TOLL-LIKE RECEPTOR 4 D299G POLYMORPHISM AND INCIDENCE OF INFECTIONS IN CIRRHOTIC PATIENTS

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Alimentary Pharmacology & Therapeutics

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Journal:	*Alimentary Pharmacology & Therapeutics*
Manuscript ID:	APT-0079-2010.R1
Manuscript Type:	Original Scientific Paper
Date Submitted by the Author:	04-Mar-2010
Complete List of Authors:	Guarner-Argente, Carlos; Hospital de la Santa Creu i Sant Pau, Department of Gastroenterology Sánchez, Elisabet; Hospital de la Santa Creu i Sant Pau, Department of Gastroenterology; CIBERehd Vidal, Sílvia; Hospital de la Santa Creu i Sant Pau, Department of Immunology Román, Eva; Hospital de la Santa Creu i Sant Pau, Department of Gastroenterology; CIBERehd Concepción, Mar; Hospital de la Santa Creu i Sant Pau, Department of Gastroenterology Poca, Maria; Hospital de la Santa Creu i Sant Pau, Department of Gastroenterology Sánchez, Diego; Hospital de la Santa Creu i Sant Pau, Department of Immunology Juárez, Cándido; Hospital de la Santa Creu i Sant Pau, Department of Immunology Soriano, Germán; Hospital de la Santa Creu i Sant Pau, Department of Gastroenterology; CIBERehd Guarner, Carlos; Hospital de la Santa Creu i Sant Pau, Department of Gastroenterology; CIBERehd
Keywords:	Cirrhosis < Hepatology, Hepatocellular carcinoma < Hepatology, Microbiology < Topics, Portal hypertension < Hepatology, Inflammation < Topics

TITLE PAGE

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RUNNING TITLE: TLR4 D299G POLYMORPHISM AND INFECTIONS IN CIRRHOSIS

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NUMBER OF TABLES: 3

NUMBER OF FIGURES: 0

ABBREVIATIONS:

TLR, toll-like receptor

PCR, protein chain reaction

RFLP, restriction fragment length polymorphism

PAMPS, pathogen-associated molecular patterns

NOD2, nucleotide-binding oligomerization domain containing 2

LPS, lipopolysaccharide

MD-2, myeloid differentiation protein 2

HIV, human immunodeficiency virus

SBP, spontaneous bacterial peritonitis

EDTA, ethylenediaminetetraacetic acid

bp, base pair

MELD, model for end stage liver disease

INR, international normalized ratio

GNB, gram-negative bacilli

GPC, gram-positive cocci

KEYWORDS: cirrhosis; bacterial infections; genetic polymorphisms; toll-like receptor 4.

SUMMARY

BACKGROUND. Toll-like receptor (TLR) 4 genetic polymorphisms, mainly D299G, have been associated with increased predisposition to infection in several populations.

AIM. To retrospectively analyze the relationship between the presence of the TLR4 D299G polymorphism and the incidence of bacterial infections in cirrhotic patients.

PATIENTS AND METHODS. We included 111 consecutive cirrhotic patients hospitalized with ascites and we determined the presence of the TLR4 D299G polymorphism by PCR-RFLP and its relationship with the incidence of previous bacterial infections. RESULTS. Ten out of 111 (9%) cirrhotic patients presented the TLR4 D299G polymorphism. The mean follow-up from first decompensation of cirrhosis until present admission was longer in D299G polymorphism patients than in wild type patients (53.8±40.7 vs 35.4±48.3 months, p=0.03). D299G polymorphism patients showed a trend towards a higher incidence of previous infections (80% vs 56.4%, p=0.19), as well as a higher number of infections (2.8±2.3 vs 1.0±1.3, p=0.01) and bacteremias (0.4±1.0 vs 0.04±0.2, p=0.02) per patient than wild type patients.

CONCLUSIONS. TLR4 D299G polymorphism could influence not only the predisposition to bacterial infections but also the evolution of the disease in cirrhotic patients. Further prospective studies in larger series of patients are warranted.

INTRODUCTION

Bacterial infections are a frequent and severe complication in cirrhosis, leading to significant morbidity and mortality[1,2]. They are mainly caused by gram-negative bacilli, although gram-positive cocci are increasingly isolated due to antibiotic prophylaxis and instrumentation[3]. The predisposition of cirrhotic patients to develop bacterial infections is mainly the result of several alterations in the mechanisms of antimicrobial defence[1]. Most of these disturbances are due to liver insufficiency, portal hypertension and bacterial translocation[1,2,4]. Mutations that alter the ability of innate immune receptors to bind to their pathogen-associated molecular patterns (PAMPS) may also affect host susceptibility to infection[5-7]. However, only one study has recently been published concerning the possible relationship between genetic factors and susceptibility to bacterial infections in cirrhotic patients[8].

Toll-like receptors (TLR) are a family of transmembrane receptors with extracellular leucine-rich repeats and an intracellular signalling domain. They are found in monocytes, macrophages and neutrophils, and they function as receptors to recognize PAMPS, such as lipopolysaccharide (LPS), lipoproteins and peptidoglycans. They therefore play a key role in the innate immune response, inducing a signal that regulates the activation of several proinflammatory genes[5-7].

In association with CD14 and its co-receptor MD-2, TLR4 triggers the inflammatory response to LPS of gram-negative bacteria, and is a key factor in eliciting the systemic inflammatory response that can lead to sepsis, organ failure, and septic shock[5,6]. Two important non-synonymous TLR4 genetic polymorphisms have been described. One is an A/G polymorphism at rs4986790 that causes the change from Asp to Gly at aminoacid position 299 (D299G) and the other is a C/T polymorphism at rs4986791 that causes the change from Thr to Ile at position 399 (T399I). These polymorphisms are

usually found in cosegregation[9] and have been shown to change the ligand-binding site of the receptor[10] because they are located close to the TLR4-MD-2 binding areas. These cosegregating TLR4 polymorphisms have been associated with blunted physiological responses to inhaled LPS[9], an increased risk of gram-negative bacteremia in patients with sepsis[11,12], invasive aspergillosis in recipients of hematopoietic-cell transplants[13], and with other infections such as malaria, brucellosis or candidiasis[6]. However, other studies do not confirm these associations[5,6]. In addition, there are conflicting results about the functional impact of TLR4 (D299G and T399I) polymorphisms on the LPS-induced cytokine response[14-16]. These reports led us to explore the relevance of the TLR4 D299G polymorphism in cirrhotic patients, and analyze its frequency and relationship with the incidence of infections in this setting.

PATIENTS AND METHODS

All cirrhotic patients with ascites and previous episodes of decompensation (ascites, variceal bleeding, encephalopathy or infection) hospitalized in the Department of Gastroenterology from April 2006 to January 2009 were considered for inclusion in the study. Cirrhosis was diagnosed by biopsy or from clinical, analytical and ultrasonographic findings.

To minimize the effect of confounding factors in the development of bacterial infections or in the course of cirrhosis, we excluded patients with human immunodeficiency virus (HIV) infection or any other immunodeficiency, those with a permanent urinary catheter, and those with advanced hepatocellular carcinoma or any other condition that had determined poor short-term prognosis at first decompensation of cirrhosis. Finally, patients referred from other centres and those not fully controlled in our centre since

first decompensation of cirrhosis were also excluded to minimize the possibility of missed infections.

We retrospectively determined the time of the first decompensation of cirrhosis (ascites, variceal bleeding, encephalopathy or infection) and the period from this time until the first day of the present hospitalization (study period). The length of the study period, demographic, clinical and analytical characteristics and incidence of previous complications were recorded at present hospitalization by patient interview and revision of medical records. The incidence and the total number of infections during the study period, the bacteria isolated and the type of infection were recorded by patient interview and careful revision of medical records and the microbiology database. Spontaneous bacterial peritonitis (SBP) was diagnosed on the basis of an ascitic fluid polymorphonuclear cell count $\geq 250/mm^3$. Ascitic fluid was inoculated into blood culture bottles for aerobic and anaerobic cultures at the bedside. A positive culture was not considered necessary for diagnosis. Bacterascites was defined as the presence of a positive culture of ascitic fluid with a polymorphonuclear count $<250/mm^3$. Bacteremia was diagnosed when blood culture was positive. If bacteremia was detected in the course of a urinary tract infection, pneumonia, SBP or other infections, it was considered secondary to these infections and was not recorded as a different infection. Urinary tract infections were diagnosed on the basis of a positive urine culture. Pneumonia, cellulitis and other infections were diagnosed using conventional criteria[3]. Secondary bacterial peritonitis and postoperative wound infections were excluded from the analysis, as TLR polymorphisms would be unlikely to influence the bacteria responsible for these infections, and their inclusion could bias the results.

Blood samples for later genetic analysis were obtained in the first day of the present admission. Patients were classified into two groups: those with the TLR4 D299G

polymorphism (D299G group) and those without (wild type group). We compared the demographic, clinical and analytical characteristics of patients and the incidence and number of infections, according to the presence or absence of TLR4 D299G polymorphism.

The study was approved by the Research Ethics Committee at Hospital de la Santa Creu i Sant Pau. All patients gave consent to be included in the study after receiving appropriate verbal and written information.

Genomic DNA extraction and polymorphism genotyping

Eight ml of peripheral blood was collected from each patient in EDTA-vacutainer tubes and centrifuged at 2000 rpm for 10 minutes. Genomic DNA was extracted from buffy-coat fraction using QIAmp DNA blood minikit (Qiagen®). The primer sequences used for TLR4 Asp299Gly genotyping were 5'- GATTAGCATACTTAGA CTACTACCTCCATG-3' and 5'-GATCAACTTCTGAAAAAGCATTCCCAC-3'. The PCR consisted of an initial denaturation at 95ºC for 3 minutes, followed by 34 cycles of 45 seconds at 95ºC, 30 seconds at 57ºC and 1 minute at 72ºC, and a final extension of 5 minutes at 72ºC. Once the amplification was confirmed, the PCR product was digested for 1 hour at 37ºC with restriction enzyme NcoI[17].

The change of A to G at position 896 produced a site for the restriction enzyme NcoI. The digested fragments of PCR amplification were analyzed by electrophoresis on 2.5% agarosa gel. One band corresponding to a 190 base pair (bp) fragment was observed in homozygous wild-type patients, two bands of 170 bp and 20 bp in patients homozygous for the mutation, and three bands of 190 bp, 170 bp and 20 bp in heterozygous patients.

Statistical analysis

Data were expressed as mean±standard deviation or frequencies. Results were analyzed using the Chi^2 test with Yates correction and Fisher exact test for qualitative variables and Mann-Whitney test for quantitative parameters. Correlations were assessed by the Spearman test. A p value <0.05 was considered statistically significant. Statistical analysis was performed with the SPSS Statistical Package (version 17.0, SPSS Inc., Chicago, IL).

RESULTS

From April 2006 to January 2009, 192 cirrhotic patients with ascites were admitted to the Department of Gastroenterology and were considered for inclusion in the study. We excluded 4 patients with HIV infection, one patient with common variable immunodeficiency, 25 patients with advanced hepatocellular carcinoma or some other condition determining poor short-term prognosis at first decompensation (one severe cardiac failure, one ovarian neoplasia and one bladder neoplasia), 3 patients with a permanent urinary catheter, and 48 patients without previous decompensation of cirrhosis or referred from other institutions. Finally, 111 patients were included in the study.

The analysis of the TLR4 D299G polymorphism in this cohort of patients (n=111) revealed 10 were heterozygous patients (DG) (9%) and 101 were wild type (DD). None of the patients were homozygous for D299G (GG). The allelic frequency of this polymorphism was 0.045.

Patients' characteristics

Patients' clinical and analytical characteristics according to the presence or absence of the TLR4 D299G polymorphism are shown in Table 1.

We did not find statistical differences in demographic characteristics, etiology of cirrhosis, degree of liver insufficiency, presence of esophageal varices, previous complications of cirrhosis, previous prophylaxis with norfloxacin or beta-blockers, or ascitic fluid total protein levels. Serum creatinine was slightly but significantly higher in patients with TLR4 D299G polymorphism. Hepatic encephalopathy was more frequently the first decompensation of cirrhosis in patients with TLR4 D299G polymorphism than in wild type patients (20% vs 1.9%, p=0.04). Patients with the polymorphism also tended to present a higher incidence of hepatic encephalopathy during the study period (70% vs 37.6%, p=0.08).

The mean time from first decompensation of cirrhosis until blood was drawn for genetic analysis at present admission was 53.8±40.7 months in patients with the TLR4 D299G polymorphism and 35.4±48.3 in the wild type group (p=0.03). Considering only patients with cirrhosis caused by hepatitis C virus (n=49, 4 patients with TLR4 D299G polymorphism), mean time was 56.0±35.4 and 33.8±50.1 months, respectively (p=0.09).

Incidence and number of infections

The incidence and number of infections are shown in Table 2. A higher number of patients with the TLR4 D299G polymorphism had previous bacterial infections (8/10, 80% vs 57/101, 56.4%), infections caused by gram-negative bacilli and infections caused by gram-positive cocci than wild type patients, although these differences did not reach statistical significance. The number of infections per patient (2.8±2.3 vs

1.0±1.3, p=0.01) was higher in the group with the TLR4 D299G polymorphism than in the wild type group. The number of infections caused by gram-negative bacilli and the number of those caused by gram-positive cocci were also higher in the former group; these differences, however, only reached statistical significance for gram-positive cocci infections (p=0.009).

Considering the different types of infections (Table 3), there was a trend towards a higher incidence and number of infections per patient in the TLR4 D299G polymorphism group for all the infection types (SBP, bacteremia, urinary infections, pneumonia and cellulitis). These differences reached statistical significance for the number of bacteremias per patient (0.4±1.0 vs 0.04±0.2, p=0.02) and the incidence of bacteremia caused by gram-positive cocci (20% vs 1.9%, p=0.04).

There was no correlation between the number of infections and time from first decompensation to the present admission (r=0.034, p=0.72).

DISCUSSION

To our knowledge, only one study evaluating whether genetic factors contribute to the high risk for bacterial infections in cirrhotic patients has been published. The study of Appenrodt et al[8] has demonstrated an association between variants of NOD2 (nucleotide-binding oligomerization domain containing 2) linked to impaired mucosal barrier function and the development of SBP and mortality in cirrhotic patients with ascites.

In the present retrospective study, we observed a trend to a higher incidence of bacterial infections and a significantly higher number of infections per patient in cirrhotic patients with ascites and the TLR4 D299G polymorphism than in wild type patients. These findings were similar for infections due to gram-negative bacilli and for those

caused by gram-positive cocci. Characteristics that could predispose patients to the development of infections, such as the degree of liver insufficiency[18], ascitic fluid total protein levels[19,20], or prophylaxis with norfloxacin[21-23] or beta-blockers[24,25] were similar in the two groups. The longer evolution of disease since first decompensation in patients with the D299G polymorphism could help to explain the higher number of infections in these patients. There was no correlation, however, between the duration of this period and the number of infections. Our findings therefore support the hypothesis that the TLR4 D299G polymorphism could favour the development of infections in cirrhotic patients.

The higher number of infections caused by gram-positive cocci in patients with this polymorphism might be considered surprising, since TLR4 is mainly involved in the response to LPS from gram-negative bacteria[5-8]. One possible explanation is that a specific TLR polymorphism with a different sensitivity to its microbial ligand could decrease the basal innate immune tone involved in the general resistance to infection[6,26-28]. This would be of special relevance in cirrhotic patients, as their immune system is frequently exposed to intestinal PAMPS due to bacterial translocation[4,29,30]. Another explanation for this finding could be the presence of other TLR4-linked polymorphisms (e.g. TLR2, that binds to ligands from grampositive cocci)[5,6] that were not determined in the present study.

We observed that the TLR4 D299G polymorphism was present in 9% of cirrhotic patients with ascites. As its frequency in non-cirrhotic populations is approximately 10%[5,6,11-13,17,31], our results suggest that the incidence of this polymorphism in cirrhotic patients is probably similar to that in the general population. We were unable to confirm this, however, as we did not have a control group to verify the incidence of the polymorphism in this population.

The incidence of encephalopathy as first decompensation was higher in TLR4 D299G polymorphism patients than in wild type patients. In addition, this complication showed a trend to a higher frequency during the study period in the former group. Polymorphisms of glutaminase have recently been observed to increase the risk of encephalopathy[32]. As the genes of glutaminase and TLR4 are located in different chromosomes (2 and 9, respectively)[31,33] it is unlikely that they cosegregate. Inflammation is increasingly involved in the pathogenesis of hepatic encephalopathy[34]. It could therefore be that a different inflammatory response related to the presence of the TLR4 D299G polymorphism favours the development of this complication. Finally, the trend to a higher incidence of encephalopathy in patients with the polymorphism could be a consequence of the higher number of infections in these patients[1,2,4,34]. This could also account for the higher serum creatinine levels in patients with the TLR4 D299G polymorphism, as they presented a tendency to a higher incidence of infections as the cause of the present hospitalization[1,2,23].

Interestingly, the time period from first decompensation to present admission was longer in TLR4 D299G polymorphism patients than in wild type patients. This difference was more marked in patients with cirrhosis due to hepatitis C virus, but it did not reach statistical significance. This finding, together with the fact that the degree of liver insufficiency was similar in the two groups, could suggest a slower evolution of cirrhosis in patients with the TLR4 D299G polymorphism. The inflammatory response to PAMPS of intestinal origin seems to play a key role in the phenomena involved in liver damage, hyperdynamic circulatory state and complications of portal hypertension in cirrhosis[4,7,29,30]. It could thus be hypothesized that the TLR4 D299G polymorphism may induce a different immune response against PAMPS such as LPS and this could account for a less severe evolution of the disease. It has recently been observed that the

presence of the TLR4 T399I and D299G polymorphisms decreases the risk of fibrosis in patients with chronic hepatitis C[35-37]. This has been considered as likely due to a reduction in TLR4-mediated inflammatory and fibrogenic signalling and a lower apoptotic threshold of activated hepatic stellate cells[38]. Therefore, in addition to clinical, analytical and hemodynamic parameters that have been shown to predict complications and mortality in cirrhotic patients[18-25,39-42], genetic factors such as TLR polymorphisms could influence the course of cirrhosis by modifying the inflammatory response.

In spite of limitations regarding the low incidence of the TLR4 D299G polymorphism and the retrospective nature of the study, our results suggest that genetic factors such as TLR4 polymorphisms could influence not only predisposition and severity of bacterial infections but also the evolution of the disease in cirrhotic patients. Further prospective studies in larger series of patients are warranted and other genetic polymorphisms and immunological mechanisms involved should be evaluated.

ACKNOWLEDGEMENTS

We thank Carolyn Newey for English language revision. This study was supported in part by grant PS09/00357 from the Instituto de Salud Carlos III, Ministerio de Ciencia e Innovación.

FINANCIAL DISCLOSURE: the authors do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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TABLES

Table 1. Characteristics of patients with the TLR4 D299G polymorphism and wild type patients.

	D299G n=10	Wild Type n=101	p
Age (yr)	65.8±10.6	66.8±12.8	0.65
Sex (male/female)	6 (60)/4 (40)	57 (56.4)/44 (43.6)	1
Alcoholic etiology	4 (40)	38 (37.6)	1
Hepatitis C virus	4 (40)	45 (44.5)	1
Child-Pugh class, A/B/C	1 (10)/9(90)/0	14(13.8)/65(64.3)/ 22(21.7)	0.86
Child-Pugh score	8.1±1	8.2±1.5	0.86
MELD score	16.9±4.3	15.9±6.1	0.25
Serum sodium (mmol/L)	135.5±6.5	135.0±5.7	0.97
Urea (mmol/L)	10.5±4.8	11.2±11.7	0.63
Creatinine (µmol/L)	130.2±31.2	115.5±73.3	**0.048**
Albumin (g/L)	28.2±5.8	28.5±5.8	0.99
INR	1.4±0.2	1.5±0.7	0.64
Ascitic fluid total protein (g/L)	11.5±4.5	14.8±6.4	0.22
Previous ascites	10 (100)	101 (100)	1
Refractory ascites	4 (40)	31 (30.7)	0.72
Previous encephalopathy	7 (70)	38 (37.6)	0.08
Previous variceal bleeding	4 (40)	36 (35.6)	0.74
Hepatocellular carcinoma	1 (10)	18 (17.9)	0.75

Norfloxacin prophylaxis	2 (20)	8 (7.9)	0.22
Beta-blockers	6 (60)	47 (46.5)	0.51
Diuretics	5 (50)	76 (75.2)	0.13
Active alcoholism	2 (20)	22 (21.7)	1
Diabetes	4 (40)	42 (41.5)	1
First decompensation			
Ascites	6 (60)	71 (70.3)	0.49
Encephalopathy	2 (20)	2 (1.9)	**0.04**
Variceal bleeding	2 (20)	23 (22.8)	1
Infection	0	5 (4.9)	1
Cause of present admission			
Ascites	1 (10)	33 (32.6)	0.17
Encephalopathy	4 (40)	17 (16.8)	0.09
Variceal bleeding	0	8 (7.9)	1
Infection	4 (40)	21 (20.7)	0.22
Other	1 (10)	22 (21.7)	0.68

Data are mean±SD or frequency (%)

Table 2. Overall incidence, number of episodes and causative bacteria of previous infections in patients with the TLR4 D299G polymorphism and wild type patients.

	D299G n=10	Wild type n=101	p
Total infections			
Patients	8 (80)	57 (56.4)	0.19
Number of infections	28	103	
Number per patient	2.8±2.3	1.0±1.3	**0.01**
Infections caused by GNB[1]			
Patients	5 (50)	31 (30.6)	0.29
Number per patient	1.5±2.1	0.5±1.1	0.09
Infections caused by GPC[2]			
Patients	5 (50)	21 (20.7)	0.05
Number per patient	1.1±1.2	0.2±0.4	**0.009**

Data are mean±SD or frequency (%)

[1]GNB: gram-negative bacilli

[2]GPC: gram-positive cocci

Table 3. Type of previous infections in patients with the TLR4 D299G polymorphism and wild type patients.

	D299G n=10	Wild type n=101	p
SBP[1]			
Patients	4 (40)	35 (34.6)	0.74
Number of SBP episodes	7	37	
Number per patient	0.7±1.2	0.3±0.5	0.60
Caused by GNB[2]	1 (10)	9 (8.9)	1
Caused by GPC[3]	2 (20)	9 (8.9)	0.26
Culture negative	2 (20)	18 (17.8)	1
Bacteremia			
Patients	2 (20)	4 (3.9)	0.09
Number of bacteremias	4	4	
Number per patient	0.4±1.0	0.04±0.2	**0.02**
Caused by GNB	1 (10)	2 (1.9)	0.25
Caused by GPC	2 (20)	2 (1.9)	**0.04**
Urinary infections			
Patients	4 (40)	25 (24.7)	0.28
Number of urinary infections	12	47	
Number per patient	1.2±2.1	0.4±1.1	0.22
Caused by GNB	3 (30)	21 (20.7)	0.45
Caused by GPC	1 (10)	7 (6.9)	0.54

Other infections			
Pneumonia	2 (20)	7 (6.9)	0.19
Bacterascites	0	3 (2.9)	1
Cellulitis	1 (10)	2 (1.9)	0.25
Secondary peritonitis	0	6 (5.9)	1

Data are mean±SD or frequency (%)

[1]SBP: spontaneous bacterial peritonitis

[2]GNB: gram-negative bacilli

[3]GPC: gram-positive cocci