

The role of the eNOS G894T and T-786C gene polymorphism in the development of ascites in cirrhosis

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Abstract. – OBJECTIVE: Increased nitric oxide (NO) production in cirrhotic patients causes splanchnic vasodilation, leading to the development of the hyperdynamic circulatory syndrome. One factor that influences plasma NO concentration is eNOS gene polymorphism; consequently, the aim of this study was to investigate whether the eNOS gene G894T and T-786C polymorphisms play any role in the development of ascites in such patients.

PATIENTS AND METHODS: Three groups were created: 70 cirrhotic patients with ascites, 69 cirrhotic participants without ascites (stable cirrhosis), and 60 healthy controls. Polymorphisms were determined using polymerase chain reaction (PCR) and melting curve analysis. The plasma nitrite (NO marker) level was measured by deploying the spectrophotometric Griess reaction.

RESULTS: Plasma nitrite levels in the cirrhosis with ascites and stable cirrhosis groups were significantly higher than in the controls ($p < 0.0001$). The frequency of GG, GT, and TT genotypes for the eNOS G894T polymorphism in the cirrhosis with ascites group was 55.7%, 38.6%, and 5.7% respectively, while in the stable cirrhosis group these figures were 60.9%, 36.2%, and 2.9%. In the controls, the distribution was 63.3%, 33.3%, and 3.3%, respectively. The frequency of TT, TC, and CC genotypes for the eNOS-786C polymorphism in the first group was 52.9%, 34.2%, and 12.9% respectively; in the second group, this was 46.4%, 42%, and 11.6%, and in the controls, 48.3%, 46.7%, and 5%. There were no significant differences in genotype and allele distributions of the eNOS-786C and eNOS G894T polymorphisms among the groups.

CONCLUSIONS: Plasma nitrite concentration is enhanced in cirrhotic patients, and there is no relationship between the G894T and eNOS-786C polymorphisms and the development of ascites.

Key Words:

Cirrhosis, Nitric oxide, Polymorphism, eNOS gene polymorphism, Ascites.

Introduction

The most common complication in the decompensated cirrhotic patient is ascites. The development of the latter is the consequence of complex pathophysiological events involving portal hypertension; subsequently, neurohumoral stimulation and splanchnic and peripheral arterial vasodilation occur. Recently, different molecules that are responsible for the splanchnic vasodilation of cirrhosis have been suggested as possible mediators, such as glucagon, substance P, prostacyclin, intestinal vasoactive peptide¹, adrenomedullin^{2,3}, and nitric oxide (NO)^{4,5}.

NO is a potent vasodilator that plays a key role in abnormal hemodynamics. According to certain studies, NO is elevated in the peripheral circulation of patients with cirrhosis^{6,7}. It originates from L-arginine by three nitric oxide synthase isoenzymes, including neuronal, inducible, and endothelial nitric oxide synthase. There are many polymorphisms in various zones of the eNOS gene on chromosome 7, which involves 26 exons⁸. A functional polymorphism in exon 7 of eNOS corresponds to a Glu-Asp change at codon 298 (also referred to as G894T). There is a tendency toward decreased eNOS enzyme activity in eNOS G894T allele carriers, compared with GG homozygotes⁹. Another polymorphism is a point mutation of thymine to cytosine at nucleotide -786 (T-786C) in the 5'-flanking area of

the eNOS gene, which could result in a significant downgrading of promoter activity¹⁰ and a significantly reduced NO level¹¹. The aim of the present study was to elucidate whether or not these two eNOS polymorphisms play any role in the development of ascites.

Patients and Methods

Study Design

This was a prospective study that was conducted at the inpatient and outpatient gastroenterology department of the Inonu University Medical Faculty from January 2012 to December 2013. The study was approved by the Ethics Committees of the institutions involved (protocol no. 2012/043) in compliance with the Declaration of Helsinki. All patients signed their informed consent before the study.

Participants

A total of 139 patients with cirrhosis (70 patients with ascites and 69 without), as well as 60 controls, were included in the study. Participants were divided into three study groups, as follows:

Group 1: Cirrhosis with Ascites. In all, 70 patients with verified cirrhosis, according to clinical, biochemical, and ultrasonographic evaluation, were recruited in this group. Ascitic fluid, which was obtained via paracentesis, was used for white blood cell count and culture to identify spontaneous bacterial peritonitis. In addition, C-reactive protein (CRP) and procalcitonin levels were studied in order to exclude other likely infections. Exclusion criteria were as follows: (1) Patients with ascitic fluid infection or other active infections; (2) Patients who had been receiving nitrates; (3) Patients with bleeding of gastrointestinal or esophageal varices; (4) Patients with known systemic diseases, such as heart failure, acute renal injury, end-stage renal disease, diabetes mellitus, hyperlipidemia, hypothyroidism, hyperthyroidism, and hematological and neoplastic disorders (including hepatocellular carcinoma). (5) Patients who had diuretic-resistant ascites; this was to ensure homogenization of the study group.

Group 2: Cirrhosis without Ascites: In this group, 69 patients were recruited who had clinical, biochemical, ultrasonographic, and

histologic diagnoses of cirrhosis that was secondary to various causes, including alcohol, viral hepatitis, autoimmune hepatitis, or unknown etiology. All patients had compensated cirrhosis without ascites.

Group 3: Control. Finally, 60 controls were recruited from the gastroenterology outpatient clinic. These participants had no liver or kidney disorders, active infections, inflammatory diseases, or malignancies.

Clinical Data

Clinical data was recorded via patient interviews. Peripheral blood samples were obtained from all participants in order to analyze blood counts, routine biochemistry, prothrombin time (INR), and eNOS gene polymorphism molecular analysis. A Child-Pugh and Model for End-Stage Liver Disease (MELD) score were calculated for all patients included in the study.

eNOS Gene Polymorphism Molecular Analysis

Isolation of Genomic DNA

Genomic DNA was isolated from peripheral blood leukocytes using a High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions, and was stored at -20°C until analysis.

Genotype Analysis

Determination of polymorphisms (G894T (GLu298Asp) (E298D) (rs 1799983)) and 786 T>C (rs2070744) was performed using LightCycler real-time PCR (Roche Diagnostics, Mannheim, Germany), with the aid of a LightSNiP assay from TIB MOLBIOL (Berlin, Germany). A 20 μ l mixed reaction solution containing 10.4 μ l of H₂O, 2 μ l of LC™ FastStart DNA Master HybProbe kit (Roche Diagnostics), 1.0 μ l of LightSNiP reagent mix, 1.6 μ l of MgCl₂ (25 mM), and 5 μ l genomic DNA, was transferred into capillary tubes. The PCR conditions were 10 minutes for initial denaturation at 95°C, 45 cycles at 95°C for 10 seconds for denaturation, 10 seconds at 60°C for annealing, and 15 seconds at 72°C for extension. In the melting point analysis, fluorescence resonance energy transfer was used to detect polymorphic sites. Melting curve analysis was performed with an initial denaturation at 95°C for 20 seconds and 40°C for 20 seconds, be-

fore slowly heating to 85°C with a ramping rate of 0.2°C/s and continuous fluorescence detection. Polymorphic, mutated, and wild-type alleles were identified by the specific melting temperature (T_m) of the resulting amplicons. T_m values for the G894T (rs 1799983) G and T alleles were 60.01°C and 65.98°C, respectively, while for the -786 T>C (rs2070744) C and T alleles they were 59.09°C and 66.17°C, respectively. The values for the respective melting temperatures were varied to be either +/- 2.5°C between different experiments. Genotype screening was performed simultaneously for all three groups.

Plasma Nitrite Assay

Plasma nitrite levels were measured via a spectrophotometric Griess reaction, as it has been shown that total nitrite is an index of endogenous nitric oxide generation^{12,13}. The procedure was partly adapted from the method described by Ozbek et al¹⁴.

Statistical Analysis

The Statistical Package for the Social Sciences SPSS17.0 (SPSS, Chicago, IL, USA) was used for all analyses. Normality for continued variables in groups was determined by the Kolmogorow-Smirnov test; normally distributed data was summarized by mean and standard deviation. Due to these variables, comparison of the groups was performed using one-way ANOVA and the Tukey method. For non-normally distributed data, median, minimum, and maximum values were used for expression, the Kruskal-Wallis test was applied for comparison of the groups, and the Conover method was used for multiple comparisons. Categorical data were presented as

numbers (n) and percentages (%). Pearson and Fisher's X^2 -tests were deployed for testing genotypic and allelic association. A value of $p < 0.05$ was considered significant.

Results

Patients

A total of 139 patients with cirrhosis (70 patients with ascites and 69 without), together with 60 controls, were included in the study. The cirrhosis with ascites group included 27 (38.6%) women and 43 (61.4%) men, while the patients with stable cirrhosis included 27 (39.1%) women and 42 (60.9%) men. There was no difference between the control group and the other groups in terms of age and sex ($p > 0.05$).

Underlying etiologies for cirrhosis were hepatitis B in 64 patients (46%), hepatitis C in 12 (8.6%), autoimmune hepatitis in 5 (3.6%), alcoholic liver disease in 33 (23.7%), other causes (Wilson disease, NASH) in 8 (5.8%), and cryptogenic cirrhosis in 17 (12.3%). The clinical characteristics of the study groups are presented in Table I.

Plasma Nitrite Levels

Nitrite levels in the cirrhosis with ascites and stable cirrhosis groups were significantly higher than in the controls ($p < 0.0001$). Meanwhile, nitrite levels in patients with eNOS G894T homozygous TT and GT genotypes were significantly lower than in respondents with the GG genotype within the cirrhosis with ascites group ($p = 0.0075$). Similarly, such levels in patients with the eNOS T786C homozygous CC genotype

Table I. Mean age and laboratory data of the study groups.

Parameters	Cirrhosis with ascites (n = 26)	Stable cirrhosis (n = 48)	Control (n = 50)	p-value
Age (years)*	55.5 ± 13.86	50.03 ± 14	53.2 ± 11.74	> 0.05
AST (U/L)*	138.65 ± 30.55 ^{a,b}	53.82 ± 8.01 ^{a,c}	25.80 ± 3.55 ^{b,c}	< 0.001
ALT (U/L)*	82.57 ± 18.85 ^{a,b}	50.15 ± 7.99 ^{a,c}	28.22 ± 2.90 ^{b,c}	< 0.001
Albumin (g/dL)*	2.45 ± 0.17 ^{a,b}	3.29 ± 0.15 ^{a,c}	4.33 ± 0.20 ^{b,c}	< 0.001
INR*	1.78 ± 0.18 ^{a,b}	1.25 ± 0.16 ^{a,c}	0.92 ± 0.05 ^{b,c}	< 0.001
Total bilirubin (mg/dL)*	6.33 ± 1.92 ^{a,b}	2.78 ± 0.79 ^{a,c}	0.82 ± 0.08 ^{b,c}	< 0.001
Child-Pough score	11.52 ± 1.65 ^b	7.55 ± 1.98 ^c	–	
MELD score*	26.35 ± 1.91 ^b	13.06 ± 1.76 ^c	–	< 0.001

AST, aspartate aminotransferase; ALT, alanine aminotransferase; MELD, model for end-stage liver disease. *Quantitative values are expressed as mean ± standard deviation. ^a $p < 0.05$ vs. control group, ^b $p < 0.05$ vs. stable cirrhosis group, ^c $p < 0.05$ vs. cirrhosis with ascites group.

Table II. Genotype and allele frequencies of eNOS polymorphisms 894G/T and T-786C in the groups.

eNOSSNPs	Genotype				Allele				
G894T	GG n (%)	GT n (%)	TT n (%)	Total n	G n (%)	T n (%)		Total n	p-value
Cirrhosis with ascites	39 (55.7)	27 (38.6)	4 (5.7)	70	105 (75)	35 (25)	–	140	$p > 0.05$
Stable cirrhosis	42 (60.9)	25 (36.2)	2 (2.9)	69	109 (79)	29 (21)	–	138	$p > 0.05$
Control	38 (63.3)	20 (33.3)	2 (3.3)	60	96 (80)	24 (20)	–	120	$p > 0.05$
T-786C	TT n (%)	TC n (%)	CC n (%)		T n (%)	C n (%)			$p > 0.05$
Cirrhosis with ascites	37 (52.9)	24 (34.2)	9 (12.9)	70	–	98 (70)	42 (30)	140	$p > 0.05$
Stable cirrhosis	32 (46.4)	29 (42.0)	8 (11.6)	69	–	93 (67.4)	45 (32.6)	138	$p > 0.05$
Control	29 (48.3)	28 (46.7)	3 (5)	60	–	86 (71.7)	34 (28.3)	120	$p > 0.05$

were markedly lower than in participants with TT and TC genotypes within the cirrhosis with ascites group ($p = 0.0116$). The plasma nitrite levels are presented in Table III.

Endothelial Nitric Oxide Synthase G894T Polymorphism

There were no significant differences in the frequency of GG, GT, and TT genotypes and mutant allele T for the eNOS G894T among all three groups ($p > 0.05$). The results of this distribution are presented in Table II.

Endothelial Nitric Oxide Synthase-786T/C Polymorphism

There were no significant differences in genotype and allele distributions of the eNOS-786C polymorphism among all three groups ($p > 0.05$) (see Table II).

Discussion

To date, this is the first study to have evaluated the relationship between eNOS gene polymorphisms and the development of ascites, despite numerous studies having investigated nitric oxide concentration in cirrhotic patients. We investigated the plasma nitrite (NO marker) concentration, as well as attempting to display its relationship with the eNOS gene polymorphism.

The development of ascites is the first and most important symptom of decompensated liver cirrhosis. Splanchnic and peripheral arterial vasodilation, together with neurohumoral activation, plays an important role in the development

of ascites. The role of NO in patients with cirrhosis and portal hypertension has long been established and is considered to be a pivotal factor for reduced mesenteric resistance. Nitric oxide, a strong vasodilator, has a very short half-life (20-30 s). Meanwhile, L-Arginine (L-Arg) is the precursor to NO production by nitric oxide synthase (NOS)¹⁵. In patients with cirrhosis, hepatic clearance of amino acids, including L-Arginine, is reduced, which may partially explain the exacerbation of NO with increased substrates^{16,17}.

Abdelmoaty et al¹⁸ determined that serum NO metabolites were three or five times higher in cirrhotic patients than in healthy controls, and three times higher in cirrhotic patients with ascites than in those without. Correspondingly, in the present investigation we determined that the highest plasma nitrite concentration was seen in cirrhotic patients with ascites, while it was significantly higher in cirrhotic patients both with and without ascites, in comparison to those in the control group.

The human eNOS gene contains 26 exons with several functional polymorphisms, which are identified in different regions of this gene. Many researchers¹⁹⁻²¹ have investigated by what mechanism these polymorphisms affect plasma NO concentrations. Tsukada et al²¹ observed a lower NO concentration in the eNOS4 a/a genotype, when compared to the b/a and b/b genotypes, in 413 healthy subjects. This suggests that the polymorphism of the eNOS4 gene results in reduced plasma NO concentration¹⁸. In another case-control study, the eNOS G894T polymorphism was determined as an independent risk factor for the development of portal hypertension in cirrhotic

Table III. Plasma nitrite levels ($\mu\text{mol/L}$) in cirrhosis with ascites, stable cirrhosis, and control groups.

Groups	Total median (min-max)	G894T median (min-max)			P	T/C 786 median (min-max)			P
		GG	GT	TT		TT	TC	CC	
Cirrhosis with ascites (n = 25)	37.3 ^a (31.5-66.0)	40.3 (34.7-66)	35.2 (31.7-47.5) ^b	32.5 ^b (31.7-33.0)	< 0.001	40.30 (34.7-66.6)	37.5 (32.7-47.5) ^c	33.2 (31.7-35) ^c	0.0116
Cirrhosis (n = 25)	35.6 ^a (30-45.50)	34.3 (31.7-47.5)	39.45 (30-42)	34.85 (32.1-37.)	0.2252	35.3 (32.7-41.3)	37.1 (30-45.5)	35.1 (32.1-37.6)	0.8529
Control (n = 20)	26.4 (23.1-36)	25.4 (24.4-36)	26.4 (25.4-29)	23.1 (23.1-23.1)	0.2251	25.9 (24.4-29.7)	27 (24.8-36)	23.7 (23.1-24.4)	0.0636

^ap < 0.05 vs. control group, ^bp < 0.05 G894T GG genotype, ^cp < 0.05 vs. T/C 786 TT genotype.

patients, due to hepatitis B infection²². However, the weakness of this study was the lack of plasma NO concentration. In the present study, we evaluated both plasma nitrite concentration and eNOS gene polymorphism. There was no significant difference between the cirrhosis groups (with and without ascites) and the control group in terms of the relationship between genotype and allele frequencies, for both polymorphisms.

In this study, we determined that plasma nitrite concentration was significantly higher in the cirrhosis patients, with and without ascites, than in the control group. In addition, this concentration was lower in the patients with eNOS G894T T/T and G/T genotypes, in comparison to those with G894T G/G in the cirrhotic group with ascites. Similar findings were observed for the eNOS T786C C/C genotype. Based on these results, we suggest that G894T and T786C polymorphisms cause a reduction in NO production.

Conclusions

The present study found no correlation between G894T and T786C polymorphisms with the development of ascites; however, plasma NO concentration is reduced as a result of these polymorphisms. Large-scale prospective studies are required to identify whether or not eNOS gene polymorphisms play a role in the development and pathogenesis of ascites.

Conflict of Interest

The authors declare that they have no conflict of interest regarding the publication of this paper.

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