Int J Clin Exp Pathol 2015;8(3):3215-3219
www.ijcep.com /ISSN:1936-2625/IJCEP0004959

Original Article

Combined effect of *tnf-α* polymorphisms and hypoxia on steroid-induced osteonecrosis of femoral head

Yaosheng Liu[1*], Weihao Jiang[1*], Shubin Liu[1], Xiuyun Su[1], Shiguo Zhou[2]

[1]*Department of Orthopedics, 307 Hospital of PLA, 8 Fengtaidongda Road, Beijing, People's Republic of China;* [2]*Statistics Room, Capital Medical University Affiliated Beijing Friendship Hospital, 95 Xuanwu District Yongan Road, Beijing, People's Republic of China.* [*]*Equal contributors.*

Received December 17, 2014; Accepted February 20, 2015; Epub March 1, 2015; Published March 15, 2015

Abstract: Objective: Tumor necrosis factor (TNF)-α is a proinflammatory cytokine, some studies reported that *TNF-α* gene plays important role in the pathogenesis of SONFH. And the polymorphisms of *TNF-α* were presented as risk factors for steroid-induced osteonecrosis of the femoral head (SONFH). Meanwhile, various environment factors involve in the pathogenesis of SONFH. Our study aimed to investigate the interaction effect of *TNF-α* polymorphisms and hypoxia factor on SONFH. Methods: 120 patients with SONFH and 100 healthy people, matched with the cases on age and sex, participated in this study. DNA was extracted from all participants. According to previous studies, genotyping of *TNF-α* polymorphisms (rs1800629, rs1799964 and rs1800630) was tested with the method of PCR-RDB (Reverse Dot Blot). Environmental factors were also chose. Logistic regression analysis was used to analyze the interaction between *TNF-α* polymorphisms and environment factors on SONFH. Results: The polymorphisms of rs1800629 and rs1800630 were significantly associated with SONFH (OR: 3.70, 9.93). Patients with hypoxia history were found higher (65.00%) compared with the healthy controls (43.00%). For the person with hypoxic history, GG and AG+AA genotypes of rs1800629 could increase their risk to suffer SONFH (OR: 2.12, 3.78). If the patients with the variant genotypes of rs1800630 experienced hypoxia state, then the risk for SONFH increased 2.41 folds. Conclusion: We concluded that the onset of SONFH was influenced by *TNF-α* and hypoxia history. There existed strong interaction between *TNF-α* and hypoxia history.

Keywords: *TNF-α*, polymorphism, hypoxia, steroid, osteonecrosis of the femoral head

Introduction

Osteonecrosis of the femoral head (ONFH) involves the pathological process of blood supply damage or bone cells death that is evoked by various factors. The disease can be divided into traumatic and non traumatic ONFH clinically. And it is generally believed that the disease is an irreversible process [1, 2]. Steroid-induced osteonecrosis of the femoral head (SONFH), a non-traumatic ONFH, is often brought about by a long-term or high-dose usage of adrenocortical hormone that is beyond the physical needs [3-6]. For the mechanism of SONFH, Weinstein et al. found that over-dose steroid could induce apoptosis of osteoblast and osteocyte, thus decrease the number of osteocyte [7]. Shibahara et al. suggested that there were mass apoptosis cells in necrotic zone and the apoptosis of osteocyte resulted in the osteonecrosis and the destruction of bone structure [8].

Apoptosis is regulated by two signaling pathways, one controlled by the TNF receptor family and the other by Bcl-2 family. As an important inflammatory factor, *TNF-α* gene was proven to associate with osteoclasts proliferation and maturation [9, 10]. In recent years, the effects of the genetic polymorphisms which exist widely in human tissues on the diseases increasingly drew the attention of the scientists and there were many researches involving the association of SNP and ONFH [11-15]. However, there are few studies focusing on the association of *TNF-α* polymorphisms and SONFH susceptibility. In addition, SONFH is also influenced by environmental factors. Guo et al. found that the age of the patients played a certain role in the incidence of ONFH [16]. Moreover, Zou et al. reported that hypoxia could enhance glucocor-

Table 1. Correlation between *TNF-α* polymorphisms and SONFH

Genotype/ allele	Case (%)	Control (%)	X^2	*P* value	OR (95% CI)
rs1800629					
GG	75 (62.5)	73 (73.0)	-	-	1
AG	26 (21.7)	22 (22.0)	0.177	0.741	1.15 (0.60-2.21)
AA	19 (15.8)	5 (5.0)	6.764	0.014	3.70 (1.31-10.43)
G	176 (73.3)	168 (84.0)	-	-	1
A	64 (26.7)	32 (16.0)	7.276	0.008	1.91 (1.19-3.07)
rs1799964					
TT	92 (76.7)	67 (67.0)	-	-	1
CT	23 (19.1)	30 (30.0)	3.351	0.067	0.56 (0.30-1.05)
CC	5 (4.2)	3 (3.0)	0.067	0.795	1.21 (0.28-5.26)
T	207(86.2)	164 (82)	-	-	1
C	33 (13.8)	36 (18)	1.490	0.222	0.73 (0.43-1.22)
rs1800630					
CC	54 (45.0)	67 (67.0)	-	-	1
AC	34 (28.3)	29 (29.0)	1.448	0.277	1.46 (0.79-2.68)
AA	32 (26.7)	4 (4.0)	21.942	0.000	9.93 (3.31-29.80)
C	142 (59.2)	163 (81.5)	-	-	1
A	98 (40.8)	37 (18.5)	25.584	0.000	3.04 (1.96-4.72)

ticoid-induced apoptosis in osteoblastic cells [17].

In our paper, we combined the effects of genetic and environmental factors to investigate the interaction of *TNF-α* polymorphisms (rs1800629, rs1799964 and rs1800630) and hypoxia in the pathogenesis of SONFH.

Materials and methods

Subjects

120 diagnosed SONFH patients were enrolled from 307 Hospital of PLA during 2008-2013. The cases included 75 males and 45 females, with the average age of 41.65 (±0.73). The clinical diagnosis and staging were performed according to the International Osteonecrosis Staging Standard made by Association Research Circulation Osseous (ARCO). 100 healthy controls were all unrelated blood donors, including 65 males and 35 females, with the average age of 41.42 (±0.84). The participants were unrelated Han Chinese and they all signed informed contract before the study.

Genomic DNA extraction

5 ml EDTA-Na2 anticoagulant was obtained, nucleated cells were separated with lymphocyte separation liquid and then genomic DNA was extracted using genomic DNA extraction kit.

Amplification of PCR-RDB

Allele specific oligonucleotide (ASO) probes with amino labeling were designed using Primer 5 software (Shanghai SANGON Biotechnology Company). Mark case and serial number were printed on the hybridization filter. And then the filter was put into 10% EDC (1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride), washed by distilled water several times and dried on the filter paper. 1 μl ASO was added on the dried filter. After 15 min, the filter was put into 0.1 mol/L NaOH, then dried for spare.

PCR amplification: The primers were designed with Primer 5 software (Shanghai SANGON Biotechnology Company). The 5' end of the primers was all labeled with Biotin. The primers of rs1800629 were 5'-GAAGTTAGAAGGAAACAGACCACAG-3' (forward), 5'-TGTGGTCTGTTTCCTTCTAACTTCC-3' (reverse); the primers of rs1799964 were 5'-AGAAGATGAAGGAAAAGTCAGGGTC-3' (forward), 5'-GACCCTGACTTTTCCTTCATCTTCT-3' (reverse); the primers of rs1800630 were 5'-TGTAGCGGCTCTGAGGAATGGGTTA-3' (forward), primer 5'-CCTGTAACCCATTCCTCAGAGCCGC-3' (reverse). PCR reaction was 50 μl with 8.0 pmol/L primers, 1 μl DNA, 2ULA Taq enzyme (Invitrogen) and distilled water. The PCR amplification was performed under the following conditions: 3 min 95°C initial denaturation followed by 35 cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 2 min, then 72°C for 5 min. PCR products (3 μL) was detected by 1% agarose gel.

RDB hybridization: The filter combining specific ASO probes were immersed in hybridization solution. Then two tubes of PCR products were added into the solution. After denaturation for 7 min in 100°C water bath, the mixed solution was put in Hybridization Oven (RobbinScientific) for 3-6 h at 42°C. After that, the filter was washed to get rid of the uncombined PCR prod-

Table 2. Analysis of SONFH-related environmental factors

Factors		Case (%)	Control (%)	*P*
Age		41.65±0.73	41.42±0.84	0.836
Sex	Male	75 (62.5)	65 (65.0)	0.779
	Female	45 (37.5)	35 (35.0)	
Hypoxia	No	42 (35.0)	57 (57.0)	0.002**
	Yes	78 (65.0)	43 (43.0)	
Methylprednisolone treatment	No	54 (45.0)	53 (53.0)	0.279
	Yes	66 (55.0)	47 (47.0)	
Leg hurt by electric shocks	No	68 (56.7)	55 (55.0)	0.892
	Yes	52 (43.3)	45 (45.0)	
Drinking	No	56 (46.7)	54 (54.0)	0.343
	Yes	64 (53.3)	46 (46.0)	

**P<0.05.

ucts. At last, the filter was left in 20 ml developing solution for 5-15 min under room temperature. Blue purple spot was recorded as positive result.

Statistics

Hardy-Weinberg equilibrium (HWE) was tested with χ^2 test. T-test was used to evaluate the age differences between cases and controls. The differences of environmental factors between cases and controls were calculated by χ^2 test. The functions of SNPs and environmental factors on SONFH susceptibility were evaluated with logistic regression (OR and 95% CI). The tests were all two-tailed, *P*-value <0.05 were considered statistically significant. All the tests were performed with SPSS 18.0.

Results

Correlation analysis between TNF-α polymorphisms and SONFH

For the polymorphisms (rs1800629, rs1799964 and rs1800630) of *TNF-α,* the genotype distribution in the control group were consistent with HWE (P: 0.07, 0.87 and 0.70). Logistic regression was used to evaluate the functions of each genotype (**Table 1**). The AA genotype frequencies of rs1800629 were found higher in the patients than that of controls (15.8% vs. 5.0%), then we found that the AA genotype and A allele were risk factors for SONFH (OR: 3.70, 1.91). Meanwhile, the AA genotype of rs1800630 increased the risk of SONFH (OR: 9.93). The A allele carriers were more likely to suffer SONFH and the risk was 3.04-folds than that of C allele. The rs1799964 polymorphism had no effects on SONFH.

Environmental factors of SONFH

We analyzed SONFH-related factors including hypoxia history, methylprednisolone treatment, leg hurt by electric shocks and drinking and found that hypoxia history was significantly associated with SONFH susceptibility (*P*=0.002) (**Table 2**). However, other environmental factors had no effects on SONFH in our study.

Interaction between TNF-α gene polymorphisms and hypoxia

Logistic regression was used to estimate the interaction of *TNF-α* gene polymorphisms and hypoxia on SONFH (**Table 3**). We found that there was remarkable interaction between polymorphisms of *TNF-α* and hypoxic history. For patients with hypoxia history, GG and variant genotypes of rs1800629 all could increase the risk to suffer SONFH (OR: 2.12, 3.78). If the patients with the variant genotypes of rs1800630 experienced hypoxic state, then the risk for SONFH increased 2.41 folds (OR: 3.41). There was no interaction of rs1799964 and hypoxia.

Discussion

For the etiology of SONFH, many scientists suggested that osteocyte apoptosis was the main cause. Apoptosis, also known as programme cell death (PCD), was well-organized cell death controlled by genes. It serves as a crucial factor in the occurrence of bone tissue, bone formation and bone remodeling. The signaling pathway related to apoptosis includes cytoplasmic pathway, mitochondrial pathway [18] and endoplasmic reticulum pathway [19, 20]. Multiple factors involve in the regulation of apoptosis.

Tumor necrosis factor (*TNF)* was one of important regulators of apoptosis. The TNF produced by macrophage as *TNF-α,* while that produced by T cell as *TNF-β.* And studies showed that there was much more significant association between *TNF-α* and apoptosis [21]. Of note, *TNF-α* was an important member of cyroplasmic pathway. For the function mechanism, sci-

Table 3. Interaction of *TNF-α* polymorphism and hypoxia

SNP	variable	Case	Control	OR (95% CI)	*P* value
rs1800629					
+	+	34	46	1	
+	-	41	27	2.054 (1.064-3.966)	0.032
-	+	8	11	0.984 (0.357-2.709)	0.791
-	-	37	16	3.129 (1.500-6.526)	0.001
rs1799964					
+	+	1	1	1	
+	-	4	2	2 (0.078-51.593)	0.752
-	+	41	56	0.732 (0.044-12.051)	0.645
-	-	74	41	1.805 (0.110-29.620)	0.890
rs1800630					
+	+	30	49	1	
+	-	24	18	2.178 (1.017-4.664)	0.067
-	+	12	8	2.450 (0.898-6.682)	0.113
-	-	54	25	3.528 (1.829-6.803)	0.000

"+" for wild genotype, "-" for mutant genotype (SNP column); "-" for hypoxia , "+" for no hypoxia (variable column).

entists have reached an agreement that *TNF-α* inhibits collagen synyhesis, AKP activity and osteocalcin synthesis [22, 23]. Moreover, Jilka reported that *TNF-α* improved osteocyte apoptosis in vitro [24]. In addition, the research indicated that *TNF-α* polymorphisms were also correlated with the risk for SONFH [12].

Hyposia was the pathophysiologic foundation of various diseases, which also regulated the genes expression, such as *Bcl-2* and *Bax*. As we all know, *Bcl-2* and *Bax* are important oncogenes that had regulation effects on cell apoptosis. Hypoxia is also the most physiological inducer of *P53* gene, which is remarkably associated with various cancers [25]. The study of Fan suggested that hypoxia-inducible factor could prevent steroid-associated osteonecrosis of the femoral head [26]. The polymorphisms of ACE, a hypoxia-related gene, were associated with steroid-induced ONFH [27].

After exploring the effects of *TNF-α* polymorphisms on SONFH, we considered the joint effects of *TNF-α* and hypoxia. The results indicated that the polymorphisms of rs1800629 and rs1800630 were significantly associated with SONFH (OR: 3.70, 9.93). For the person with hypoxic history, GG and AG+AA genotypes of rs1800629 all could increase their risk to suffer SONFH (OR: 2.12, 3.78). If patients with the variant genotypes of rs1800630 experienced hypoxic state, then the risk for SONFH increased 2.41 folds. So we concluded that there was significant interaction of *TNF-α* and hypoxia on SONFH.

With the limitations of sample size and SNP variety, it is difficult to completely illuminate the interaction of genes and environment factors. At present, there are few studies in this field and more systematic studies with multiple environment factors needs to be conducted, which will contribute to uncover the effects of gene polymorphisms and environmental factors on SONFH.

Acknowledgements

The work is supported by application study of Capital clinical characteristics of China No Z121107001012093.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Shubin Liu, Department of Orthopedics, 307 Hospital of PLA, 8 Fengtaidongda Road, Beijing 100071, China. Tel: 0086-10-66947316; Fax: 0086-10-66947316; E-mail: shubnnliu@126.com

References

[1] Jones JP Jr. Fat embolism, intravascular coagulation, and osteonecrosis. Clin Orthop Relat Res 1993; 294-308.

[2] 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.

[3] Fisher DE and Bickel WH. Corticosteroid-induced avascular necrosis. A clinical study of seventy-seven patients. J Bone Joint Surg Am 1971; 53: 859-873.

[4] Pfeiffer M and Griss P. [Craniocerebral trauma and aseptic osteonecrosis. Steroid-induced sequelae after therapy of brain edema]. Unfallchirurg 1992; 95: 284-287.

[5] Mont MA and Hungerford DS. Non-traumatic avascular necrosis of the femoral head. J Bone Joint Surg Am 1995; 77: 459-474.
[6] Kuribayashi M, Fujioka M, Takahashi KA, Arai Y, Hirata T, Nakajima S, Yoshimura N, Satomi Y, Nishino H, Kondo K, Fukushima W, Hirota Y and Kubo T. Combination analysis of three polymorphisms for predicting the risk for steroid-induced osteonecrosis of the femoral head. J Orthop Sci 2008; 13: 297-303.
[7] Weinstein RS, Jilka RL, Parfitt AM and Manolagas SC. Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. J Clin Invest 1998; 102: 274-282.
[8] Shibahara M, Nishida K, Asahara H, Yoshikawa T, Mitani S, Kondo Y and Inoue H. Increased osteocyte apoptosis during the development of femoral head osteonecrosis in spontaneously hypertensive rats. Acta Med Okayama 2000; 54: 67-74.
[9] Dai CY, Chuang WL, Lee LP, Chen SC, Hou NJ, Lin ZY, Hsieh MY, Wang LY, Chang WY and Yu ML. Associations of tumour necrosis factor alpha promoter polymorphisms at position -308 and -238 with clinical characteristics of chronic hepatitis C. J Viral Hepat 2006; 13: 770-774.
[10] Strand V and Kavanaugh AF. The role of interleukin-1 in bone resorption in rheumatoid arthritis. Rheumatology (Oxford) 2004; 43: iii10-iii16.
[11] Hirata T, Fujioka M, Takahashi KA, Arai Y, Asano T, Ishida M, Kuribayashi M, Akioka K, Okamoto M, Yoshimura N, Satomi Y, Nishino H, Fukushima W, Hirota Y, Nakajima S, Kato S and Kubo T. ApoB C7623T polymorphism predicts risk for steroid-induced osteonecrosis of the femoral head after renal transplantation. J Orthop Sci 2007; 12: 199-206.
[12] Samara S, Kollia P, Dailiana Z, Chassanidis C, Papatheodorou L, Koromila T and Malizos KN. Predictive role of cytokine gene polymorphisms for the development of femoral head osteonecrosis. Dis Markers 2012; 33: 215-221.
[13] Peng KT, Huang KC, Huang TW, Lee YS, Hsu WH, Hsu RW, Ueng SW and Lee MS. Single nucleotide polymorphisms other than factor V Leiden are associated with coagulopathy and osteonecrosis of the femoral head in Chinese patients. PLoS One 2014; 9: e104461.
[14] Shang X, Luo Z, Li X, Hu F, Zhao Q and Zhang W. Meta-analysis of Factor V Leiden G1691A polymorphism and osteonecrosis of femoral head susceptibility. Biomed Rep 2013; 1: 594-598.
[15] Opdecam P, Halleux P, Coutelier L and Blaimont P. [Research methods in stresses appearing in bone after osteosynthesis]. Acta Orthop Belg 1974; 40: 665-681.
[16] Guo KJ, Zhao FC, Guo Y, Li FL, Zhu L and Zheng W. The influence of age, gender and treatment with steroids on the incidence of osteonecrosis of the femoral head during the management of severe acute respiratory syndrome: a retrospective study. Bone Joint J 2014; 96-B: 259-262.
[17] Zou W, Yang S, Zhang T, Sun H, Wang Y, Xue H and Zhou D. Hypoxia enhances glucocorticoid-induced apoptosis and cell cycle arrest via the PI3K/Akt signaling pathway in osteoblastic cells. J Bone Miner Metab 2014; [Epub ahead of print].
[18] Bodmer JL, Schneider P and Tschopp J. The molecular architecture of the TNF superfamily. Trends Biochem Sci 2002; 27: 19-26.
[19] Kaufman RJ. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Dev 1999; 13: 1211-1233.
[20] Patil C and Walter P. Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. Curr Opin Cell Biol 2001; 13: 349-355.
[21] Horowitz SM and Purdon MA. Mechanisms of cellular recruitment in aseptic loosening of prosthetic joint implants. Calcif Tissue Int 1995; 57: 301-305.
[22] Zheng MH, Wood DJ and Papadimitriou JM. What's new in the role of cytokines on osteoblast proliferation and differentiation? Pathol Res Pract 1992; 188: 1104-1121.
[23] Frost A, Jonsson KB, Nilsson O and Ljunggren O. Inflammatory cytokines regulate proliferation of cultured human osteoblasts. Acta Orthop Scand 1997; 68: 91-96.
[24] Jilka RL, Weinstein RS, Bellido T, Parfitt AM and Manolagas SC. Osteoblast programmed cell death (apoptosis): modulation by growth factors and cytokines. J Bone Miner Res 1998; 13: 793-802.
[25] An WG, Kanekal M, Simon MC, Maltepe E, Blagosklonny MV and Neckers LM. Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. Nature 1998; 392: 405-408.
[26] Fan L, Li J, Yu Z, Dang X and Wang K. Hypoxia-inducible factor prolyl hydroxylase inhibitor prevents steroid-associated osteonecrosis of the femoral head in rabbits by promoting angiogenesis and inhibiting apoptosis. PLoS One 2014; 9: e107774.
[27] Hong JM, Kim TH, Kim HJ, Park EK, Yang EK and Kim SY. Genetic association of angiogenesis- and hypoxia-related gene polymorphisms with osteonecrosis of the femoral head. Exp Mol Med 2010; 42: 376-385.