Association of eNOS 27-bp VNTR, 894G > T and 786T > C polymorphisms with susceptibility to Legg-Calve-Perthes Disease in Iranian children

Mohammad Reza Azarpira^a, Mohammad Mahdi Ghilian^{a,*}, Mohammad Reza Sobhan^b, Masoud Mahdinezhad-Yazdi^b, Kazem Aghili^c, Hossein Ahrar^c, Hossein Neamatzadeh^{d,e}

^a Bone and Joint Diseases Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

^b Department of Orthopedics, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

^c Department of Radiology, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

^d Department of Medical Genetics, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

^e Mother and Newborn Health Research Center, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

ARTICLE INFO

Keywords:

Legg-Calve-Perthes disease
Nitric oxide synthase
Polymorphism

ABSTRACT

Background: The aim of this study was to analyze the association of eNOS polymorphisms with risk of Legg-Calve-Perthes Disease (LCPD).

Methods: The study comprised of 45 LCPD patients and 55 controls. The eNOS polymorphisms were genotyped with PCR and by PCR-RFLP.

Results: The eNOS 894G > T and –786T > C polymorphisms were significantly associated with an increased risk of LCPD. However, there was no significant association between eNOS 27-bp VNTR polymorphism and LCPD risk.

Conclusion: Our results suggest that the eNOS 894G > T and –786T > C polymorphisms may be a risk factor for LCPD in Iranian children, but not 27-bp VNTR polymorphism.

1. Introduction

Legg-Calve-Perthes disease (LCPD) is an idiopathic osteonecrosis of the femoral head epiphysis in children ≤15 years of age, with a maximum diagnosis at age between five and eight year.^{1–3} LCPD is usually unilateral and occurs five times more commonly in boys than in girls.⁴ The incidence of LCPD varies within certain ethnicities, partly depending on latitude.^{1,4} The incidence ranges from 0.4/100,000 to 29.0/100,000 children < 15 years of age. The etiology and underlying pathophysiology of LCPD are still not completely understood, which makes it impossible to create prevention strategies or to identify individuals at risk. However, maternal smoking during pregnancy, small stature, skeletal retardation, and low birth weight seems to be the key pathogenetic causes of LCPD.^{2,5}

Generally, it seems LCPD is usually not caused by genetic factors, but there are some cases where LCPD affects multiple family members. In addition, in a few familial cases an autosomal dominant inheritance has been reported.^{6,7} This complexity makes it very difficult to predict whether or not an individual will inherit LCPD. Understanding genetic basis of LCPD is yet to be achieved, although this field has been increasing in recent years.⁷ Some studies have examined the relationship

between genetic diversity and LCPD susceptibility. Evidence is growing that genetic variations in the COL2A1,^{8,9} eNOS,¹⁰ TLR4, IL-6,¹¹ α, FV, FII, and MTHFR¹² genes to be responsible on the likelihood of developing LCPD.

The eNOS (MIM: 163729; GenBank ID: 4846) gene is located on chromosome 7q35-36, comprises 26 exons, and spanning 21 kb.¹³ The eNOS gene is one of the factors that regulate the expression of the endothelial cell gene. Three polymorphisms of the eNOS gene have been identified, including a T786C (rs2070744) polymorphism in the promoter region, a G894T (rs1799983) polymorphism in exon 7 and a variable number of tandem 4a4b repeats (rs61722009) in intron 4.^{14,15} Studies results showed that each polymorphism can influence the expression or functional activity of eNOS enzyme.¹⁶

Recently, Zhao et al. have been carried out a study to investigate the relationship between eNOS-4b/a polymorphism and the risk of LCPD in a Chinese population. Their results demonstrated that eNOS polymorphisms may be involved in the etiology of LCPD.¹⁰ However, the number of studies conducted to examine the eNOS polymorphism is not sufficient. Especially, there is a lack of investigation on different ethnicities. Therefore, in the present research, we hypothesize that the eNOS 27-bp VNTR, Glu298Asp and T-786C polymorphisms may have an

* Corresponding author.

E-mail address: mmghilian@gmail.com (M.M. Ghilian).

<https://doi.org/10.1016/j.jor.2019.02.024>

Received 8 September 2018; Accepted 17 February 2019

Available online 26 February 2019

0972-978X/ © 2019 Prof. PK Surendran Memorial Education Foundation. Published by Elsevier, a division of RELX India, Pvt. Ltd. All rights reserved.

effect on development LCPD. In order to verify the hypothesis, a case-control study was conducted to investigate the association of eNOS polymorphisms and susceptibility to LCPD in Iranian children.

2. Materials and methods

2.1. Study population

The study was approved by the ethics committee and written informed consent was obtained from parents of all subjects. Forty-five patients with LCPD including 27 males and 18 females with a mean age of 8.52 ± 3.22 years (with a median age at disease onset being 5, range: 2–12 years) were consecutively enrolled in this study between January 2013 and April 2017. LCPD was diagnosed according to the standard clinical criteria, ultrasonographic examination and radiographic signs.¹² Fifty-five age, sex and race matched controls subjects including 32 males and 23 females (with a mean age of 9.22 ± 2.28 years) with no history of orthopedic disease were recruited from the general population. All participants were recruited from different region of Iran.

2.2. Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using a using a Roche kit (Mannheim, Germany) in accordance with the manufacturer's protocol and Extracted DNA was labeled and suspended in Tris buffer and stored at -20°C until used. The 27-bp VNTR polymorphism was genotyped by polymerase chain reaction (PCR); and the eNOS 894G > T and 786T > C polymorphisms were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique.

2.3. The eNOS VNTR ab polymorphism

The eNOS gene intron 4, 27-bp VNTR polymorphism was detected by PCR by using the following primers 5'-AGGCCC TATGGTAGTGC CTT-3' (forward) and 5'-TCTCTTA GTGCTGTGGTCAC-3' (reverse). Polymerase chain reaction (PCR) consisted of 0.5 mM of each primer (Sigma), 0.2 mM each of the dNTP (Fermentas, Lithuania), 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2.0 mmol/L MgCl₂, 1.5 units of Taq DNA polymerase (Invitrogen), and 50–100 ng of genomic DNA. The PCR reactions are performed using a Thermal Cycler at the following conditions: initial denaturation at 94°C for 5 min, 32 cycles of denaturation at 94°C for 1 min, annealing temperature at 58°C for 1 min, and extension at 72°C for 1 min followed by last extension at 72°C for 3 min. PCR products were electrophoresed on 3% agarose gel. The eNOS intron 4 a/b polymorphism "a" allele contained four 27bp repeats giving rise to a PCR product of 393 bp, whereas the "b" allele contained five 27bp repeats, yielding a PCR product of 420 bp. Thus, the eNOS intron 4 a/b polymorphism results in three genotypes: aa homozygous (aa), bb homozygous (bb), and ab heterozygous (ab).

2.4. The eNOS 894G > T polymorphism

Genotyping of eNOS 894G > T polymorphism by with the restriction fragment length polymorphism (RFLP) technique was performed mainly according to the protocols described previously. The eNOS 894G > T polymorphism was amplified using the following primers 5'-AAGGCAGGAGACAGTGGATGG-3' (forward) and 5'-CCCAGTCAAT CCCTTTGGTGCT CA-3' (reverse). PCR reactions were carried out in a 20 μL volume containing 50 ng of genomic DNA, 1x PCR buffer (Qiagen, Hilden, Germany), 3.0 mM MgCl₂, 0.5 mM of each primer, 0.2 mM of each dNTPs (Fermentas, Lithuania) and 2.4 U of Taq DNA polymerase (Invitrogen). After an initial 5 min at 94°C , DNA was amplified by 40 cycles of 30 s at 94°C , 30 s at 66°C , 30 s at 72°C , and a final extension step of 8 min at 72°C . After amplification the polymerase chain reaction

(PCR) products were digested by 5 units of MboI restriction endonuclease at 37°C for 3 h. The presence of a G at position 894 (AA: aspartic acid; codon 298) generated a unique 206 bp fragment, while the 206 bp fragment was divided into unique 119 bp and 87 bp fragments when position 894 contains a T.

2.5. The eNOS –786T > C polymorphism

The eNOS –786T > C Polymorphism was determined by polymerase chain reaction (PCR) followed by restriction-enzyme digestion. Primers were 5'-ATGCTCCCACCAGGGCATCA-3' (forward) and 5'-GTC CTTGAGTCTGACATTAGGG-3' (reverse). PCR was in 25 μL containing 10 μM each primer, 10 μM each dNTP (Fermentas, Lithuania), 50 μM MgCl₂, 1.5 units Taq polymerase (Invitrogen), and 20 ng/ μL genomic DNA. PCR conditions were 94°C for 6 min, followed by 36 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 8 min. Amplified DNA fragments were digested with 1.0 unit of NgOAIIV (Promega) restriction enzyme and the reaction buffer, incubated at 37°C for 4 h. Fragments were visualized by 2% agarose gel electrophoresis. The eNOS –786T allele has no NgOAIIV cleavage site, whereas the PCR product is cleaved into two fragments of 203 bp and 33 bp in the presence of the –786C allele.

2.6. Statistical analysis

The Statistical Package for the Social Sciences (SPSS) software package version 19.0 (SPSS, Inc., Chicago, IL, USA) was used for the statistical analysis of data. Hardy-Weinberg equilibrium (HWE) for each eNOS polymorphism among the controls was tested using a goodness-of-fit χ^2 test. The eNOS polymorphisms genotypes and allele frequencies in patients with LCPD were compared to control subjects and the significance of differences calculated using χ^2 test. The association between eNOS polymorphisms and risk LCPD was evaluated by multivariate unconditional logistic regression analyses with odds ratios (ORs) and 95% confidence intervals (95% CIs). $P < 0.05$ was considered to indicate a statistically significant result.

3. Results

The general characteristics of the LCPD cases and controls are summarized in Table 1. For all participants, DNA analyses of eNOS gene polymorphisms were performed. There were no significant differences between cases and controls regarding age and gender (Table 1).

3.1. eNOS 27-bp VNTR polymorphism

The distribution of the 27-bp VNTR polymorphism aa genotype in cases and controls were similar. There were slight differences in the frequency of the 27-bp VNTR polymorphism genotypes among cases and control subjects, as shown in Table 2. All the three homozygous variants were control subjects. The V27-bp VNTR polymorphism bb, ba and aa genotypes did not associated with a significantly increased risk of LCPD.

Table 1
General characteristics of the LCPD cases and controls.

	Cases (n = 45)	Control (n = 55)	P-value
Age ($\pm$ SD)	8.52 ± 3.22	9.22 ± 2.28	> 0.05
Gender			
Male	27 (60.0)	32 (58.2)	> 0.05
Female	18 (40.0)	23 (41.8)	> 0.05
Site of LCPD			
Right	21 (46.7)		
Left	16 (35.5)		
Bilateral	8 (17.8)		

Table 2
Genotype frequencies of eNOS polymorphisms between LCPD patents and controls.

Genotypes	Cases (n = 45)	Control (n = 55)	OR (95% CI)	P-value
eNOS 27-bp VNTR				
Genotype				
bb	27 (60.0)	42 (76.4)	1.000 (reference)	
ba	18 (40.0)	13 (23.6)	2.154 (0.910–5.099)	0.081
aa	0 (0.0)	0 (0.0)	NA	NA
Allele				
b	72 (80.0)	97 (88.2)	1.000 (reference)	
a	18 (20.0)	13 (11.8)	1.865 (0.859–4.052)	0.115
Dominant (bb vs. ab + aa)			0.464 (0.196–1.099)	0.081
Recessive (bb + ab vs. aa)			NA	NA
eNOS 894G > T				
Genotype				
GG	19 (42.2)	40 (72.8)	1.000 (reference)	
GT	24 (53.3)	14 (25.4)	3.347 (1.440–7.778)	0.005
TT	2 (4.5)	1 (1.8)	2.512 (0.220–28.634)	0.458
Allele				
G	62 (68.9)	94 (85.5)	1.000 (reference)	
T	28 (31.1)	16 (14.5)	2.653 (1.327–5.305)	0.006
Dominant (GG vs. GT + TT)			0.274 (0.119–0.633)	0.002
Recessive (GG + GT vs. TT)			0.398 (0.035–4.539)	0.458
eNOS –786T > C				
Genotype				
TT	16 (35.6)	25 (45.5)	1.000 (reference)	
TC	22 (48.9)	29 (52.7)	0.858 (0.390–1.887)	0.703
CC	7 (15.5)	1 (1.8)	9.947 (1.175–84.209)	0.036
Allele				
T	54 (60.0)	79 (71.8)	1.000 (reference)	
C	36 (40.0)	31 (28.2)	1.699 (0.940–3.071)	0.079
Dominant (TT vs. TC + CC)			0.662 (0.295–1.487)	0.318
Recessive (TT + TC vs. CC)			0.101 (0.012–0.851)	0.035

OR: Odds Ratio; CI: Confidence Interval; NA: Not Applicable.

3.2. eNOS 894G > T polymorphism

The genotype frequencies of the eNOS 894G > T polymorphism GG, GA and AA in the cases were 0.73, 0.25, and 0.02, respectively while the corresponding frequencies in the control subjects were 0.81, 0.17, and 0.02, respectively. As shown in Table 2, cases were more likely to carriers the AG genotype and T allele than the controls. The heterozygous GA genotype was associated with a significant increased risk of LCPD (OR = 3.347, 95% CI 1.440–7.778, P = 0.005), compared with the homozygous mutant TT genotype (OR = 2.512, 95% CI 0.220–28.634, P = 0.458). The eNOS 894T allele was associated with a significantly increased risk of LCPD (OR = 2.653, 95% CI 1.327–5.305, P = 0.006). We also found a significantly increased LCPD risk with eNOS 894G > T under dominant genetic model (GG vs. GT + TT; OR = 0.274; 95% CI, 0.119–0.633; P = 0.002).

3.3. eNOS –786T > C polymorphism

The association of allele frequencies and carriage rates of eNOS –786T > C polymorphism is also shown in Table 2. The genotype frequencies of the eNOS –786T > C polymorphism TT, TC and CC genotypes in the cases were 35.6, 48.9 and 0.15.5, respectively. While the corresponding frequencies in the control subjects were 45.5, 52.7 and 0.1.8, respectively. As shown in Table 2, the homozygote mutant genotype (CC) of eNOS –786T > C polymorphism was associated with a significantly increased risk of LCPD (OR = 9.947, 95% CI 1.175–84.209, p = 0.036). We also found a significantly increased LCPD risk with eNOS –786T > C polymorphism under recessive genetic model (TT + TC vs. CC; OR = 0.101; 95% CI, 0.012–0.851; P = 0.035).

4. Discussion

Recently, several epidemiological studies revealed that there are

various polymorphisms on eNOS gene and these mutations might be a risk factor for several diseases including Coronary artery disease,¹⁷ orthopedic disease¹⁰ and malignancies.¹⁸ The eNOS can produce nitric oxide (NO) from L-arginine in the endothelial cell. NO, as an important endothelium-derived factor, is synthesized in the endothelial cells and plays a central role in regulating a wide spectrum of functions in the cardiovascular system, including vasorelaxation, limiting platelet aggregation, ischaemia-reperfusion injury, vascular smooth muscle cell migration and proliferation, as well as platelet aggregation.^{19,20} To better clarify the role of eNOS 27-bp VNTR, 894G > T and 786T > C polymorphism in LCPD risk, we conducted a case-control study.

The present study involved LCPD patients with age matched healthy controls from an Iranian genetically homogenous population. Our results suggest that the two eNOS 894G > T and –786T > C polymorphisms are associated with increased risk of LCPD. The few available studies supporting this evidence include a recent study conducted by Zhao et al.¹⁰ in Chinese population. Regarding to the functional role of NO in regulating angiogenesis in human, it is possible that eNOS 894G > T and –786T > C polymorphisms might be positively correlated with development LCPD by affecting NO synthesis.

Over the last few years several polymorphisms of the eNOS gene have been identified, and their association with orthopedic disease has been explored. Several studies revealed a statistically significant correlation of eNOS –786T > C polymorphism with neuralgia-inducing cavitation osteonecrosis,²¹ osteoporosis,²² idiopathic as well as secondary multifocal idiopathic osteonecrosis of the head of the femur.^{23,24} To the best of our knowledge this is the first study analyzing eNOS –786T > C polymorphism with LCPD. This study revealed a significantly higher percentage of homozygote mutant genotype (CC) of the eNOS –786T > C polymorphism in LCPD as compared with controls, indicating that T786C polymorphism is associated with susceptibility to LCPD patients in Iranian children. The –786T > C polymorphism in the promoter region of eNOS gene is associated with the reduction of eNOS promoter transcription rate and significantly

reduced promoter activity, leading to endothelial dysfunction and reduced NO production in the vascular endothelium. It seems an impairment of NO production by eNOS -786T > C polymorphism may play in the development of LCPD.

The eNOS 894G > T polymorphism, located at exon 7, leads to an amino acid substitution and subsequently leading an immature protein, in which a G > T substitution at exon 7 leads to Glu > Asp substitution at position 298.²⁵ The eNOS 894G > T polymorphism can lead to low expression levels and activity of eNOS. Therefore, the TT genotype of eNOS 894G > T polymorphism caused a diminished and slower aggregation of erythrocytes, as this genotype has been shown to blunt endothelium-mediated vasodilatation and implicated as a risk factor in numerous cardiovascular conditions hypertension, coronary spasm, myocardial infarction, and coronary artery disease. We have found that the heterozygous GA genotype of eNOS 894G > T was significantly associated increased risk of LCPD (OR = 3.347, 95% CI 1.440–7.778, P = 0.005), compared with the homozygous mutant TT genotype (OR = 2.512, 95% CI 0.220–28.634, P = 0.458). Similar results for the associations of the GA genotype of eNOS 894G > T and LCPD risk were reported in a Chinese population.¹⁰

The eNOS 27-bp VNTR polymorphism has been found to be associated with altered plasma NO levels. This polymorphism also has been associated with many vascular diseases including hypertension, diabetic retinopathy, and diabetic nephropathy in various populations.²⁶ Notably, our results were different from a previous study by Zhao et al.,¹⁰ which revealed that no significant association between eNOS 27-bp VNTR polymorphism and LCPD risk. Moreover, in the present study, frequency of 27-bp VNTR polymorphism genotypes was not significantly higher in control group than LCPD patients. Therefore, we suggest that the eNOS 27-bp VNTR polymorphism may not function as a protective factor LCPD risk.

This study has two main advantages: First, this is the second case-control study on eNOS polymorphisms association with LCPD susceptibility worldwide. Second, in this study the LCPD patients are from the different region of Iran, therefore the potential selection bias excluded. However, limited power is a common problem in studies of genetic polymorphisms, and this study was limited by the small sample size, which needs further investigations. In addition, gene-gene and gene-environment interactions might influence LCPD susceptibility. However, no appropriate information was available to test this.

5. Conclusions

This study results showed that the eNOS 894G > T and -786T > C polymorphisms may affect susceptibility to LCPD in the Iranian children, but not eNOS 27-bp VNTR polymorphism. Considering the sample size is small, well-designed large-scale studies are needed to further warranted our results.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

There was no support or funding for this study. We would like to acknowledge all the participants in the research.

References

- Perry DC, Bruce CE, Pope D, Dangerfield P, Platt MJ, Hall AJ. Legg-Calvé-Perthes disease in the UK: geographic and temporal trends in incidence reflecting differences in degree of deprivation in childhood. *Arthritis Rheum.* 2012;64(5):1673–1679.
- Hailer YD, Nilsson O. Legg-Calvé-Perthes disease and the risk of ADHD, depression, and mortality. *Acta Orthop.* 2014;85(5):501–505.
- Azarpira MR, Ghilian MM, Sobhan MR, et al. Association of MTHFR and TNF-α genes polymorphisms with susceptibility to Legg-Calvé-Perthes disease in Iranian children: a case-control study. *J Orthop.* September 2018;15(4):984–987.
- Perry DC, Machin DMG, Pope D, et al. Racial and geographic factors in the incidence of Legg-Calvé-Perthes' disease: a systematic review. *Am J Epidemiol.* 2012;175(3):159–166.
- Loder RT, Skopelja EN. The epidemiology and demographics of Legg-Calvé-Perthes' disease. *ISRN Orthop.* 2011;2011:1–14.
- Al-Omran AK, Sadat-Ali M. Legg-calve-perthes disease in two generations of male family members: a case report. *J Orthop Surg.* 2013;21(2):258–261.
- Leroux J, Abu Amara S, Lechevallier J. Legg-Calvé-Perthes disease. *Orthop Traumatol Surg Res.* 2018;104(1):S107–S112.
- Li N, Yu J, Cao X, et al. A novel p. Gly630Ser mutation of COL2A1 in a Chinese family with presentations of Legg-Calvé-Perthes disease or avascular necrosis of the femoral head. *Zhou Z. PLoS One.* 2014;9(6):e100505.
- Kannu P, Irving M, Aftimos S, Savarirayan R. Two novel COL2A1 mutations associated with a Legg-Calvé-Perthes disease-like presentation. *Clin Orthop Relat Res.* 2011;469(6):1785–1790.
- Zhao Y, Liao S, Lu R, Dang H, Zhao J, Ding X. Endothelial nitric oxide synthase gene polymorphism is associated with Legg-Calvé-Perthes disease. *Exp Ther Med.* 2016;11(5):1913–1917.
- Szrentić S, Spasovski V, Spasovski D, et al. Association of gene variants in TLR4 and IL-6 genes with Perthes disease. *Srp Arh Celok Lek.* 142(7-8):450–456.
- Szrentić S, Nikčević G, Spasovski D, et al. Predictive genetic markers of coagulation, inflammation and apoptosis in Perthes disease—Serbian experience. *Eur J Pediatr.* 2015;174(8):1085–1092.
- Sezgin N, Tekin A, Atac FB, Verdi H, Sezgin AT. Endothelial nitric oxide synthase gene polymorphisms in patients with slow coronary flow. *Interv Med Appl Sci.* 2017;9(3):117–122.
- Chang J, Pan F, Tang Q, et al. eNOS gene T786C, G894T and 4a4b polymorphisms and male infertility susceptibility: a meta-analysis. *Andrologia.* 2017;49(4):e12646.
- Xie X, Shi X, Xun X, Rao L. Endothelial nitric oxide synthase gene single nucleotide polymorphisms and the risk of hypertension: a meta-analysis involving 63,258 subjects. *Clin Exp Hypertens.* 2017;39(2):175–182.
- Yao H-X, Ma F-Z, Tan Y-Y, Liu L-Y. Endothelial nitric oxide synthase gene polymorphisms and risk of erectile dysfunction: an updated meta-analysis of genetic association studies. *Int J Surg.* April 2018.
- Rai H, Parveen F, Kumar S, Kapoor A, Sinha N. Association of endothelial nitric oxide synthase gene polymorphisms with coronary artery disease: an updated meta-analysis and systematic review. *Ai J. PLoS One.* 2014;9(11):e113363.
- Gao X, Wang J, Wang W, Wang M, Zhang J. eNOS genetic polymorphisms and cancer risk. *Medicine (Baltim).* 2015;94(26):e972.
- Favero G, Paganelli C, Buffoli B, Rodella LF, Rezzani R. Endothelium and its alterations in cardiovascular diseases: life style intervention. *BioMed Res Int.* 2014;2014:801896.
- Sukhovershin RA, Yepuri G, Ghebremariam YT. Endothelium-derived nitric oxide as an antiatherogenic mechanism: implications for therapy. *Methodist Debakey Cardiovasc J.* 2015;11(3):166–171.
- Glueck CJ, McMahon RE, Bouquot JE, Khan NA, Wang P. T-786C polymorphism of the endothelial nitric oxide synthase gene and neuralgia-inducing cavitation osteonecrosis of the jaws. *Oral Surgery, Oral Med Oral Pathol Oral Radiol Endodontology.* 2010;109(4):548–553.
- Gu Z, Zhang Y, Qiu G. Promoter polymorphism T-786C, 894G→T at exon 7 of endothelial nitric oxide synthase gene are associated with risk of osteoporosis in Sichuan region male residents. *Int J Clin Exp Pathol.* 2015;8(11):15270–15274.
- Glueck CJ, Freiberg RA, Boppana S, Wang P. Thrombophilia, hypofibrinolysis, the eNOS T-786C polymorphism, and multifocal osteonecrosis. *J Bone Jt Surgery-American.* 2008;90(10):2220–2229.
- Zhou ZC, Gu SZ, Wu J, Liang QW. VEGF, eNOS, and ABCB1 genetic polymorphisms may increase the risk of osteonecrosis of the femoral head. *Genet Mol Res.* 2015;14(4):13688–13698.
- Zhao C, Yan W, Zu X, et al. Association between endothelial nitric oxide synthase 894G > T polymorphism and prostate cancer risk: a meta-analysis of literature studies. *Tumor Biol.* 2014;35(12):11727–11733.
- Ma Z, Chen R, Ren H-Z, Guo X, Guo J, Chen L. Association between eNOS 4b/a polymorphism and the risk of diabetic retinopathy in type 2 diabetes mellitus: a meta-analysis. *J Diabetes Res.* 2014;2014:549747.