Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Genetic variation in *TNFA* predicts protection from severe bacterial infections in patients with end-stage liver disease awaiting liver transplantation

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Background & Aims: Augmented susceptibility to infections increases mortality in patients with end-stage liver disease (ESLD). We sought to determine the contribution of selected genetic variants involved in inflammatory signalling downstream of the Toll-like receptor 4 (TLR4) to severe bacterial infections (SBIs) in patients with ESLD.

Methods: We retrospectively assessed incidence of SBIs in 336 adult ESLD patients enlisted for orthotopic liver transplantation (OLT) and genotyped them for *TLR4* c.+1196C/T, *CD14* c.−159C/T, *TNFA* c.−238G/A, *TNFA* c.−863C/A, *IL1B* c.−31C/T and *IL1RN* variable number of tandem repeats allelic variants. Principal findings were validated in an independent cohort of 332 ESLD patients.

Results: Thirty-four percent of patients from the identification cohort and 40% of patients from the validation cohort presented with SBI while enlisted for OLT. The presence of the variant allele *TNFA* c.−238A (rs361525) was associated with lower serum levels of TNF-α, and with significantly decreased risk of SBI in both cohorts. Multivariate analysis showed that the relative protection from SBI associated with this allele almost completely negated the increased susceptibility to SBI owed to advanced ESLD. Although not predictive of overall mortality, the presence of the *TNFA* c.−238A allele was associated with a complete prevention of SBI-related pre-transplant deaths.

Conclusions: Our results suggest that genetic variability in inflammatory signalling is associated with the development of SBI in patients with ESLD. Specifically, we identified the importance of the *TNFA* c.−238A allele as a strong predictor of protection from SBI, and as a genetic marker associated with significantly improved pre-transplant survival in patients with SBI.

Keywords: Liver cirrhosis; Inflammatory signalling; Toll-like receptor 4; Innate immune cells; Neutrophil exhaustion.

Received 1 May 2013; received in revised form 5 December 2013; accepted 6 December 2013; available online 18 December 2013

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Abbreviations: SBI, severe bacterial infections; ESLD, end-stage liver disease; OLT, orthotopic liver transplantation; TLR, toll-like receptors; *TLR4*, toll-like receptor 4; LPS, lipopolysaccharide; SNP, single nucleotide polymorphism; TNF-α, tumour necrosis factor-alpha; IL-1β, interleukin-1 beta; *TNFA*, tumour necrosis factor alpha; *IL1B*, interleukin 1 beta; *IL1RN*, interleukin 1 receptor antagonist; VNTR, variable number of tandem repeats; IL-1RA, interleukin-1 receptor antagonist; SBP, spontaneous bacterial peritonitis; EASL, European Association for the Study of the Liver; CRP, C-reactive protein; PCR, polymerase chain reaction; HWE, Hardy-Weinberg equilibrium; CV, coefficient variability; OR, odds ratio; CI, Confidence Interval; MELD, model for end-stage liver disease.

Introduction

Severe bacterial infections (SBIs) represent the second leading cause of death in patients with end-stage liver disease (ESLD) waitlisted for orthotopic liver transplantation (OLT) [1,2], with mortality approaching 10% in European registries [3] and 23% in the US [4]. The high mortality attributed to SBIs in this patient category is driven by the impaired antimicrobial response associated with ESLD [5,6], and by progression of liver failure that is accelerated by severe infection [1,2]. Cirrhotic patients are prone to develop SBIs because of compromised antimicrobial defence caused by liver synthetic failure, portal hypertension and bacterial translocation from the gut [1,2,5,7,8], in conjunction with altered function of immune cells, including impaired opsonising and neutrophil phagocytic capacity [9,10]. Therefore, early identification of patients with ESLD at risk for SBIs is of paramount importance, but indicators predicting the development of SBIs are missing.

Toll-like receptors (TLRs) play a key role in innate immune responses by recognition of a broad range of microbial components and triggering signals critical for antimicrobial defences [11–13]. Although substantially conserved across species, TLRs

show a genetic variability that modulates their downstream signalling, potentially determining individual susceptibility to infections [14].

It has been shown that the variant p.399Ile of *TLR4* corresponding to the nucleotide substitution *TLR4* c.+1196C/T (rs4986791) changes the ligand-binding site of the receptor [15] and, in one study with limited sample size, predisposed cirrhotic patients to infections [16]. Plasma concentration of CD14 is affected by the promoter polymorphism c.−159C/T (rs2569190) [17], which influences expression of the protein [18] and the risk of death in patients with sepsis [19]. SNPs at the positions c.−863 (rs1800630) and c.−238 (rs361525) of the *TNFA* promoter independently influence transcription of this gene [20–22].

The Interleukin 1 gene cluster on chromosome 2 contains genes *IL1B* and *IL1RN* encoding the pro-inflammatory IL-1β and the anti-inflammatory IL-1 receptor antagonist (IL-1RA) [23]. The variant c.−31T (rs1143627) in the *IL1B* promoter increases the transcriptional activity of this gene [24]. The second intron of *IL1RN* contains a variable number of tandem repeats (VNTR) 86-bp long. Allele 2 (*IL1RN*∗2) increases the concentration of IL-1β *in vitro* [25] and increases mortality in septic patients [26].

The above mentioned studies [16,19,26,27] demonstrated significant associations between genetic variants and susceptibility to bacterial infections. However, these studies were performed mainly on limited numbers of non-cirrhotic patients and were not validated. Therefore, we decided to evaluate the contribution of genetic variants in the TLR4 pathway to the development of SBI in large, well characterised independent cohorts of cirrhotic patients with ESLD enlisted for OLT in two centres.

Patients and methods

Patients and definition of severe bacterial infections

Identification cohort

Three hundred and thirty-six patients with liver cirrhosis with Child-Pugh class B and C were enrolled and retrospectively screened for the occurrence of one or more episodes of extrahepatic SBIs during their time on the waiting list and 270 days before enlistment to include also patients with a recent episode of SBI. These patients were sorted out of 708 adult cirrhotic patients who were enlisted for OLT in Prague between February 1995 and June 2010. Patients with Child-Pugh class A and patients with acute liver failure were excluded. Patients with Caroli disease and primary and secondary sclerosing cholangitis were excluded as well, since intrahepatic bacterial complications are characteristic for the natural course of these diseases.

SBIs were defined as the following bacterial infections requiring hospitalisation and treatment with intravenous antibiotics:

(a) Spontaneous bacterial peritonitis (SBP), diagnosis of which was based on neutrophil cell count exceeding 250/mm^3 and/or positive culture of ascitic fluid if secondary causes of peritonitis were excluded (EASL guidelines [28]).
(b) Urinary tract infections diagnosed on the basis of clinical findings (dysuria, fever), pyuria (leukocytes >10/mm^3) and positive urine culture [29].
(c) Pneumonia, diagnosis of which was determined by clinical symptoms (cough, expectoration, and fever), positive chest X-ray and positive bacteriological finding in sputum [30].
(d) Skin and soft tissue infection, diagnosis of which was established by local cutaneous findings (blush, tumefaction, and pain) and leukocytosis [31].
(e) Bacterial infection of unknown origin defined as a positive blood culture with serum C-reactive protein (CRP) level ⩾70 mg/L,

All clinical data were collected from hospitalisation and outpatient medical records archived at our centre.

Validation cohort

The validation cohort (n = 332 cirrhotic patients enlisted for OLT) was selected from the 522 adult patients evaluated for OLT between September 1995 and April 2011 in Erasmus MC-University Medical Centre, Rotterdam, The Netherlands. The selection process was based on the same criteria as in the identification cohort. In 332 selected patients, SBIs were defined according to the same definitions as used in the identification cohort from Prague.

A higher rate of Child-Pugh B patients was observed in the validation cohort because of the Dutch policy to enlist patients for OLT when they had Child-Pugh score 8 (B) or higher. Another reason to enlist patients with cirrhosis staged as Child-Pugh B was the presence of hepatocellular carcinoma. Due to progression of liver disease on the waiting list, most patients had Child-Pugh score C at the moment they were transplanted.

Genotyping

Patients were genotyped for *TLR4* c.+1196C/T, *CD14* c.−159C/T, *TNFA* c.−238G/A, *TNFA* c.−863C/A, *IL1B* c.−31C/T and *IL1RN* VNTR (UniSTS:156109) allelic variants, as described in [32], using specific primers and PCR conditions shown in Supplementary Table 1. In order to minimise genotyping errors, blank control wells were left on the PCR plates and two operators, unaware of the status of the samples, performed the genotype assignment independently. After testing for Hardy-Weinberg equilibrium (HWE), allele frequencies were checked for consistency with data from the population of European ancestry (Utah Residents with Northern and Western European Ancestry) from the HapMap database [33].

Primary assessment of associations between allelic frequencies and SBIs was performed in the identification cohort from Prague, and positive associations were confirmed in the validation cohort from Rotterdam. The study was approved by the institutional Research Ethics Committee of both participating centres. Written informed consent with DNA sampling was obtained from all patients and the study conformed to the declaration of Helsinki Ethical Guidelines.

Determination of serum levels of TNF-α

Serum levels of TNF-α were determined in blood samples taken from patients at the moment of liver transplantation, i.e., in patients with no physical and laboratory signs of infection. The samples were frozen immediately after serum separation and stored at −80 °C. In the identification cohort, TNF-α was assessed in serum samples of 199 patients, out of which 179 patients were homozygotes for the *TNFA* c.−238G allele and 20 patients were heterozygotes. Additional 36 samples (12 samples of patients carrying the *TNFA* c.−238GA genotype and 24 samples of homozygotes for *TNFA* c.−238G) came from the validation cohort.

Quantitative determination of TNF-α was performed with the Quantikine HS ELISA human TNF-α immunoassay (R&D Systems, Abingdon, UK). All standards, controls and samples were analysed in duplicates and the duplicate readings were averaged. Duplicates with coefficient variability (CV) higher than 50% (5 heterozygotes and 25 homozygotes) were excluded and the remaining 27 heterozygotes were then matched in age, sex, and underlying diseases with 81 of the 188 non-excluded heterozygotes.

Statistical analysis

Data are presented as mean and standard deviation, as median and range, or as frequencies, as appropriate. HWE and differences in genotype frequencies between patients with SBI and controls were analysed using two-sided χ^2 testing. Using standard formulas based on two-by-two tables [34], we calculated basic epidemiology statistics and evaluated the preventable fractions among the population and among the exposed. *t* test or Mann-Whitney tests were used for comparisons of the means. Due to the testing of multiple statistical hypotheses, Bonferroni correction was used in the identification cohort. Cox regression analysis was used to calculate hazard ratio and 95% confidence interval (CI). Significant risk factors from univariate analysis were entered into the multivariate Cox regression analysis, which was performed with a forward stepwise approach. Wald statistics was employed in the regression module to evaluate the relative contribution of significant variables to SBI. Kaplan-Meier analysis with log-rank test was performed to evaluate survival. A p value <0.05 was considered statistically significant throughout the study. Statistical analysis was performed using JMP 9.0.0. and SPSS 13.0 programs.