Genetic dilemma: eNOS gene intron 4a/b VNTR polymorphism in sepsis and its clinical features in Turkish children

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SUMMARY: Çelik Ü, Yıldızdaş D, Alhan E, Çelik T, Attila G, Sertdemir Y, Tepe T. Genetic dilemma: eNOS gene intron 4 a/b VNTR polymorphism in sepsis and its clinical features in Turkish children. Turk J Pediatr 2008; 50: 114-119.

The role of endothelial nitric oxide synthase gene intron 4 a/b (eNOS4a/b) variable number of tandem repeats (VNTR) polymorphism in various diseases was investigated. We investigated whether this polymorphism is associated with susceptibility to sepsis and its clinical features such as acute respiratory distress syndrome (ARDS), multiorgan dysfunction syndrome (MODS) and shock. eNOS4a/b VNTR polymorphism was determined by the polymerase chain reaction in 100 children with sepsis and in 134 healthy controls. The genotype distribution of eNOS4 was not different between the patients and controls (p=0.44). There was no statistically significant association between genotypes/allele frequency and outcomes like mortality, MODS, ARDS, and shock (p>0.05).

This is the first study that evaluates the effect of eNOS4a/b polymorphism in sepsis. We were unable to show a relationship between eNOS gene intron 4 a/b VNTR polymorphism and MODS, ARDS, mortality and shock. Larger studies that do research on the interaction of such genes are needed to clarify the association between eNOS4a/b polymorphism and sepsis.

Key words: eNOS gene intron 4a/b VNTR polymorphism, sepsis, acute respiratory distress syndrome, septic shock, multiorgan dysfunction syndrome, mortality.

Sepsis is a major disease entity with important clinical and economic implications. It is the host's reaction to infection and is characterized by a systemic inflammatory response[1]. The presence of acute respiratory distress syndrome (ARDS), shock and multiorgan dysfunction syndrome (MODS) may affect the prognosis of sepsis. However, clinical progress might be different even when the infection cases are caused by the same agent. It is difficult to foresee the variety of clinical features that will develop in each patient. Although there have been significant improvements in intensive care unit facilities, mortality of sepsis and sepsis-related entities is still high. Mortality rates in ARDS are decreasing, with several recent studies reporting mortality in the order of 20-40% rather than the early descriptions of this disease, in which a mortality of 40-60% or higher was frequently cited[2].

On the other hand, many studies have been undertaken into the pathogenesis of sepsis, ARDS and MODS[2,3]. These studies show a variety of cytokines and inflammatory agents as having important roles, with nitric oxide (NO) the leading one. NO is thought to play a key role in the pathogenesis of sepsis and ARDS[4-6]. The major NO synthase (NOS) isoforms identified can be broadly grouped together as constitutive NOS (cNOS) and inducible NOS (iNOS). cNOS includes nNOS (neuronal NOS or NOS1) and eNOS (endothelial NOS or NOS3). In the vascular endothelium, NO has key functions in the relaxation of vascular smooth muscle, and it is especially an important vasodilator for local blood flow[7,8]. eNOS also regulates vascular tone and interactions between leukocytes and endothelium. It has been characterized with life-threatening organ failure attributable to

a combination of excessive inflammation and disruption of integrity of the microvascular endothelium[9]. Reduced NO synthesis could be the result of the variants of eNOS gene. eNOS is encoded by a gene located on chromosome 7q35-3b, comprising 2b exons that span 21 kb, and is expressed by the endothelium[10,11]. Miyahara et al.[12] suggested that five tandem repeats of a 27 bp consensus sequence of intron 4 might serve as markers for the eNOS gene. Wang et al.[13] reported that this gene has a common larger allele and a smaller allele; the larger allele with five tandem 27-bp repeats was designated as 'b', and the smaller allele with four tandem 27-bp repeats was designated as 'a'. The eNOS4 variable number of tandem repeats (VNTR) polymorphism has a functional significance. It has been reported that eNOS4 VNTR polymorphism may be responsible for plasma NO levels. Recently, Tsukada et al.[14] reported a strong association between an allele of the eNOS4 and decreased plasma NO levels. Analysis of the eNOS gene polymorphism may be a useful tool for studying the relation between NO and sepsis. The aim of this study was to investigate whether the eNOS4a/b VNTR polymorphism is associated with susceptibility to sepsis and its clinical features such as shock, ARDS, and MODS, and mortality.

Material and Methods

This study was conducted in the pediatric intensive care unit (PICU) of Çukurova University Hospital, Adana, Turkey. This unit has 16 beds and serves as a referral center for pediatric patients with medical and surgical problems in southern Turkey. Between June 2005 to June 2006, 184 patients with sepsis were admitted to our PICU. Patients previously admitted to other hospitals and who had been given antibiotics were not included (84 patients). The remaining 100 Turkish children with sepsis and 134 healthy Turkish adults were enrolled in this study. Neonatal patients were excluded. The control group consisted of 134 unrelated healthy adult volunteers without renal, metabolic, infectious or cardiac disease.

Nine patients had cyanotic congenital heart disorder, 22 had neurologic disorder, 6 had congenital immune deficiency, 10 had hematologic malignancy, 2 had congenital pulmonary anomaly, 3 had chronic renal insufficiency, and 3 had burn. Forty-three patients had nosocomial sepsis.

Definitions

Nosocomial infection was defined according to Centers for Disease Control (CDC) criteria[15]. Sepsis was defined as systemic inflammatory response syndrome (SIRS) caused by an infection. SIRS was characterized by the presence of at least two of the following four criteria, one of which must be abnormal temperature or leukocyte count:

- Core temperature of >38.5°C or <36°C.
- Tachycardia, defined as a mean heart rate >2 SD above normal for age in the absence of external stimulus, chronic drugs, or painful stimuli; or otherwise unexplained persistent elevation over a 0.5- to 4-hr time period OR for children <1 yr old: bradycardia, defined as a mean heart rate <10th percentile for age in the absence of external vagal stimulus, beta-blocker drugs, or congenital heart disease; or otherwise unexplained persistent depression over a 0.5-hr time period.
- Mean respiratory rate >2 SD above normal for age or mechanical ventilation for an acute process not related to underlying neuromuscular disease or the receipt of general anesthesia.
- Leukocyte count elevated or depressed for age (not secondary to chemotherapy-induced leukopenia) or >10% immature neutrophils.

Septic shock was defined as sepsis and cardiovascular dysfunction[16]:

Criteria for cardiovascular dysfunction

Despite administration of isotonic intravenous fluid bolus ≥40 ml/kg in 1 hr:

Decrease in blood pressure (BP) (hypotension) <5th percentile for age or systolic BP <2 SD below normal for age or need for vasoactive drug to maintain BP in normal range (dopamine >5 µg/kg/min or dobutamine, epinephrine, or norepinephrine at any dose) or two of the following: Unexplained metabolic acidosis: base deficit >5.0 mEq/L, increased arterial lactate >2 times upper limit of normal, oliguria: urine output <0.5 ml/kg/hr, prolonged capillary refill: >5 secs, and core to peripheral temperature gap >3°C.

Criteria for respiratory dysfunction

PaO_2/FiO_2 <300 in absence of cyanotic heart disease or preexisting lung disease or $PaCO_2$ >65 torr or 20 mmHg over baseline $PaCO_2$

or proven need or >50% FiO_2 to maintain saturation ≤92% or need for nonelective invasive or noninvasive mechanical ventilation.

Criteria for neurologic dysfunction

Glasgow Coma Score ≤11 or acute change in mental status with a decrease in Glasgow Coma Score ≥3 points from abnormal baseline.

Criteria for hematologic dysfunction

Platelet count <80,000/mm^3 or a decline of 50% in platelet count from highest value recorded over the past 3 days (for chronic hematology/oncology patients) or international normalized ratio >2.

Criteria for renal dysfunction

Serum creatinine ≥2 times upper limit of normal for age.

Criteria for hepatic dysfunction:

Total bilirubin >4 mg/dl (not applicable for newborn) or alanine aminotransferase (ALT) 2 times upper limit of normal for age

The American–European Consensus Conference Committee criteria were used to diagnose ARDS: (i) acute onset, (ii) bilateral infiltrates on chest radiography, (iii) PaO_2/FiO_2 less than 200, and (iv) absence of clinical evidence for left-sided heart failure[17].

Although blood cultures were negative in community-acquired sepsis, in 30 of 43 patients with nosocomial sepsis there were positive blood culture results. Ten patients were positive for *Acinetobacter baumannii*, 8 for *Pseudomonas aeruginosa*, 2 for *Klebsiella pneumoniae*, 5 for *Staphylococcus aureus*, 2 for *Staphylococcus warneri*, 2 for *Candida albicans* and only 1 for *Candida non albicans*.

Blood samples were drawn via an arterial line and femoral catheter within the first hour of admission to PICU before administration of inotropic agents. All patients were treated with broad-spectrum antibiotics, appropriate fluid and inotropics. Depending on culture-antibiogram results, the antibiotics given at first-line were changed with the proper antibiotics.

ACE Genotyping

Blood samples were collected from patients and controls into EDTA tubes. Genomic DNA from leukocytes was purified according to the method of Miller[18]. The eNOS gene intron 4.27 bp VNTR polymorphism was detected by polymerase chain reaction (PCR) according to the method described by Wang et al.[13]. The template DNA was amplified using the following primers (forward) 5'AGG CCC TAT GGT AGT GCC TTT3'- and 5'TCT CTT AGT GCT GTG CTC AC- 3'. These primers (25 pmol of each) were added to a mixture containing 0.2 µmol/L each of the dATP, dCTP, dGTP, dTTP, 5 µl of 10X Cetus buffer (pH 8.3), 5 µl of DMSO (100%) and 0.5 units of Taq DNA polymerase (Perkin Elmer Cetus) in a final volume of 50 µl. The PCR was initiated with a denaturation by first heating the samples for 5 min at 94°C. Thirty-five cycles of denaturation for 1 min at 94°C, annealing for 1 min at 56°C and primer extension for 2 min at 72°C and a last extension for 5 min at 72°C was applied for amplification. PCR products of NO gene locus were examined by agarose gel electrophoresis (2% NuSieve agarose-agarose) at 150 V for 30 min and visualized at room temperature under UV after ethidium bromide staining.

All protocols and procedures were reviewed and approved by the local Ethics Committee of the Faculty of Medicine.

Statistical Methods

The data was analyzed with the SPSS package program (version 15.0 Chicago, IL, 2006). Allelic frequencies were calculated by the gene-counting method. A chi-square test was used to test the expected type frequencies, assuming Hardy–Weinberg equilibrium. The genotype/allele frequencies in patients were compared to those in the control subjects using the chi-square test. Data were expressed as mean ± SD. Results were considered statistically significant if the p values were less than 0.05.

Results

During the study period, 100 children met the inclusion criteria. The mean age of 68 (68%) male and 32 (32%) female patients was 54±51.6 months (1-144). The mean age of the 83 (62%) male and 51 (38%) female controls was 42.1±13.38 years (Table I).

In the patient group, 33 (33%) patients were assessed with ARDS. Fifty-five (55%) of the patients survived, while 45 (45%) did not. MODS was found in 52 (52%) and shock in 55 (55%) patients.

Table I. Demographic Features and Genotypes of the Patient and Control Groups

	Age	Female (n) %	Male (n) %	aa genotype (n) %	ab genotype (n) %	bb genotype (n) %
Patients	1-144 months	32 32	68 68	4 4	28 28	68 68
Controls	15-55 years	51 38	83 62	2 2	35 26	97 72

The genotype frequencies in controls were in agreement with those predicted by the Hardy–Weinberg equilibrium (Table II). The most frequently observed genotype was b/b in all subjects (70%). The eNOS genotype distribution was aa 4 (4%), ab 28 (28%), bb 68 (68%) in the patient group and aa 2 (2%), ab 35 (26%), bb 97 (72%) in the control group. There was no statistically significant difference between patients and the control group in genotype distribution (p=0.44).

Table II. Distribution of Genotypes and Allele Frequencies of eNOS4 in Patients and Controls

Gene	Control n %	Sepsis n %
bb	97 72	68 68
ab	35 26	28 28
aa	2 2	4 4
Total	134	100

Allele	Control n %	Sepsis n %
b	229 85.4	164 82
a	39 14.5	36 18
Total	268 100	200 100
p (b)	0.854	0.820

Test for heterogeneity between control and sepsis for genotype: chi-square=1.64, DF=2, p=0.44.
Test for heterogeneity between control and sepsis for b allele frequency: chi-square=0.35, DF=1, p=0.556.
Test for goodness of fit for Hardy-Weinberg equilibrium (control group) with P(b)=0.854: chi-square=0.338, DF=2, p=0.844.
Test for goodness of fit for Hardy-Weinberg equilibrium (sepsis group) with P(b) from control group: chi-square=2.41, DF=2, p=0.299.

The frequency of a allele was 18% (36/200) and 14.5% (39/268) in the patient and control groups, respectively, while the frequency of b allele was 82% (164/200) and 85.4% (229/268), respectively. Carrying the a or b alleles was not found to increase the risk of having sepsis (p=0.37) (Table II).

Thirty-seven (54.4%) of the 68 with bb genotype and 18 (64.2%) of the 28 with ab genotype had shock. None of the 4 with aa genotype was found to have shock. There was no statistically significant association between septic shock and genotype (p=0.056) (Fig. 1).

When the mortality rate of the patients was evaluated, 37 (54.4%) of the 68 with bb genotype, 17 (60.7%) of the 28 with ab genotype and 1 (25%) of the 4 with aa genotype died. There was no statistically significant difference between mortality and genotype (p=0.39) (Fig. 1). In the mortal group, the frequency of a allele was 52.8% (19/36) and of b allele was 55.5% (91/164) (Fig. 2). Carrying the a or b allele was not found to increase the risk of death (p=0.8).

Twenty-one (30.9%) of the 68 with bb genotype, 10 (35.7%) of the 28 with ab genotype and 2 (50%) of the 4 with aa genotype were found to have ARDS. In this group, the frequency of a allele was 38.9% (14/36) and of b allele was 31.7% (52/164). There was no statistically significant difference between ARDS and genotype/allele frequency (p=0.69, p=0.43, respectively) (Figs. 1, 2).

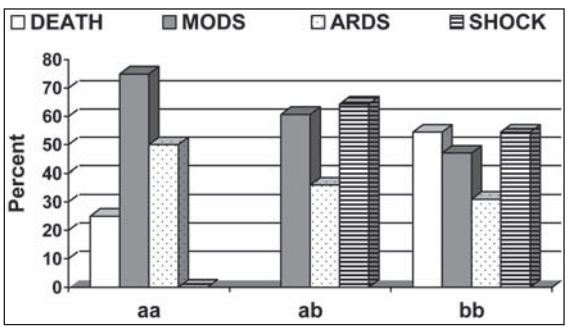

Fig. 1. The relationship between genotype polymorphism and death, MODS, ARDS, and shock. [MODS: Multiorgan dysfunction syndrome. ARDS: Acute respiratory distress syndrome.]

Thirty-two (47%) of the 68 with bb genotype, 17 (60.7%) of the 28 with ab genotype and 3 (75%) of the 4 with aa genotype were found

to have MODS. Allele frequency in this group was 63.9% (23/36) for a allele and 49.4% (81/164) for b allele. There was no statistically significant difference between MODS and genotype/allele frequency (p=0.126, p=0.14, respectively) (Figs. 1, 2).

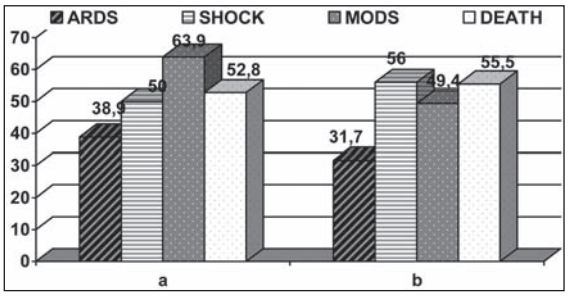

Fig. 2. The relationship between allele frequency and death, MODS, ARDS, and shock.
[MODS: Multiorgan dysfunction syndrome.
ARDS: Acute respiratory distress syndrome.]

Discussion

The response to infection is variable between different patients. This variability has been attributed to many factors including the virulence or the load of etiological agent or the length of period between onset of symptoms and initiation of treatment. Genetic differences in the inflammatory response may play a role in determining outcome from serious infection.

Animal models and human clinical data in sepsis provide evidence of an increase in NO release in sepsis[19-21]. Proinflammatory cytokines associated with sepsis, especially interleukin (IL)-1, tumor necrosis factor (TNF), and endotoxin (lipopolysaccharide) increase NO production. Consequently, NO has been implicated in the pathogenesis of the hypotension and organ failure attributable to severe sepsis[22]. In light of the function of NO as an endogenous vasodilator, observations of reduced vascular tone following the administration of endotoxin and proinflammatory cytokines suggest a pathogenic role for NO during sepsis. In addition to its adverse hemodynamic effects in sepsis, NO overproduction has other physiologic and cellular consequences. Patients in sepsis often have dangerously low blood pressure, which is thought to be caused in part by the overproduction of NO. The a allele of the eNOS has also been related to low NO metabolite levels, and subjects homozygous for the a allele exhibit 20% lower levels of NO metabolites than subjects with the bb genotype[14]. We investigated whether polymorphism of eNOS4 a/b might be associated with tendency to sepsis and its clinical progress.

In our study, we did not find a relationship between septic shock and genotype. Septic shock was not observed in aa genotype with the exception of a few patients in this group. aa genotype is an independent risk factor in cardiovascular disease such as atherosclerosis. Cotter el al.[23] reported that L-NMMA NO inhibitor administration in patients with cardiogenic shock was safe and had favorable clinical and hemodynamic effects. However, in recent studies involving animals and humans, suppression of excessive amounts of NO in sepsis has not proven to be of significant benefit. There is still controversy as to whether inhibition of NO is beneficial or harmful, on whether NO needs to be suppressed, and on which isoform is really important. In this study, we could not find an answer to the question of which patients benefit from the NO inhibition treatment. eNOS gene 4a/b polymorphism does not seem to be effective in sepsis shock. On the other hand, the rates of MODS were 75% and 47% in patients with aa and bb genotypes, respectively. The rate of MODS seemed to increase with that of the a allele (the frequency of a allele was 63.9% [3/36] and of b allele was 49.4% [81/164]) and NO might be an important factor in the involvement of multiple organs, but we could not show a statistically significant difference between MODS and gene polymorphism in this study. We suggest that larger studies are needed to clarify this relationship.

We did not find any correlation between the development of ARDS and genotype distribution. Hickling et al.[24] reported that only 14% of deaths from ARDS were directly related to hypoxemia. It is commonly believed that pediatric patients with ARDS are more likely to die from cardiorespiratory failure than other causes (i.e. children die from ARDS while adults die with ARDS). While a number of authors have reported improvements in oxygenation and pulmonary vascular resistance in pediatric patients with ARDS treated with inhaled NO, there has been no demonstrated improvement in survival with NO therapy. In a multicenter randomized controlled trial of

NO versus placebo gas in 108 children with acute hypoxemic respiratory failure (defined as an OI>15 in two blood gases within 6 hr), NO administration improved oxygenation but had no effect on mortality (42% for NO vs. 43.4% for control patients)[25]. A similar result has been reported for adults[26]. In our study, a allele was not determined as a risk factor for ARDS.

In conclusion, we found no relationship between eNOS 4a/b gene polymorphism and shock, ARDS, MODS, or mortality. However, the results of the present study are only a preliminary report. Larger studies are needed to clarify the relationship between eNOS gene intron 4 a/b VNTR and sepsis, septic shock, ARDS, MODS and mortality.

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