variants (R702W, G908R, and 1007 fs) in patients with cirrhosis. There was no significant association of R702W and G908R with SBP patients or patients with sterile ascites. However, they found a statistically significant association between 1007 fs and SBP (odds ratio 6.1; $p = 0.031$). They also found that the frequency of G908R (20.0 %) was increased in patients with culture-positive SBP (odds ratio 16.8; $p < 0.001$). We found a significant association between G908R frequency and culture-positive ascitic fluid ($p = 0.021$). Ascitic fluid that was culture-positive was detected in 11 patients, with two of these patients containing the G908R variant (18.2 %). Bruns et al. [6] reported that neutrophil migration and the clearance of microorganisms in ascitic fluid might be impaired for certain NOD2 variants. Our results appear to correspond to this hypothesis.

Serum IL-6 levels in SBP patients were higher than in those without SBP ($p = 0.005$). Suliman et al. [19] reported that IL-6 levels in serum were significantly higher in patients with SBP than in patients with sterile ascites. Moreover, about 48 (40 %) SBP patients with cirrhosis developed renal and hepatic impairment and had significantly higher plasma IL-6 levels when infection was diagnosed. Navasa et al. [20] reported that patients who developed renal impairment showed significantly higher plasma and ascitic fluid IL-6 levels at diagnosis of SBP infection. Reiberger et al. [21] reported that patients with severe portal hypertension had increased IL-6 levels. They also reported that NOD2 risk variants were associated with elevated markers of BT. Based on these previous findings, the measurement of serum IL-6 in SBP patients with cirrhosis is recommended, given that it has diagnostic and prognostic significance.

The bacterial endotoxin LPS promotes the synthesis of LBP in the liver and forms an LPS–LBP complex. Tang and Chen [8] reported that serum LBP levels were significantly higher in cirrhosis patients with SBP than in cirrhosis patients without SBP. Reiberger et al. [21] reported a significant correlation between LBP levels and portal pressure with all markers of gastroduodenal/intestinal permeability. Albillos et al. [22] reported that plasma LBP levels were significantly higher in cirrhotic patients with ascites than those in cirrhotic patients without ascites, and healthy controls. In a separate study, they also reported that patients with ascites and cirrhosis, but without evidence of bacterial infection, exhibited increased serum LBP levels and were four times more likely to have a severe bacterial infection during follow-up than patients with normal LBP levels [23]. In contrast to these previous results, we found that serum LBP levels in SBP patients were significantly lower than those without SBP ($p < 0.001$). Chen et al. [24] investigated LBP in cirrhotic patients with severe sepsis and showed that low LBP levels upon admission to an intensive care unit were associated with impaired liver reserve, multiple organ dysfunction, and increased mortality in a clinical setting. They reported that LBP might have dual effects with respect to modulating the innate immune response. It has been shown that LBP plays dual concentration-dependent roles in the pathogenesis of sepsis. Low levels of LBP enhance the LPS-induced activation of mononuclear cells, whereas acute-phase increases in LBP concentrations inhibit LPS-induced cellular stimulation [24–26]. It has been reported that serum LBP levels are low in patients with sepsis [27, 28]. Patients with decompensated cirrhosis have an increased risk of developing multiple organ failure, along with sepsis leading to death. Our results suggest that LBP levels in SBP patients have a similar profile to those in patients with sepsis. Upregulation of LBP might be compromised in cases where the synthetic capacity of the liver is impaired, making this interpretation difficult. It has been reported that impaired synthesis of LBP by diseased livers can make cirrhotic patients even more susceptible to toxic bacterial products [24]. In our study, the majority of patients in both groups had advanced liver disease. Therefore, lower LBP levels in the SBP group could be related to a decreased capacity for LBP synthesis.

Endotoxins are an important trigger of TNF-α release, which itself is rather short-lived, but can be indirectly assessed by measuring the sTNFαRs p55 and p75. Levels of sTNFαR can be used as a surrogate marker of TNF-α and its activation [7, 29, 30]. In our study, sTNFαR levels in the SBP group were higher than those in the non-SBP group, although this difference was not statistically significant. Serum levels of LBP, IL-6, and sTNFαR were significantly higher in patients infected with hepatitis C virus and human immunodeficiency virus, and in hepatitis C virus-infected patients with decompensated cirrhosis compared with those who had compensated liver disease [31]. Trebicka et al. [7] reported that sTNFαR levels correlated with severity of liver dysfunction. Rodríguez-Ramos et al. [32] reported that serum levels of p55 and p75 were higher in patients with SBP compared with cirrhotic controls. These previous findings in conjunction with our results suggest that increased serum levels of sTNFαRs could be an appropriate indirect marker for BT in patients with cirrhosis.

We did not observe a statistically significant difference serum IL-6, sTNFαR, and LBP levels between patients with and without NOD2 risk variants. However, these parameters in patients with G908R variant were lower than in those without NOD2 risk variants. These results suggest that G908R variant might play some role on these parameters.

Cirrhosis has also been associated with decreased activity of the reticuloendothelial system, one of the most relevant defense systems against bacteremia and other infections acquired hematogenously. The presence of porto-systemic shunts and the decreased phagocytic capacity of Kupffer cells are associated with the development of bacteremia and SBP [33]. The altered clearance capacity of the reticuloendothelial system affects viable bacteria and bacterial products, such as endotoxins or bactDNA. The presence of bactDNA fragments is a direct and sensitive marker of BT. BactDNA appears to play a direct role in the inflammatory response associated with BT and is a predictor of mortality in patients with cirrhosis and ascites [4]. Bruns et al. [34] also found that bactDNA detection in patients with severe liver dysfunction (MELD score > 15) is associated with increased mortality. We observed a significant difference in bactDNA detection in ascitic fluid between SBP and non-SBP groups ($p = 0.027$). There was also a significant difference with respect to detection of bactDNA in the ascitic fluid of patients with NOD2 risk variants ($p = 0.021$).

In conclusion, the frequent detection of bactDNA in the ascitic fluid of SBP patients with the NOD2 risk variant p.G908R suggests a strong association between NOD2 risk variants and BT in patients with SBP. Analysis of many more NOD2 risk alleles, instead of a single risk allele, would be more beneficial in definitively determining an association between NOD2 and SBP. Increased serum IL-6 levels and the detection of bactDNA in ascitic fluid could be considered a surrogate marker for BT in cirrhotic patients.

Compliance with ethical standards

Conflict of interest No conflict of interest.

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