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# Association between functional polymorphisms in the nitric oxide synthase 3 gene and pediatric acute respiratory distress syndrome

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**ABSTRACT.** Nitric oxide mediates multiple physiological functions, including neurotransmission, immune regulation, angiogenesis, antiplatelet activity, and surfactant maturation or secretion. Mice deficient in the nitric oxide synthase 3 (*NOS3*) gene displayed defective lung vascular development and fatal respiratory distress. Polymorphisms in *NOS3* have been reported to be associated with respiratory distress syndrome (RDS). The role of *NOS3* polymorphisms as a risk factor for pediatric acute respiratory distress syndrome (PARDS) was evaluated by analyzing the possible functional single nucleotide polymorphisms (SNPs) in the regulatory and coding regions of *NOS3*. Samples from 216 PARDS patients and 225 healthy control subjects were genotyped

at 4 SNP loci (rs11771443 and rs3918188 in the promoter region, rs1799983 in exon 7, and rs7830 at the intron24-exon23 boundary). Statistically significant differences were observed in the allelic or genotypic frequencies of the rs1799983 and rs11771443 polymorphisms in *NOS3*. The T and G alleles of rs1799983 and rs11771443 were associated with a significantly higher risk of PARDS compared to the C allele (P = 0.030) and the T allele (P = 0.004), respectively. Strong linkage disequilibrium was observed in one block (D' > 0.9). Subjects with PARDS displayed significantly fewer T-C haplotypes (P = 0.013) in block 1 (rs1799983-rs11771443). These findings indicate that *NOS3* polymorphisms play a definitive role in PARDS, and therefore may be useful for future genetic or neurobiological studies on RDS.

**Key words:** Pediatric acute respiratory distress syndrome; Nitric oxide synthase 3; Single nucleotide polymorphisms

## INTRODUCTION

Acute respiratory distress syndrome (ARDS) is an important cause of acute respiratory failure, which is often associated with multiple organ failure (Rubenfeld et al., 2005). The risk factors for ARDS include infection, alcohol abuse, cigarette smoke exposure, and race (Moss et al., 1996). Genetic susceptibility may also be an important determinant of ARDS incidence (Matthay et al., 2012).

Nitric oxide (NO) is a modulator of apoptotic and inflammatory cascades and endothelial permeability. NO synthesis is catalyzed by the nitric oxide synthase (NOS) enzyme family, which includes several neuronal (NOS1), inducible (NOS2), and endothelial (eNOS, NOS3) synthases (Nikkari et al., 2015). The most important member of the NOS family that is involved in the basal release of vascular NO is eNOS. Endogenous NO plays a critical role in decreasing pulmonary vascular resistance and improving ventilation-perfusion matching after birth, enabling the reversal of pulmonary hypertension (Han et al., 2004). eNOS-deficient mice display major defects in lung morphogenesis (Sung et al., 2015) that cause respiratory distress and death within the first hours of life in a majority of animals (Han et al., 2004). The role of eNOS in lung development, which may influence the clinical syndromes of neonatal respiratory distress, remains to be elucidated (Han et al., 2004). Wu et al. (2014) suggested that eNOS uncoupling contributes to superoxide production and barrier dysfunction in the lung microvasculature after exposure to lipopolysaccharides. The results of this study also implicated the Nox2-mediated eNOS-S-glutathionylation in LPS-induced eNOS uncoupling in the lung microvasculature (Wu et al., 2014). A study of the SNP rs1799983 in *NOS3* showed a significant increase in the GG genotype and G allele frequencies in groups with respiratory distress syndrome (Shen et al., 2014). Additional studies must be conducted to determine if functional SNPs in *NOS3* modulate the risk of disease by themselves or by correlating with other causative SNPs, and their effect on other populations.

Based on the growing relevance of NOS3 in RDS, we hypothesized that the functional variants of *NOS3* might contribute significantly to the predisposition to pediatric acute respiratory distress syndrome (PARDS). In this study, functional SNPs in the promoter region, 5'- and 3'-untranslated regions, exons, and the intron-exon boundary of *NOS3* were

systematically screened. We investigated the association between four SNPs in *NOS3* (rs11771443 and rs3918188 in the promoter region, rs1799983 in exon 7, and rs7830 in the intron24-exon23 boundary) and the risk of PARDS in a Chinese population, and the potential pathogenesis of PARDS patients expressing potentially functional SNPs.

## MATERIAL AND METHODS

### Subjects

Two hundred and sixteen unrelated subjects with PARDS were recruited from the Shaanxi Provincial People's Hospital and Xi'an Children's Hospital between March 2010 and April 2015. RDS was characterized by a need for supplemental oxygen, a chest radiograph consistent with RDS, and the need for continuous positive airway pressure or mechanical ventilation within the first 24 h of life. The control group was composed of 225 unrelated healthy individuals recruited from the Medical Examination Center of Shaanxi Provincial People's Hospital. Subjects who suffered from asthma or showed symptoms of allergy were excluded. The exclusion criteria included the presence of congenital anomalies, severe infection, and inherited metabolic disorders. Clinical data of all individuals, including the gestational age, gender, birth weight, and maternal and neonatal clinical histories, were obtained from the patient medical records. All participants belonged to the Han Chinese ethnicity and were genetically unrelated individuals from the Henan Province of China. The study complied with the guidelines of the local medical Ethics Committee, and written informed consent was obtained from all participants recruited to this study.

### Genotyping

Peripheral blood (3-5 mL) was obtained from all patients in tubes coated with EDTA. Genomic DNA was extracted from blood leukocytes using an EZNA™ Blood DNA Midi kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer protocols. Primers for PCR and single-base extension were designed using the Assay Designer software package (Sequenom Inc., San Diego, CA, USA) (Table 1). SNP genotyping was performed using the SNaPshot SNP technology according to the manufacturer protocols. Primary data were analyzed by GeneMapper 4.1 (Applied Biosystems, Foster City, CA, USA). The genotypes were determined based on the nucleotide present at the SNP site, as visualized by one or two differently colored peaks in the chromatograms. For quality control, 5% of the recruited subjects were randomly genotyped twice by researchers in a blind manner, with a reproducibility of 100%.

**Table 1.** Primer sequences used to genotype the single nucleotide polymorphisms (SNPs) in the extracellular nitric oxide synthase (*eNOS*) gene.

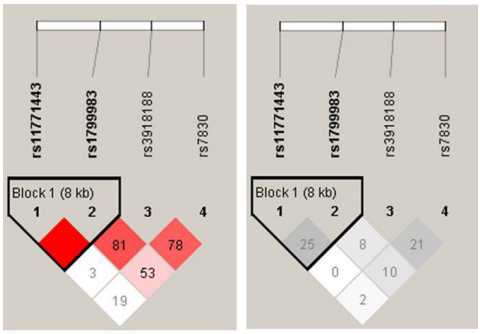
| SNPs | Forward primers | Reverse primers | Extension primers |
|---|---|---|---|
| rs1799983 | ACGTTGGATGAAACGGTCGCTTCGACGTG | ACGTTGGATGACCTCAAGGACCAGCTCGG | TGCAGGCCCCAGATGAG |
| rs11771443 | ACGTTGGATGCGTCTGTGGGCGTAACATC | ACGTTGGATGGAAGGATCAGGCCCACAATG | CCGCTGGGCTGATGTA |
| rs3918188 | ACGTTGGATGAAAAGTGGGAGCAAGGCACA | ACGTTGGATGACTTCACTGAGACTGAAGGG | AGGCACACGTACAAGGGC |
| rs7830 | ACGTTGGATGCTGTCCCTAGATTGTGTGAC | ACGTTGGATGCGGCTGCATGACATTGAGAG | CCTTCAGGCAGTCCTTTAGTCC |

## Statistical analysis

The genotype and allele frequencies for each individual polymorphism, as well as the Hardy-Weinberg equilibrium of controls, were calculated using the chi-square test. The association between polymorphisms and heroin addiction was assessed by the Pearson chi-square test. The odds ratio (OR) and 95% confidence interval (CI) was calculated by binary logistic regression. The P values were calculated based on codominant, dominant (for the rare allele), heterosis, and recessive (for the rare allele) models of inheritance. The pairwise linkage disequilibrium (LD) statistics (D' and $r^2$) and haplotype frequencies were compared using Haploview 4.0, in order to construct haplotype blocks defined by D' > 0.9 and $r^2$ > 0.8. All statistical analyses were carried out using SPSS 20.0 (SPSS Inc., Chicago, IL, USA).

## RESULTS

The controls showed no significant deviation from the HWE for any of the SNPs. LD analyses of the patient and control data revealed that the rs1799983 and rs11771443 polymorphisms are located in haplotype block 1. The haplotype structure and pairwise LD values ($r^2$) are shown in Figure 1. The structure of the haplotype blocks and pairwise LDs calculated for each patient group were roughly similar to those of the controls. The genotype distributions, allelic frequencies, and haplotypes of control and patient groups, together with the results of statistical analysis, are summarized in Tables 2 and 3.




D' in controls $r^2$ in controls

**Figure 1.** Linkage disequilibrium plot of single nucleotide polymorphisms in *NOS3* and in controls.

The rs1799983 genotype distribution was weakly linked to PARDS. The frequency of the G allele was significantly higher in PARDS patients than in the healthy controls (P = 0.030; OR = 6.143, 95%CI = 4.669-8.082). Additionally, we observed a strong correlation between the rs11771443 allele frequencies and PARDS. PARDS subjects also exhibited a significantly higher frequency of the T allele (P = 0.004; OR = 3.057, 95%CI = 2.398-3.897) compared to the controls. The differences retained statistical significance after Bonferroni correction (P < 0.0125).

Furthermore, strong linkage disequilibrium (LD) was observed in rs1799983-rs11771443 (D' > 0.9). The control subjects showed a significantly higher number of T-C haplotypes (P = 0.013; OR = 0.619, 95%CI = 0.424-0.903) in block 1. These differences retained statistical significance after Bonferroni correction.

**Table 2.** Genotypic and allelic frequencies of *NOS3* polymorphisms in the controls and patients.

| Variable | MAF | Cases (N = 216) | | Controls (N = 225) | | HWE | P value | OR (95%CI) |
|---|---|---|---|---|---|---|---|---|
| | | N | % | N | % | | | |
| rs1799983 | 0.140 | | | | | 0.152 | 0.001 | |
| GG | | 134 | 62.0 | 169 | 75.1 | | 0.003 | 0.541 (0.360-0.815) |
| GT | | 80 | 37.0 | 49 | 21.8 | | 0.000483 | 2.113 (1.388-3.216) |
| TT | | 2 | 0.9 | 7 | 3.1 | | 0.126 | 0.291 (0.060-1.417) |
| G allele | | 348 | 80.6 | 387 | 86.0 | | 0.030 | 6.143 (4.669-8.082) |
| T allele | | 84 | 19.4 | 63 | 14.0 | | | |
| rs11771443 | 0.391 | | | | | 0.118 | 0.024 | |
| TT | | 66 | 30.6 | 89 | 39.6 | | 0.048 | 0.672 (0.453-0.997) |
| GT | | 90 | 41.7 | 96 | 42.7 | | 0.832 | 0.960 (0.658-1.401) |
| GG | | 60 | 27.8 | 40 | 17.8 | | 0.013 | 1.779 (1.131-2.799) |
| T allele | | 222 | 51.4 | 274 | 60.9 | | 0.004 | 3.057 (2.398-3.897) |
| G allele | | 210 | 48.6 | 176 | 39.1 | | | |
| rs3918188 | 0.444 | | | | | 0.134 | 0.262 | |
| GG | | 68 | 31.5 | 75 | 33.3 | | 0.678 | 0.919 (0.617-1.370) |
| GA | | 111 | 51.4 | 100 | 44.4 | | 0.145 | 1.321 (0.909-1.922) |
| AA | | 37 | 17.1 | 50 | 22.2 | | 0.180 | 0.723 (0.451-1.161) |
| G allele | | 247 | 57.2 | 250 | 55.6 | | 0.628 | 3.335 (2.621-4.244) |
| A allele | | 185 | 42.8 | 200 | 44.4 | | | |
| rs7830 | 0.307 | | | | | 0.716 | 0.548 | |
| GG | | 92 | 42.6 | 107 | 47.6 | | 0.295 | 0.818 (0.562-1.191) |
| GA | | 101 | 46.8 | 98 | 43.6 | | 0.499 | 1.138 (0.782-1.657) |
| AA | | 23 | 10.6 | 20 | 8.9 | | 0.534 | 1.222 (0.650-2.295) |
| G allele | | 285 | 66.0 | 312 | 69.3 | | 0.286 | 3.939 (3.085-5.029) |
| A allele | | 147 | 34.0 | 138 | 30.7 | | | |

MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium; OR, odds ratio; CI, confidence interval.

**Table 3.** Haplotype in block 1 frequencies and the results of their association with risk of pediatric acute respiratory distress syndrome.

| Haplotype | Cases [N (%)] | Controls [N (%)] | Statistics | | | |
|---|---|---|---|---|---|---|
| | | | $\chi^2$ | P | OR | 95%CI |
| T-C | 106 (40.074) | 137 (60.889) | 6.218 | 0.013 | 0.619 | 0.424-0.903 |
| G-C | 67 (31.019) | 56 (24.889) | 2.059 | 0.151 | 1.357 | 0.894-2.062 |
| G-T | 38 (17.593) | 32 (14.222) | 0.937 | 0.333 | 1.288 | 0.771-2.149 |

OR, odds ratio; CI, confidence interval.

## DISCUSSION

Several polymorphisms in *NOS3* were identified in this study, among which, three common functional polymorphisms, the promoter -786T>C (rs2070744) SNP (Aggarwal et al., 2010), the 4b/4a (rs61722009) variable number of tandem repeats (27-bp repeat) in intron 4 (Singh et al., 2010; Rahimi et al., 2013), and the functional Glu298Asp (rs1799983) exon 7 variant (a G-to-T substitution at position 894 resulting in the replacement of glutamic acid with aspartic acid at codon 298) (Yu et al., 2006; Turan et al., 2010), were investigated. Recent clinical trials have proven the beneficiary effects of inhaled NO on respiratory syndromes (Schreiber et al., 2003; Ballard et al., 2006). Our results provide direct evidence that a genetic change in *NOS3* is linked to PARDS, with the relevant markers being mapped to different locations in *NOS3*. Moreover, we identified the signals and haplotypes associated with these changes in this region.

Godfrey et al. (2007) studied the functional effects of the rs1799983 polymorphism in young healthy volunteers and showed an association between this locus and blunted

endothelial-dependent vasodilation, possibly resulting from decreased NO synthesis. In this case-control association study, the T allele of *NOS3* rs1799983 was weakly associated with an increased risk of PARDS. The rs1799983 polymorphism was suggested to be associated with altered NOS3 enzyme activity, reduced NO production, and blunted endothelial-dependent vasodilation (Wang et al., 2000). Recent studies have reported significant gestational age-related differences between RDS and the control groups in terms of the Glu298Asp polymorphism. Therefore, the development of RDS was associated with alterations in the eNOS Glu298Asp genotype frequencies in the Turkish population (Demirçubuk et al., 2013). Although the precise molecular mechanism underlying our observations is unclear, one possible explanation is that the variants of *NOS3* may lead to altered gene expression in PARDS patients. We also observed a significantly higher frequency of the G allele of the *NOS3* rs11771443 polymorphism in PARDS subjects. The differences retained statistical significance after Bonferroni correction. To our knowledge, this is first report associating the rs11771443 polymorphism in the promoter with PARDS. The rs2070744 SNP in the promoter region of eNOS has been observed in patients with vascular disease (Guang-da et al., 2005), recurrent miscarriage (Luo et al., 2013), and glaucoma (Liao et al., 2011). Therefore, our observations strongly suggest that this SNP could be a useful marker in the long-term monitoring of PARDS.

An analysis of the genetic interactions among polymorphisms revealed a strong LD. Haplotype analysis revealed that significantly more T-C haplotypes (in block 1) were observed in controls. These results indicate that patients with the T-C haplotype of *NOS3* were less prone to PARDS. To some extent, this finding further supports the potential role of *NOS3* polymorphisms in PARDS.

The results of this study are subject to certain limitations. For example, as *NOS3* expression was not analyzed *in vivo*, the results of this study must be interpreted cautiously. Moreover, further independent research is required to validate our results.

In summary, our study demonstrated that SNPs in *NOS3*, which may alter its expression, may partially influence PARDS susceptibility (by playing a role in the development and progression of PARDS) in a Chinese Han population.

## Conflicts of interest

The authors declare no conflict of interest.

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