Association between NOS3 polymorphisms and osteonecrosis of the femoral head

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\textbf{ABSTRACT}

\textbf{Purpose:} This study aimed to detect the association between nitric oxide synthase 3 (NOS3) gene polymorphisms (rs1799983 and rs3918181) and the susceptibility to osteonecrosis of the femoral head (ONFH).

\textbf{Methods:} Total 88 ONFH patients (55 non-traumatic ONFH and 33 traumatic ONFH) and 90 healthy controls were recruited in this case–control study. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was adopted for genotyping NOS3 rs1799983 and rs3918181 polymorphisms. \chi^2 \text{ test was used to calculate differences in genotype and allele frequencies of NOS3 gene polymorphisms between the cases and controls. Relative risk of ONFH was represented using odds ratios (ORs) with corresponding 95\% confidence intervals (CIs).}

\textbf{Results:} The T allele of the polymorphism rs1799983 showed significantly different frequencies between ONFH patients and control groups (\( p = .046 \)) and carrying this allele significantly decreased the disease risk (OR = 0.521, 95\%CI = 0.272–0.997), especially for non-traumatic ONFH (OR = 0.408, 95\%CI = 0.179–0.929, \( p = .029 \)). But genotype frequencies of the polymorphism rs1799983 had no obvious difference between the compared two groups (\( p > .05 \) for all). There was no remarkable association between NOS3 rs3918181 polymorphism and NOFH risk (\( p > .05 \) for all).

\textbf{Conclusions:} NOS3 rs1799983 polymorphism is obviously associated with ONFH and its T allele may be a protective factor against ONFH occurrence in Chinese Han population.

\textbf{Introduction}

Osteonecrosis of the femoral head (ONFH), a common joint disease, is caused by the collapse of subchondral bones due to reduced blood supply to femoral head [1]. ONFH often occurs in individuals aged between 20 and 50 years old. This refractory diseases sees high disability rate [2]. Recent years, due to changes in lifestyle, alcoholism and wide applications of hormone drugs, the incidence of ONFH exhibits rising tendency [3]. However, the pathogenesis of ONFH has not been fully elucidated. Growing evidences have demonstrated that ONFH is a complex disease regulated by the combination of multiple factors [4], such as trauma, genetic factors [5], abuse of hormone drugs [6], and excessive drinking [7]. With advancements in molecular techniques, genetic factors have been generally accepted to play critical roles in the etiology of non-trauma ONFH [8]. A variety of genes have been confirmed to be involved in ONFH pathogenesis, such as MTHFR, CYP3A4, CYP2D6, CYP2C19, ABCB1/MDR1, and nitric oxide synthase 3 (NOS3) gene [9–12].

Endothelial nitric oxide synthase (eNOS) encoded by NOS3 gene is mainly produced by vascular endothelial cells. eNOS is responsible for the synthesis and release of nitric oxide (NO). It promotes angiectasis and inhibits platelet aggregation [13]. Polymorphisms in NOS3 gene can influence the expression and structure of the protein, thus impairing enzyme activity. The dysregulation of eNOS increases vascular tension, which damages angiogenesis ability, increases blood clots risk, and reduces bone mineral density [14]. NOS3 gene polymorphisms show close associations with several diseases caused by vascular lesions, such as acute myocardial infarction, essential hypertension, coronary heart disease and avascular necrosis of femoral head [15–18].

Given close relationship between blood supply and ONFH, we speculated that NOS3 gene polymorphisms might influence individual susceptibility to ONFH. The present study was designed to explore genetic association of NOS3 gene polymorphisms (rs1799983 and rs3918181) with ONFH risk in a Chinese Han population.

\textbf{Materials and methods}

\textbf{Study subjects}

Our study included 88 ONFH patients who were diagnosed through clinical examination and X-ray detection in
Eighty-ninth Hospital of the Chinese People’s Liberation Army. According to pathogenic factors, the patients were divided into two groups: non-traumatic ONFH (55) and traumatic ONFH (33). Non-traumatic ONFH was caused by environmental factors, such as glucocorticoid induction, chemotherapy or alcohol poisoning. The healthy individuals (90) who received health check-ups in the physical examination center of Eighty-ninth Hospital of the Chinese People’s Liberation Army were categorized into the control group. The controls had no disease or malignancy histories. All the individuals were Chinese Han population, and had no blood association with each other. This study was granted by the Ethics Committee in Eighty-ninth Hospital of the Chinese People’s Liberation Army. Besides, data collection and blood sampling were approved by all the participants, following the regulations of the ethics.

**Blood DNA extraction**

A total of 5 mL peripheral blood was collected for every participant, and processed with EDTA. Genome DNA was extracted from these blood specimens using DNA extraction kit (TianGen, Beijing, China) according to the manufacturer’s instructions. Then, the obtained DNA samples were stored at −20 °C fridge.

**Genotyping**

In this study, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used for genotyping eNOS polymorphisms. According to corresponding base sequences for NOS3 rs1799983 and rs3918181 polymorphisms in GenBank database, we employed Primer Premier 5.0 software to design PCR primers. Primer information of each polymorphism was shown in Table 1.

| SNP          | Primer sequence                        | Tm (°C) | Restriction enzyme | Restriction fragment |
|--------------|----------------------------------------|---------|-------------------|---------------------|
| rs1799983    | F: 5'-CATAGGCTCACCCCCAGAA-3'           | 60      | Sdul              | TT: 206 bp;         |
|              | R: 5'-AGTCATCCCTTGTGGCTC-3'            |         |                   | GG: 124 bp;         |
|              |                                        |         |                   | GT: 206 bp;         |
|              |                                        |         |                   | AA: 216 bp;         |
|              |                                        |         |                   | AG: 216 bp, 150bp, 66bp |
| rs3918181    | F: 5'-CAACAGTGAGGTAATCTCA-3'           | 60      | Rsal              | AG: 216 bp, 150bp, 66bp |

**Genotype analysis**

Restriction enzymes were adopted to digest the obtained PCR amplification products. Total volume of digested system was 20 μL, including restriction enzyme 2 μL, PCR products 10 μL, 10× Buffer 2 μL, and ddH2O 6 μL. The digested system were blended at 37 °C with water bath overnight. Later, 4 μL digested products were detected with 2% agarose gel electrophoresis.

**Statistical analysis**

SPSS 18.0 statistical software was applied for data calculations. Continuous variables were expressed as x ± S. Hardy–Weinberg equilibrium (HWE) was used to check genetic equilibrium goodness for the control group. χ² test and Fisher’s exact test were taken to examine the frequencies of genotypes and alleles of NOS3 gene polymorphisms (rs1799983 and rs3918181) in the two groups. Odds ratios (ORs) with 95% confidence intervals (95%CIs) were utilized to represent relative risk of ONFH. p < .05 means statistical significance.

**Results**

**HWE examination**

Relevant result showed that for rs1799983 and rs3918181, genotype distribution in the controls were all consistent with HWE, suggesting the good representativeness of the study population.

**Analysis of clinical information for the cases**

As shown in Table 2, there was no significant differences in gender or age distribution between the non-traumatic ONFH patients and traumatic ONFH patients (p > .05 for both). In addition, the two groups did not show statistical differences in smoking, diabetes, coronary heart disease, thrombosis, hypertension or hyperlipidemia (p > .05 for all).

**Analysis of allele frequencies of NOS3 polymorphisms**

The frequency of the T allele of NOS3 rs1799983 polymorphism was apparently higher in the controls than in the cases (p = .046), indicating this allele might be correlated with decreased risk of ONFH (OR = 0.521, 95%CI = 0.272–0.997). Additionally, the frequency of the T allele was also significantly higher in controls than in non-traumatic ONFH (p = .029), but not in traumatic ONFH, which demonstrated that T
NOS3 polymorphisms and ONFH susceptibility. Similar results were observed for non-traumatic and traumatic ONFH \( p > .05 \), Table 4. When it came to genotype frequencies of the rs3918181 polymorphism, no apparent difference was detected between the two groups either \( p > .05 \), Table 4.

### Discussion

ONFH is characterized by the death of osteocytes and bone marrow. This recalcitrant disease could lead to the destruction of the hip joint. The main cause of ONFH is blood supply insufficiency in local femoral head, which promotes bone tissue necrosis and eventually leads to bone cortex collapse and cartilage damage. According to its causes, ONFH could be divided into traumatic and non-traumatic sub-types. Major risk factors for ONFH include fracture, drinking and hormone application [19–22].

Until now, the replacement of artificial hip joint represents an effective way to relieve pain and improve joint functions among ONFH cases. However, artificial joint costs highly, with limited service life time [23]. ONFH has brought about serious torment and heavy financial burden to the patients. Therefore, early diagnosis and prevention are critical ways for the settlement of ONFH. However, due to unclear etiology, it is difficult to realize such operations in clinic. As we all know, not all of the individuals exposing to risk factors would eventually subject to ONFH. This phenomenon is determined by individual susceptibility which is closely related to genetic information. DNA is the basic carrier of human genetic information whose characteristics cannot be changed along with either time or space. Gene polymorphism is an important reason for differences in individual susceptibility to diseases, and single nucleotide polymorphism (SNP) stands for a most common form [24–28].

To our knowledge, NO, an important signal molecule in blood vessel, participates in the process of coagulation, fibrinolysis, revascularization and thrombosis, and plays a vital role in anti-inflammation, antioxidant and the prevention of the settlement of ONFH. However, due to unclear etiology, it is difficult to realize such operations in clinic. As we all know, not all of the individuals exposing to risk factors would eventually subject to ONFH. This phenomenon is determined by individual susceptibility which is closely related to genetic information. DNA is the basic carrier of human genetic information whose characteristics cannot be changed along with either time or space. Gene polymorphism is an important reason for differences in individual susceptibility to diseases, and single nucleotide polymorphism (SNP) stands for a most common form [24–28].
impose critical impacts on osteoarticular diseases. eNOS encoded by NOS3 gene may influence the synthesis of NO. Besides, alterations in NOS3 gene may cause cardiovascular disease and bone disease [34–36]. In human beings, NOS3 gene is located on the 7th chromosome q36 with 29 exons and 28 introns, encoding 1203 amino acids. Studies have found several SNPs in NOS3 gene, such as T-786C and A-922G in the promoter region, and Glu298Asp in exon 7 [37–39]. In this study, we analyzed the association of NOS3 gene rs1799983 and rs3918181 polymorphisms with the susceptibility to ONFH.

We found that the T allele of rs1799983 was significantly less frequent in ONFH patients than in healthy controls, suggesting the T allele might act as a protective factor against the occurrence of ONFH. Additionally, the T allele also decreased the risk of non-traumatic ONFH by 0.408 fold, but not for traumatic ONFH. Genotypes of the polymorphism rs1799983 had no significant association with the susceptibility to ONFH. Meanwhile, the genotypes and alleles of rs3918181 SNP were not related to the development of ONFH either.

Age and gender showed no obvious differences among non-traumatic and traumatic ONFH patients and healthy controls. Genotype distributions of the two SNPs did not deviate from HWE in controls. All of the results guaranteed the representativeness of the controls. However, the simple size in this study was small, and final results were not adjusted. So well-designed studies are necessary to further explore the pathogenesis of ONFH.

Disclosure statement

No potential conflict of interest was reported by the authors.

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