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

YULONG ZHAO, SHIJIE LIAO, RONGBIN LU, HAO DANG, JINMIN ZHAO and XIAOFEI DING

Department of Orthopedic Trauma and Hand Surgery, The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi 530021, P.R. China

Received December 24, 2014; Accepted February 11, 2016

DOI: 10.3892/etm.2016.3111

Abstract. The aim of this study was to assess the association of 27-bp variable 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.

Correspondence to: Professor Xiaofei Ding, Department of Orthopedic Trauma and Hand Surgery, The First Affiliated Hospital of Guangxi Medical University, 6 Shuangyong Road, Nanning, Guangxi 530021, P.R. China
E-mail: dxfeicsgk2014@163.com

Abbreviations: CI, confidence interval; eNOS, endothelial nitric oxide synthase; HWE, Hardy-Weinberg equilibrium; LCPD, Legg-Calvé-Perthes disease; NO, nitric oxide; OR, odds ratio; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; VNTR, variable number tandem repeat

Key words: Legg-Calvé-Perthes disease, nitric oxide synthase, gene polymorphism

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-1 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'-AGGCCCTATGGTAGTGCC TTT-3' and reverse 5'-TCTCTTAGTGCTGTGGTCAC-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 $MgCl_2$, 0.1 units Taq polymerase (Tiangen Biotech Co., Ltd.), 10 μM each primer and 100 ng genomic DNA. The reaction mixture was heated to 94˚C (3 min), followed by 30 cycles of 94˚C (30 sec), 58˚C (30 sec), and 72˚C (1 min), and then a final extension at 72˚C (5 min). The PCR products were analyzed by electrophoresis in 2% agarose gels at 80 V for 40 min. A band size of 393 bp indicated a polymorphic genotype (aa, 4 repeats). A 420-bp fragment represented the wild-type genotype (bb, 5 repeats). Two fragments at 393 bp and 420 bp indicated a heterozygous genotype ab (Fig. 1). The PCR products were sequenced using an ABI3730XL genetic analyzer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) (Fig. 2). The eNOS G894T polymorphism in exon 7 was determined by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis. The forward primer was 5'-AAGGCAGGAGACAGTGGATGG-A-3' and the reverse primer was 5'-CCCAGTCAATCCCTTTGGTGC TCA-3'. PCR was conducted to amplify 0.1 μg genomic DNA. The 25 μl reaction mixture contained 500 μM each dNTP, 20 mM Tris-HCl (pH 8.3), 100 mM KCl, 3 mM $MgCl_2$, 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 *Ban*II 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 (Beijing Solarbio 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).

Table I. General characteristics of the study subjects.

Variable	LCPD (n=80)	Healthy control (n=100)	P-value
Gender, n (%)			
Male	70 (87.5)	85 (85.0)	>0.05
Female	10 (12.5)	15 (15.0)	>0.05
Age, years[a]	7.3±2.0	6.8±2.1	>0.05
Site of LCPD, n (%)			
Right	38 (47.5)		
Left	30 (37.5)		
Bilateral	12 (15.0)		
Surgery			
Yes	49		
No	31		

[a]Mean ± standard deviation. LCPD, Legg-Calvé-Perthes disease.

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

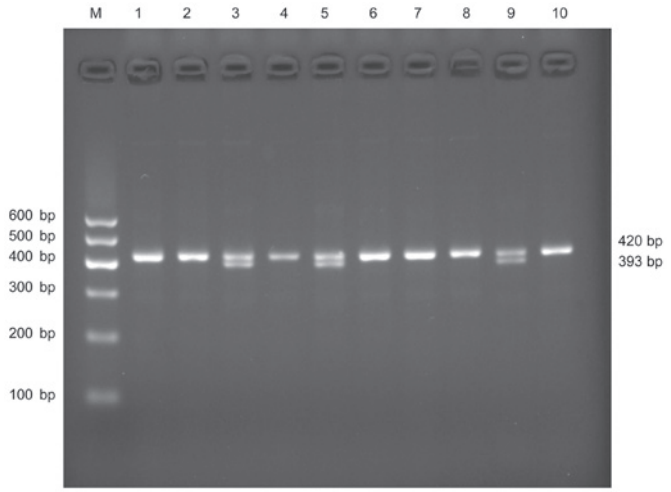

Figure 1. Agarose gel electrophoresis result of eNOS 27-bp VNTR polymorphism. Fragments containing 420 bp (eNOS b allele) and 393 bp (eNOS a allele) can be visualized. Lanes 1, 2, 4, 6, 7, 8 and 10 show the bb genotype; lanes 3, 5 and 9 show the ab genotype; lane M: DNA marker. The rare aa genotype was not found in patients with LCPD and healthy controls. eNOS, endothelial nitric oxide synthase; VNTR, variable number tandem repeat.

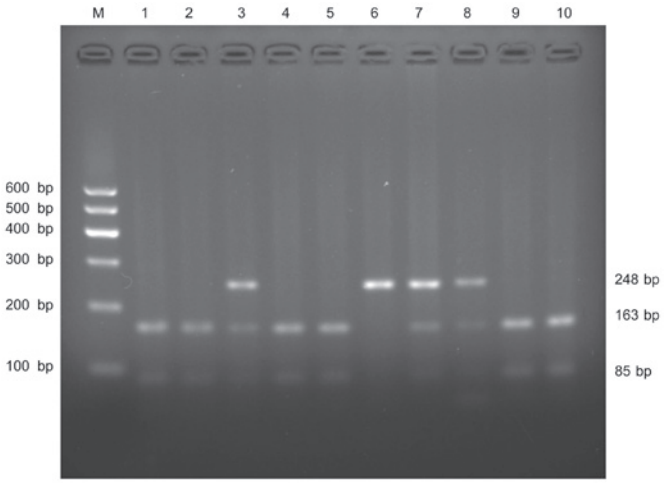

Figure 3. Agarose gel electrophoresis result of eNOS G894T polymorphism provided by PCR-RFLP. The PCR product was digested with *Ban*II restriction enzyme and visualized on a 2.5% agarose gel. Lanes 1, 2, 4, 5, 9, 10 show wild type GG genotype (163 and 85 bp); lanes 3,7,8 show heterozygous GT (248, 163 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.

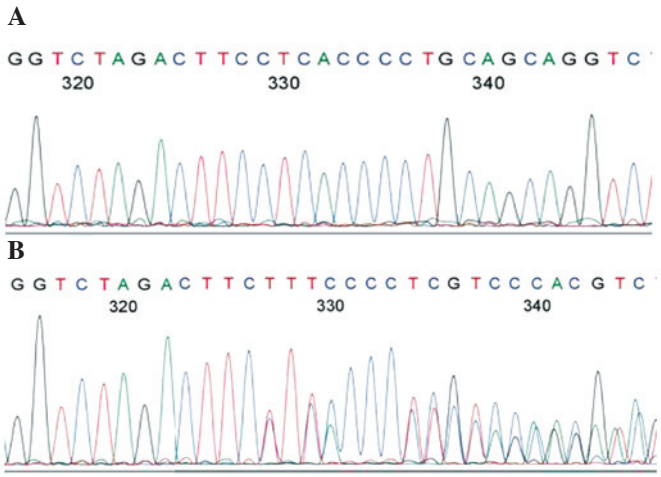

Figure 2. Sequence diagrams of the PCR amplification products of the eNOS 27-bp VNTR polymorphism. Sequence analysis of (A) bb genotype and (B) ab genotype. PCR, polymerase chain reaction; eNOS, endothelial nitric oxide synthase; VNTR, variable number tandem repeat.

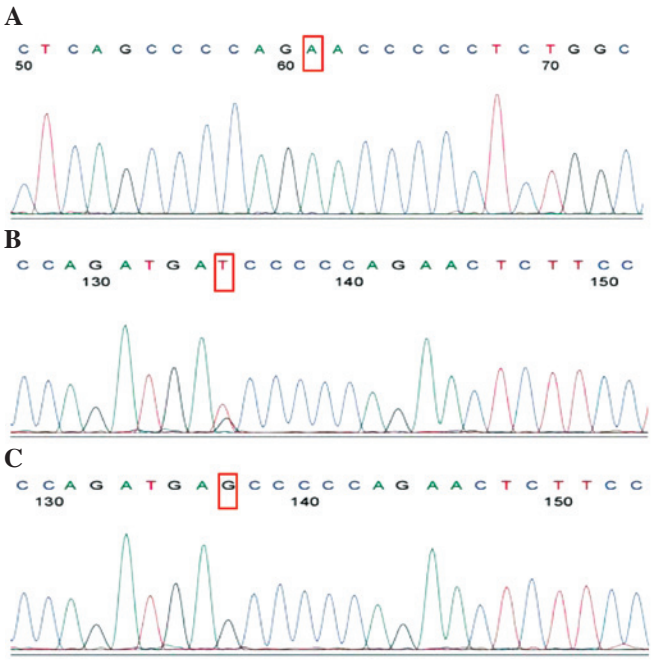

Figure 4. Sequence diagrams of the PCR amplification products of the eNOS G894T polymorphism. Sequence analysis of tbe (A) TT genotype. (B) GT heterozygous genotype and (C) GG genotype. PCR, polymerase chain reaction; eNOS, endothelial nitric oxide synthase.

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

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

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

eNOS, endothelial nitric oxide synthase; VNTR, variable number tandem repeat; LCPD, Legg-Calvé-Perthes disease.

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.

NO, synthesized by eNOS, has various roles in the vasculature, which include the regulation of vascular tone and blood pressure via vasodilation (26). NO also been suggested to be involved in the modulation of angiogenesis (27). In a previous study, it was observed that the development of angiogenesis in response to limb ischemia was severely reduced in mice lacking the eNOS gene (28). Another study demonstrated that the presence of a 27-bp VNTR polymorphism in intron 4 and G894T polymorphism in exon 7 can cause changes in eNOS expression and enzymatic activity; the NO levels in the plasma of subjects with the a allele of eNOS in intron 4 were significantly lower than those without the a allele (29). The G894T polymorphism causes a structural change in the eNOS protein that is associated with impaired eNOS activity (30). Therefore, allele a and G894T gene polymorphisms can lead to low expression levels and activity of eNOS. The present study also reveals that the 27-bp VNTR polymorphism in intron 4 and G894T polymorphism in exon 7 may be risk factors for LCPD, and indicates that NO produced by constitutively expressed eNOS may play a protective role in the pathogenesis of LCPD. The eNOS enzyme is constitutively expressed in vascular endothelial cells. Endothelial cells are important in the pathogenesis of diseases that involve thrombosis and/or inflammation (31). Changes in hemodynamics (22), vascular thrombotic tendency due to hypercoagulability (32), and hypofibrinolysis (9,33,34) have all been suggested to be significant factors in the pathogenesis of LCPD. Intron 4 and G894T polymorphisms of eNOS have been shown to decrease NO levels in human plasma (35,36). As a result, polymorphisms of the eNOS gene and resultant reductions in the quantity of synthesized NO are likely to lead to different types of vascular diseases, as previously mentioned; these diseases may share some common risks through inheritance or exposure. Polymorphisms of the eNOS gene have been indicated to be risk factors for avascular necrosis of the femoral head (20), an adult disease that has a similar pathogenesis to LCPD. The finding in the present study that eNOS polymorphism is associated with the pathogenesis of LCPD is, therefore, not remarkable.

The present study has certain limitations: As all of the samples are from the same hospital, potential selection bias cannot be fully excluded. Also, in consideration of the low incidence of LCPD, the sample size was small, and the rare aa genotype was not found in the subjects investigated.

In summary, to the best of our knowledge, this is the first report investigating an association between eNOS gene

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

1. Shah H: Perthes disease: Evaluation and management. Orthop Clin North Am 45: 87-97, 2014.
2. Kim HK: Pathophysiology and new strategies for the treatment of Legg-Calvé-Perthes disease. J Bone Joint Surg Am 94: 659-669, 2012.
3. Cheng TL, Murphy CM, Cantrill LC, Mikulec K, Carpenter C, Schindeler A and Little DG: Local delivery of recombinant human bone morphogenetic proteins and bisphosphonate via sucrose acetate isobutyrate can prevent femoral head collapse in Legg-Calve-Perthes disease: A pilot study in pigs. Int Orthop 38: 1527-1533, 2014.
4. Perthes G: Concerning arthritis deformans juvenilis. 1910. Clin Orthop Relat Res 451: 17-20, 2006.
5. Legg AT: An obscure affection of the hip joint. 1910. Clin Orthop Relat Res 451: 11-13, 2006.
6. Calvé J: On a particular form of pseudo-coxalgia associated with a characteristic deformity of the upper end of the femur. Clin Orthop Relat Res 451: 14-16,2006.
7. Wiig O, Terjesen T, Svenningsen S and Lie SA: The epidemiology and aetiology of Perthes' disease in Norway. A nationwide study of 425 patients. J Bone Joint Surg Br 88: 1217-1223, 2006.
8. Alpaslan AM, Aksoy MC and Yazici M: Interruption of the blood supply of femoral head: An experimental study on the pathogenesis of Legg-Calve-Perthes disease. Arch Orthop Trauma Surg 127: 485-491, 2007.
9. Glueck CJ, Freiberg RA and Wang P: Role of thrombosis in osteonecrosis. Curr Hematol Rep 2: 417-422, 2003.
10. Marsden PA, Schappert KT, Chen HS, Flowers M, Sundell CL, Wilcox JN, Lamas S and Michel T: Molecular cloning and characterization of human endothelial nitric oxide synthase. FEBS Lett 307: 287-293, 1992.
11. Marsden PA, Heng HH, Scherer SW, Stewart RJ, Hall AV, Shi XM, Tsui LC and Schappert KT: Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. J Biol Chem 268: 17478-17488, 1993.
12. Fairchild TA, Fulton D, Fontana JT, Gratton JP, McCabe TJ and Sessa WC: Acidic hydrolysis as a mechanism for the cleavage of the Glu (298)->Asp variant of human endothelial nitric-oxide synthase. J Biol Chem 276: 26674-26679, 2001.
13. Lembo G, De Luca N, Battagli C, Iovino G, Aretini A, Musicco M, Frati G, Pompeo F, Vecchione C and Trimarco B: A common variant of endothelial nitric oxide synthase (Glu298Asp) is an independent risk factor for carotid atherosclerosis. Stroke 32: 735-740, 2001.
14. Abdel-Aziz TA and Mohamed RH: Association of endothelial nitric oxide synthase gene polymorphisms with classical risk factors in development of premature coronary artery disease. Mol Biol Rep 40: 3065-3071, 2013.
15. Balligand JL, Feron O and Dessy C: eNOS activation by physical forces: From short-term regulation of contraction to chronic remodeling of cardiovascular tissues. Physiol Rev 89: 481-534, 2009.
16. Doshi AA, Ziolo MT, Wang H, Burke E, Lesinski A and Binkley P: A promoter polymorphism of the endothelial nitric oxide synthase gene is associated with reduced mRNA and protein expression in failing human myocardium. J Card Fail 16: 314-319, 2010.
17. Levinsson A, Olin AC, Björck L, Rosengren A and Nyberg F: Nitric oxide synthase (NOS) single nucleotide polymorphisms are associated with coronary heart disease and hypertension in the INTERGENE study. Nitric Oxide 39: 1-7, 2014.
18. Bellini MH, Figueira MN, Piccoli MF, Marumo JT, Cendoroglo MS, Neto MC, Dalboni MA, Batista MC, Goes MA and Schor N: Association of endothelial nitric oxide synthase gene intron 4 polymorphism with end-stage renal disease. Nephrology (Carlton) 12: 289-293, 2007.
19. Spoto B, Benedetto FA, Testa A, Tripepi G, Mallamaci F, Maas R, Boeger RH and Zoccali C: An additive effect of endothelial nitric oxide synthase gene polymorphisms contributes to the severity of atherosclerosis in patients on dialysis. Am J Hypertens 20: 758-763, 2007.
20. Zheng L, Wang W, Ni J, Li Z and Xiao T: The association of eNOS gene polymorphism with avascular necrosis of femoral head. PloS One 9: e87583, 2014.
21. Ferguson AB Jr: Segmental vascular changes in the femoral head in children and adults. Clin Orthop Relat Res: 291-298, 1985.
22. Launder WJ, Hungerford DS and Jones LH: Hemodynamics of the femoral head. J Bone Joint Surg Am 63: 442-448, 1981.
23. Woratanarat P, Thaveeratitharm C, Woratanarat T, Angsanuntsukh C, Attia J and Thakkinstian A: Meta-analysis of hypercoagulability genetic polymorphisms in Perthes disease. J Orthop Res 32: 1-7, 2014.
24. Aksoy MC, Aksoy DY, Haznedaroglu IC, Sayinalp N, Kirazli S and Alpaslan M: Enhanced tissue factor pathway inhibitor response as a defense mechanism against ongoing local microvascular events of Legg-Calve-Perthes disease. Pediatr Hematol Oncol 22: 391-399, 2005.
25. Aksoy MC, Aksoy DY, Haznedaroglu IC, Sayinalp N, Kirazli S and Alpaslan M: Thrombomodulin and GFC levels in Legg-Calve-Perthes disease. Hematology 13: 324-328, 2008.
26. Gamboa A, Okamoto LE, Diedrich A, Choi L, Robertson D, Farley G, Paranjape S and Biaggioni I: Sympathetic activation and nitric oxide function in early hypertension. Am J Physiol Heart Circ Physiol 302: H1438-H1443, 2012.
27. Cooke JP: NO and angiogenesis. Atheroscler Suppl 4: 53-60, 2003.
28. Murohara T, Asahara T, Silver M, Bauters C, Masuda H, Kalka C, Kearney M, Chen D, Symes JF, Fishman MC, *et al*: Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. J Clin Invest 101: 2567-2578, 1998.
29. Tsukada T, Yokoyama K, Arai T, Takemoto F, Hara S, Yamada A, Kawaguchi Y, Hosoya T and Igari J: Evidence of association of the ecNOS gene polymorphism with plasma NO metabolite levels in humans. Biochem Biophys Res Commun 245: 190-193, 1998.
30. Yoshimura M, Yasue H, Nakayama M, Shimasaki Y, Sumida H, Sugiyama S, Kugiyama K, Ogawa H, Ogawa Y, Saito Y, *et al*: A missense Glu298Asp variant in the endothelial nitric oxide synthase gene is associated with coronary spasm in the Japanese. Hum Genet 103: 65-69, 1998.
31. Horstman LL, Jy W, Jimenez JJ and Ahn YS: Endothelial microparticles as markers of endothelial dysfunction. Front Biosci 9: 1118-1135, 2004.
32. Eldridge J, Dilley A, Austin H, EL-Jamil M, Wolstein L, Doris J, Hooper WC, Meehan PL and Evatt B: The role of protein C, protein S and resistance to activated protein C in Legg-Perthes disease. Pediatrics 107: 1329-1334, 2001.
33. Hunt DM, Holmes Z, Pickering W and Cohen H: Correspondence. J Bone Joint Surg Am 80: 604-606, 1998.
34. Van Veldhuizen PJ, Neff J, Murphey MD, Bodensteiner D and Skikne BS: Decreased fibrinolytic potential in patients with idiopathic avascular necrosis and transient osteoporosis of the hip. Am J Hematol 44: 243-248, 1993.
35. de Marco KC, Antunes LM, Tanus-Santos JE and Barbosa F Jr: Intron 4 polymorphism of the endothelial nitric oxide synthase (eNOS) gene is associated with decreased NO production in a mercury-exposed population. Sci Total Environ 414: 708-712, 2012.
36. Saini V, Bhatnagar MK and Bhattacharjee J: Endothelial nitric oxide synthase Glu298Asp (G894T) gene polymorphism in coronary artery disease patients with type 2 diabetes mellitus. Diabetes Metab Syndr 6: 106-109, 2012.