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The genetic association between *PON1* polymorphisms and osteonecrosis of femoral head

A case-control study

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Abstract

The purpose of this study was to investigate the relationship between Paraoxonase-1 (PON1) gene rs662, rs854555 polymorphisms and osteonecrosis of the femoral head (ONFH) in Han population, northern China.

Polymerase chain reaction-restriction fragment length polymorphism was used to determine genotypes of *PON1* polymorphisms in 84 patients with ONFH and 96 healthy persons. χ^2 test was used to compare distribution differences of genotype, allele, and haplotype between the case and control groups. The odds ratio (OR) and 95% confidence interval (CI) were calculated to reveal the effects of *PON1* polymorphisms on risk of ONFH, and the results were adjusted using logistic regression analysis. The linkage disequilibrium and haplotype analysis were performed with haploview software.

That people carrying AA genotype of rs662 were easier to be attacked by ONFH than GG genotype carriers (OR = 2.53, 95% CI = 1.05–6.07, $P = .038$). Meanwhile, the frequency of A allele in the case group was significantly higher than the controls and it was a risk factor for ONFH (OR = 1.56, 95% CI = 1.03–2.38, $P = .038$). The A-A haplotype frequency of rs854555-rs662 in *PON1* was significantly correlated to the increased susceptibility to ONFH (OR = 2.74, 95% CI = 1.28–5.84).

The rs662 polymorphism in *PON1* may be associated with ONFH susceptibility, but not rs854555 in Han population, northern China. Additionally, haplotype is also a nonignorable risk factor.

Abbreviations: AGE = agarose gel electrophoresis, CI = confidence interval, EDTA-2Na = disodium ethylene diamine tetraacetic acid, HDL = high-density lipoprotein, HWE = Hardy–Weinberg equilibrium, LD = linkage disequilibrium, LDL = low-density lipoprotein, ONFH = osteonecrosis of the femoral head, OR = odds ratio, PON1 = paraoxonase-1, RCR-RFLP = polymerase chain reaction-restriction fragment length polymorphism, SNP = single-nucleotide polymorphism, WML = white matter lesions.

Keywords: haplotype, ONFH, polymorphism, *PON1*

1. Introduction

Osteonecrosis of the femoral head (ONFH) is a clinical common progressive disease with high disability rate, which is caused by the disruption of blood supply to femoral head.[1,2] Ischemia, necrosis, collapse are the different pathologic stages of ONFH, ischemia is reversible, but the latter 2 are not.[3] ONFH is usually divided into traumatic and nontraumatic ONFH, the former is caused by traumatic, but the etiology of latter is unclear. It is generally considered that nontraumatic ONFH results from the combined action of various factors, including genetic and environmental factors.[4–6] Its pathological process is very complicated.

Paraoxonase-1 (PON1) is encoded by *PON1* gene which is located on chromosome 7q21.3-q22.1, composed of 354 amino acids.[7,8] It is mainly synthesized in liver in humans and secreted into blood mostly in a way of combining with high-density lipoprotein (HDL).[9] PON1 can hydrolyze various substrates, such as organophosphate, aromatic ester, lactone, low-density lipoprotein (LDL), and cholesterol.[10] It prevents the body against oxidative damage and lipid peroxidation, and PON1 also takes part in innate immunity, even regulation of endoplasmic reticulum stress, cell proliferation, and apoptosis.[11,12]*PON1* play important roles in multiple biochemical pathways, and its abnormal expression is correlated with several human diseases, such as organophosphorus intoxication, cardiovascular and cerebrovascular diseases.[13–15] Besides, PON1 could be employed as a biomarker for inflammation and kidney diseases.[16,17]

Lipids metabolic disturbance is an important pathogenesis of ONFH. Apolipoprotein A and B are 2 key proteins for lipid metabolism which are involved in the onset of ONFH.[18,19] What is more, PON1 protein combined with HDL in serum forms an octamer that is a vital component of apolipoprotein A1. Genetic variants of *PON1* may influence white matter lesions (WML). Furthermore, WML is observed in more than 50% of patients with ONFH.[20] Therefore, we speculated that PON1 mutations might be correlated with the development and progression of ONFH, but the relative studies are few.

In the present study, 2 single-nucleotide polymorphisms (SNPs) of *PON1* (rs854555, rs622) were selected to investigate the

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relationship between *PON1* polymorphisms and ONFH risk in Han population of north China.

2. Materials and methods

2.1. The selection of the case and control groups

A case-control study was conducted. All subjects signed the informed consents before sample collection. Afterward, the professionally trained epidemiological investigators took charge to collect blood samples. This research was authorized by the Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University. The process of sample collection was conducted according to the national ethics criteria of human genome research.

The case group included 84 patients with ONFH who were selected from the clinical inpatients in Orthopaedic Department of Shandong Provincial Hospital Affiliated to Shandong University and diagnosed by pathobiology during January, 2012 to December, 2013. These patients were excluded, if the following conditions presented: the diagnosis was not clear; without complete data; had the history of trauma in the hip joint; with infectious disease of the hip joint and tumor.

The controls were all healthy people from the medical examination center of Shandong Provincial Hospital Affiliated to Shandong University, too, during the study period. People who once suffered from cardiovascular and cerebrovascular diseases, tumors would be excluded. The subjects were all Han population of north China and they had no blood relationship between each other.

3. Methods

3.1. DNA extraction

Three milliliters peripheral venous blood was collected from all subjects who kept fasting for 12 hours and placed in the tube containing disodium ethylene diamine tetraacetic acid (EDTA-2Na) anticoagulation. According to the manufacturer's instructions, peripheral blood leucocyte genome DNA was extracted using Beijing TIANGEN biochemical blood genome DNA extraction kit, and then stored in −20°C refrigerator for standby application.

3.2. PON1 genotyping

The genotypes of *PON1* were determined by the method of polymerse chain reaction-restriction fragment length polymorphism. The PCR primers were designed using Primer Premier 5.0 software based on rs662 and rs854555 sequences of *PON1* in NCBI Genebank database, and synthesized in Shanghai Sangon biotech Co, Ltd. The detailed sequences are listed in Table 1.

PCR amplification was a volume of 25 μL reaction system including 2.5 μL 10 × buffer, 1.5 μL dNTP mixture, 1.0 μL forward primers, 1.0 μL reverse primers, 1.0 μL Taq DNA polymerase, 1.0 μL DNA template, 1.5 μL MgCl2 and 15.5 μL sterilization ddH_2O. The amplification reaction was performed in PCR thermal cycler, and the reaction conditions were as follows: 94°C predegeneration for 4 minutes, followed by a total of 30 cycles including 94°C degeneration for 30 seconds, 69°C/57°C annealing for 30 seconds, 72°C extension for 30 seconds, then finally 72°C extension for 10 minutes.

Table 1

Primer sequences of *PON1* gene in rs662, rs854555.

	SNP	Primer sequence	Tm (°C)
rs662	For.	5′-TTGAATGATTGTTGCTGTGGGACCTGAG-3′	69°C
	Rev.	5′-CGACCACGCTAAACCCAAATACATCTCCCAG-3′	
rs854555	For.	5′-GCATAGAACACGCATGATCT-3′	57°C
	Rev.	5′-TTCTGGCAGAAACTGGCTCG-3′	

PON1 = paraoxonase-1, SNP = single-nucleotide polymorphism.

Enzyme digestion reaction system was a volume of 20 μL solution, including 3.0 μL restriction enzyme (*Hinf*I for rs662 and *Nla*III for rs854555), 10 μL PCR products, 1.0 μL 10 × buffer solution, 6.0 μL double distilled water. And then the mixture was digested for 16 hours in a water bath of 37°C. The enzyme-digested products were separated by 3% agarose gel electrophoresis (AGE) and observed the final outcome in imaging system.

3.3. Statistical analysis

PASW Statistics 18.0 software was used for data analysis. The continuous variables were denoted by $\bar{x} \pm s$, and their comparison between 2 groups was performed by Student *t* test. The categorical data were shown as n and %. The frequencies of genotype and allele in *PON1* polymorphisms were gained by direct counting. χ^2 test was used to check whether the genotype distributions matched Hardy–Weinberg equilibrium (HWE) in the control group. The distribution differences of the genotype, allele, and haplotype between the 2 groups were tested by χ^2 test. The effects of *PON1* polymorphisms on ONFH risk were evaluated through odds ratio (OR) with 95% confidence interval (CI), and the results were adjusted to age and gender by logistic regression analysis. Linkage disequilibriuln (LD) and haplotypes between *PON1* rs662, rs854555 polymorphisms were analyzed by haploview software. $P < .05$ was considered the statistically significant difference.

4. Results

4.1. General conditions of research objects

A total of 84 ONFH cases and 96 healthy individuals were collected in our study. There were 59 men and 37 women in the control group. The average age of the control group were 50.9 ± 8.63 years, with the age range of 26 to 79 years old. The case group included 50 males and 34 females, and their age range was 22 to 76, with the average age of 51.58 ± 9.12 years. The control and case groups were matched in gender and age ($P > .05$ for both). According to the etiology of ONFH, 24 (58.57%) cases were divided into idiopathic subtype, 27 (32.14%) patients were diagnosed with corticosteroids ONFH, while 33 (39.29%) patients were diagnosed with alcohol ONFH. Furthermore, 36 (42.86%) cases were at Association Research Circulation Osseous (ARCO) stage II, and 48 (57.14%) patients were at ARCO stages III and IV (Table 2).

The genotypes distribution of *PON1* polymorphisms conformed to HWE in the control group ($P > .05$), which indicated our study groups had a good representativeness (Table 3).

4.2. The genotypes distribution of PON1 polymorphisms in case and control groups and the relationship with ONFH risk

The results of genotype and allele distributions in *PON1* rs662, rs854555 SNPs between the case and control groups were

Table 2

The basic characteristics of case and control groups.

Characteristics	Case, n = 84 (%)	Control, n = 96 (%)	*P* values
Age, y	51.58 ± 9.12	50.90 ± 8.97	.609
Gender			.791
Male	50 (59.52)	59 (61.46)	
Female	34 (40.48)	37 (38.54)	
Etiology			
Idiopathic	24 (28.57)	–	–
Corticosteroids	27 (32.14)	–	–
Alcohol	33 (39.29)	–	–
ARCO staging			
II	36 (42.86)	–	–
III, IV	48 (57.14)	–	–

ARCO = Association Research Circulation Osseous.

displayed in Table 3. The frequencies of rs662 GG, AG, AA genotypes were 27.38%, 48.81%, 23.81% in cases and 38.54%, 47.92%, 13.54% in controls respectively. AA genotype had a significantly higher frequency in the case group than that of in the control group ($P = .039$), and so was A allele ($P = .040$). AA genotype and A allele of rs662 polymorphism were correlated with increased risk of ONFH, compared with GG genotype and G allele (AA vs GG: OR = 2.48, 95% CI = 1.04–5.91; A vs G: OR = 1.55, 95% CI = 1.02–2.36). Furthermore, the association was also significant after adjustments (AA vs GG: OR = 2.53, 95% CI = 1.05–6.07, $P = .038$; A vs G: OR = 1.56, 95% CI = 1.03–2.38, $P = .039$) (Table 3).

AA, AC, CC genotype frequencies of *PON1* rs854555 were 32.14%, 45.24%, 22.62%, and 30.21%, 43.75%, 26.04% in case and control groups, respectively, and A, C allele frequencies were 54.76%, 45.24% and 52.08%, 47.92% in case and control groups respectively, which demonstrated that the genotype and allele frequency distributions of rs854555 between 2 groups had no statistical significance ($P > .05$ for all).

4.3. Haplotype analysis of PON1 rs662, rs854555 polymorphisms

The LD and haplotype analysis of *PON1* rs662 and rs854555 were performed by haploview software (D' = 0.95). Four haplotypes were detected in *PON1* rs854555, rs662, namely A-G, C-A, C-G, A-A and the relative information was displayed in Table 4. The data showed that the distributions of A-A haplotype had obvious difference in 2 groups ($P = .01$), which indicated that it could increase the risk of ONFH occurrence (OR = 2.74, 95% CI = 1.28–5.84).

Table 4

Analyses of LD and haplotypes in *PON1* rs662, rs854555 polymorphisms.

Haplotype SNP1-SNP2	Case 2n = 168 (%)	Control 2n = 192 (%)	χ^2	*P*	OR (95% CI)
A-G	67 (39.88)	88 (45.83)	–	–	1.00
C-A	56 (33.33)	60 (31.25)	0.68	.41	1.23 (0.76–1.99)
C-G	20 (11.91)	32 (16.67)	0.36	.55	0.82 (0.43–1.56)
A-A	25 (14.88)	12 (6.25)	7.09	.01	2.74 (1.28–5.84)

Notes: SNP1: rs854555; SNP2: rs662.
LD = linkage disequilibrium, PON1 = paraoxonase-1, SNP = single-nucleotide polymorphism.

5. Discussion

ONFH is a bone destructive disease, resulting in hip pain and dysfunction. Its procession is collapse of the femoral head and results in heavy life and economic burden for patients and their family. The incidence of females is higher than that of males, which is related to high incidence of hyperlipidemia in postmenopausal women.[3,21] But up to now, there are no effective treatments for ONFH cases. The pathology of non-traumatic ONFH is complex, with the involvements of various factor, including heredity and environment, especially alcohol consumption and the application of hormones.[22,23] However, not all the cases exposing to the risk factors will develop ONFH, suggesting the pivotal roles of genetic factors in individual susceptibility.

PON is a group of enzymes which play an important role in the hydrolysis of organophosphates and lactones. This multigene family includes 3 members: *PON1*, *PON2*, *PON3* and they have the similar structure.[24] *PON1* is the first discovered member in *PON* family and is also the most extensively studied. As a hydrolase of lipids and lactones, PON1 is considered to be involved in the development of ONFH via inducing lipid

Table 3

Frequency comparisons of genotypes and alleles in *PON1* gene polymorphisms.

Genotype/allele	Case, n = 84 (%)	Control, n = 96 (%)	*P*	OR (95% CI)	*P**	OR (95% CI)*
rs662						
GG	23 (27.38)	37 (38.54)	–	1.00	–	1.00
AG	41 (48.81)	46 (47.92)	.291	1.43 (0.73–2.80)	.294	1.43 (0.73–2.81)
AA	20 (23.81)	13 (13.54)	.039	2.48 (1.04–5.91)	.038	2.53 (1.05–6.07)
G	87 (51.79)	120 (62.50)	–	1.00	–	1.00
A	81 (48.21)	72 (37.50)	.040	1.55 ((1.02–2.36)	.038	1.56 (1.03–2.38)
P_{HWE}		0.83				
rs854555						
AA	27 (32.14)	29 (30.21)	–	1.00	–	1.00
AC	38 (45.24)	42 (43.75)	.935	0.97 (0.49–1.93)	.948	0.98 (0.49–1.94)
CC	19 (22.62)	25 (26.04)	.616	0.82 (0.37–1.81)	.610	0.81 (0.37–1.80)
A	92 (54.76)	100 (52.08)	–	1.00	–	1.00
C	76 (45.24)	92 (47.92)	.611	0.90 (0.59–1.36)	.606	0.90 (0.59–1.36)
P_{HWE}		0.23				

HWE = Hardy–Weinberg equilibrium.
* The results were adjusted to age and gender using logistic regression analysis.

metabolism disorder. On the one hand, PON1 combined with HDL is an important component in apolipoprotein A1 which is a key protein in lipid metabolism. Normal lipid metabolism is a protective factor from avoiding ONFH occurrence, or else, the result is unmanageable. On the other hand, *PON1* had been proved to play an important role in the development of WML in young people, in the meanwhile, WML is observed in about more than 50% ONFH cases. Therefore, to investigate the genetic association of *PON1* polymorphisms with ONFH may provide a new insight into the etiology of ONFH.

The study about the association between *PON1* polymorphism and ONFH susceptibility has been conducted in previous years. In 2007, Hadjigeorgiou et al[25] researched the role of *PON1* polymorphisms in ONFH with/without WML in Greek population and the common polymorphisms M55L, R192Q were selects, the results indicated that *PON1* 192QQ genotype carriers had a significant increased risk to suffer from ONFH and WML, but not M55L polymorphism. In Chinese Han population, Wang et al[26] conducted a case-control study about *PON1* rs662 (R192Q) polymorphism and ONFH risk and also gained a similar conclusion that rs662 was associated with the susceptibility to steroid-induced ONFH. However, the steroid users without developing ONFH were employed as control. Therefore, the genetic association of *PON1* polymorphisms with ONFH in general Chinese Han population remained unclear.

In the present study, *PON1* rs662, rs*854555* polymorphisms were selected to explore the association of *PON1* polymorphisms with ONFH susceptibility. The patients diagnosed with ONFH were recruited as case group, while the gender and age matched healthy individuals served as control. The result reflected that AA genotype of rs662 exhibited significant difference in the case and control groups, suggesting that AA genotype could increase the susceptibility to ONFH. It was also found that the distribution of A allele in the healthy control group was much lower than that of the cases, which showed that A allele was also correlated with increased risk of ONFH. The genotype and allele distribution of *PON1* poymorphisms were different in various races and regions.[27,28] The present study demonstrated that re662 might be a genetic factor for the onset of ONFH. However, genotypes and allele frequencies of rs*854555* polymorphism had no statistically significant difference between 2 groups. In addition, the result was checked by the correlation between the haplotypes in *PON1* rs662, rs*854555* polymorphisms and ONFH, A-A haplotype was discovered to obviously increase the risk suffering from ONFH.

In summary, the correlation between *PON1* rs662 polymorphism and ONFH risk was supported in Han population of north China in this study. But rs*854555* is not found in the independent relationship with ONFH. Meanwhile, there are several limitations in our study. First, the sample size was relatively small. Second, the environmental factors were not considered in our study. Third, the mechanisms underlying *PON1* SNPs with ONFH development still remained poorly known. Therefore, further well-designed researches should be conducted to verify our study in extended sample size and various populations.

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