Int J Clin Exp Pathol 2017;10(5):5964-5970
www.ijcep.com /ISSN:1936-2625/IJCEP0048628

Original Article

VEGF and eNOS genetic polymorphisms and their interactions with steroid and the risk of osteonecrosis of the femoral head

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Received January 11, 2017; Accepted February 22, 2017; Epub May 1, 2017; Published May 15, 2017

Abstract: This study investigated the effects of vascular endothelial growth factor (VEGF) -634G>C and endothelial nitric oxide synthase (eNOS) 894G>T genetic polymorphisms, their gene-gene interaction and their interactions with steroid use on the risk of osteonecrosis of the femoral head (ONFH). An age-matched case-control study was conducted with 174 cases and 174 controls. VEGF and eNOS genotypes were determined by polymerase chain reaction (PCR) and sequencing. The effects were evaluated by the adjusted odds ratio (OR) and 95% confidence interval (CI) derived from logistic regression analyses. VEGF CC and eNOS GT genotypes were both found to be independently related to the increased risk for ONFH, with ORs of 4.53 (95% CI 1.20-17.10) and 2.53 (95% CI 1.23-5.22), respectively. Further, compared with individuals having both VEGF GG and eNOS GG, the adjusted OR (95% CI) for those with both VEGF CC and eNOS GT was 4.87 (2.33-10.18) in all subjects. A statistically significant gene-gene interaction existed between the two polymorphisms (P<0.001). Furthermore, statistically significant interactions between the two polymorphisms and steroid use were found (VEGF P<0.001 and eNOS P=0.001). Heavy steroid users with VEGF CC and with eNOS GT both had significantly increased risks for ONFH, with ORs of 4.00 (95% CI 2.04-7.84) and 3.46 (95% CI 1.84-6.50), respectively. In conclusion, our results suggest a synergism between VEGF -634G>C and eNOS 894G>T genetic polymorphisms and their gene-environment interactions with steroid use in the ONFH risk.

Keywords: VEGF, eNOS, single nucleotide polymorphisms, osteonecrosis of the femoral head, steroid, association

Introduction

Osteonecrosis of the femoral head (ONFH) is a common disease of hip joint that frequently causes disability. In the world, nearly 20 million populations suffer from ONFH, with an approximate 8.12 million non-traumatic ONFH patients in 2013 in China alone [1]. The etiology of ONFH has yet to be fully disclosed, although its clinical features have been defined. Genetic factors and their interactions with environments may play a critical role in the development of ONFH. Trauma, steroid use, alcohol abuse and vascular injury all seem to play roles among the environment-related risk factors. With regards to genetic factors, several gene polymorphisms might potentially influence the individual susceptibility to ONFH [2-8]. In non-traumatic ONFH, steroid use has been considered as a leading cause [9, 10]. Some previous studies demonstrate that only some individuals develop ONFH within several months of steroid therapy, suggesting that genetic factors may alter susceptibility or resistance to the steroid-related ONFH.

Among the hypotheses about the ONFH pathology, ischemic injury, which results from decreased blood vessel number and reduced blood supply, appears to be the most convincing [10, 11]. Vascular endothelial growth factor (VEGF), as a known angiogenic factor, has a crucial role in angiogenesis and osteogenesis. It has been reported that VEGF is highly expressed in the edematous place around necrotic area of ONFH [12]. Thus VEGF and ONFH may be closely associated. Nitric oxide (NO) is a very important biomolecule in keeping vascular homeostasis

and regulating blood flow. NO is involved in many physiologic processes, including thrombosis, angiogenesis and bone turnover [13]. NO is produced by the catalysis of NO synthases (NOS). In adult bone, endothelial NOS (eNOS) is the predominant isoform of NOS. Thus, eNOS may have a marked effect on ONFH.

In view of the role of VEGF and eNOS above mentioned, both VEGF and eNOS genes are considered as plausible biological candidates for ONFH. -634G>C (rs2010963) in the 5'-untranslated region of VEGF gene and 894G>T (rs1799983) in exon 7 of eNOS gene are two important functional genetic polymorphisms, and they have significant effects on VEGF and eNOS levels, respectively [14, 15]. Previous studies have reported associations between the two polymorphisms and ONFH [2-7]. However, their effects on the ONFH risk according to the levels of steroid use are poorly understood. Also, no study has reported the interaction between the two genetic polymorphisms in the ONFH risk. Moreover, the role of individual gene need be further investigated and confirmed.

This study aimed to investigate the effects of VEGF -634G>C and eNOS 894G>T polymorphisms, their gene-gene interaction and their interactions with steroid use on the ONFH risk.

Materials and methods

Study population

The study subjects consisted of 174 cases and 174 controls. Cases were ONFH patients. ONFH was diagnosed by checking osteonecrosis in anteroposterior and frog view X-rays of both hips and (or) magnetic resonance imaging [16]. Exclusion criteria of cases were as follows: (i) those with traumatic ONFH, hip joint dislocation, and other hip-related diseases. (ii) those that had marked familial hereditary diseases. Controls were selected from non-ONFH (no disease history of ONFH) individuals matched to cases for age (±3 years). Individuals with hip-related diseases and those who had marked familial hereditary diseases were excluded from controls. In the study, all participants were unrelated Chinese Han male and from Central China. Mean age was 45.9±9.0 years in the cases and 45.9±8.9 years in the controls. All protocols involving human were approved by the Ethical Committee of Pu Ai Hospital of Tongji Medical College of Huazhong University of Science and Technology and written informed consent was obtained from each subject. The study follows international and national regulations in accordance with the Declaration of Helsinki.

Data collection

Cases and controls were interviewed using a standard questionnaire to obtain information on demographic variables, residence place, clinical history of ONFH and other diseases, family history of diseases, alcohol drinking and steroid use (including steroid type, use duration and dose). Family history of diseases referred to offspring, siblings and parents. Based on the definition of systematic steroid use [9], steroid use were classified accordingly: non-user (never user), moderate user and heavy user. Heavy users were defined as individuals who had a history of intake of ≥2 g of steroid or its equivalent within a period of 3 months [9]. Moderate users were defined as individuals other than heavy users. Alcohol drinking was also divided into three categories: non-drinker, moderate drinker and heavy drinker. Heavy drinkers were defined as individuals drinking alcoholic beverages 5 days or more (50 g ethanol or more on each occasion) per week. Moderate drinkers were defined as individuals other than heavy drinkers.

DNA extraction and genotyping

Laboratory technicians were blinded to control and case status. Genomic DNA was isolated from peripheral blood samples using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA quality and concentration were assessed by a Nanodrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA).

Genotyping of VEGF and eNOS polymorphisms were detected by PCR and sequencing. Primer sequences for VEGF -634C>G: 5'-GGA-TTT-TGG-AAA-CCA-GCA-GA-3' (forward), 5'-CTG-TCT-GTC-TGT-CCG-TCA-GC-3' (reverse); Primer sequences for eNOS +894G>T: 5'-AAG-GCA-GGA-GAC-AGT-GGA-TG-3' (forward), 5'-GTT-GGG-GTG-TGG-GAT-CAG-3' (reverse). PCR was performed in a volume of 25 μl, with 50 ng of genomic DNA, 6 pmol of each primer, 1×Taq

Table 1. Steroid use and alcohol drinking status among cases (n=174) and controls (n=174)

	Cases n (%)	Controls n (%)	OR (95% CI)
Alcohol drinking			
Never drinker	43 (24.7)	74 (42.5)	1.00 (Reference)[a]
Moderate drinker	57 (32.8)	45 (25.9)	2.22 (1.29-3.83)
Heavy drinker	74 (42.5)	55 (31.6)	2.38 (1.41-4.00)
Steroid use			
Never user	40 (23.0)	60 (34.5)	1.00 (Reference)[b]
Moderate user	54 (31.0)	69 (39.7)	1.53 (0.65-3.60)
Heavy user	80 (46.0)	45 (25.8)	4.67 (1.05-20.72)

Note: OR = Odds ratio; CI = Confidence interval. ORs were assessed using logistic regression analyses. [a]ORs adjusted for age. [b]ORs adjusted for age and alcohol drinking.

Table 2. Genotype distributions of VEGF and eNOS polymorphisms and their effects on risk of osteonecrosis of the femoral head

	Cases n (%)	Controls n (%)	Model 1[a] OR (95% CI)	Model 2[b] OR (95% CI)
VEGF				
GG	42 (24.1)	70 (40.2)	1.00 (Reference)	1.00 (Reference)
GC	84 (48.3)	84 (48.3)	1.71 (1.04-2.80)	1.87 (0.74-4.74)
CC	48 (27.6)	20 (11.5)	4.13 (2.15-7.96)	4.53 (1.20-17.10)
eNOS				
GG	111 (63.8)	146 (83.9)	1.00 (Reference)	1.00 (Reference)
GT	53 (30.5)	23 (13.2)	3.08 (1.77-5.36)	2.53 (1.23-5.22)
TT	10 (5.7)	5 (2.9)	2.61 (0.87-7.86)	2.13 (0.64-7.08)

Note: OR = Odds ratio; CI = Confidence interval. ORs were assessed using logistic regression analyses. [a]Model 1 adjusted for age. [b]Model 2 adjusted for age, steroid use and alcohol drinking.

polymerase buffer (1.5 mM $MgCl_2$) and 0.3 U of Amplitaq polymerase (Perkin Elmer, Foster City, CA, USA). PCR was run on a thermal-cycler gradient PCR (Eppendorf AG, Hamburg, Germany) for 5 min at 95°C; followed by 38 cycles for 1 min at 95°C for DNA denaturation, 1 min at 62°C (for VEGF) and 64°C (for eNOS) for annealing, and 1 min at 72°C for extension; followed by a final extension for 10 min at 72°C. PCR products of 224 bp for VEGF and 319 bp for eNOS were analyzed on a 1.5% agarose gel stained by ethidium bromide. Sequencing was performed using an ABI 3770 analyser (Applied BioSystems, Foster City, CA, USA) with a Taq-Dye deoxy-terminator cycle sequencing kit (Applied BioSystems, Foster City, CA, USA). The sequence data were analyzed by Chromas V.2 software (Technelysium, Tewantin, Queensland, Australia).

Statistical analysis

SPSS 19.0 statistical package (SPSS, Chicago, IL, USA) was used to perform the statistical analyses. A *P*-value <0.05 was considered statistically significant. For controls, the accordance of genotype frequencies with the Hardy-Weinberg equilibrium was examined with the χ^2-test. The effects of environment factors, individual gene, and gene-gene and gene-environment interactions on the ONFH risk were evaluated by the adjusted odds ratios (ORs) and 95% confidence interval (CI) derived from logistic regression analyses. Possible risk factors for ONFH were considered as confounders. Interaction terms were introduced when conducting gene-gene and gene-environment interaction analyses. Stratified logistic regression analyses were performed, when assessing the interactions between genes and steroid use. In individual gene and gene-gene interaction analyses, GG genotype (VEGF GG or eNOS GG) and the combination of the two GG were used as reference groups, respectively. For gene-environment interaction analyses, the reference group was GG carriers not exposed to steroid use.

Results

Table 1 shows steroid use and alcohol drinking status among controls and cases. Significant differences were seen between the two groups for both steroid use and alcohol drinking. The ORs for ONFH in heavy drinkers and moderate drinkers were both increased compared with never drinkers (age-adjusted OR, 2.38; 95% CI, 1.41-4.00 and 2.22; 1.29-3.83, respectively). The OR for ONFH in heavy steroid users was increased compared with never users (age and alcohol drinking-adjusted OR, 4.67; 95% CI, 1.05-20.72).

Table 2 shows the genotype distributions of VEGF and eNOS, and their ORs and 95% CI for ONFH. Genotype frequencies for the two polymorphisms were in accordance with the Hardy-

Table 3. Effects of the combinations of VEGF and eNOS genotypes on risk of osteonecrosis of the femoral head (case/control)

		VEGF		
		GG	GC	CC
eNOS	GG	42/70	69/74	0/2
		1.00 (reference)[a]	1.55 (0.94-2.57)	NE
	GT	0/0	15/10	38/13
		NE	2.50 (1.03-6.07)	4.87 (2.33-10.18)
	TT	0/0	0/0	10/5
		NE	NE	3.33 (1.07-10.42)
P-interaction			<0.001	

Note: [a]Odds ratios (95% confidence intervals) assessed using logistic regression analyses adjusted for age, steroid use and alcohol drinking. *P* value derived from logistic regression analysis. NE, not estimated because of no case in this category.

Weinberg equilibrium in controls (VEGF, P= 0.86; eNOS, P=0.41). The genotype frequencies of GG, GC and CC of VEGF were 24.1, 48.3 and 27.6% among cases and 40.2, 48.3 and 11.5% among controls, respectively. A significantly increased risk of ONFH was observed with CC relative to GG (age, steroid use and alcohol drinking-adjusted OR, 4.53; 95% CI, 1.20-17.10). The genotype frequencies of GG, GT and TT of eNOS were 63.8, 30.5 and 5.7% among cases and 83.9, 13.2 and 2.9% among controls, respectively. A significantly increased risk of ONFH was observed with GT relative to GG (confounder-adjusted OR, 2.53; 95% CI, 1.23-5.22).

On analysis of the combination of VEGF and eNOS polymorphisms in all subjects, a statistically significant gene-gene interaction was found between the two polymorphisms (P< 0.001). Increased risks were observed in subjects having both VEGF GC and eNOS GT, both VEGF CC and eNOS GT and both VEGF CC and eNOS TT, and the adjusted ORs (95% CI) compared with those having both VEGF G/G and eNOS G/G were 2.50 (1.03-6.07), 4.87 (2.33-10.18) and 3.33 (1.07-10.42), respectively (**Table 3**). Increased risk was particularly significant in the subjects with the combination of VEGF CC and eNOS GT genotypes.

The impacts of VEGF and eNOS polymorphisms in combination with steroid use are presented in **Table 4**. The gene-environment interactions between VEGF and eNOS polymorphisms and steroid use were both statistically significant (P<0.001 for VEGF and P=0.001 for eNOS, respectively). For VEGF gene, on the whole, the risk of ONFH appeared consistently increased with the increases of steroid use level as well as C allele number, and was particularly significant in the heavy steroid users with CC genotype. For eNOS gene, similar results (the risk increased with the increases of steroid level and T allele number) were observed. The impacts of VEGF and eNOS polymorphisms in heavy steroid users were both higher than that in moderate and never users. The ORs of heavy steroid users with VEGF GC genotype and with VEGF CC genotype were 1.78 (95% CI 0.93-3.41) and 4.00 (95% CI 2.04-7.84), respectively, when compared with never users with VEGF GG genotype. The ORs of heavy steroid users with eNOS GT genotype and with eNOS TT genotype were 3.46 (95% CI 1.84-6.50) and 3.00 (95% CI 0.95-9.43), respectively, when compared with never users with eNOS GG genotype.

Discussion

The present study evaluated for the first time the effects on ONFH of VEGF -634G>C and eNOS 894G>T genetic polymorphisms simultaneously. VEGF CC and eNOS GT genotypes were both significantly associated with the ONFH risk. The risk was more pronounced when the two genotypes were combined. The risk was possibly increased with the increases of VEGF C and eNOS T allele numbers. This study also investigated for the first time that the interaction between VEGF and eNOS genes and steroid use in the ONFH risk in a Chinese Han population. Heavy steroid users with the unfavorable genotypes had significant increased risks, suggesting gene-environment interactions between the two genes and steroid use.

Concerning the interpretation of our results, Limitations should be taken into account. Firstly, the sample size of this study limits the ability to explore some individual or combined effects, such as eNOS TT genotype, the interaction between eNOS TT and VEGF CC genotypes and the interaction between eNOS TT genotype and steroid use, which highlights the

Table 4. Effects of VEGF and eNOS genotypes in combination with steroid use on risk of osteonecrosis of the femoral head (case/control)

Steroid use	VEGF			eNOS		
	GG	GC	CC	GG	GT	TT
Never user	40/60	0/0	0/0	40/60	0/0	0/0
	1.00 (reference)[a]	NE	NE	1.00 (reference)[a]	NE	NE
Moderate user	2/10	52/57	0/2	54/69	0/0	0/0
	0.30 (0.062-1.44)	1.37 (0.79-2.37)	NE	1.17 (0.69-2.01)	NE	NE
Heavy user	0/0	32/27	48/18	17/17	53/23	10/5
	NE	1.78 (0.93-3.41)	4.00 (2.04-7.84)	1.50 (0.69-3.28)	3.46 (1.84-6.50)	3.00 (0.95-9.43)
P-interaction		<0.001			0.001	

Note: [a]Odds ratios (95% confidence intervals) stratified by steroid use levels. Odds ratios and *P* value were assessed using logistic regression analyses adjusted for age and alcohol drinking. NE, not estimated because of no case in this category.

need to enlarge the sample size to confirm our results. In this study, we tried to keep the study population as homogeneous as possible, and all subjects are Chinese Han adult male and from Central China, which increases the statistical power of our study population. One of the main confounding factors in genetic association studies comes from population stratification, since the stratification factors, such as the ethnicity, themselves may be related to the allele frequencies and to a specific disease as well. Secondly, information bias may exist in all case-control studies, which results in biased ORs related to the results of gene-environment interactions.

The present study showed an association of individual VEGF -634G>C polymorphism with ONFH, which is in keeping with the result of a recent meta-analysis [12] that suggests that VEGF CC genotype may be a risk factor for ONFH. The meta-analysis also shows the association of VEGF CC polymorphism with ONFH is more evident in males compared with females. Thus, our study on males is possibly more meaningful than on females. Our study also showed an association between individual eNOS 894G>T polymorphism and ONFH. The result is not consistent to a previous study on a Korean population [2]. However, it is consistent to the other two studies on Chinese populations that indicate that eNOS GT and eNOS TT genotype frequencies are significantly higher in ONFH patients compared to controls [6, 7].

Our results about genotype combinations suggest a significant biological interaction between VEGF and eNOS genes in the ONFH risk. VEGF promotes angiogenesis through lots of pathway [17]. While a lot of effects of VEGF may be achieved through eNOS/NO [18]. VEGF, through special biochemical pathway, acts on Ser1177 or Ser1179 in eNOS to active eNOS [19, 20], and therefore produces NO to dilate vessels and spur angiogenesis. Angiotrophic effect of VEGF would weaken, and even disappear when eNOS is lacking in a study on eNOS knocked-out mouse [21]. Thus, the fluency of VEGF and eNOS gene combination may be more obvious than individual gene as the cooperative effect of eNOS and VEGF mentioned above.

Our results showed that both VEGF -634G>C and eNOS 894G>T polymorphisms modified the relationship between steroid use and the ONFH risk. Individuals with VEGF or eNOS unfavorable genotype might be particularly susceptible to ONFH when exposed to steroid environment. Steroid has been considered as the most common factor leading to non-traumatic ONFH. Previous studies have reported that steroid could directly damage endothelial cells, causing capillary rarefaction and thrombi formation of femoral head, which severely decreases blood supply in the trabecular bone [10, 22]. Therefore, functional VEGF -634G>C and eNOS 894G>T genetic polymorphisms, through influencing VEGF and eNOS levels to adjust angiogenesis and blood supply, have effects on the association of steroid use with the ONFH risk. In addition, steroid could decrease the expression of VEGF as well as eNOS [23, 24], which may also be related to the variation of effects of different genotypes on the ONFH risk when exposed to steroid environment.

In conclusion, VEGF CC and eNOS GT genotypes may be associated with an increased ONFH risk, particularly when the two genotypes were present simultaneously. Heavy steroid users

with the unfavorable genotypes may have a significant increased risk for ONFH. Our results suggest a synergism between VEGF -634G>C and eNOS 894G>T genetic polymorphisms and their interactions with steroid use in the ONFH risk. Clearly, as our study is based on a limited sample size, it is necessary that larger prospective studies confirm our results.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (no. 81401834), the Natural Science Foundation of Hubei Province (no. 2014CFB363) and the Health and Family Planning Commission Program of Wuhan City (no. WX14B18).

Disclosure of conflict of interest

None.

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