Association between the Glu298Asp and T(-786)C polymorphisms of the endothelial nitric oxide synthase gene and respiratory distress in preterm neonates

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Genetic polymorphisms in the gene that codes for endothelial nitric oxide synthase (eNOS) have been associated with less nitric oxide availability and with various cardiovascular diseases in humans. The objective of this study was to analyze the genotype distributions and allele frequencies for the Glu298Asp (G894T) and T(-786)C polymorphisms of the eNOS gene among neonates with respiratory distress in comparison to healthy control subjects. Fifty premature neonates with respiratory distress and 55 neonates without any respiratory problem were included in the study. Genomic DNA from all the neonates was analyzed by polymerase chain reaction. A polymerase chain reaction–restriction fragment length polymorphism analysis of eNOS gene polymorphisms was performed, and the results were compared. There were no significant differences between the groups regarding either genotype distributions or the allele frequencies for the Glu298Asp and T(-786)C polymorphisms. These results suggest that eNOS Glu298Asp and T(-786)C polymorphisms are not associated with development of respiratory distress.

Key words: endothelial nitric oxide synthase gene polymorphisms, preterm infant, respiratory distress.

Nitric oxide (NO) is synthesized by conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS). In humans, three isoforms of NOS have been identified: endothelial (eNOS), inducible (iNOS), and neuronal (nNOS)¹. In endothelial cells, NO is synthesized by eNOS (NOS 3), which is located on chromosome 7q35–36². Several specific allelic variations of the gene have been identified. One such polymorphism is the Glu298Asp (G894T) polymorphism in exon 7, which results in the substitution of glutamate to aspartate at amino acid position 298 (Glu298Asp)³. This variant has been associated with reduced endothelial responses to NO in vivo in humans⁴ and with various cardiovascular diseases, such as myocardial infarction and hypertension⁵. The other polymorphism in the promoter region, T(-786)C, is a point mutation and has been identified in the 5’ flanking region of the eNOS gene. This variant results in a significant reduction in the eNOS gene promoter activity⁵,⁶. In animal experiments, it has been shown that endogenous NO plays a major role in postnatal pulmonary adaptation, regulation of pulmonary vascular tonus, and development of pulmonary edema⁷,⁸. In humans, endogenous production of NO is vital for the decrease in pulmonary vascular resistance after birth⁹. Furthermore, a few clinical trials in preterm infants reported that inhaled NO had beneficial effects on respiratory outcomes, i.e. NO significantly reduced the risk of death or chronic lung disease, or the requirement for supplemental oxygen therapy¹⁰,¹¹. Therefore, we hypothesized that eNOS genetic polymorphisms may play a role in the development of respiratory distress (RD) in preterm infants. Involvement of other
Genetic factors in respiratory distress syndrome (RDS) has been suggested. There has been no study investigating the role of eNOS polymorphisms in RDS in premature neonates. The aim of this study was to evaluate the distribution of eNOS G894T and T(-786)C gene polymorphisms in premature infants with and without RDS within the first 24 hours of life.

**Material and Methods**

**Subjects**

One hundred and five premature infants who were admitted to the neonatal intensive care unit at Hacettepe University İhsan Doğramacı Children’s Hospital, with gestational age under 37 weeks, were included in the study. The premature neonates with RDS during the first 24 hours of life constituted the patient group (gestational age 26-36 weeks, birth weight 600-3270 grams) and those without any respiratory problem constituted the control group (gestational age 28-37 weeks, birth weight 760-2900 grams). All the premature infants were treated with antenatal corticosteroids, and none received prophylactic surfactant treatment. Exclusion criteria were major congenital anomalies, sepsis, intrauterine infections, and inherited metabolic disorders. The study was approved by the Hacettepe University Human Ethics Committee, and informed consent was obtained from parents of all newborns.

The criteria for the diagnosis of RDS were the presence of a respiratory rate of more than 60 per minute and dyspnea characterized by intercostal, subcostal or suprasternal retraction, grunting, nasal flare, and cyanosis. The patient group consisted of neonates with RDS (n=29), wet lung (n=9), and primary pulmonary hypertension (PPH) (n=12). Diagnosis of RDS in preterm infants was based on the presence of typical clinical and radiological signs. The diagnosis of wet lung was confirmed by the presence of RDS occurring after 1-2 hours of life and resolving within 2-3 days, with the typical radiological findings. PPH was diagnosed by the presence of mean pulmonary artery pressure >30 mmHg echocardiographically, radiologically normal lungs, and no evidence of parenchymal lung disease or any cardiac anomaly. Duration of hospitalization, mechanical ventilation and oxygen use, number of surfactant use, and mortality in the RD group were recorded.

**DNA Isolation and Genotyping**

Venous blood samples were taken into Vacutainer tubes containing disodium ethyl endiaminetetraacetic acid (EDTA). Total genomic DNA was isolated by using QIAamp DNA blood kit (Qiagen, Hilden, Germany) and stored at –20°C. The Glu298Asp and T(-786)C polymorphisms in the eNOS gene were analyzed with polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) as described previously. Genotyping was conducted in a blinded fashion.

**Statistical Analysis**

Average results are expressed as the means ± standard error of the means (SEM). Statistical analysis was performed using SPSS for Windows (version 11.0). For between-group analyses, the Mann-Whitney U or unpaired Student’s t-test was used; for calculation of the significance of differences in genotype and allele frequencies, the chi-square test was used. Tests and P values were two-tailed. A value of p<0.05 was considered statistically significant.

**Results**

The RD group consisted of 50 preterm infants; four of the 50 infants (8.0%) died in the follow-up period due to RDS. The control group

| Table I. Demographic Characteristics of Infants With and Without Respiratory Distress (RD) |
|---------------------------------|-----------------|-----------------|-------------|
| Gestational age (weeks) | Control (n=55) | RD (n=50) | P value |
| 1 | 33.2 ± 0.3 | 32.1 ± 0.5 | 0.0705 |
| Birth weight (g) | 1890 ± 81 | 1829 ± 110 | 0.6516 |
| Apgar score (5th min) | 8.7 ± 0.2 | 7.3 ± 0.4 | 0.0008 |
| Male/female ratio | 27/28 | 30/20 | 0.2624 |
| Mode of birth (V/CS) | 10/45 | 8/42 | 0.7670 |

1Mean ± SEM

V: Vaginal delivery. CS: Cesarean section.
Table II. Frequencies of Genotypes for the Endothelial Nitric Oxide Synthase (eNOS) Glu298Asp (G894T) Polymorphism and G and T Alleles in Premature Newborns With RD and the Control Group

<table>
<thead>
<tr>
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<th>Genotypes n (%)</th>
<th>Alleles n (%)</th>
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<tbody>
<tr>
<td></td>
<td>GG</td>
<td>GT</td>
</tr>
<tr>
<td>Control</td>
<td>55</td>
<td>30 (54.6)</td>
</tr>
<tr>
<td>RD</td>
<td>50</td>
<td>35 (70.0)</td>
</tr>
</tbody>
</table>

RD: Respiratory distress.

The eNOS G894T genotypes and G and T allele frequencies in the RD and control groups are presented in Table II. GG, GT and TT genotype frequencies were 54.6%, 43.6%, and 1.8% in the control group, and 70.0%, 28.0%, and 2.0% in the RD group, respectively. The difference in genotype distributions did not reach statistical significance (p=0.25). The frequencies of G and T alleles were calculated from the genotype frequencies in both groups. The frequencies of the G allele in the control and patient groups were 76.4% (CI95% = 68.5–84.3%) versus 84.0% (CI95% = 76.8–91.2%), respectively, and those of the T allele were 23.6% (CI95% = 15.7–31.5%) versus 16.0% (CI95% = 8.8–23.2%), respectively. As with the genotype distribution, allele frequencies were not statistically different between the control and study groups (p=0.17).

Table III. Frequency (%) of Endothelial Nitric Oxide Synthase (eNOS) Gene T(-786)C Polymorphism Genotypes and C and T Alleles in Premature Newborns With RD and the Control Group

<table>
<thead>
<tr>
<th></th>
<th>Genotypes n (%)</th>
<th>Alleles n (%)</th>
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<tbody>
<tr>
<td></td>
<td>CC</td>
<td>CT</td>
</tr>
<tr>
<td>Control</td>
<td>55</td>
<td>6 (10.9)</td>
</tr>
<tr>
<td>RD</td>
<td>50</td>
<td>5 (10.0)</td>
</tr>
</tbody>
</table>

RD: Respiratory distress.

This study investigated the association between genetic variants and development of RD using a healthy control group and a patient group selected from preterm infants.

no significant impact of genotype on disease susceptibility was observed (OR=0.5143, CI95%=0.230-1.150; p>0.05).

The eNOS gene T(-786)C genotypes and C and T alleles in the RD and control groups are presented in Table III. The incidences of CC, CT, and TT genotypes were found as 10.9%, 52.7% and 36.4%, respectively, in the control group, and 10.0%, 44.0% and 46.0%, respectively, in the RD group (p>0.05). The difference in genotype distributions did not reach statistical significance (p=0.60). The frequencies of C and T alleles were calculated from the genotype frequencies in both groups. The frequencies of the C allele in the control and patient groups were 37.3% (CI95% = 28.3–46.3%) versus 32.0% (CI95% = 22.9–41.1%), respectively, and those of T allele were 62.7% (CI95% = 53.7–71.7%) versus 68.0% (CI95% = 58.9–77.1%), respectively. Allele frequencies were not different between the control and study groups (p=0.42). The calculated OR for RD susceptibility across genotypes was 1.263 (CI95% = 0.7133-2.235, p>0.05).

Discussion

This study investigated the association between genetic variants and development of RD using a healthy control group and a patient group selected from preterm infants.
Respiratory distress is a common problem in preterm infants. While non-pulmonary problems can also manifest with RD, pulmonary diseases are the most common causes of RD, among which the most frequent presentation is RDS. Other causes of RD in preterm infants include pneumonia, wet lung, PH, and upper airway obstruction. Studies have been conducted to better understand the etiopathogenesis and genetic influences in RD and to develop new management strategies. Among the genetic factors examined, variants in the genes that code for surfactant proteins and other genes have been studied. In both animals and humans, endogenous NO is crucial for maturation of pulmonary structure and functions. NO mediates a wide variety of physiological functions, including neurotransmission, immune cell cytotoxicity, vascular smooth muscle relaxation, angiogenesis, and surfactant maturation/secretion. Research in animals has suggested that NO inhalation decreases lung injury by reducing inflammation and improving surfactant function and also promotes lung growth. In humans, a few trials have demonstrated that inhaled NO could improve tissue oxygenation in preterm infants with severe respiratory failure and bronchopulmonary dysplasia. In view of these findings, we aimed to investigate whether or not eNOS genetic polymorphisms are associated with development of RD in preterm infants. To our knowledge, there have been no previous reports examining the association between the Glu298Asp (G894T) or T(-786)C polymorphisms of the eNOS gene and RD in preterm neonates. Therefore, this is the first such study to examine this association in preterm infants.

Many reports in the literature and a few meta-analyses have revealed that T(-786)C and Glu298Asp polymorphisms of the eNOS gene are associated with increased incidence of cardiovascular diseases. In examination of functional consequences of the genomic variants, the Glu298Asp polymorphism was associated with a blunted endothelial-dependent vasodilation in healthy subjects, possibly due to decreased NO synthesis. It has also been demonstrated that healthy pregnant women who carried the Glu298Asp polymorphism had reduced flow-mediated dilatation of the brachial artery, which is an NO-dependent response. Thus, these findings suggest that subjects homozygous for the Asp298 allele generate low NO and may be more susceptible to endothelial dysfunction. A functional effect for the –786T3C promoter polymorphism has also been proposed, and the T(-786)C variant has been associated with reduced placental eNOS mRNA levels. Additionally, lower serum nitrite/nitrate levels have been found in individuals with the –786C variant. T(-786)C base substitution decreases promoter activity by 50%, thereby leading to suppressed eNOS protein expression.

Our view was the possibility that defective alleles may contribute to the increased risk of RDS; however, the results did not support this view.

A major limitation of our study is the small sample size for comparing the control and patient groups. Small sample sizes may lead to false-negative results in association studies. In order to have a power of 80%, hundreds of patients in each group were needed to reach a reliable conclusion. Considering that the study population consists of premature infants with RD, it would be unlikely to collect such a high number of samples at a single center. We were able to recruit only 50 patients in a one-year period.

In conclusion, our data suggest the lack of an association between the Glu298Asp (G894T) or T(-786)C polymorphism of the eNOS gene and the development of RD in the Turkish population. This association could be examined in other studies with larger sample sizes.

REFERENCES


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