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Study of role of common NOD2 gene polymorphisms in Indian patients with decompensated liver cirrhosis

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ABSTRACT

Background: Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) gene polymorphisms promote intestinal bacterial translocation (BT). Three common NOD2 gene polymorphisms (R702W, G908R and 1007fs) were recently identified as important genetic risk factors for the occurrence of spontaneous bacterial peritonitis (SBP) and also for poor prognosis in western patients of liver cirrhosis. Our aim was to see the association of these three common NOD2 gene polymorphisms with SBP and other major complications of liver cirrhosis, along with disease prognosis, in Indian population cohort.

Methods: Ninety seven consecutive patients of decompensated liver cirrhosis and 45 healthy controls were recruited. Baseline clinical and laboratory parameters were recorded. Child-Turcotte-Pugh (CTP) and Model for End-Stage Liver Disease (MELD) scores were calculated as disease severity scores. All patients and healthy controls underwent genetic testing for the three common NOD2 gene polymorphisms. Association of these NOD2 gene polymorphisms with major complications of cirrhosis as well as with disease severity, and mortality over 6-months was studied.

Results: Out of the three common NOD2 gene polymorphisms studied, only 1007fs polymorphism was identified in six (6.18%) cirrhotic patients. None of the healthy controls showed any of the three polymorphisms. There was no statistically significant association between the presence of 1007fs polymorphism and major complications of cirrhosis or with disease severity and mortality over 6-months.

Conclusions: Common NOD2 gene polymorphisms are not significantly associated with SBP and other major complications of liver cirrhosis or with poor prognosis.

KEYWORDS: Liver cirrhosis, NOD2 polymorphism, spontaneous bacterial peritonitis.

Introduction

Complications related to liver cirrhosis still remain an important cause of mortality worldwide. Ascites, variceal bleeding, spontaneous bacterial peritonitis (SBP), hepatic encephalopathy (HE), hepatorenal syndrome (HRS) and hepatocellular carcinoma (HCC) constitute major complications of cirrhosis.¹ Development of these complications is associated with decreased long-term survival for patients with cirrhosis. For example, in-hospital mortality for the first episode of SBP ranges from 10% to 50%²⁻⁵ and 1-year mortality after the first episode of SBP has been reported to be 31% to 93%.⁶⁻⁸ Similarly, median survival after development of type-1 HRS is less than 2 weeks and after type-2 HRS is approximately 6 months.⁹ Various modifiable and non-modifiable risk factors are involved in the pathogenesis of these complications.

Pathological bacterial translocation (BT) has been termed the “Achilles heel” in cirrhosis¹⁰ and is reported to play a crucial role in the pathogenesis of various complications of cirrhosis like SBP, HRS, HE and variceal bleeds.¹¹⁻¹⁴ Bacterial translocation is defined as translocation of bacteria and/or bacterial products (lipopolysaccharides, peptidoglycans, muramyl-dipeptides, bacterial DNA etc.) from the gut to mesenteric lymph nodes (MLNs).¹⁵ BT, together with porto-systemic shunting and reduced hepatic clearance of bacterial products, leads to endotoxemia, with further worsening of hemodynamic parameters in cirrhosis.¹⁶ The three main mechanisms involved in BT are small intestinal bacterial overgrowth (SIBO), increased intestinal permeability and impaired host defense.¹⁷ Alterations in both local (intestinal) and systemic immunity promote BT in cirrhosis. Some of these alterations are genetically driven. NOD2 protein which plays an important role in the intestinal innate immunity, recognizes bacterial molecules (peptidoglycans) and stimulates an immune reaction to limit the entry of bacteria across gut by activation of NF κ B (nuclear factor-kappa beta) signaling.¹⁸ Hence, polymorphisms of NOD2 gene may serve as a genetic risk factor for development of complications related to BT in patients with liver cirrhosis.

Three common NOD2 gene polymorphisms (R702W, G908R and 1007fs) have been recently identified as important genetic risk factors for the development of SBP and reduced survival in studies conducted in western populations.^{19,20} These studies have also reported an increased risk of HCC and recent variceal bleed among patients with NOD2 variants. Similar studies have not been done in the Indian population till date to see the association of common NOD2 gene polymorphisms with SBP or any other major complications of liver cirrhosis, that involve BT in their pathogenesis. Therefore, we conducted this study to find out association of the three common NOD2 gene polymorphisms with major complications of cirrhosis including SBP, HE, HRS, variceal bleeding and HCC as well as with disease severity and prognosis in a cohort of Indian patients with liver cirrhosis.

Methods

This was a hospital-based prospective study conducted between September 2014 and December 2015 in a tertiary-care hospital in central India. Ninety seven consecutive patients with decompensated liver cirrhosis of age \geq 18 years, admitted with various complications were recruited for the study, along with 45 age and gender matched healthy controls. Patients younger than 18 years, pregnant women, those with secondary bacterial peritonitis, ascites of mixed etiology, or extra-hepatic malignancy, patients on long-term antibiotic therapy and those who did not give consent to participate in the study were excluded. The institutional ethical committee approved our study protocol. The study followed the Helsinki Declaration of 1975, as revised in 2000 and 2008, concerning human and animal rights.

Detailed clinical interview and physical examination were performed at the time of admission. Biochemical and serological testing in form of complete hemogram, liver function tests, renal function tests, serum electrolytes and viral markers (HBsAg & Anti HCV) were performed. Diagnosis of cirrhosis was based on clinical, laboratory, ultrasonography & endoscopic criteria.²¹ The development of complications such as variceal hemorrhage, ascites, hepatic encephalopathy (HE), jaundice, or hepatocellular carcinoma (HCC)

characterizes decompensated cirrhosis.²² Relevant tests were performed to establish the etiology of cirrhosis according to standard criteria. For ascitic fluid cell count, 2.5 ml ascitic fluid was collected in an EDTA vial prior to antibiotic administration, and counts were manually determined microscopically. The concentration of protein and albumin was determined by routine laboratory analysis. For ascitic fluid culture, 10 ml of ascitic fluid was collected in a blood culture bottle at the patients' bedside. Diagnosis of SBP was made when ascitic fluid polymorphonuclear (PMN) count was $\geq 250/\mu\text{l}$ in the absence of a surgically treatable intra-abdominal source of infection [according to European association for the study of liver (EASL) guideline].²³ HRS and HE were diagnosed using the previously validated standard criteria.^{24,25} Diagnosis of acute on chronic liver failure (ACLF) was made according to criteria established by Asian Pacific Association for the study of liver (APASL)²⁶ and diagnosis of HCC was made according to American association for the study of liver disease (AASLD) criteria.²⁷ The severity of the underlying liver disease was assessed according to the Child-Turcotte-Pugh (CTP) score^{28,29} and model for

end-stage liver disease (MELD) score.³⁰ All patients and healthy controls underwent genetic testing for the three common NOD2 gene polymorphisms (R702W, G908R and 1007fs). Genomic DNA was extracted from EDTA-anticoagulated blood using a membrane based extraction kit. 1007fs and R702W polymorphisms were detected by amplified-refractory mutation system (ARMS) – polymerase chain reaction (PCR) using primers shown in **Table 1**. G908R polymorphism was detected by polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) method using primer and restriction enzyme as shown in **Table 1**.

Difference in frequency of common NOD2 gene variants in patients with cirrhosis and healthy controls was noted, and association of these polymorphisms with major complications of cirrhosis including SBP, HRS, HE, variceal bleed and HCC was assessed. All cirrhotic patients were subdivided into two clinically relevant severity groups according to their CTP (<10 or ≥ 10) and MELD (≤ 21 or >21) score in order to see the association of NOD2 gene polymorphisms with disease severity. All cases were followed up for at least 6-months from admission, and mortality over this period was noted.

Table 1: Primers, PCR product sizes, restriction enzymes and digestion product sizes for the three common NOD2 polymorphisms studied.

	Forward and reverse primer sequences	PCR product size	Restriction enzyme	Digestion product size
<i>R702W</i>	Forward Wild: 5' ATCTGAGAAGGCCCTGCTCC 3' Forward Mutant: 5' ATCTGAGAAGGCCCTGCTCT 3' Common Reverse: 5' CCCACACTTAGCCTTGATG 3'	439bp	-	-
<i>G908R</i>	Forward: 5' CCCAGCTCCTCCCTCTTC 3' Reverse: 5' AAGTCTGTAATGTAAAGCAC 3'	380bp	HhaI	GG=380bp GR=380bp+138bp+242bp RR=130bp+242bp
<i>1007fs</i>	Forward Wild: 5' CAGAAGCCCTCCTGCA GGCCCT 3' Forward Mutant: 5' CAGAAGCCCTCCTGCAGGCCCT3' Common Reverse: 5' TCTTCAACCACATCCCCATT 3'	333bp	-	-

PCR – Polymerase chain reaction, NOD2 – Nucleotide binding oligomerization domain containing protein 2.

Baseline patient characteristics were reported either as median and range for continuous variables or as frequency for discrete variables. All data was organized in Microsoft excel and analyzed using MedCalc software trial version (version 15.6, MedCalc software bvba, Ostend, Belgium). Chi-square test was done to identify association of complications encountered in cirrhosis with the frequency of common NOD2 gene polymorphisms. Statistical tests were based on two-tailed probability. P-value < 0.05 was taken as significant.

Results

We studied 97 consecutive adult patients, admitted with various complications related to decompensated liver cirrhosis in the gastroenterology department of our hospital. Baseline clinical and laboratory parameters are shown in **Table 2**. Majority of patients were male (84.53%) and had alcohol-related cirrhosis (57.73%) followed by hepatitis B-related cirrhosis (20.62%). Ascites (70.10%) and variceal bleed (45.36%) were the most common presentations followed by SBP (23.71%), HE (19.58%) and HRS (12.37%). Ascitic fluid culture was positive in 11 patients. Microbiological profile of ascitic fluid was as follows: *Klebsiella Pneumoniae* (4 patients), *Escherichia Coli* (1 patient), Coagulase-negative *Staphylococcus Aureus* (3 patients), *Enterococci* (2 patients) and *Candida Albicans* (1 patient). Out of the three common NOD2 gene polymorphisms studied, only 1007fs polymorphism was identified in six (6.18%) patients of cirrhosis but none of our healthy controls showed any NOD2 gene polymorphism. On subgroup analysis of our data we did not find statistically significant difference in the frequency of the 1007fs polymorphism in patients with or without complications like SBP, HE, variceal bleed, HRS, ACLF or HCC as shown in **Table 3**. Furthermore, we did not find any significant association of 1007fs polymorphism with either severity of liver cirrhosis as assessed by CTP and MELD scores or mortality over 6-months as shown in **Table 4**.

COX regression analysis was done for identifying the risk factors associated with mortality over 6 months and we found that only MELD and SBP have high hazard

ratio, 1.137 (95%CI 1.054-1.227) and 5.178 (95%CI 2.212-12.120) respectively as shown in **Table 5**.

Discussion:

The present study showed that common NOD2 gene polymorphisms (R702W, G908R and 1007fs) are infrequent in Indian patients of decompensated liver cirrhosis as well as in healthy population. This finding is in concordance with results of earlier studies from India conducted in patients with inflammatory bowel disease. Substantial work has been done in India in the past to see the role of NOD2 gene polymorphisms in patients of inflammatory bowel disease. In a large study conducted in northern India by Juyal et al in 2007, over 298 patients with ulcerative colitis, 25 patients with Crohn's disease and 262 healthy controls reported that the above three common NOD2 gene polymorphisms were infrequent. Only G908R polymorphism was found in 2 patients with ulcerative colitis.³¹ Similarly, a study from southern India by Pugazhendhi et al. among 82 patients of Crohn's disease and 149 controls did not show any of the three common NOD2 gene polymorphisms in patients as well as in healthy controls.³²

In the present study we did not find statistically significant association of the three common NOD2 gene polymorphisms with any major complication of decompensated liver cirrhosis including SBP, variceal bleeding, HE, HRS, ACLF or HCC. The severity of cirrhosis, assessed by CTP and MELD scores, as well as mortality over 6months were also independent of the presence of NOD2 gene polymorphism. This is in contrast to earlier reports from western populations. Appenrodt et al had reported that NOD2 variants are genetic risk factors for death and SBP in patients of liver cirrhosis. The occurrence of SBP was increased significantly (P-value = 0.008) in carriers of NOD2 variants (OR = 3.06).¹⁹ Bruns et al further validated these findings and showed that NOD2 variants increase the risk of culture-positive SBP and bacterial ascites in western populations. They had also reported that carriers of common NOD2 variants have reduced survival as compared to normal wild type genotype. In their study, occurrence of HCC and recent variceal bleed were more common in patients with NOD2 gene variant.²⁰

Table 2: Baseline characteristics of patients with cirrhosis.

Total number of study patients (n)	97
Median age (years), (Range)	47.5 (26 – 80)
Gender (Male/Female)	82/15
Etiology of cirrhosis, n (%)	
Alcohol	56 (57.73)
Hepatitis B	20 (20.62)
Autoimmune	2 (2.06)
NASH	6 (6.18)
Cryptogenic	13 (13.40)
Presentation, n/total (%)	
Ascites	68/97 (70.10)
Variceal bleed	44/97 (45.36)
HE	19/97 (19.58)
SBP	23/97 (23.71)
HRS	12/97 (12.37)
ACLF	14/97 (14.43)
HCC	7/97 (7.22)
Laboratory parameters	
Median Hemoglobin (g/dl), (range)	8.4 (4.9 – 14.8)
Median TLC ($\times 1000/\mu\text{l}$), (range)	5.7 (1 – 29.8)
Median Platelets ($\times 1000/\mu\text{l}$), (range)	237 (21 – 382)
Median Creatinine (mg/dl), (range)	0.75 (0.52 – 4.58)
Median Sodium (mEq/L), (range)	135 (119 – 146)
Median AST (IU/L), (range)	42.5 (20 – 879)
Median ALT (IU/L), (range)	35 (14 – 279)
Median Bilirubin (mg/dl), (range)	0.85 (0.34 – 27.74)
Median Albumin (g/dl), (range)	2.49 (1.38 – 3.63)
Median PT (seconds), (range)	21 (14 – 48)
Median CTP score (range)	9.5 (6 – 14)
Median MELD score (range)	12.5 (6 – 39)
Minor allele frequency of three NOD2 gene polymorphisms in total study patients (%)	
R702W	0.00
G908R	0.00
1007fs	6.18
Patients died over 6-months, n/total (%)	26/97 (26.80)

SBP-Spontaneous bacterial peritonitis, HE-Hepatic encephalopathy, HRS-Hepatorenal syndrome, ACLF-Acute on chronic liver failure, HCC-Hepatocellular carcinoma, TLC-Total leucocyte count, AST-Aspartate aminotransferase, ALT-Alanine aminotransferase, PT-Prothombin time, CTP-Child Turcotte Pugh, MELD-Model for End Stage Liver Disease.

Table 3: Minor allele frequency of 1007fs polymorphism associated with presence or absence of different complications of cirrhosis.

	Frequency of 1007fs polymorphism (%)	OR, (95% CI)	P-value
SBP	8.7	1.667 (0.285-9.746)	0.567
No SBP	5.4		
HE	10.5	2.176 (0.368-12.872)	0.381
No HE	5.1		
Variceal bleed	2.3	0.223 (0.025-1.987)	0.145
No variceal bleed	9.4		
HRS	8.3	1.455 (0.155-13.631)	0.741
No HRS	5.9		
ACLF	7.1	1.200 (0.130-11.114)	0.872
No ACLF	6		
HCC	14.3	2.833 (0.284-28.292)	0.356
No HCC	5.6		

SBP-Spontaneous bacterial peritonitis, HE-Hepatic encephalopathy, HRS-Hepatorenal syndrome, ACLF-Acute on chronic liver failure, HCC-Hepatocellular carcinoma, OR-Odds ratio, CI-Confidence interval.

Table 4: Minor allele frequency of 1007fs polymorphism associated with severity of cirrhosis (as assessed by CTP and MELD score) and outcome over 6-months.

	Frequency of 1007fs polymorphism (%)	OR, (95% CI)	P-value
CTP score < 10	2.5%	3.750 (0.421-33.400)	0.207
CTP score ≥ 10	8.8%		
MELD score ≤ 21	46.3%	0.922 (0.160-5.311)	0.927
MELD score > 21	5.9%		
Patients survived over 6- months	34.2%	2.957 (0.557-15.685)	0.185
Patients died over 6-months	11.5%		

CTP-Child Turcotte Pugh, MELD-Model for End Stage Liver Disease, OR-Odds ratio, CI-Confidence interval.

The failure to find an association between common NOD2 polymorphisms and complications of liver cirrhosis in Indian patients can be explained by the following reasons. First, the pathogenesis of complications in liver cirrhosis is multifactorial and hence inability to find a particular genetic change in a small group of patients is acceptable. The second important fact is the difference in the prevalence of NOD2 gene polymorphisms among different races and in different parts of the world. The three common NOD2 gene polymorphisms are found in up to 30% patients with Crohn's disease in western populations³³ but are rare in Asian countries.^{34,35}

Table 5: Cox Regression 6-month survival analysis.

Variables	Hazards Ratio	P-Value	95% CI
Age	0.996	0.873	0.952-1.043
Gender	0.479	0.313	0.115-1.998
MELD Score	1.137	0.001	1.054-1.227
CTP Score	0.992	0.963	0.714-1.380
SBP	5.178	<0.0001	2.212-12.120
ACLF	0.742	09.603	0.241-2.284
NOD2 (1007fs) gene polymorphism	1.133	0.861	0.282-4.542

MELD-Model for End Stage Liver Disease, CTP-Child Turcotte Pugh, SBP-Spontaneous bacterial peritonitis, ACLF-Acute on chronic liver failure, CI-Confidence interval.

These observations suggest that although the clinical profile of our patients is similar to patients in western studies, the three common NOD2 gene polymorphisms are infrequent and are not associated with major complications in our patients with liver cirrhosis. It is possible that other NOD2 gene polymorphisms or different genetic factors are involved in the development of complications in Indian patients with liver cirrhosis. This warrants further research in larger populations. Our study is limited by its relatively small sample size, a single study center and examination of only 3 NOD2 gene polymorphisms.

In conclusion, the three common NOD2 gene polymorphisms are infrequent and are not associated with SBP and other major complications of decompensated liver cirrhosis or with poor prognosis in our Indian population cohort.

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