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Original Article

The association between intron 4 polymorphism of endothelial nitric oxide synthase gene (eNOS) and osteonecrosis of femoral head: a meta-analysis

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Abstract: The polymorphism of the endothelial nitric oxide synthase gene (eNOS) has been correlated with susceptibility to osteonecrosis of the femoral head (ONFH). The aim of the present study was to derive a more precise and evidence-based estimation of the relationship between the intron 4 polymorphism of eNOS and ONFH by performing a meta-analysis. We searched related articles in Pubmed, and Web of Science published up to October 2015 that met our predefined criteria. The association between eNOS intron 4 polymorphism and ONFH risk was assessed by odds ratio (OR) with the corresponding 95% CI. Three eligible articles from 6 case-control studies with 296 cases and 329 controls were identified. Overall, pooled analysis indicated a significant association between eNOS intron 4 polymorphism and ONFH risk (for a vs. b: OR=2.931, 95% CI 2.093-4.103, P<0.001; for aa vs. bb: OR=7.515, 95% CI 1.679-33.635, *P*=0.008; for ab vs. bb: OR=2.709, 95% CI 1.925-3.814, *P*<0.001; for aa+ab vs. bb: OR=2.792, 95% CI 2.010-3.880, *P*<0.001; for aa vs. ab+bb: OR=5.926, 95% CI 1.336-26.293, *P*=0.019). No evidence of publication bias was observed. In conclusion, this meta-analysis suggested that eNOS intron 4 polymorphism was a risk factor for ONFH. This finding needs further confirmation by trans-regional multicenter study with large sample in different ethnic populations.

Keywords: Endothelial nitric oxide synthase gene, polymorphism, osteonecrosis, risk factor, meta-analysis

Introduction

Osteonecrosis of the femoral head (ONFH) is a devastating bone disease in which cellular death within the femoral head occurs as a result of interruption of the blood supply to the anterior-superior-lateral region [1]. As the disease frequently occurs in young adult patients, between the ages of 20 and 50, it results in a substantial socioeconomic burden [2]. However, the etiology and pathogenesis of ONFH are not revealed and genetic risk factors have been not extensively investigated as yet.

Nitric oxide (NO) is a biomolecule involved in a variety of physiologic processes, including angiogenesis, thrombosis, and bone turnover, which have been proven to be related to the pathogenesis of osteonecrosis [3-5]. NO is produced by NO synthases (NOS), which catalyze the conversion of L-arginine to L-citrulline and NO. There are three isoforms of NOS: neuronal (nNOS), induced (iNOS) and endothelial (eNOS) [6]. It has been reported that eNOS is the predominant isoform of NOS expressed in normal adult bone [7, 8]. Most of the attention has focused on a single nucleotide polymorphism in the promoter region (T-786-C), a single nucleotide polymorphism in exon 7 (Glu296Asp) and a variable number of tandem repeats in intron 4. In the 27-bp repeat in intron 4, two alleles have been identified, the larger of which, eNOS4b, has five tandem 27-bp repeats [GAAGTCTAGACCTGCTGC(A/G)GGGGTGAG] and the smaller, eNOS4a, has four repeats. It was revealed that NO levels were significantly lower in the subjects with the 4a allele than in those without the presence of the 4a allele [9]. Recent studies have reported an association between ONFH and genetic polymorphisms in eNOS intron 4 polymorphism. Koo et al. [10] reported a statistically significant association between

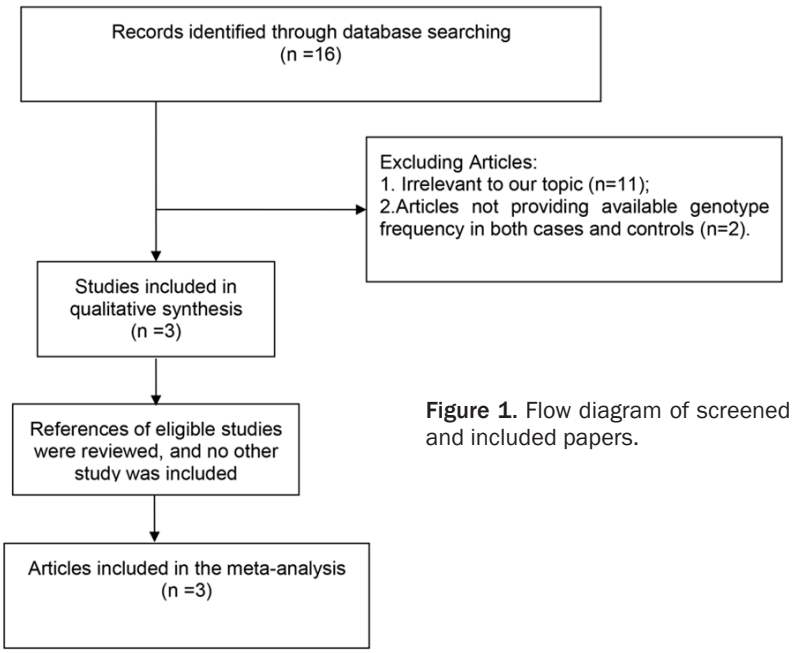

Figure 1. Flow diagram of screened and included papers.

the eNOS intron 4 polymorphism and the incidence of idiopathic ONFH patients, but no relationship was found in secondary ONFH patients. Gagala et al. [5] demonstrated a relationship between the eNOS intron 4 polymorphism and the incidence of both idiopathic and secondary ONFH patients. Zheng et al. suggested that the eNOS intron 4 polymorphism played a role in the pathogenesis of ONFH in 125 Chinese patients [11]. Meta-analysis is a well established statistical tool that serves for integration of data from independent studies in order to formulate more general conclusions. The aim of this study was to assess the association of the eNOS intron 4 polymorphism with the risk of ONFH by conducting a meta-analysis of individual datasets from all eligible studies.

Methods

Search strategy

We searched related articles written in English in PubMed, and Web of Science published up to October 2015. Literature searches were performed using the keywords: ("endothelial nitric oxide synthase gene" OR eNOS) AND polymorphism AND osteonecrosis. Reference lists of all included studies were screened to identify potentially eligible studies. Emails were sent to the authors of identified studies for additional information if necessary.

Selection criteria

Titles and abstracts were screened for subject relevance. Studies that could not be definitely excluded based on abstract information were also selected for full text screening. Two authors selected eligible studies for inclusion possibility independently, where there was a disagreement for study inclusion, a discussion was held by third author to reach a consensus. Eligible studies had to meet the following criteria: (1) human study; (2) case-control study; (3) studies evaluating the association between eNOS intron 4 polymorphism and ONFH risk; (4) studies providing available genotype frequency in both cases and controls; (5) sufficient data available to estimate an odds ratio (OR) with its 95% CI; (6) there was no deviation from Hardy-Weinberg equilibrium (HWE) among the controls.

Data extraction and quality assessment

Information was extracted from all included studies independently by two authors according to the inclusion criteria. The following information was extracted from each included study: first author's family name, year of publication, country, total numbers of cases and controls, genotyping method and genotyping information.

The methodological quality of the included studies was independently and appraised twice by two authors. The qualities of all included studies were assessed using the Newcastle-Ottawa Scale [12]. Each study is judged on eight items, categorized into three groups: the selection of the study groups; the comparability of the groups; and the ascertainment of either the exposure or outcome of interest for case-control or cohort studies respectively. Stars awarded for each quality item serve as a quick visual assessment. Stars are awarded such that the highest quality studies are awarded up to nine stars. Studies were graded as good

Table 1. Characteristics of subjects in eligible studies

Studies	Country	Detection method	ONFH						Healthy control					HWE	Quality score
			No.	aa	ab	bb	a (frequency)	Type of ONFH	No.	aa	ab	bb	a (frequency)		
Koo 2006-1	Korea	PCR-RFLP	50	0	9	41	0.090	Idiopathic	103	0	5	98	0.024	Yes	7
Koo 2006-2	Korea	PCR-RFLP	53	0	5	48	0.047	Secondary	103	0	5	98	0.024	Yes	7
Gagala 2013-1	Poland	Taqman assay	45	1	16	28	0.200	Idiopathic	100	1	16	83	0.090	Yes	6
Gagala 2013-2	Poland	Taqman assay	23	2	9	12	0.282	Secondary	100	1	16	83	0.090	Yes	6
Zheng 2014-1	China	PCR-RFLP	65	2	11	52	0.115	Idiopathic	126	0	6	120	0.024	Yes	7
Zheng 2014-2	China	PCR-RFLP	60	0	8	52	0.067	Secondary	126	0	6	120	0.024	Yes	7

PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism.

Table 2. Meta-analysis of the association between eNOS intron 4 polymorphism and ONFH

Genetic models	Test of association			Heterogeneity		Effects model
	OR	95% CI	*P*	I^2	*P*	
a vs. b	2.931	2.093-4.103	<0.001	0.0%	0.758	Fix-effect model
aa vs. bb	7.515	1.679-33.635	0.008	0.0%	0.711	Fix-effect model
ab vs. bb	2.709	1.925-3.814	<0.001	0.0%	0.922	Fix-effect model
aa+ab vs. bb	2.792	2.010-3.880	<0.001	0.0%	0.848	Fix-effect model
aa vs. ab+bb	5.926	1.336-26.293	0.019	0.0%	0.708	Fix-effect model

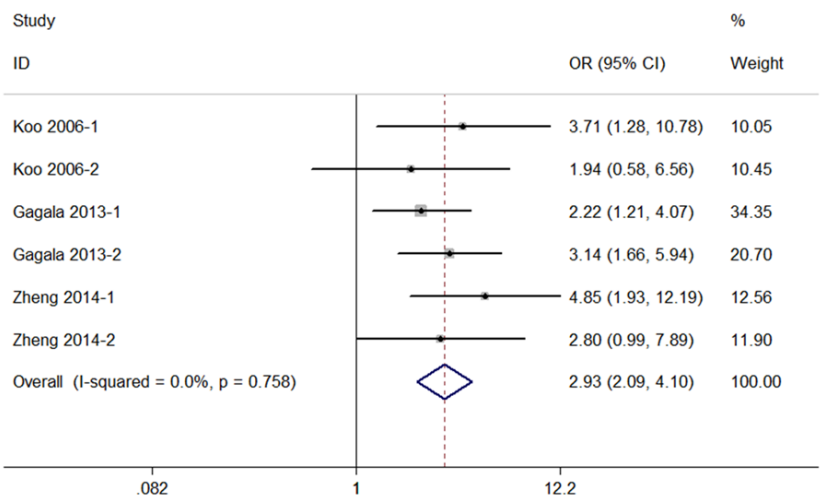

Figure 2. Forest plot of ORs in the additive model (a vs. b) for the association between eNOS intron 4 polymorphism and ONFH risk. The combined OR and 95% confidence intervals (CIs) were calculated using the fix-effects model.

quality if they awarded 6 to 9 stars; fair if they awarded 3 to 5 stars; and poor if they awarded less than 3 stars.

Statistical analysis

The strength of the association between eNOS intron 4 polymorphism and ONFH risk was assessed by an odds ratio (OR) with the corresponding 95% CI. The statistical significance of OR was analyzed by a Z test, and *P* less than 0.05 was considered as statistically significant. Heterogeneity was tested through the Chi-square and I-square tests. The meta-analysis was considered as homogeneous if the I^2 value was greater than 50% and the *p* value was less than 0.05. The OR were calculated using either fixed-effects models or, in the presence of heterogeneity, random-effects models. We estimated with the dominant model (aa+ab vs. bb) and recessive model (aa vs. ab+bb) and then evaluated a codominant model (aa vs. bb and ab vs. bb). We also estimated the risks of the additive model (a vs. b). Publication bias was examined visually in a funnel plot of log [OR] against its standard error (SE), and the degree of asymmetry was tested by Begg's test (P< 0.05 was considered a significant publication bias). The stability of the study was detected by sensitivity analysis, through re-meta-analysis with one involved study excluded each time. All statistical analyses were performed with Stata version 11.0 (StataCorp, College Station, TX, USA).

Results

Literature search

The literature search yielded a total of 16 primary studies, of which 13 were excluded for one of the following reasons: (1) irrelevant to our topic (n=11), (2) studies not providing available genotype frequency of eNOS intron 4 polymorphism in both cases and controls (n=2). Overall, 3 articles from 6 case-control studies with 296 cases and 329 controls were included in the present meta-analysis [5, 10, 11]. A flow diagram of the study selection process is presented in **Figure 1**.

Study characteristics and quality assessment

By geographic location, 3 articles with 6 case-control studies were conducted in 3 different countries (Poland, China, and Korea). The earliest study was published in 2006, and the latest in 2014. The number of subjects with ONFH in each study ranged from 23 to 65. 4 included case-control studies used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to detect genotypes, while the other 2 case-control studies used Taqman

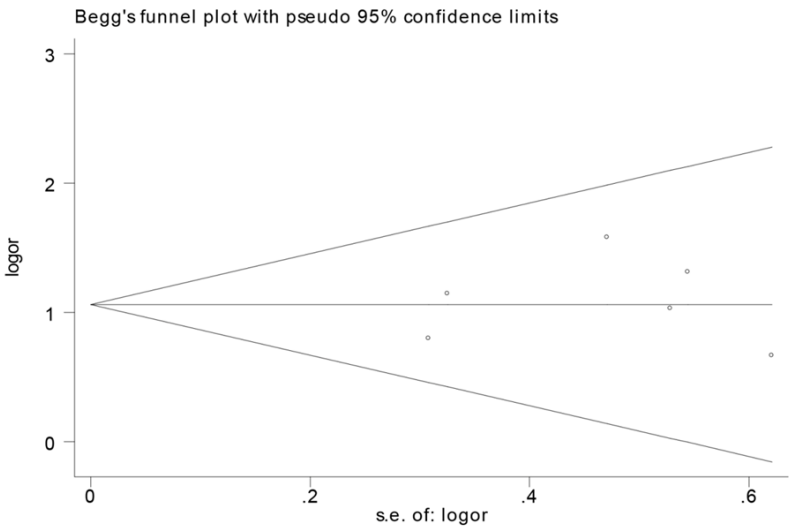

Figure 3. Begg's funnel plot of publication bias in selection of studies on the association between eNOS intron 4 polymorphism and ONFH risk in the additive model (a vs. b).

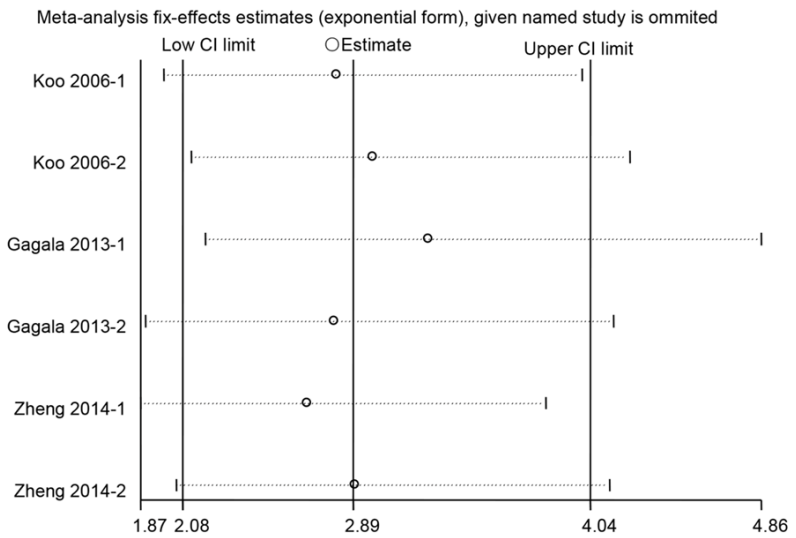

Figure 4. The stability of the study was detected through sensitivity analysis.

assay. The overall study quality averaged 6.7 stars on a scale of 0 to 9. The characteristics of the included studies and the results of the quality assessment were listed in **Table 1**.

Association between eNOS intron 4 polymorphism and ONFH

The results of the meta-analysis and heterogeneity test are listed in **Table 2**. We analyzed the heterogeneity of five genetic models for all 6 case-control studies, and found no significant amount of heterogeneity (I^2=0%, P>0.05). Thus, the fix-effect model was used for synthesis of the data in the five genetic models. When all the studies were pooled into meta-analysis, a significant association appeared between eNOS intron 4 polymorphism and ONFH risk (**Figure 2**) (for a vs. b: OR =2.931, 95% CI 2.093-4.103, P<0.001; for aa vs. bb: OR=7.515, 95% CI 1.679-33.635, *P*=0.008; for ab vs. bb: OR=2.709, 95% CI 1.925-3.814, P< 0.001; for aa+ab vs. bb: OR=2.792, 95% CI 2.010-3.880, P<0.001; for aa vs. ab+bb: OR=5.926, 95% CI 1.336-26.293, *P*=0.019) (**Table 2**).

Publication bias and sensitivity analysis

Publication bias was determined by Begg's test and visualization of funnel plot, and there was no evidence of publication bias (*P*= 0.851) (**Figure 3**). Sensitivity analysis showed that excluding any one involved study from the pooled analysis did not vary the results substantially (**Figure 4**).

Discussion

Although ONFH has been widely recognized as a pathological state with multiple etiologies, the exact pathogenesis of osteonecrosis remains to be elucidated [13]. The vascular hypothesis appears to be the most persuasive among several confounding pathogenic mechanisms for ONFH, hypothesizing that a decrease in the local blood flow in the femoral head, owing to vascular obstruction by any means, plays an important role in the pathogenesis of ONFH [14, 15]. There are some studies in human and animal models of ONFH, indicating that vascular abnormalities have resulted in thrombosis associated with abnormal thrombophilic coagu-

lopathy and hypofibrinolysis and embolism, which contribute to the development of ONFH [16-18]. As a biomolecule involved in angiogenesis, NO is an intracellular messenger which plays an important role in homoeostasis, vascular system and bone turnover [19-21]. NO inhibits platelet activation, adhesion and aggregation [22]. It has been revealed that NO is a vasodilatation molecule, and the deficiency of NO causes vasospasm [23]. Studies have found that impairment in NO production diminishes angiogenesis [24]. NO also controls microvascular permeability [25]. It has been reported that NO could even regulate bone mass and bone turnover through effects on osteoclast and osteoblast activity and inhibit osteoclasis [25-28].

NO is synthesized from L-arginine by three isoforms of synthases (NOS): neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). It has been demonstrated that the presence of 27-bp repeat polymorphism in intron 4 could result in a change in eNOS expression and enzymatic activity [9]. For example, plasma NO levels in subjects with the 4a allele of eNOS were significantly lower than those without the 4a allele. A hallmark of endothelial function is the synthesis and release of NO, which provides local regulation of vasomotor tone and anti-thrombotic actions [29, 30]. eNOS is constitutively expressed in vascular endothelium [31]. The polymorphism in intron 4 polymorphisms of eNOS has been demonstrated to reduce NO levels in human plasma [9]. Thus, polymorphisms of eNOS gene and resultant reduction of NO synthesis should lead to vascular abnormalities [11, 32, 33]. Indeed, many studies have observed associations between genetic polymorphisms in the eNOS gene and vascular diseases, for example ONFH, which is a phenomenon involving the disruption of vascular supply to the femoral head [11, 34].

In the present study, we conducted a meta-analysis to assess the association between intron 4 polymorphisms of eNOS with ONFH. The results of the meta-analysis indicated a significant association between the intron 4 polymorphisms of eNOS with the risk of ONFH. Although many etiological factors contributed to pathogenesis of ONFH and the pathologic changes of bone marrow in ONFH were almost similar, it is generally accepted that the interruption of the circulation of blood is the final common pathway for the development of ONFH [34, 35]. Tsukada et al. [9] found that the polymorphism in intron 4 of eNOS could reduce the plasma NO level, which is involved in angiogenesis. Therefore, we speculated that the decreased NO expression caused by polymorphism in intron 4 of eNOS could affect angiogenesis, repair processes, the progression, and outcome of ONFH.

To the best of our knowledge, this is the first meta-analysis to estimate the association between intron 4 polymorphism of eNOS with ONFH. We made sure to minimize the bias by means of study procedure. Not only did we search Pubmed, and Web of Science to identify potential studies, but also we manually examined all reference lists from relevant studies. Publication bias was also absent, as determined by visualization of funnel plot and Begg's test. However, the possible limitation of our study must be considered. Only 3 eligible articles from 6 case-control studies with 296 cases and 329 controls were included in the meta-analysis. Considering the possibly small effect size of this genetic polymorphism to ONFH and the relatively small sample size in each study, the discrepancy will become apparent because a single study may have been underpowered to detect a small but real association. This finding needs further confirmation by trans-regional multicenter study with large sample.

Conclusions

This meta-analysis supports a significant association between eNOS intron 4 polymorphism and ONFH risk. This finding needs further confirmation by trans-regional multicenter study with large sample in different ethnic populations.

Disclosure of conflict of interest

None.

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