

## Original Article

# Gene-disease association study of interleukin-(IL-6/12) and tumor necrosis factor- $\alpha$ gene polymorphisms with risk of osteonecrosis of the femoral head induced by steroids in a Chinese Han population

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**Abstract:** Subjective: Osteonecrosis of the femoral head (ONFH) is a bone disease in which cellular death happens within the femoral head due to damage of blood supply to the anterior-superior-lateral part of the femoral head. Numerous studies of genetic epidemiology have assessed the association of pro-inflammation cytokine gene polymorphisms and risk of ONFH in different populations, but conflicting results have been obtained due to the heterogeneity of genetic backgrounds among the populations. The present study recruited 248 ONFH patients and 226 matched healthy controls from Hainan General Hospital to evaluate the influence of interleukin-(IL-6) rs1800795, IL-12 rs3212227, and tumor necrosis factor-(TNF- $\alpha$ ) rs1800629 polymorphisms on ONFH patients. Single nucleotide polymorphism locus was genotyped using PCR-RFLP. Results: Genotypic and allelic frequencies of TNF- $\alpha$  and IL-6 did not show significant differences between TC and normal controls. However, frequencies of wild (AA) and homozygous mutant (CC) genotype IL-12 rs3212227 genotypes in cases and controls were found more in controls (43% and 5.7% respectively), but that of the heterozygous genotype was higher (60.15%) in cases with ONFH patients. Conclusion: Although no relationship between IL-6 and TNF- $\alpha$  genotypes or alleles and ONFH susceptibility was revealed, this study first identified that IL-12 rs3212227 AC genotype confers genetic susceptibility to ONFH in a Chinese population.

**Keywords:** Osteonecrosis of femoral head, IL-6, IL-12, TNF- $\alpha$ , polymorphism, susceptibility

## Introduction

Osteonecrosis of the femoral head (ONFH) is a bone disease in which cellular death happens within the femoral head due to damage of blood supply to the anterior-superior-lateral part of the femoral head [1-3]. Without effective intervention, most cases will develop into a collapse of the femoral head and eventually degenerative arthritis of the hip [4, 5]. Although many treatments have been developed to relieve or reverse the course of this illness, none of them have been satisfactory in resolving this intractable medical condition [6-8]. Invention of total hip arthroplasty is a milestone in management of ONFH. However, patients often require multiple increasingly difficult surgeries over the course of a lifetime as the average age at presentation is very young [9, 10].

Among ONFH caused by different etiologies, steroid-induced ONFH has attracted vast attention for the following reasons [11]. First, there is a growing trend of its morbidity. Some epidemic studies have suggested that it has become the leading cause of ONFH. Second, it typically happens in young patients, the worst cases to handle. Third, preventative methods have been effective according to laboratory trials. Even if patients receive the same steroid administration regime, not every patient develops ONFH. It is easy to reach the conclusion that steroid sensitivity differs among different people. Hence, it is imperative for the medical community to bring about substantial advances in prevention [12].

In China, there are approximately 7 million people with ONFH. Moreover, new cases have

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**Table 1.** Characteristics of controls and patients with ONFH

Characteristics	Steroid-induced ONFH	Non-steroid-induced ONFH	P-value
Gender (M/F)	108/140	112/114	0.431
Age (years)	45.94 ± 2.120	43.46 ± 3.257	0.569
BMI (kg/m <sup>2</sup> )	24.70 ± 4.19	25.85 ± 4.96	0.271
TC (mmol/L)	5.24 ± 0.78	4.17 ± 1.08	0.007
TG (mmol/L)	2.5 ± 0.26	1.3 ± 0.14	0.002
HDL-C (mmol/L)	1.53 ± 0.48	1.42 ± 0.29	0.08
LDL-C (± 0.48)	3.05 ± 0.68	2.43 ± 0.41	< 0.001

Note: BMI = Body mass index; TC = Total cholesterol; LDL-C = Low-density lipoprotein-cholesterol; HDL-C = High-density lipoprotein-cholesterol; TG = Triglycerides.

reached to 100-300 thousand each year [13]. Recent studies have suggested that abnormal lipid metabolism is a main pathogenesis of osteonecrosis. Hyperlipidemia affects the microcirculation of the femoral head, resulting in femoral necrosis from multiple links, such as affecting blood coagulation solvent systems, bone fat embolisms, and formation of bone micro-thrombosis [14, 15]. Although the pathogenic mechanisms of steroid-induced ONFH have not been fully elucidated, recent studies have demonstrated that the development of this disease may be associated with blood coagulation in the femoral head, mainly caused by damage of the plasma lipid metabolism, as well as the tendency for blood to congeal [15].

Numerous findings have provided evidence that the inflammatory process is an important pathological factor associated with various malignancies [17, 18]. Nucleotide variations in gene encoding inflammatory molecules, such as interleukin (IL)-1 $\beta$ , IL-6, IL-12, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), may influence their biological activities, influencing the risk of ONFH [19-21]. For example, both experimental and clinical data have indicated that brain expression and plasma and cerebrospinal fluid levels of IL-6 may affect plaque formation, cognitive decline, or dementia, both in cross-sectional and longitudinal follow-up studies [17]. The IL-6 gene in humans is located on chromosome 7 (7p21). The (-174 C/G), also known as (rs1800795) polymorphism in the promoter region of the IL-6 gene, has been reported to affect IL-6 gene transcription rates and IL-6 plasma levels in ONFH patients, implicating its role in the development of ONFH [18]. Additionally, clinical research has suggested

that there is a correlation between IL-12 SNPs and levels of serum IL-12 with disease severity in ONFH patients. Many studies have focused mainly on 3'UTR, -1188A/C (rs321-2227) in the IL-12 gene [19].

Several polymorphisms in the promoter region of TNF- $\alpha$  have been associated with levels of TNF- $\alpha$  expression. Of these, TNF- $\alpha$  -308G/A (also referred to as rs1800629) has been studied the most. It involves the substitution of a guanine (G) by an adenine (A) and has been associated

with an increase in TNF- $\alpha$  expression levels [17]. Until now, numerous studies of genetic epidemiology have assessed the association of pro-inflammation cytokine gene polymorphisms and risk of cancer in different populations, but conflicting results have been obtained due to the heterogeneity of genetic backgrounds among populations. Furthermore, this supports the need for replication studies among all ethnic groups.

Taking together the association of pro-inflammation cytokines and ONFH, the aim of the present study was to evaluate the influence of gene polymorphisms of IL-6, IL-12, and TNF- $\alpha$  on the susceptibility to ONFH patients in a Chinese population.

## Patients and methods

### Ethics statement

The Medical Ethics Committee of Hainan General approved this study. Written informed consent conforming to the tenets of the Declaration of Helsinki was obtained from each participant prior to the study.

### Participants

A total of 248 patients with steroid-induced ONFH and 226 non-steroid-induced subjects were consecutively enrolled in the Department of Joint Surgery, Hainan General Hospital, between 2013 to 2018. ONFH diagnoses were established by the evidence of osteonecrosis through magnetic resonance imaging (MRI) in Stage 1 of the Association Research Circulation Osseous (ARCO) classification system, along

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**Table 2.** Genotype and allele frequency of IL-6 rs1800795, IL-12 rs3212227, and TNF- $\alpha$  rs1800629 and Pearson's Chi-squared test in ONFH patients and normal controls

Genotype/Allele	Patients (n = 248)	Controls (n = 226)	P-value	OR (95% CI)
IL-6 rs1800795	HWE*P = 0.59	HWE P = 0.25		
GG	147	146	0.621	0.905 (0.626-1.320)
GC	83	69	0.057	0.822 (0.681-1.484)
CC	18	11	0.132	4.560 (0.556-37.412)
G	377	361	0.334	0.841 (0.498-1.192)
C	119	91		
IL-12 rs3212227	HWE*P = 0.21	HWE P = 0.77		
AA	106	81	0.0531	0.805 (0.526-1.220)
AC	127	140	0.025	1.749 (1.104-2.417)
CC	15	5	0.0539	0.972 (0.653-1.521)
A	339	302	0.235	0.571 (0.328-0.981)
C	157	150		
TNF- $\alpha$ rs1800629	HWE*P = 0.42	HWE P = 0.53		
GG	172	169	0.539	0.869 (0.542-1.392)
GA	68	51	0.931	1.258 (0.787-2.010)
AA	8	6	0.330	3.358 (0.347-32.543)
G	412	389	0.336	0.813 (0.524-1.267)
A	84	63		

\*Chi-squared test for deviation from the Hardy-Weinberg equilibrium (a value of  $P < 0.001$  is regarded as a deviation from the HWE).

with plain radiographs in Stages 2, 3, and 4. Control subjects were defined as follows. They had no hip pain and anteroposterior and frog leg lateral pelvic radiographs did not show any lesions with a sclerotic margin or subchondral collapse consistent with ONFH. Cases with a demonstrable history of direct trauma or the possibility of a combination of many causes were excluded. Steroid-induced osteonecrosis was defined by a history of taking prednisolone (1,800 mg) or an equivalent over 4 weeks with nephritic syndrome, systemic lupus erythematosus, rheumatoid arthritis, allergic asthma, or organ transplantation.

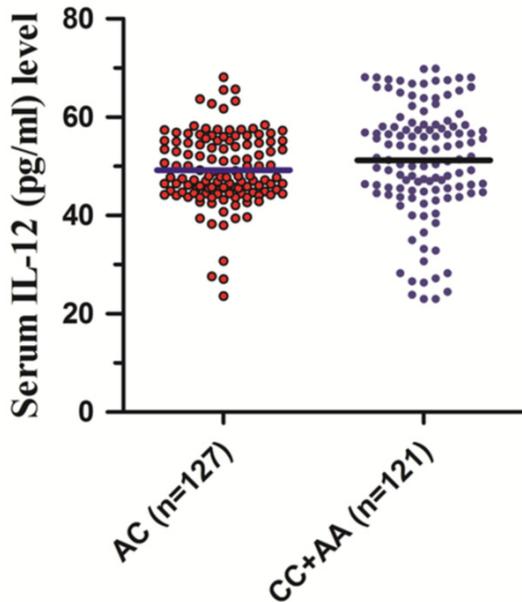
### Genotyping

Two SNPs (rs7412 C/T and rs429358 T/C), reportedly associated with plasma lipid levels in the ApoE gene, were selected for the present study. Genome DNA from whole blood cells of each sample was extracted using the Blood Genomic DNA Miniprep Kit (Axygen, USA), according to manufacturer instructions. DNA samples were stored at  $-20^{\circ}\text{C}$  for further analysis. SNPs rs7412 C/T and rs429358 T/C were genotyped using PCR-RFLP. The genomic region encompassing polymorphisms were amplified

using the following primers: ApoE (rs7412) F: 5'-GCAAGCTGCGTAAGCGGCTCC-3'; R: 5'-TCGC-GGATGGCGCTGAGG-3'. rs429358 F: 5'-CGGG-CACGGCTGTCCAAG-3'; R: 5'-CGCGGTACTGCAC-CAGGC-3'. Polymerase chain reaction products were generated in a 10  $\mu\text{L}$  reaction volume containing 50 ng genomic DNA, 1  $\times$  PCR buffer, 2 mmol/L  $\text{MgCl}_2$ , 0.2 mmol/L of each dNTP, 1  $\mu\text{mol/L}$  of each primer, and 0.25 U Taq DNA polymerase (Invitrogen Corporation, Carlsbad, CA). Cycling conditions consisted of an initial denaturation step at  $94^{\circ}\text{C}$  for 5 minutes, followed by 35 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 seconds, annealing at  $60^{\circ}\text{C}$  for 30 seconds, and extension at  $72^{\circ}\text{C}$  for 30 seconds, with a final elongation step at  $72^{\circ}\text{C}$  for 1 minute. Polymerase chain reaction products were digested with 2 U *NcoI* restriction enzyme at  $37^{\circ}\text{C}$ , in line with manufacturer instructions (New England BioLabs, Ipswich, MA).

### Statistical analysis

Data are statistically presented in terms of mean  $\pm$  standard deviation (SD) or frequencies (number of cases) and percentages, as required depending on their distribution. Hardy-Weinberg equilibrium (HWE) was assessed for each vari-



**Figure 1.** Comparison of serum levels of IL-12 in ONFH patient group under the genotypes of rs3212227 polymorphisms. AC genotype of rs10504813 in patients (n = 127) and AA and CC genotypes of rs3212227 in patients with ONFH (n = 121).

ant to identify the deviation. Differences of the genotypes and alleles of detected genes between patients and normal controls were evaluated using Pearson's Chi-squared test. Exact test was used instead when the expected frequency was less than 5. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated. Unpaired Student's t-test or Mann-Whitney U-tests were used for comparisons between two groups. Statistical analysis of data was performed using SPSS software package 18.0 (SPSS Inc. USA). *P*-values less than 0.05 indicate statistical significance.

### Results

In this study, 248 steroid-induced ONFH patients (108 males and 140 females) and 226 controls (112 males and 114 females) with non-steroid-induced ONFH were screened for rs7412 C/T and rs429358 T/C polymorphisms using PCR-RFLP methods. No statistically significant differences were observed in the distribution of age, sex, body mass index (BMI), GLU, and HDL-C between the two groups (**Table 1**). However, there were significant differences in TG, TC, and LDL-C between these two groups.

First, frequencies of genotypes and alleles of IL-6 rs1800795, IL-12 rs3212227, and TNF- $\alpha$

rs1800629 were detected in case and control groups. HWE of rs1800795, rs3212227, and rs1800629 in patients and controls are listed in **Table 2**. Results showed that allelic distribution of detected SNPs were not deviated from HWE in both case and control populations. IL-6 rs1800795 in the study population were as follows: 7.4% CC, 33.5% CG, and 59.1% GG for the case study group and 4.9% CC, 30.4% GC, and 64.7% GG for the controls, indicating that genotype distributions were similar between case and control groups. Moreover, genomic analysis did not reveal differences between ONFH patients and healthy controls in allelic frequencies at the -174 position for the IL-6 gene promoter. Similarly, genotypic and allelic frequencies of rs1800629 did not show any significant differences between ONFH and normal controls. Next, genotypic and allelic frequencies of rs1800629 were detected in ONFH patients and normal controls (**Table 2**). Genotypic and allelic frequencies of rs1800629 between cases and healthy controls did not show significant differences.

In contrast, the frequency of wild (AA) and homozygous mutant (CC) genotype IL-12 rs3212227 genotypes was found more in controls (43% and 5.7% respectively), but that of the heterozygous genotype was higher (60.15%) in cases with ONFH patients. Significant risk of ONFH was observed for AC (OR = 1.74, 95% CI = 1.10-2.41, *p* = 0.025) genotype of IL-12. Genomic analysis did not reveal any differences in allelic frequencies of the IL-12 (A/C) gene between ONFH patients and healthy controls. Furthermore, results revealed that, among ONFH patients (**Figure 1**), rs10504813 AC genotypes exhibited significantly higher IL-12 serum levels than AA and TT genotypes ( $53.21 \pm 1.63$  vs  $49.43 \pm 1.52$ , *P* < 0.001).

### Discussion

Steroids exhibit diverse activities in multiple organs and hypercortisolism may cause various disorders, including ONFH, a common complication induced by high dose administration of steroids. The pathogenesis seems to be multifactorial and is still unclear. Recent studies have demonstrated that both hypo-fibrinolysis and corresponding abnormal lipid metabolism are major features in the development of steroid-induced ONFH [22, 23]. Genetic variations of genes implicated in these pathophysiological processes have been hypothesized to be asso-

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ciated with steroid-induced ONFH [24, 25]. Gene single nucleotide polymorphisms (SNP) have been thought to alter the expression or influence certain genes. Thus, SNPs could be associated with altered risks of multiple diseases [26-34]. The important roles of pro-inflammatory cytokines during tumor development and prognosis have increasingly gained interest. Several lines of evidence point to the involvement of ApoE in pathogenesis of ONFH [35-40].

Experimental and clinical data have indicated that brain expression and plasma and cerebrospinal fluid levels of IL-6 may affect plaque formation, cognitive decline, or dementia both in cross-sectional and longitudinal follow-up studies [41]. The present study did not find any evidence of an association between IL-6 (-174 C/G) polymorphisms and ONFH in the China population sample. The distribution of the studied polymorphism was in accord with that observed in countries at the same geographic latitude, but different when geographical longitude was considered. Previous studies (12 case-control and 2 prospective studies) assessing the connection between IL-6 polymorphisms and risk of ONFH have brought equivocal results. Faltraco et al. reported a risk reducing association of IL-6 C allele in ONFH. Yamaguchi et al. found that G/G polymorphisms were associated with increased risk of ONFH [18]. In other studies regarding Italian populations, IL-6 C allele increased the risk of ONFH, C/C genotype increased the risk of ONFH in women, and the G/G genotype was lower in ONFH than in healthy controls [42].

IL-12 is an important antitumor cytokine that plays important role in the development and progression of cancer. Variations in the DNA sequence lead to altered IL-12 production. This can alter an individual's susceptibility to cancer. The IL-12 3'UTR A > C polymorphism is a functionally important SNP that alters IL-12 production. It has been reported as a potential biomarker for risk of numerous diseases, such as hepatitis, psoriasis, Barrett's esophagus, asthma, and arteritis. More importantly, genetic variations in IL-12 were revealed to affect susceptibility to multiple sclerosis, another neurodegenerative disease with evident inflammatory responses. The potential roles of IL-12 in ONFH pathogenesis, as well as the involvement of

IL-12 polymorphisms, have been involved in the predisposition to many inflammatory diseases. In the present study, increased frequencies of IL-12 rs3212227 AA and CC homozygous genotype was seen among controls, but that of the heterozygous AC genotype was higher in cases with ONFH. Thus, a significant risk of ONFH was observed for AC genotype of IL-12 rs3212227, consistent with the results of Yuan [19].

TNF- $\alpha$  gene is located in the class III region of the human major histocompatibility complex (MHC) on chromosome 6p21 [21]. Among several single nucleotide polymorphisms (SNPs) identified in TNF- $\alpha$ , TNF- $\alpha$  rs1800629 has been the most extensively studied. The A allele of this polymorphism can lead to high binding affinity of nuclear factors to the TNF promoter, resulting in a high level of transcription activity and secretion levels of TNF- $\alpha$ . Therefore, it was suggested to have a significant functional effect [19]. A variety of SNPs located in the promoter region of TNF- $\alpha$  genes have been investigated in ONFH patients by different groups, with contrasting results [43]. Thus, the present study aimed to better define the roles of TNF- $\alpha$  polymorphisms in ONFH. Present results suggested that genotypic distributions of TNF- $\alpha$  rs1800629 were almost the same in the cases and control groups. TNF gene polymorphisms and the risk of digestive system cancers have been long discussed and many previous studies have been reported. TNF- $\alpha$  gene on chromosome 6p21.3 encoding. TNF and epidermal growth factor (EGF) are well-known stimuli of cyclooxygenase (COX)-2 expression and TNF stimulates transactivation of EGF receptor (EGFR) signaling to promote survival in colon epithelial cells [20]. TNF- $\alpha$  308 promoter polymorphism is a biallelic G to A polymorphism and the TNF- $\alpha$  A allele has been associated with increased levels of TNF in plasma. Although studies have reported that TNF can modify the risk of ONFH, the exact roles of TNF as a gastric carcinogen remain controversial. The present study investigated the association between TNF polymorphisms and susceptibility to ONFH in a Chinese Han population.

### Conclusion

Present results indicate a lack of association between pro-inflammatory cytokine SNPs and steroid-induced ONFH in a Chinese Han popula-

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tion sample, suggesting that genetic, clinical, and population heterogeneity are probably responsible for contradictory results in association studies.

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## Disclosure of conflict of interest

None.

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