Endothelial nitric oxide synthase gene polymorphism is associated with Legg-Calvé-Perthes disease

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Abstract. The aim of this study was to assess the association of 27-bp number tandem repeat (VNTR) polymorphism in intron 4 and G894T polymorphism in exon 7 of the endothelial nitric oxide synthase (eNOS) gene with Legg-Calvé-Perthes disease (LCPD), and to provide a scientific basis for further research into the pathogenic mechanism. A total of 80 patients with LCPD and 100 healthy subjects were recruited in this case-control study. The 27-bp VNTR and G894T polymorphisms of the eNOS gene were genotyped using polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism, respectively, followed by agarose gel electrophoresis and DNA sequencing. Allelic and genotypic frequencies were computed in the two groups and subjected to statistical analysis. For the 27-bp VNTR polymorphism, individuals with LCPD showed a higher frequency of the ab genotype [27.5 vs. 14%; odds ratio (OR), 2.33; 95% confidence interval (CI), 1.10-4.92; P=0.024]. For the G894T polymorphism, the LCPD case group showed a higher frequency of the heterozygous genotype GT than the healthy control group (35 vs. 17%; OR, 2.67; 95% CI, 1.33-5.36; P=0.005). The results indicate that these eNOS gene polymorphisms may be a risk factor for LCPD. The 27-bp VNTR polymorphism in intron 4 and G894T polymorphism in exon 7 may be involved in the etiology of LCPD.

Introduction

Legg-Calvé-Perthes disease (LCPD) is a noninflammatory, aseptic, self-limiting, idiopathic, avascular necrosis of the femoral head, occurring in childhood, typically between the ages of 4 and 10 years and causing permanent femoral head deformity and premature osteoarthritis (1-3). The disease was first described by three independent authors in the year 1910: Perthes in Germany (4), Legg in the United States (5) and Calvé in France (6). The incidence of LCPD varies between 5.1 and 16.9 per 100,000 in various regions of the world (2,7). The etiology of LCPD remains unclear, which makes it impossible to create prevention strategies or to identify individuals at risk. A number of possible causes have been proposed, including trauma, an inflammatory process, vascular occlusion, hemodynamic alterations, endothelial injury, thrombophilia and insulin-like growth factor-I pathway abnormality (2,8,9).

Nitric oxide (NO) is a multifunctional biomolecule synthesized by three isoforms of the nitric oxide synthase (NOS) enzyme, namely endothelial NOS (eNOS, also known as NOS III), inducible NOS (iNOS or NOS II) and neuronal NOS (nNOS or NOS I). NO participates in numerous physiological processes, including angiogenesis, thrombosis, coagulation and fibrinolysis. The eNOS gene is located on the long arm of chromosome 7 (7q35-36), has 26 exons and 25 introns, and encodes a protein of 1,203 amino acids (10,11). The most examined and functionally associated polymorphisms are G894T in exon 7 and 27-bp variable number tandem repeat (VNTR) polymorphism in intron 4, respectively (12,13). A number of studies have observed associations between genetic polymorphisms in the eNOS gene and cardiovascular diseases, including coronary artery disease, chronic heart failure, hypertension, atherosclerosis, stroke, renal diseases and avascular necrosis of the femoral head, mainly in adult patients (14-20). Numerous studies have indicated that disruption of the blood supply to the femoral head is a key pathogenic event resulting in bone necrosis (2,21,22). Thrombophilia and/or decreased fibrinolysis are possible mechanisms that have been investigated as potential causes of LCPD (23-25). It may be speculated that sequence variations in the eNOS gene could influence nitric oxide production, and thereby affect the progression of LCPD. To the best of our knowledge, no study has yet investigated the association between eNOS polymorphism and the pathogenesis of LCPD.
Therefore, in the present study, the aim was to examine a relevance of the 27-bp VNTR polymorphism in intron 4 and G894T polymorphism in exon 7 of the eNOS gene by conducting an analysis of 80 LCPD Chinese patients and 100 healthy controls.

Materials and methods

Study subjects. A total of 80 patients with LCPD (70 boys and 10 girls, aged between 4 and 10 years) were consecutively enrolled in this study at the Department of Orthopedics of the First Affiliated Hospital of Guangxi Medical University (Nanning, China) from October 2012 to September 2014. LCPD was diagnosed on the basis of radiography and clinical presentation. Magnetic resonance imaging (MRI) was used as a supplementary technique to identify the changes of the femoral head in the early stage of LCPD. None of the patients with LCPD had historical or clinical evidence of vascular diseases, such as atherosclerosis, arterial thrombosis, coronary artery disease, hypertension, renal diseases, ischemic heart diseases, cerebrovascular diseases, or coexisting disorders that may be associated with eNOS polymorphism. In addition, 100 healthy age- and gender-matched children were included as controls, none of whom had LCPD or clinical symptoms suggesting LCPD. This study was approved by the Clinical Research Ethics Committee of the First Affiliated Hospital of Guangxi Medical University. Written informed consent was obtained from all participants or their parents.

DNA extraction and genotyping. For all subjects, peripheral blood samples were collected into 2 ml EDTA tubes. Genomic DNA was extracted from whole blood using a commercial kit (DP318-03; Tiangen Biotech Co., Ltd., Beijing, China), in accordance with the manufacturer's protocol. The amplification of the eNOS 27-bp VNTR polymorphism of intron 4 was performed by polymerase chain reaction (PCR) using the following primers: Forward 5'-AGGCCCTATGGTATGGC TTT-3' and reverse 5'-TCTCTTAGTGCTGTCAC-3'. The PCR reactions were performed in a total volume of 25 µl, which contained containing 500 µM each dNTP, 20 mM Tris-HCl (pH 8.3), 100 mM KCl, 3 mM MgCl₂, 0.1 units Taq polymerase, and 10 µM sense and antisense primers. PCR was performed by denaturation at 94°C for 3 min, followed by 30 cycles (30 sec at 94°C, 30 sec at 65°C and 60 sec at 72°C) and a final extension at 72°C for 5 min. This PCR amplification yielded a 248-bp fragment. Following amplification, PCR products were digested for 6 h using 2 units of BanI restriction enzyme (Takara Biotechnology Co., Ltd., Dalian, China) at 37°C. The final products of eNOS G894T polymorphism including GG, GT and TT genotypes had band sizes of 163 bp/85 bp, 248 bp/163 bp/85 bp and 248 bp, respectively. The digestion products were separated by electrophoresis on GoldView I-stained (Tiangen Science & Technology Co., Ltd., Beijing, China) 2.5% agarose gel (Fig. 3) with DNA marker I (Tiangen Biotech Co., Ltd.). The eNOS G894T polymorphisms were also sequenced using the ABI3730XL genetic analyzer (Fig. 4).

Statistical analysis. Continuous data are presented as the mean ± standard deviation. Categorical data are expressed in percentages. The allelic frequencies were calculated by the chromosome counting method. The genotype frequencies for eNOS polymorphism among patients and healthy controls were assessed for Hardy-Weinberg equilibrium (HWE) using the Pearson's Chi-square method. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using relevant 2x2 contingency tables. The data was analyzed using the SPSS statistical software package (version 16.0; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant result.

Results

General characteristics of the study subjects. In this study, 80 LCPD patients and 100 controls were included in the final analysis. LCPD was more common in boys than in girls. The general characteristics of the study population are summarized in Table I. There were more boys than girls in both the

<table>
<thead>
<tr>
<th>Variable</th>
<th>LCPD (n=80)</th>
<th>Healthy control (n=100)</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Gender, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>70 (87.5)</td>
<td>85 (85.0)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Female</td>
<td>10 (12.5)</td>
<td>15 (15.0)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Age, yearsa</td>
<td>7.3±2.0</td>
<td>6.8±2.1</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Site of LCPD, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>38 (47.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>30 (37.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bilateral</td>
<td>12 (15.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surgery</td>
<td></td>
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<tr>
<td>Yes</td>
<td>49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>31</td>
<td></td>
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</tr>
</tbody>
</table>

*aMean ± standard deviation. LCPD, Legg-Calvé-Perthes disease.
patient group (87.5% boys) and the gender-matched pediatric control group (85% boys). There were no significant differences between the groups regarding age and gender.

**eNOS gene polymorphisms in the case and control groups.**

The genotypic and allelic frequencies of the two eNOS gene polymorphisms in the case and control groups are shown in Table II. The genotype frequencies of the analyzed polymorphism were in HWE. The genotypic and allelic frequencies of the two eNOS gene polymorphisms differed between the control and patient groups. Representative electrophoresis results and sequence diagrams for the eNOS 27-bp VNTR polymorphism are shown in Figs. 1 and 2. The LCPD case group showed a higher frequency of the ab genotype than the healthy control group (27.5 vs. 14%; OR, 2.33; 95% CI, 1.10-4.92; P=0.024). The frequency of the a allele was significantly higher in the LCPD case group than in the healthy controls (13.8 vs. 7%; OR, 2.12; 95% CI, 1.05-4.29; P=0.034). Representative electrophoresis results and sequence diagrams for the eNOS G894T polymorphism are shown in Figs. 3 and 4. The heterozygous genotype GT was observed in 35% of the patients (28/80) and 17% (17/100) of the controls (OR, 2.67; 95% CI, 1.33-5.36; P=0.005). The frequency of allele T

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**Figure 1.** Agarose gel electrophoresis result of eNOS 27-bp VNTR polymorphism. Fragments containing 420 bp (eNOS b allele) and 393 bp (eNOS a allele) can be visualized. Lanes 1, 2, 4, 6, 8 and 10 show the bb genotype; lanes 3, 5 and 9 show the ab genotype; lane M: DNA marker. The rare aa genotype was not found in patients with LCPD and healthy controls. eNOS, endothelial nitric oxide synthase; VNTR, variable number tandem repeat.

**Figure 2.** Sequence diagrams of the PCR amplification products of the eNOS 27-bp VNTR polymorphism. Sequence analysis of (A) bb genotype and (B) ab genotype. PCR, polymerase chain reaction; eNOS, endothelial nitric oxide synthase; VNTR, variable number tandem repeat.

**Figure 3.** Agarose gel electrophoresis result of eNOS G894T polymorphism provided by PCR-RFLP. The PCR product was digested with BanII restriction enzyme and visualized on a 2.5% agarose gel. Lanes 1, 2, 4, 5, 9, 10 show wild type GG genotype (163 and 85 bp); lanes 3, 7, 8 show heterozygous GT (248, 163 and 85 bp); and lane 6: homozygous TT (248 bp). Lane M: DNA marker. eNOS, endothelial nitric oxide synthase; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism.

**Figure 4.** Sequence diagrams of the PCR amplification products of the eNOS G894T polymorphism. Sequence analysis of the (A) TT genotype. (B) GT heterozygous genotype and (C) GG genotype. PCR, polymerase chain reaction; eNOS, endothelial nitric oxide synthase.
in the LCPD case group (20%) was greater than that of the healthy control group (10.5%), and the difference in frequency distribution between alleles was significant (OR, 2.13; 95% CI, 1.18‑3.87; P=0.011).

Discussion

The precise pathogenetic basis of LCPD is currently unknown. Repeated interruptions of the vascular blood supply to the femoral head, which results in ischemic necrosis, appears to be a key pathogenic event causing pathological and subsequent structural changes to the growing femoral head (2,21,22). However, at present, the exact pathogenetic basis of the reductions in blood flow to the femoral head vasculature in patients with LCPD is unclear. LCPD is considered to be a multifactorial disease, in which genetic and environmental factors play a role (2). Since genetic background is a determinant of LCPD, extensive genomics research is required to identify polymorphic loci and elucidate the pathogenesis. eNOS gene polymorphisms have been investigated as potential risk factors in numerous vascular diseases, as aforementioned (14‑20). To the best of our knowledge, no studies have investigated the role of eNOS gene polymorphism in the risk of LCPD. In the present study, the association between eNOS gene polymorphism and the risk of LCPD was analyzed in children from the Guangxi province of China, and it was found that the frequency of an a allele and ab genotype in intron 4 and allele 894T, GT genotype in exon 7 were significantly higher in patients with LCPD than in healthy controls.

Table II. Comparison of genotypic distributions and allele frequencies of the two eNOS gene polymorphisms between the LCPD and healthy control groups.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype/Allele</th>
<th>LCPD (n=80)</th>
<th>Healthy control (n=100)</th>
<th>OR</th>
<th>95% CI</th>
<th>P‑value</th>
</tr>
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<tr>
<td>eNOS 27 bp VNTR, n (%)</td>
<td>bb</td>
<td>58 (72.5)</td>
<td>86 (86.0)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ab</td>
<td>22 (27.5)</td>
<td>14 (14.0)</td>
<td>2.33</td>
<td>1.10‑4.92</td>
<td>0.024</td>
</tr>
<tr>
<td></td>
<td>aa</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>138 (86.2)</td>
<td>186 (93.0)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>22 (13.8)</td>
<td>14 (7.0)</td>
<td>2.12</td>
<td>1.05‑4.29</td>
<td>0.034</td>
</tr>
<tr>
<td>eNOS G894T, n (%)</td>
<td>GG</td>
<td>50 (62.5)</td>
<td>81 (81.0)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>28 (35.0)</td>
<td>17 (17.0)</td>
<td>2.67</td>
<td>1.33‑5.36</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>2 (2.5)</td>
<td>2 (2.0)</td>
<td>1.62</td>
<td>0.22‑11.87</td>
<td>0.632</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>128 (80.0)</td>
<td>179 (89.5)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>32 (20.0)</td>
<td>21 (10.5)</td>
<td>2.13</td>
<td>1.18‑3.87</td>
<td>0.011</td>
</tr>
</tbody>
</table>

eNOS, endothelial nitric oxide synthase; VNTR, variable number tandem repeat; LCPD, Legg-Calvé-Perthes disease.
polymorphism and the risk of LCPD. The results support the role of eNOS in the pathogenesis of LCPD. However, as the sample size of this study was relatively small, additional studies with larger cohorts are required in order to confirm the observed association.

References