

Apolipoprotein A1 and neuronal nitric oxide synthase gene polymorphisms and hormone-related osteonecrosis of the femoral head

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Abstract. – **OBJECTIVE:** This study was designed to investigate the influence of several polymorphisms in neuronal NOS genes and apolipoprotein A1 on the hormone-related osteonecrosis of the femoral head (ONFH) risk.

PATIENTS AND METHODS: Peripheral blood mononuclear cell (PBMCs) samples extracted from hormone-related ONFH patients and controls were used to amplify fragments of the apolipoprotein A1 and neuronal NOS exon 7 and intron 4 using specific PCR primers. The products were analyzed by DNA sequencing and mapped to find the genotype distributions.

RESULTS: The proportions of G/G, G/T and T/T on NOS exon 7 in hormone-related ONFH patient group and control group were 68%, 27%, 5% and 81%, 16%, and 3% respectively. The proportions of G/T and T/T in the experimental group were significantly higher than those in the control group ($p < 0.05$). The proportions of b/b, a/b and a/a on NOS intron 4 in hormone-related ONFH patient group and control group were 85%, 13%, 1% and 96%, 5% and 0% respectively. The proportion of a/b in the experimental group was much higher than in the control group. The distribution of A/A, G/A and G/G in the apolipoprotein gene in control and experimental groups were 19%, 33%, 71% and 42%, 20%, 38% respectively. In this case, the experimental group's A/A genotype was significantly higher than the control's genotype.

CONCLUSIONS: In our study group, several polymorphisms of the neuronal NOS gene and apolipoprotein A1 genes were significantly associated with hormone-related ONFH. These results suggest that those gene polymorphisms might be involved in the occurrence and development of ONFH.

Key Words

Hormone-related ONFH, Neuronal NOS gene, Apolipoprotein A1.

Introduction

Osteonecrosis of the femoral head (ONFH) is a common incapacitating orthopedic disease. It is difficult to detect during the early stages, due to non-specific clinical symptoms, which usually results in delayed treatment and prognosis¹⁻³. ONFH begins in the femoral head and neck first and progresses distally. Then bone cells and bone marrow cells die through apoptosis. Next, abnormalities of the femoral head resulting in osteoarthritis ensue. The disease can occur in all age groups, but is more common in young adults⁴⁻⁶. The pathogenesis of hormone-related ONFH is not clear yet. A history of hormonal drug abuse or primary diseases altering the hormonal balance may play a role. The prevalence of hormone-related ONFH has reached 40% in the general population these years⁷⁻¹⁰. Nitric oxide is synthesized by human neuronal nitric oxide synthase (NOS) and it is one of the essential biological factors involved in the pathogenesis of the disease. Some studies have shown that vascular endothelial NO production is influenced by NOS gene polymorphisms^{11,12}.

Another work has shown a role for abnormal lipid metabolism in the pathological evolution of osteonecrosis. Given the unclear pathogenesis of hormone-related osteonecrosis and the resulting poor treatment effects, clinical studies have focused on determining the best time for treatment, and in finding early diagnosis tools¹³⁻¹⁵. ONFH is an important cause of morbidity in the population³. Fortunately, with the development of molecular biology tools, the pathological analysis of the damaged tissues has become possible. In this study, we selected apolipoprotein A and neuronal nitric oxide

Table I. Neuronal NOS and apolipoprotein A1 primers synthesized.

Gene name	Locus	PCR primer
Neuronal NOS	Intron 4, 27 bp repeats	Upstream: 5'-AGGCCCTATGGTAGTGCCTT 3' Downstream: 5'-TCTCTTAGTGCTGTGGTCAC -3'
Neuronal NOS	Exon 7, G894T	Upstream: 5'-AAGGCAGGAGACAGTGGAGGT -3' ˆ Downstream: 5'-CCAGTCAATCCCCI'1-I'GGTGCTCA -3' ˆ
Apo A1	75bp	5' ˆ: AGGGACAGAGCTGATCCTTGA ACTCTTAAG; 3' ˆ: TTAGGGGACACCTAGCCCTCAGGAAGAGCA.

synthase (due to their close relation to the lipid and lipoprotein metabolism) for analyzing the relation of the presence of gene polymorphisms and the development of hormone-related ONFH.

Patients and Methods

Patients

A hormone-related ONFH group was selected from cases examined from January 2014 to December 2015 in Affiliated Hospital of North Sichuan Medical College. Patients with hip trauma histories were excluded. The total of 150 cases included 54 male and 96 female patients. The average age was 52.4 years with a standard deviation of 13.9 years. All patients included signed informed consents. 5 ml blood samples were drawn from each subject using EDTA anticoagulant test tubes. Additionally, samples from healthy donors without ONFH were used as controls. PBMCs were isolated and frozen on the same day for later further analyses. This study was approved by the Ethics Committee of Affiliated Hospital of North Sichuan Medical College. Signed written informed consents were obtained from the patients and/or guardians.

Methods

DNA concentration and purity testing

Total DNA was isolated from each of the patients' samples using standard techniques¹⁶⁻¹⁸. Briefly, DNA was extracted after cell lysis. The recovered DNA sample was detected by 0.8% agarose gel electrophoresis and UV spectrophotometry to confirm purity and assess concentration. Briefly, 1 µl of sample was used for electrophoresis (1X TBE, EB, 80 MV, 1.5 h electrophoresis). Pictures were taken under the FR-200 and UV-visible analysis device and quantified by comparing to a standard marker (λ -DNA/*EcoRI* + *HindIII*).

All DNA samples satisfactory for analysis displayed a significant absorption peak at OD260 (an OD260 value of 1 is equivalent to approximately

50 µg/ml of double-stranded DNA, or 40 µg/ml of single stranded DNA) OD260/OD280 ratio ranges from 1.7 to 1.9 were obtained.

PCR

Specific primers were used to amplify apolipoprotein A1 and neuronal NOS exon 7 and intron 4 (Table I). Each reaction tube included 1.5 µL of 10X Buffer (15 mM MgCl₂), 1 µL MgCl₂ (25 mM), 0.5 µL dNTP (10 mM), 0.5 µL forward primer (10 µM), 0.5 µL reverse primer (10 M), 1 µL cDNA template, and 0.15 µL HotStar-Taq enzyme (5 U/L). Water was added to 15 µL. The reaction conditions were the following: 95°C pre-denaturing for 15-30 minutes, 55°C annealing for 30 seconds, 72°C extension for 1 minute, 40 cycles, 72°C reaction for 5 minutes. The DNA was recovered using a PCR product extraction kit (MACHEREY-NAGEL, Bethlehem, PA, USA) using agarose gel electrophoresis^{19,20}. DNA sequencing and mapping for genotypes' distribution were used to analyze the product sequences.

Results

Genomic DNA Extraction Results

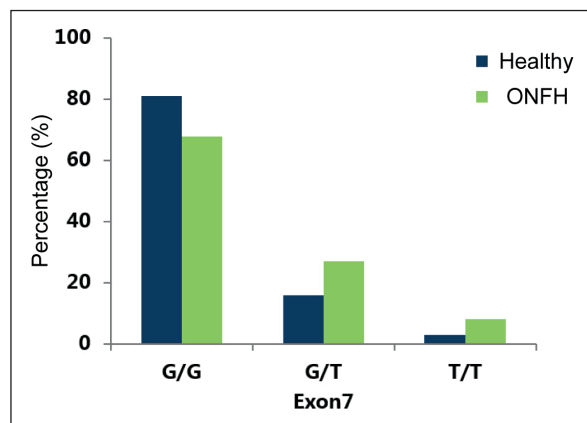
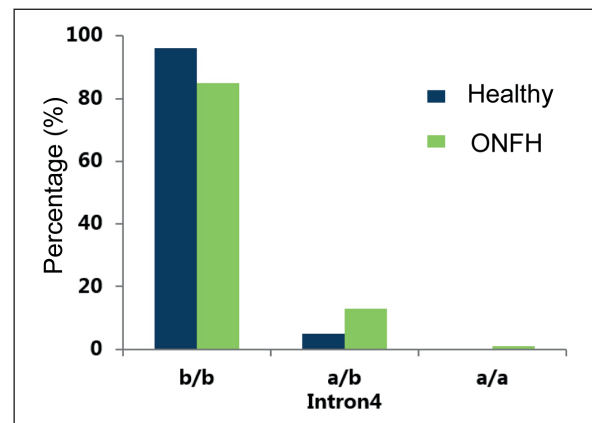
DNA was isolated successfully from peripheral blood samples of patients with ONFH and controls.

Gene Distribution on NOS Exon 7 and Intron 4 in Hormone-related ONFH Patient Group and Control Group

The proportions of G/G, G/T and T/T on NOS exon 7 in hormone-related ONFH patient group were 68%, 27%, 5% and those of the control group were 81%, 16%, and 3% respectively. The proportions of G/T and T/T in the experimental group were higher than those in the control group ($p < 0.05$) (Table II, Figure 1). Additionally, the proportions of b/b, a/b and a/a on NOS intron 4 in the hormone-related ONFH patient group were 85%, 13%, and 1% and those in the control group were 96%, 5%, and 0%

Table II. Proportions of G/G, G/T and T/T on NOS exon 7 in hormone-related ONFH patients and in controls.

Group	Genotypes of exon 7			Total
	G/G	G/T	T/T	
Control	122 (81%)	24 (16%)	4 (3%)	150
Experimental	102 (68%)	40 (27%)	8 (5%)	150


Figure 1. Distribution of G/G, G/T and T/T on NOS exon 7 in hormone-related ONFH patients and in controls.

Figure 2. Distribution of b/b, a/b and a/a in intron 4 in hormone-related ONFH patients and in controls.

respectively. The proportion of a/b in the experimental group was much higher than that in the control group ($p < 0.05$) (Table III, Figure 2).

Distribution of Polymorphisms on the Apolipoprotein A1 gene-75bp Region

The distributions of A/A, G/A and G/G polymorphisms on the Apolipoprotein in control and experimental groups were 19%, 33%, 71% and 42%, 20%, 38% respectively. The experimental group's A/A genotype was significantly higher than the control's genotype (Table IV, Figure 3).

Discussion

In this study, we found that the neuronal NOS exon 7 in the experimental group has a higher ratio of G/T and T/T genotypes than the control

group ($p < 0.05$), and also that the neuronal NOS intron 4 in the experimental group has a significantly higher a/b genotype than the control group. This suggests that the neuronal NOS gene mutation could be related to the development of ONFH. Another finding showed that the ONFH patients had higher A/A genotype proportions on the apolipoprotein A1 gene 75bp, compared to the control group, while the G/A genotype frequency was lower in the affected patients. This suggests that the G/A mutation on Apo A1 promoter might be one of the predisposing factors for ONFH. However, whether this relation is relevant to the lipid metabolism of ONFH patients remains to be confirmed.

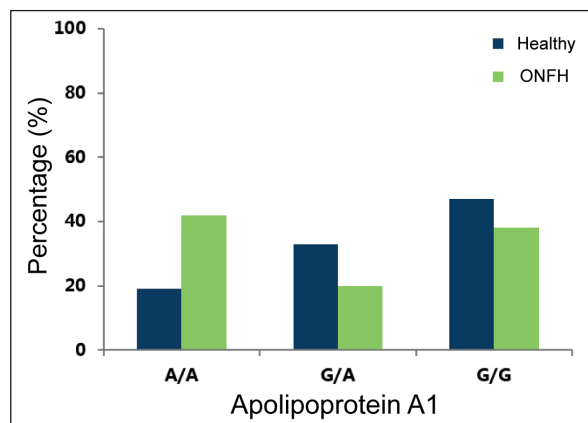
This study also found that a hormone-induced lipid metabolism disorder is a risk factor for ONFH. Scholars believe that glucocorticoid will increase liver lipid significantly, which leads to

Table III. Proportions of G/G, G/T and T/T in intron 4 in hormone-related ONFH patients and in controls.

Group	Genotypes of intron 4			Total
	b/b	a/b	a/a	
Control	142 (96%)	8 (5%)	0 (0%)	150
Experimental	128 (85%)	20 (13%)	2 (1%)	150

Table IV. Distribution of A/A, G/A and G/G polymorphisms on the apolipoprotein A1 gene promoter region.

Group	Genotypes (%)			Total
	A/A	G/A	G/G	
Control	29 (19%)	50 (33%)	71 (47%)	150
Experimental	63 (42%)	30 (20%)	57 (38%)	150

**Figure 3.** Distribution of A/A, G/A and G/G polymorphisms on the apolipoprotein A1 gene promoter region.

the change of a series of indicators, including triglycerides, cholesterol, low and very low-density lipoprotein. These indicators keep going up, and eventually increase the risk of hyperlipidemia, thereby increasing the lipid content of the bone cells in the long-term, which in turn induce osteoblast apoptosis²²⁻²⁴.

Conclusions

Although it is generally believed that a long-term use of high doses of hormones, triggering the lipid metabolism abnormalities, is the main reason for ONFH, there are still many unanswered questions. Especially the impact on blood vessel production of the presence of fat globules in the femoral bone marrow has not been taken into consideration; therefore, further studies are needed to provide more reliable evidence²⁵.

Conflict of Interests:

The Authors declare that they have no conflict of interests.

References

- GUPTA AK, RASTOGI S, NATH R. Internal fixation and muscle pedicle bone grafting in femoral neck fractures. *Indian J Orthop* 2008; 42: 39-42.
- NAKAMURA F, FUJIOKA M, TAKAHASHI KA, UESHIMA K, ARAI Y, IMAHORI Y, ITANI K, NISHIMURA T, KUBO T. Evaluation of the hemodynamics of the femoral head compared with the ilium, femoral neck and femoral intertrochanteric region in healthy adults: Measurement with positron emission tomography (PET) *Ann Nucl Med* 2005; 19: 549-555.
- GUPTA A. The management of ununited fractures of the femoral neck using internal fixation and muscle pedicle periosteal grafting. *J Bone Joint Surg Br* 2007; 89: 1482-1487.
- LECROY CM, RIZZO M, GUNNESON EE, URBANIAK JR. Free vascularized fibular bone grafting in the management of femoral neck nonunion in patients younger than fifty years. *J Orthop Trauma* 2002; 16: 464-472.
- SANDHU HS, SANDHU PS, KAPOOR A. Neglected fractured neck of the femur: A predictive classification and treatment by osteosynthesis. *Clin Orthop Relat Res* 2005; 431: 14-20.
- BIAN Y, QIAN W, LI H, ZHAO RC, SHAN WX, WENG X. Pathogenesis of glucocorticoid-induced avascular necrosis: a microarray analysis of gene expression in vitro. *Int J Mol Med* 2015; 36: 678-684.
- LI JG, LIN JJ, WANG ZL, CAI WK, WANG PN, JIA Q, ZHANG AS, WU GY, ZHU GX, NI LX. Melatonin attenuates inflammation of acute pulpitis subjected to dental pulp injury. *Am J Transl Res* 2015; 7: 66-78.
- CHEN D, ZHAO M, MUNDY GR. Bone morphogenetic proteins. *Growth Factors* 2004; 22: 233-241.
- CELIK A, TEKIS D, SAGLAM F, TUNALI S, KABAKCI N, OZAKSOY D, MANISAL M, OZCAN MA, MERAL M, GULAY H, CAMSARI T. Association of corticosteroids and factor V, prothrombin, and MTHFR gene mutations with avascular osteonecrosis in renal allograft recipients. *Transplant Proc* 2006; 38: 512-516.
- CHONG B, HEGDE M, FAWKNER M, SIMONET S, CASSINELLI H, COKER M, KANIS J, SEIDEL J, TAU C, TUYSUZ B, YUKSEL B, LOVE D. Idiopathic hyperphosphatasia and TNFRSF11B mutations: relationships between phenotype and genotype. *J Bone Miner Res* 2003; 18: 2095-2104.
- CUNDY T, HEGDE M, NAOT D, CHONG B, KING A, WALLACE R, MULLEY J, LOVE DR, SEIDEL J, FAKNER M, BANOVIC T, CALLON KE, GREY AB, REID IR, MIDDLETON-HARDIE CA, CORNISH J. A mutation in the gene TNFRSF11B encoding osteoprotegerin causes an idiopathic hyperphosphatasia phenotype. *Hum Mol Genet* 2002; 11: 2119-2127.
- ESTRADA K, STYRKARSDOTTIR U, EVANGELOU E. Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. *Nat Genet* 2012; 44: 491-501.

- 13) GLUECK CJ, FREIBERG RA, FONTAINE RN, TRACY T, WANG P. Hypofibrinolysis, thrombophilia, osteonecrosis. *Clin Orthop Relat Res* 2001; 386: 19-33.
- 14) JORGENSEN HL, KUSK P, MADSEN B, FENGER M, LAURITZEN JB. Serum osteoprotegerin (OPG) and the A163G polymorphism in the OPG promoter region are related to peripheral measures of bone mass and fracture odds ratios. *J Bone Miner Metab* 2004; 22: 132-138.
- 15) SAMARA S, DAILIANA Z, VARITIMIDIS S, CHASSANIDIS C, KOROMILA T, MALIZOS KN, KOLLIA P. Bone morphogenetic proteins (BMPs) expression in the femoral heads of patients with avascular necrosis. *Mol Biol Rep* 2013; 40: 4465-4472.
- 16) SOUCACOS PN, BERIS AE, MALIZOS K, KOROPILIAS A, ZALAVRAS H, DAILIANA Z. Treatment of avascular necrosis of the femoral head with vascularized fibular transplant. *Clin Orthop Relat Res* 2001; 386: 120-130.
- 17) TULI R, SEGHA TOLESLAMI MR, TULI S, WANG ML, HOZACK WJ, MANNER PA, DANIELSON KG, TUAN RS. A simple, high-yield method for obtaining multipotential mesenchymal progenitor cells from trabecular bone. *Mol Biotechnol* 2003; 23: 37-49.
- 18) WHYTE MP, MILLS BG, REINUS WR, PODGORNICK MN, ROODMAN GD, GANNON FH, EDDY MC, McALISTER WH. Expansile skeletal hyperphosphatasia: a new familial metabolic bone disease. *J Bone Miner Res* 2000; 15: 2330-2344.
- 19) LEIBBRANDT A, PENNINGER JM. RANK/RANKL: regulators of immune responses and bone physiology. *Ann N Y Acad Sci* 2008; 1143: 123-150.
- 20) KAMIYA N. The role of BMPs in bone anabolism and their potential targets SOST and DKK1. *Curr Mol Pharmacol* 2012; 5: 153-163.
- 21) KEARNS AE, KHOSLA S, KOSTENIUK PJ. Receptor activator of nuclear factor kappaB ligand and osteoprotegerin regulation of bone remodeling in health and disease. *Endocr Rev* 2008; 29: 155-192.
- 22) KOSTENIUK PJ. Osteoprotegerin and RANKL regulate bone resorption, density, geometry and strength. *Curr Opin Pharmacol* 2005; 5: 618-625.
- 23) ZALAVRAS CG, MALIZOS KN, DOKOU E, VARTHOLOMATOS G. The 677C->T mutation of the methylene-tetrahydrofolate reductase gene in the pathogenesis of osteonecrosis of the femoral head. *Haematologica* 2002; 87: 111-112.
- 24) ZUPAN J, KOMADINA R, MARC J. The relationship between osteoclastogenic and anti-osteoclastogenic pro-inflammatory cytokines differs in human osteoporotic and osteoarthritic bone tissues. *J Biomed Sci* 2012; 19: 28.
- 25) LI X, YI W, JIN A, DUAN Y, MIN S. Effects of sequentially released BMP-2 and BMP-7 from PELA microcapsule-based scaffolds on the bone regeneration. *Am J Transl Res* 2015; 7: 1417-1428.