Int J Clin Exp Pathol 2016;9(10):10670-10675
www.ijcep.com /ISSN:1936-2625/IJCEP0027770

Original Article

Nitric oxide of genetic variants is associated with alcohol-induced osteonecrosis risk of the femoral head in a Han population

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Received March 8, 2016; Accepted May 24, 2016; Epub October 1, 2016; Published October 15, 2016

Abstract: Purpose: Osteonecrosis of the femoral head (ONFH) is a disease in which cell death in the bone occurs. This study investigated the association the tagging SNPs (tSNPs) genetic variants for the NOS3 gene associated with alcohol-induced ONFH in a Han Chinese population. Methods: A total of 209 alcohol-induced ONFH patients and 300 controls were recruited in this case-control study. We genotyped 4 tSNPs in the *NOS3* and evaluated their association with alcohol-induced ONFH using the χ^2 test and genetic model analysis. Results: From the association test using the logistic regression model, The minor allele "T" frequency of rs3918184 showed a significant risk effect on alcohol-induced ONFH (P = 0.026, OR = 1.42; 95% CI = 1.04-1.95) and the genotype "G/G" of rs743506 showed an increased risk (P = 0.034, OR = 2.47, 95% CI = 1.05-5.79). Additionally, haplotype analysis revealed that the rs743506, rs743507 have tight linked. Conclusion: These results suggested that the minor allele of rs3918184, rs743506 (T and G, respectively) contributes to an increase in the risk of alcohol-induced ONFH in the Chinese Han populations. And subsequently the *NOS3* gene polymorphism with the pathogenesis of the alcohol-induced ONFH needed to be validation in a large sample.

Keywords: *NOS3*, alcohol-induced ONFH, tSNP, genetics polymorphism, Han

Introduction

Osteonecrosis of the femoral head (ONFH) frequently leads to progressive collapse of the femoral head followed by a degenerative arthritis of the hip joint [1]. It may lead to a subsequent collapse and secondary degenerative arthritis of the hip [2]. Recent data suggest that ONFH is a multisystemic disease rather than a disease of the femoral head [3]. As the disease frequently occurs in young adult patients, between the ages of 20 and 50, it results in a substantial socioeconomic burden [4]. Although many risk factors, such as steroids, alcoholism, coagulation defects, storage diseases, marrow infiltrating diseases, and some autoimmune diseases, associated with osteonecrosis have been found, the pathogenesis has not been completely elucidated [5-9]. ONFH in twins and a clustering of cases in families imply that genetic factors are involved [10, 11]. And recent studies have explored associations between genetic mutations and polymorphisms and ONFH. Some studies have investigated the association between genetic predisposition and development of ONFH.

Nitric oxide (NO) is a multifunctional biomolecule and serves as an important signal in physiologic processes including angiogenesis, thrombosis, and bone turnover, which are known to be related to the pathogenesis of osteonecrosis [12]. eNOS is the predominant isoform expressed in normal bone. Recently, it has been reported that the incidence of osteocyte apoptosis is increased in the femoral head during ON, regardless of etiological factors [13]. The majority of our studies focused on gene polymorphisms affecting the ONFH. Identification of genetic variants that convey an

Table 1. PCR primers

SNP_ID	1st-PCR primer sequences	2nd-PCR primer sequences	UEP sequences
rs3918227	ACGTTGGATGTGAGTGCCGTTCATTGTGTG	ACGTTGGATGTTCATAATAGCCCCGACCTG	gGTCACCAACAAGAGAATG
rs3918184	ACGTTGGATGCCATCGAGAAACATTACCCG	ACGTTGGATGCTTGAATCCCTGACCTCAGC	gggagTACAGGCGTGAGCCACCA
rs743506	ACGTTGGATGGAGCAAGCTAGATTGCTAGG	ACGTTGGATGAAATGCACCCCCACCAAAAG	tcccgCCCTCTGGGCTCCTCTCC
rs743507	ACGTTGGATGAAAGTCCCCCCTGGACTTTC	ACGTTGGATGAGAATCCAGCCATGAATTCC	cctcAAGCACCAGTGCATGTC

Table 2. Basic information of candidate tSNPs in this study

SNP ID	Gene name	Chromosome	Alleles *A/B*	MAF		p-HWE	*P* value	OR (95% CI)
				Case	Control			
rs3918227	*NOS3*	7q36.1	*A/C*	0.065	0.077	0.687	0.463	0.83 (0.51-1.36)
rs3918184	*NOS3*	7q36.1	*T/C*	0.357	0.318	0.894	0.194	1.19 (0.91-1.55)
rs743506	*NOS3*	7q36.1	*G/A*	0.275	0.233	0.872	0.130	1.25 (0.94-1.66)
rs743507	*NOS3*	7q36.1	*C/T*	0.267	0.232	0.746	0.211	1.20 (0.90-1.61)

A/B stands for minor/major alleles on the control sample frequencies. *HWE:* Hardy-Weinberg equilibrium*; MAF:* minor allele frequency; OR: odds ratio; SNP: single-nucleotide polymorphisms.

additional risk will also help to personalize the way we deliver care, both in the prevention and treatment of osteonecrosis.

Materials and methods

Subjects

Two hundred and nine patients with alcohol-induced ONFH were consecutively enrolled at the Third Hospital of Anshan, Anshan and Honghui Hospital, Xi'an Jiaotong University College of Medicine from 2014 to 2015. Patients were diagnosed using anteroposterior and lateral pelvic radiographs and magnetic resonance images. According to the etiological factors of ONFH, we selected 209 alcohol-induced ONFH cases, and patients with a history of ethanol consumption of at least 800 mg per week were categorized under alcohol-induced osteonecrosis [14]. Patients with a demonstrable history of direct trauma, or with a possible combination of causes were excluded. The control subjects were defined by the following criteria: a lack of hip pain and by the absence of any lesions with a sclerotic margin or subchondral collapse consistent with ONFH in anteroposterior and frog leg lateral pelvic radiographs. Final 300 unrelated healthy individuals were selected as control group. All persons related to patients were excluded from the control group. All individuals provided informed consent for their participation in the study and this study was approved by The Third Hospital of Anshan, Anshan and Honghui Hospital, Xi'an Jiaotong University College of Medicine.

DNA isolation and genotyping

Four SNPs from NOS3 genes were selected, which previously published to be associated with ONFH, and with minor allele frequency (MAF) > 5% in the HapMap CHB (Chinese Han Beijing) population. We taking 5 ml peripheral blood from each subject and using the GoldMag-Mini Whole Blood extracted Genomic DNA Purification Kit (GoldMag Co. Ltd. Xi'an City, China). DNA concentration was measured by NanoDrop 2000 (Gene Company Limited). We used Sequenom MassARRAY Assay Design 3.0 Software to design Multiplexed SNP MassEXTEND assay [15]. We performed Sequenom MassARRAY RS1000 to genotype the SNPs using the standard protocol recommended by the manufacturer [15]. Finally, Data management and analysis was performed by Sequenom Typer 4.0 Software [15, 16]. Laboratory personnel were blinded to the genotyping results of all samples.

Statistical analysis

Microsoft Excel and SPSS 19.0 statistical package (SPSS, Chicago, IL) were used to perform statistical analyses. The Odds ratios were calculated and a logistic regression was used to obtain adjusted Ors and 95% CIs. Comparisons between variables were performed using Chi-squared test or paired samples t-test. A $P <$

Table 3. Relationship between candidate SNPs and alcohol-induced osteonecrosis of the femoral head (adjusted by gender + age)

SNP ID	Model	Genotype	Control (N, %)	Case (N, %)	OR (95% CI)	*P*	AIC	BIC
rs3918227	Codominant	C/C	256 (85.3%)	184 (88%)	1.00	0.85	520.9	542
		C/A	42 (14%)	23 (11%)	0.84 (0.43-1.62)			
		A/A	2 (0.7%)	2 (1%)	1.22 (0.15-9.79)			
	Dominant	C/C	256 (85.3%)	184 (88%)	1.00	0.65	519	535.9
		C/A-A/A	44 (14.7%)	25 (12%)	0.86 (0.46-1.63)			
	Recessive	C/C-C/A	298 (99.3%)	207 (99%)	1.00	0.84	519.1	536.1
		A/A	2 (0.7%)	2 (1%)	1.25 (0.16-9.97)			
	Log-additive	---	---	---	0.90 (0.51-1.59)	0.73	519.1	536
rs3918184	Codominant	C/C	140 (46.7%)	89 (43%)	1.00	0.084	513.9	535.1
		C/T	129 (43%)	88 (42.5%)	1.44 (0.91-2.25)			
		T/T	31 (10.3%)	30 (14.5%)	2.01 (1.00-4.07)			
	Dominant	C/C	140 (46.7%)	89 (43%)	1.00	0.044*	512.8	529.7
		C/T-T/T	160 (53.3%)	118 (57%)	1.55 (1.01-2.36)			
	Recessive	C/C-C/T	269 (89.7%)	177 (85.5%)	1.00	0.12	514.4	531.3
		T/T	31 (10.3%)	30 (14.5%)	1.70 (0.87-3.31)			
	Log-additive	---	---	---	1.42 (1.04-1.95)	0.026*	511.9	528.8
rs743506	Codominant	A/A	177 (59%)	113 (54.1%)	1.00	0.068	515.8	537
		A/G	106 (35.3%)	77 (36.8%)	1.23 (0.79-1.93)			
		G/G	17 (5.7%)	19 (9.1%)	2.68 (1.12-6.42)			
	Dominant	A/A	177 (59%)	113 (54.1%)	1.00	0.12	516.8	533.7
		A/G-G/G	123 (41%)	96 (45.9%)	1.39 (0.91-2.13)			
	Recessive	A/A-A/G	283 (94.3%)	190 (90.9%)	1.00	0.034*	514.7	531.6
		G/G	17 (5.7%)	19 (9.1%)	2.47 (1.05-5.79)			
	Log-additive	---	---	---	1.43 (1.02-2.01)	0.037*	514.8	531.8
rs743507	Codominant	T/T	176 (59.3%)	112 (53.9%)	1.00	0.15	510.2	531.3
		T/C	104 (35%)	81 (38.9%)	1.37 (0.87-2.14)			
		C/C	17 (5.7%)	15 (7.2%)	2.10 (0.84-5.25)			
	Dominant	T/T	176 (59.3%)	112 (53.9%)	1.00	0.088	509	525.9
		T/C-C/C	121 (40.7%)	96 (46.1%)	1.45 (0.94-2.23)			
	Recessive	T/T-T/C	280 (94.3%)	193 (92.8%)	1.00	0.17	510.1	527
		C/C	17 (5.7%)	15 (7.2%)	1.86 (0.76-4.54)			
	Log-additive	---	---	---	1.41 (0.99-2.00)	0.055	508.3	525.2

**P* value ≤ 0.05 indicates statistical significance; OR: odd ratio; CI: confidence interval; AIC: Akaike's Information criterion; BIC: Bayesian Information criterion.

0.05 was considered statistically significant. The validation of each SNP frequency in control subjects was tested for departure from Hardy-Weinberg Equilibrium (HWE) using an exact test. The *p*-value was generated using a two-sided test.

Results

In order to investigate the association of *NOS3* gene polymorphisms with respect to alcohol-induced ONFH, we performed four polymorphic sites in the *NOS3* gene from public databases by considering their allele frequencies and positions, and analyzed these polymorphisms in 209 alcohol-induced ONFH patients and 300 control subjects. All alleles were observed in alcohol-induced ONFH patient and control groups. As listed in **Table 1**, a multiplexed SNP MassEXTEND assay was designed with the Sequenom MassARRAY Assay Design 3.0 Software. **Table 2** shows the distribution of *NOS3* gene polymorphisms in both groups; all genotype distributions were coherent with the

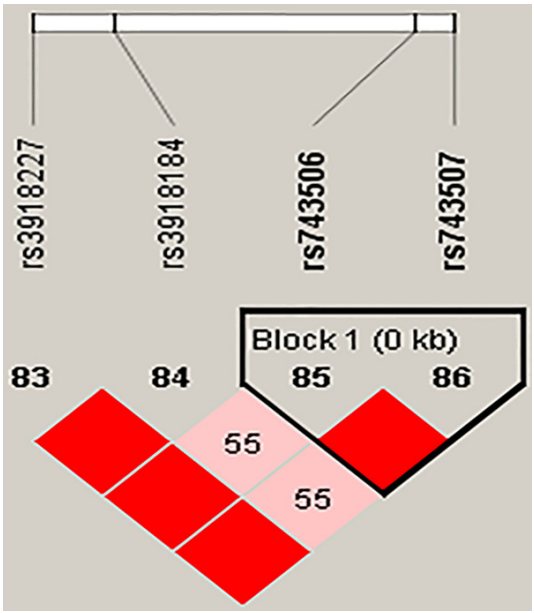

Figure 1. Haplotype block map for the NOS3 tSNPs genotyped in this study. The linkage disequilibrium between two SN Ps is indicated by standardized D' (red boxes).

assumption of Hardy-Weinberg equilibrium in the controls of this study ($P < 0.01$). As show in **Table 2**, there was no evidence of association between *NOS3* polymorphisms and alcohol-induced ONFH. In **Table 3**, from the association test using the logistic regression model, the rs3918184, rs743506 SNPs of theNOS3 gene were significantly associated with a risk of ONFH in the analysis models. The minor allele "T" frequency of rs3918184 showed a significant risk effect on ONFH ($P = 0.026$, OR = 1.42; 95% CI = 1.04-1.95) and in Dominant model (C/T-T/T) ($P = 0.044$, OR = 1.55, 95% CI = 1.01-2.36). Additionally, the genotype "G/G" of rs743506 showed an increased risk in the Recessive model ($P = 0.034$, OR = 2.47, 95% CI = 1.05-5.79), meanwhile in log-additive model showed significant ($P = 0.037$, OR = 1.43, 95% CI = 1.43-2.01).

Since the pattern of LD is highly structured into conserved blocks of sequence separated by hotspots of recombination [17], the final function of a conserved haplotype may be the result of interaction among polymorphisms within the block. We analyzed linkage LDs among the *NOS3* SNPs. In **Figure 1** the results suggested that the rs743506, rs743507 have tight linked. We calculated the haplotype with two loci within the LD block associated with ONFH. However, there were no differences found in the haplotype block controls and patients with ONFH risk.

Discussion

Many studies have shown that there have many factors, such as steroid usage, alcoholism, infections, coagulation defects, and some autoimmune diseases are closely related with ONFH susceptibility [6, 8, 9]. However, etiological and pathological mechanisms of ONFH have not yet been thoroughly investigated. Recently, many studies have been carried out to determine a number of candidate genes have been identified in an attempt to determine the genetic factors involved in the pathogenesis of ONFH [13, 18]. Our data suggest for the first time that the *NOS3* gene genetic polymorphisms and alcohol-induced ONFH risk in Han Chinese.

A number of studies have been carried out to determine the associations between genetic polymorphisms in the *eNOS* gene and vascular diseases, including coronary artery disease or myocardial infarction, hypertension, stroke, and renal diseases [19, 20].

Nitric oxide (NO) has been identified to be a biomolecule that is involved in a variety of physiologic processes that could induce non-traumatic osteonecrosis, including angiogenesis, thrombosis, and bone turnover [12, 21]. NO is produced by NO synthases (NOS), which catalyze the conversion of L-arginine to Lcitrulline and NO. There are 3 protein forms of nitric oxide synthase (NOS): neuronal NOS, endothelial NOS (eNOS), and inducible NOS (iNOS) [22]. It was reported that the predominant isoform of nitric oxide synthases expressed in normal adult bone is endothelial nitric oxide synthase *eNOS* gene deficiency attenuates vascular reactivity, increases platelet recruitment, reduces mobilization of endothelial progenitor cells, impairment of angiogenesis, and deceases bone volume and bone formation rate in mice [23]. There were limited data concerning effects of ethanol on the expression level or activity of constitutive NOS and the results were discrepantly reported according to the experimental design. Furthermore, it was proposed that there have two polymorphic sites of the *eNOS* gene, the 27-bp repeat polymorphism in intron

4 and G894T polymorphism in exon 7, are associated with the altered function of this gene [24-26]. And Koo et al indicated the 27-bp repeat polymorphism in intron 4 was significantly associated with ONFH in Korean patients, however the G894T site polymorphisms was not significant associated with ONFH [4].

The HapMap data showed the *NOS3* gene to be located at the edge of a region of elevated linkage disequilibrium that extends at least 45 kb upstream of the gene, while linkage disequilibrium downstream of the *NOS3* gene breaks down abruptly. We selected four tSNPs for NOS3, and our results found rs39918184, rs743506 were associated with ONFH risk. There is no functional data related to the *eNOS* Tag SNPs, rs39918184 and rs743506. However, according to the position of the two sites, it is possible that it modulates *NOS3* gene expression, once the region has important physiological and pathological roles in the regulation of the mRNA level.

Conclusions

Our findings demonstrate a risk effect of the *NOS3* (rs39918184 of the T and rs743506 of the G) against the development of alcohol-induced ONFH for the first time in the Han Chinese. However there is no functional data related to the *eNOS* Tag SNPs. Therefore studies are necessary to elucidate molecular mechanisms in the future studies.

Acknowledgements

This work was supported by the National 863 High-Technology Research and Development Program (No. 2012AA02A519). The authors declare that they have no competing interests. We thank all of the participants for their involvement in this study.

Disclosure of conflict of interest

None.

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References

[1] Atsumi T and Kuroki Y. Role of impairment of blood supply of the femoral head in the pathogenesis of idiopathic osteonecrosis. Clin Orthop Relat Res 1992; 22-30.

[2] Assouline-Dayan Y, Chang C, Greenspan A, Shoenfeld Y and Gershwin ME. Pathogenesis and natural history of osteonecrosis. Semin Arthritis Rheum 2002; 32: 94-124.

[3] Hadjigeorgiou GM, Karantanas AH, Zibis A, Dardiotis E, Aggelakis K, Papadimitriou A and Malizos K. Increased frequency of white matter lesions in patients with osteonecrosis (WMLeOn) of the femoral head. Eur J Radiol 2004; 50: 278-284.

[4] Koo KH, Lee JS, Lee YJ, Kim KJ, Yoo JJ and Kim HJ. Endothelial nitric oxide synthase gene polymorphisms in patients with nontraumatic femoral head osteonecrosis. J Orthop Res 2006; 24: 1722-1728.

[5] Abu-Shakra M, Buskila D and Shoenfeld Y. Osteonecrosis in patients with SLE. Clin Rev Allergy Immunol 2003; 25: 13-24.

[6] Fisher DE. The role of fat embolism in the etiology of corticosteroid-induced avascular necrosis: clinical and experimental results. Clin Orthop Relat Res 1978; 68-80.

[7] Hirota Y, Hirohata T, Fukuda K, Mori M, Yanagawa H, Ohno Y and Sugioka Y. Association of alcohol intake, cigarette smoking, and occupational status with the risk of idiopathic osteonecrosis of the femoral head. Am J Epidemiol 1993; 137: 530-538.

[8] Jones LC, Mont MA, Le TB, Petri M, Hungerford DS, Wang P and Glueck CJ. Procoagulants and osteonecrosis. J Rheumatol 2003; 30: 783-791.

[9] Wang Y, Li Y, Mao K, Li J, Cui Q and Wang GJ. Alcohol-induced adipogenesis in bone and marrow: a possible mechanism for osteonecrosis. Clin Orthop Relat Res 2003; 213-224.

[10] Nobillot R, Le Parc JM, Benoit J and Paolaggi JB. Idiopathic osteonecrosis of the hip in twins. Ann Rheum Dis 1994; 53: 702.

[11] Glueck CJ, Glueck HI, Welch M, Freiberg R, Tracy T, Hamer T and Stroop D. Familial idiopathic osteonecrosis mediated by familial hypofibrinolysis with high levels of plasminogen activator inhibitor. Thromb Haemost 1994; 71: 195-198.

[12] Calder JD, Buttery L, Revell PA, Pearse M and Polak JM. Apoptosis--a significant cause of bone cell death in osteonecrosis of the femoral head. J Bone Joint Surg Br 2004; 86: 1209-1213.

[13] Hadjigeorgiou G, Dardiotis E, Dardioti M, Karantanas A, Dimitroulias A and Malizos K. Genetic association studies in osteonecrosis

of the femoral head: mini review of the literature. Skeletal Radiol 2008; 37: 1-7.
[14] Martin-Algarra RV, Polache A, Fernandez-Villalba E, Pla Delfina JM and Guerri C. Influence of chronic alcohol intake on intestinal taurine and antipyrine transport in pregnant rats. Alcohol Clin Exp Res 1998; 22: 463-467.
[15] Gabriel S, Ziaugra L and Tabbaa D. SNP genotyping using the Sequenom MassARRAY iPLEX platform. Curr Protoc Hum Genet 2009; 2: 2 12.
[16] Thomas RK, Baker AC, Debiasi RM, Winckler W, Laframboise T, Lin WM, Wang M, Feng W, Zander T, MacConaill L, Lee JC, Nicoletti R, Hatton C, Goyette M, Girard L, Majmudar K, Ziaugra L, Wong KK, Gabriel S, Beroukhim R, Peyton M, Barretina J, Dutt A, Emery C, Greulich H, Shah K, Sasaki H, Gazdar A, Minna J, Armstrong SA, Mellinghoff IK, Hodi FS, Dranoff G, Mischel PS, Cloughesy TF, Nelson SF, Liau LM, Mertz K, Rubin MA, Moch H, Loda M, Catalona W, Fletcher J, Signoretti S, Kaye F, Anderson KC, Demetri GD, Dummer R, Wagner S, Herlyn M, Sellers WR, Meyerson M and Garraway LA. High-throughput oncogene mutation profiling in human cancer. Nat Genet 2007; 39: 347-351.
[17] Goldstein DB. Islands of linkage disequilibrium. Nat Genet 2001; 29: 109-111.
[18] Glueck CJ, Freiberg RA and Wang P. Heritable thrombophilia-hypofibrinolysis and osteonecrosis of the femoral head. Clin Orthop Relat Res 2008; 466: 1034-1040.
[19] Carreras-Torres R, Kundu S, Zanetti D, Esteban E, Via M and Moral P. Genetic risk score of NOS gene variants associated with myocardial infarction correlates with coronary incidence across Europe. PLoS One 2014; 9: e96504.
[20] Tikkanen I and Fyhrquist F. Nitric oxide in hypertension and renal diseases. Ann Med 1995; 27: 353-357.
[21] Pan L, Song K, Hu F, Sun W and Lee I. Nitric oxide induces apoptosis associated with TRPV1 channel-mediated Ca(2+) entry via S-nitrosylation in osteoblasts. Eur J Pharmacol 2013; 715: 280-285.
[22] Li H and Poulos TL. Structure-function studies on nitric oxide synthases. J Inorg Biochem 2005; 99: 293-305.
[23] Albrecht EW, Stegeman CA, Heeringa P, Henning RH and van Goor H. Protective role of endothelial nitric oxide synthase. J Pathol 2003; 199: 8-17.
[24] Philip I, Plantefeve G, Vuillaumier-Barrot S, Vicaut E, LeMarie C, Henrion D, Poirier O, Levy BI, Desmonts JM, Durand G and Benessiano J. G894T polymorphism in the endothelial nitric oxide synthase gene is associated with an enhanced vascular responsiveness to phenylephrine. Circulation 1999; 99: 3096-3098.
[25] Tesauro M, Thompson WC, Rogliani P, Qi L, Chaudhary PP and Moss J. Intracellular processing of endothelial nitric oxide synthase isoforms associated with differences in severity of cardiopulmonary diseases: cleavage of proteins with aspartate vs. glutamate at position 298. Proc Natl Acad Sci U S A 2000; 97: 2832-2835.
[26] Tsukada T, Yokoyama K, Arai T, Takemoto F, Hara S, Yamada A, Kawaguchi Y, Hosoya T and Igari J. Evidence of association of the ecNOS gene polymorphism with plasma NO metabolite levels in humans. Biochem Biophys Res Commun 1998; 245: 190-193.