Combination analysis of NOS3, ABCB1 and IL23R polymorphisms with alcohol-induced osteonecrosis of the femoral head risk in Chinese males

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ABSTRACT

Background: Common variants of multiple genes played a crucial role in osteonecrosis of the femoral head (ONFH) onset which was proved by many previous reports. We hypothesized that polymorphisms in NOS3, ABCB1 and IL23R were related to individual differences in alcohol sensitivity and the development of alcohol-induced ONFH.

Methods: In this case-control study, we evaluated 8 SNPs in three genes in the Chinese Han population including 355 male cases and 355 healthy male controls. These SNPs were genotyped by Sequenom MassARRAY RS1000. To identify their relationship with alcohol-induced ONFH susceptibility using $\chi^2$ test and genetic model analysis.

Results: We found an association with alcohol-induced ONFH susceptibility for 4 SNPs (rs743506, rs3918184, rs13233308 and rs6693831) in three genes after adjusted by age. The genotype “G/A” of rs743506 in NOS3 gene acts as a risk factor in genotype ($P = 0.003$), dominant ($P = 0.048$), recessive ($P = 0.005$) and additive model($P = 0.006$); The genotype “T/C” of rs3918184 in NOS3 gene acts as a risk factor in genotype ($P = 0.012$) and recessive model ($P = 0.009$); The genotype “T/C” of rs13233308 in ABCB1 gene acts as a risk factor in genotype ($P = 0.038$) and additive model($P = 0.041$); The genotype “T/C” of rs6693831 in IL23R gene acts as a protective factor in genotype model ($P = 0.046$).

Conclusions: This study provides evidence for three alcohol-induced ONFH susceptibility genes (NOS3, ABCB1 and IL23R) in Chinese males and polymorphisms of them may be associated with alcohol-induced ONFH risk.

INTRODUCTION

Osteonecrosis of the femoral head (ONFH) indicates a debilitating disorder that the bone collapses, happening in a special anatomical site of femoral head and resulting in osteocyte death [1, 2]. It mainly affects younger males who is between 30 and 50 years old. In China, there are seven million patients with osteonecrosis and be found annually new patients to increase by 150,000 to 200,000 patients [3]. The seventy-five to ninety percent cases of ONFH have been associated with various risk factors, involving alcohol
abuse, corticosteroid use, hip trauma and smoking [4]. Increasing of adipose vesicles in the blood circulating is induced these risk factors, adding lipid deposition in osteocytes of the femoral head and resulting in embolism, which influence finally blood flow [5].

Current evidence suggests that being related to the risk of ONFH and eight SNPs in three genes of our research can be identified through the genome-wide association studies [1, 6, 7]. Nitric oxide is synthesised from L-arginine and has three isoforms of synthases (NOS): endothelial (eNOS) neuronal (nNOS) and inducible(iNOS). The eNOS is expressed in normal adult bone as a constitutive isoform [8]. A previous study in Korean patients indicated that idiopathic osteonecrosis of the femoral head was significantly associated with polymorphism of eNOS gene, but it also indicate that polymorphism in intron 4 of eNOS was not significant differences [9]. Glueck et al. found that the pathogenesis of idiopathic ONFH are related with the intron 4 of eNOS polymorphism and synthetic loss of nitric oxide outcome in African American and Caucasian patients. The ABCB1, is also called adenosine triphosphate-binding cassette B1, can encode the transport protein and made an significant influence on distribution and absorption in the mammalian body [10]. So far a few single nucleotide polymorphisms (SNPs) of ABCB1 gene have been identified, of which mutations in exon 21 (G2677T) and exon 26 (C3435T) are associated with alteration of P-gp expression or function, as recently reviewed by Sakaeda et al. and Fromm et al. [11, 12]. Interleukin 23 receptor (IL23R) gene are associated with alcohol-induced ONFH in Chinese males.

### RESULTS

In the current study, a total of 355 male cases (median age at diagnosis 44.91 ± 9.85 years) and 355 healthy male controls (median age 46.02 ± 9.61 years) were included. The basic characteristics of cases and controls were showed in Table 1. The primers of the 8 candidate SNPs are revealed in Table 2. Eight SNP loci(rs6693831, rs790631, rs4148749, rs10808072, rs13233308, rs3918227, rs3918184 and rs743506) in the NOS3, ABCB1 and IL23R were evaluated in this study. SNP ID, HWE P value, allele A/B, MAF control/case, odds ratio, 95% confidence interval and P value were listed in Table 3. Using x² test, we found significant differences in frequency of alleles and rs13233308 in the ABCB1 gene and rs743506 in the NOS3 gene were associated with increased risk of alcohol-induced ONFH risk by allele model analysis (rs13233308, P = 0.032, odds ratio [OR]: 1.26, 95% confidence interval [CI]: 1.02-1.55 and rs743506, P = 0.006, OR: 1.39, 95%CI: 1.09-1.78). All of the SNPs were in Hardy–Weinberg equilibrium (HWE) in the control population of this study.

In this study, the whole of patients were male and we then further analyzed the association of the NOS3, ABCB1 and IL23R gene polymorphisms with alcohol-induced ONFH patients adjusted by age in Table 4. The “G/A” of rs743506 increased alcohol-induced ONFH risk was found by the genotype model (P = 0.004, OR, 2.57; 95% CI, 1.36-4.86 crude; P = 0.003, OR, 2.66; 95% CI, 1.40-5.05 adjusted by age), the dominant model (P = 0.048, OR, 1.35; 95% CI, 1.01-1.82 adjusted by age), the recessive model (P = 0.006, OR, 2.40; 95% CI, 1.28-4.49 crude; P = 0.005, OR, 2.48; 95% CI, 1.32-4.65 adjusted by age) and the additive model (P = 0.008, OR, 1.38; 95% CI, 1.09-1.75 crude; P = 0.006, OR, 1.39; 95% CI, 1.10-1.78 adjusted by age), analysis respectively. The “T/C” of rs3918184 increased alcohol-induced ONFH risk was found by the genotype model (P = 0.016, OR, 1.86; 95% CI, 1.12-3.08 crude; P = 0.012, OR, 1.91; 95% CI, 1.15-3.18 adjusted by age) and the recessive model (P = 0.013, OR, 1.85; 95% CI, 1.14-2.99 crude; P = 0.009, OR, 1.89; 95% CI, 1.17-3.08 adjusted by age), analysis respectively. The “T/C” of rs13233308 increased alcohol-induced ONFH risk was showed by the genotype model (P = 0.031, OR, 1.59; 95% CI, 1.04-2.43 crude; P = 0.038, OR, 1.57; 95% CI, 1.03-2.39 adjusted by age), the

| Table 1: Characteristics of 355 alcohol-induced ONFH male subjects |
|-----------------------|-----------------------|-----------------------|
| Parameters            | Cases                | Controls              | P value |
| Age [mean±SD]         | 44.91 ± 9.85         | 46.02 ± 9.61          | NS      |
| Sex [male]            | 355                  | 355                   |         |
| BMI (kg/m²)           |                      |                       |         |
| ≥25                   | 53                   | 61                    | NS      |
| < 25                  | 302                  | 294                   | NS      |

SD, standard deviation: NS, notsignificant: BMI, body mass index.
The results of the association between the \textit{NOS3} haplotype and alcohol-induced ONFH risk were listed in Table 5. Haplotype “CT” in Block 1 was found to be associated with increased alcohol-induced ONFH risk ($P = 0.044$, OR, 1.26; 95% CI, 1.01-1.58 adjusted by age) (Figure 1).

\textbf{DISCUSSION}

Non-traumatic ONFH has been diffusely awakened as a pathological define with numerous etiologies, the precise
Table 4: Association between single-nucleotide polymorphisms and risk of alcohol-induced ONFH based on logistic test

| SNP ID   | Model  | Genotype | OR (95% CI)          | p value | †OR (95% CI)          | †p value |
|----------|--------|----------|----------------------|---------|----------------------|----------|
| rs6693831| Genotype | T/C      | 0.72(0.53-0.98)      | 0.041*  | 0.73(0.53-0.99)      | 0.046*   |
|          | Dominant | T/C      | 0.79(0.59-1.07)      | 0.121   | 0.80(0.59-1.08)      | 0.150    |
|          | Recessive| T/C      | 1.57(0.83-2.94)      | 0.163   | 1.67(0.88-3.15)      | 0.120    |
|          | Additive | T/C      | 0.92(0.72-1.17)      | 0.493   | 0.93(0.73-1.19)      | 0.570    |
| rs790631 | Genotype | C/T      | 2.05(0.18-2.27)      | 0.560   | 1.96(0.17-2.18)      | 0.582    |
|          | Dominant | C/T      | 1.23(0.76-1.99)      | 0.393   | 1.26(0.77-2.03)      | 0.356    |
|          | Recessive| C/T      | 2.01(0.81-2.22)      | 0.571   | 1.92(0.17-2.13)      | 0.593    |
|          | Additive | C/T      | 1.24(0.78-1.94)      | 0.358   | 1.25(0.79-1.97)      | 0.328    |
| rs4148749| Genotype | C/G      | 0.74(0.31-1.79)      | 0.511   | 0.74(0.31-1.78)      | 0.498    |
|          | Dominant | C/G      | 0.97(0.71-1.34)      | 0.871   | 0.98(0.71-1.35)      | 0.902    |
|          | Recessive| C/G      | 0.74(0.31-1.78)      | 0.507   | 0.73(0.31-1.77)      | 0.492    |
|          | Additive | C/G      | 0.95(0.72-1.25)      | 0.724   | 0.95(0.72-1.26)      | 0.745    |
| rs1080872| Genotype | G/A      | 0.99(0.56-1.57)      | 0.812   | 0.96(0.57-1.61)      | 0.874    |
|          | Dominant | G/A      | 1.01(0.74-1.35)      | 0.983   | 1.01(0.74-1.35)      | 0.982    |
|          | Recessive| G/A      | 0.93(0.57-1.51)      | 0.774   | 0.95(0.58-1.55)      | 0.845    |
|          | Additive | G/A      | 0.98(0.78-1.23)      | 0.906   | 0.99(0.79-1.24)      | 0.941    |
| rs1323308| Genotype | T/C      | 1.59(1.04-2.43)      | 0.031*  | 1.57(1.03-2.39)      | 0.038*   |
|          | Dominant | T/C      | 1.43(1.00-2.04)      | 0.049*  | 1.41(0.99-2.01)      | 0.060    |
|          | Recessive| T/C      | 1.29(0.93-1.81)      | 0.124   | 1.29(0.92-1.79)      | 0.140    |
|          | Additive | T/C      | 1.26(1.02-1.55)      | 0.033*  | 1.25(1.01-1.54)      | 0.041*   |
| rs3918227| Genotype | A/C      | 1.00(0.17-7.14)      | 1.000   | 1.06(0.14-7.59)      | 0.955    |
|          | Dominant | A/C      | 1.00(0.63-1.57)      | 1.000   | 1.01(0.64-1.58)      | 0.971    |
|          | Recessive| A/C      | 1.00(0.14-7.13)      | 1.000   | 1.06(0.15-7.17)      | 0.955    |
|          | Additive | A/C      | 1.00(0.65-1.52)      | 1.000   | 1.01(0.66-1.54)      | 0.963    |
| rs3918184| Genotype | T/C      | 1.86(1.12-3.08)      | 0.016*  | 1.91(1.15-3.18)      | 0.012*   |
|          | Dominant | T/C      | 1.15(0.86-1.55)      | 0.346   | 1.16(0.86-1.56)      | 0.322    |
|          | Recessive| T/C      | 1.85(1.14-2.99)      | 0.013*  | 1.89(1.17-3.08)      | 0.009*   |
|          | Additive | T/C      | 1.23(0.99-1.54)      | 0.061   | 1.25(0.99-1.55)      | 0.051    |
| rs743506 | Genotype | G/A      | 2.57(1.36-4.86)      | 0.004*  | 2.66(1.40-5.05)      | 0.003*   |
|          | Dominant | G/A      | 1.33(0.99-1.79)      | 0.058   | 1.35(1.01-1.82)      | 0.048*   |
|          | Recessive| G/A      | 2.40(1.28-4.49)      | 0.006*  | 2.48(1.32-4.65)      | 0.005*   |
|          | Additive | G/A      | 1.38(1.09-1.75)      | 0.008*  | 1.39(1.10-1.78)      | 0.006*   |

OR, odds ratio; 95% CI, 95% confidence interval; *P < 0.05, statistical significance. †Adjusted by age.

Table 5: Haplotype analysis results of rs3918227 and rs3918184 in NOS3 gene

| Haplotype | freq(case) | freq(control) | †OR(95%CI) | †P    |
|-----------|------------|---------------|------------|-------|
| CT        | 0.340      | 0.293         | 1.26(1.01-1.58) | 0.044* |
| AC        | 0.063      | 0.063         | 1.12(0.72-1.73) | 0.610  |

Freq, frequency; OR, odd ratio; CI, confidence interval.
*P < 0.05, statistical significance: †Adjusted by age
pathogenesis of osteonecrosis leaves to be evaluated. Alcoholism and chronic steroid use were considered to be the essential risk factors induced osteonecrosis. Genetic mutation of alcohol-metabolizing enzyme genes has been associated with alcohol-induced osteonecrosis and polymorphism of NOS3, ABCB1 and IL23R gene are related with developing osteonecrosis [6, 15–17].

The NOS3 gene located in chromosome 7q36.1 involves two SNPs (“G/A” in rs743506 and “T/C” in rs3918184) related with increased alcohol-induced ONFH risk. Endothelial nitric oxide synthase is the remarkable NOS isoform expressed in normal bone and eNOS adjusts bone resorption and formation and oestrogen are dependent on NO production via eNOS in the skeletal system [18]. Excessive NO production was found in various rheumatic diseases, including rheumatoid arthritis, systemic lupus erythematosus, osteoarthritis and vasculitis [8]. Güler S et al. found the genotype “GA” of rs743506 showed risk effect was associated with aura in migraine patients [19]. It proved that mutation of rs743506 would affect function of NO in cerebral blood flow regulation and include the activation of nociceptors in the vascular system and the release of vasoactive neuropeptides in the neurogenic inflammatory response [20]. Meanwhile, Juan P et al. found genotype “T/C” of rs3918184 be related with increased risk of hypertension, preeclampsia and stroke [21]. Combination with our research, rs743506 and rs3918184 associated with increased with alcohol-induced ONFH risk and it may also be effect NO in the femoral head blood flow regulation. The NOS is fundamentally expressed in vascular endothelium and had been proved to shorten NO norms in human plasma [22]. Our study suggests that the genotype “GA” of rs743506 and genotype “T/C” of rs3918184 polymorphisms may be risk factors for alcohol-induced ONFH, and NO brought in fundamentally decided eNOS may make a significant effect on the pathogenesis of alcohol-induced ONFH.

The ABCB1 gene, also known as Multidrug-resistant transporter-1(MDR1), was located in chromosome 7q21.12 and the genotype “T/C” of rs13233308 related with increased alcohol-induced ONFH risk. This gene spans 4.5 kb encoding a 1280

![Figure 1: Haplotype-Block Map for NOS3 Based on rs3918227, rs3918184 and rs743506.](image)

Linkage disequilibrium (LD) among single nucleotide polymorphisms (SNPs) analyzed in chromosome 7q. LD is indicated by standard color schemes with bright red for very strong LD (LD > 2, D’= 1) and pink red (LD > 2, D’< 1) for intermediate LD.
In this case–control study, some limitations were intrinsic and must be marked. First, to avoid selection bias, alcohol-induced ONFH cases and healthy controls came from the same hospital. However, this bias not necessarily to be of consequence because of demographic variables. Secondly, the sample size (355 male cases and 355 male controls) is not large enough in our work. We performed a power analysis and just found the power of rs743506 in NOS3 gene was 0.91. The power of seven surplus SNPs was < 0.75. We considered that the number of samples was small in association studies and confirming our finding required a larger sample size [28].

Our research provides a new evidence for a relationship between NOS3, ABCB1 and IL23R gene and alcohol-induced ONFH in Chinese males onset, which may shed light on the etiology of alcohol-induced ONFH. Functional studies are further required to evaluate the correlation of genotype and phenotype in a large cohort of various ethnicities.

**MATERIALS AND METHODS**

**Ethics statement**

The use of the protocol in this study was strictly affirmed to the principles expressed in the Declaration of Helsinki and were approved by the Ethical Committee of Zhengzhou Traditional Chinese Medicine Traumatology Hospital. Informed consent was signed from all of the participants.

**Study participants**

Three hundred and fifty male patients with alcohol-induced ONFH were consecutively enrolled in this study at the Zhengzhou Traditional Chinese Medicine Traumatology Hospital from June 2014 to January 2016. Patients with a history of ethanol consumption of at least 400 ml per week were categorized under alcohol-induced osteonecrosis [29]. The development of alcohol-induced ONFH was diagnosed according to assessment by X-rays, magnetic resonance imaging (MRI), and bone scans [2]. The ONFH was present in one hip in 135 patients and in both hips in 220 patients (440 hips). Patients with a demonstrable history of direct trauma or with the possibility of a combination of causes were excluded.

We also enrolled 355 healthy male controls between July 2014 and January 2016 based on medical examination at Zhengzhou Traditional Chinese Medicine Traumatology Hospital. The controls had a history of ethanol consumption of at least 400 ml per week, however,
they had no alcohol-induced ONFH and other related diseases, no history of thromboembolic events and no symptoms of hip disease. All participants were restricted to Chinese Han population who lived in Zhengzhou city and its surrounding areas.

**Genotyping**

Genomic DNA was extracted and purified from the whole blood of all the participants using a kit (GoldMag, China) and their concentration was measured by spectrometry (DU530UV/VIS spectrophotometer; Beckman Instruments, Fullerton, CA). All PCR reactions were operated in a 25ul volume containing 10pmol of each primer, 1X PCR buffer, 10mM dNTP, 25 ng of genomic DNA, and 1U of Taq DNA polymerase (Solgent, Daejeon, Korea). The conditions of PCR amplification were as follows: initial denaturation at 95°C for 15 min followed by 35 cycles of denaturation at 95°C for 20 sec, annealing at 55°C for 40 sec, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. SNP genotyping was performed on the SEQUENOM MassARRAY_Analyzer 4 (Sequenom, Inc., San Diego, CA, USA) using genomic DNA in a single multiplex reaction. According to the manufacturer’s instructions, the primers for polymerase chain reaction amplification and single base extension were designed by Sequenom Assay Design 3.0 software (Sequenom, San Diego, CA, USA)[30]. SNP genotyping using the standard protocol recommended by the manufacturer was performed by Sequenom MassARRAY RS1000. Data analyses and management were conducted by Sequenom Typer 4.0 Software [30, 31].

**Statistical analysis**

Microsoft Excel and SPSS 16.0 statistical package (SPSS, Chicago, IL) were used to perform statistical analyses. All P values in these studies were two-sided, and P = 0.05 was considered the threshold of whether statistical significance was achieved or not. A significant departure of genotype frequency from Hardy–Weinberg equilibrium (HWE) for each SNP was estimated using SNPStats (http://bioinfo.iconcologia.net/snpstats/start.htm). The genotype frequencies of cases and controls were calculated using χ2 test [32]. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were determined using unconditional logistic regression analysis with adjustment by age [33].

The four genetic models (Genotype, Dominant, Recessive and Additive) were applied using PLINK software (http://pngu.mgh.harvard.edu/purcell/plink/) to assess the association of SNPs with the risk of alcohol-induced ONFH.

**Abbreviations**

NOS3, nitric oxide synthase 3; ABCB1, adenosine triphosphate-binding cassette B1; IL23R, interleukin 23 receptor; PCR, polymerase chain reaction; UEP, unextended mini-sequencing primer; SNPs: single nucleotide polymorphisms; MAF: minor allele frequency; HWE: Hardy - Weinberg equilibrium; OR: Odds ratio; CI: confidence interval.

**Author contributions**

YW collected and analyzed the data, and drafted the manuscript; XY conceived the idea and critically revised the manuscript; JS and YZ collected and analyzed the data; LP and JZ participated in the design of the study and performed the statistical analysis. GW and JW participated in its design and coordination, and funded the study. All authors read and approved the final manuscript.

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**CONFLICTS OF INTEREST**

The authors declare that they have no competing interests.

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