

# Association of SREBP2 gene polymorphisms with the risk of osteonecrosis of the femoral head relates to gene expression and lipid metabolism disorders

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**Abstract.** Although lipid metabolism disorders have been recognized as a primary factor of osteonecrosis of the femoral head (ONFH), the molecular pathogenesis remains unclear. Sterol regulatory element-binding protein 2 (SREBP2) specifically regulates cholesterol and fatty acid metabolism to maintain lipid homeostasis. To explore the roles of the SREBP2 gene in the development of ONFH, the authors analyzed the gene polymorphism and gene expression of three tag single nucleotide polymorphisms of the SREBP2 gene, the serum lipids levels, and their associations with ONFH development in 182 ONFH patients and 179 healthy controls. The results demonstrated that the stage IV proportions of ONFH patients carrying the rs2267439CT or CC genotype were significantly higher and lower than the stage III proportions of ONFH patients ( $P=0.039$ ), respectively. The serum triglyceride, low-density lipoprotein (LDL)-c levels, and LDL-C/high-density lipoprotein (HDL)-C ratio in the ONFH group were significantly increased compared to those in the control group ( $P=0.01$ ,  $P=0.005$ ,  $P=0.0001$ ) while the HDL-C level of ONFH group was remarkably lower than that of the control group ( $P=0.0001$ ). Association analysis further revealed that the LDL-c levels of the rs226744 GG and AG genotype carriers were statistically higher than that of the AA genotype carriers ( $P=0.039$ ,  $P=0.05$ ). These results demonstrated that the gene polymorphism of SREBP2 not only significantly associated with the clinical phenotypes of

ONFH but also closely related to lipid metabolism disorder. The results indicated that SREBP2 gene polymorphism and function may play key roles in the development of ONFH.

## Introduction

Osteonecrosis of the femoral head (ONFH) is a complex disorder caused by interactions between genetic factors and environmental factors (1). It is characterized by a complicated pathological process, is difficult to diagnose in early stage, and eventually requires operation for artificial joint replacement. ONFH incidence has increased in recent decades. It is estimated that 20,000-30,000 new patients in the United States (2) and 150,000 to 200,000 new patients in China are diagnosed with ONFH annually, respectively. It is very important to elaborate the ONFH molecular pathogenesis and to identify its molecular targets to decrease the incidence. The polymorphisms of multiple genes have been suggested to be associated with ONFH risk. These polymorphisms are thought to cause disequilibrium with other genes, affecting ONFH occurrence, or to play a synergistic role in the development of ONFH by interfering with the expression of other genes (3-6). The ONFH patients often occur with a severe lipid metabolism disorder and the adipose accumulation in the impaired bone marrow cavity. Thus, lipid metabolism disorder has generally been regarded as a critical factor in ONFH pathogenesis, but the molecular mechanism of the adipose accumulation remains unclear.

Sterol regulatory element-binding protein 2 (SREBP2), a member of the SREBPs nuclear transcription factor family, binds to the sterol regulator, the promoter/enhancer in the lipid synthetic enzyme gene, to activate the target gene transcription and the genes expression of the cholesterol biosynthesis pathway, playing a key role in lipid homeostasis (7,8). Considering the critical role of the SREBP2 gene in lipid homeostasis, the authors' research selected three Tag SNPs of the SREBP2 gene as molecular targets and investigated their genotypes, haplotypes, the serum SREBP2 expression and lipids level of ONFH patients, and their associations with the clinical phenotypes and the development of ONFH.

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**Key words:** ONFH, SREBP2, gene polymorphism, gene expression, serum SREBP2 level, clinical phenotypes

Table I. Primers and probes of target SNPs of SREBP2 with LDR.

dbSNP name	Primers <sup>a</sup>	Probe <sup>b</sup>	Product size <sup>c</sup>
rs2267439 [C/T]	Sense: AGCAGTTTTATTCAATTAG AAC Antisense: TGTTC AACAGGTACAC AGGTG	TC: TTTTTTTTAAATTGTCCGTATCCTTCTGGA TTCTC TT: TTTTTTTTTTTTAAATTGTCCGTATCCTTCT GGATTCTT TR: P-GTCTGAACATTGCTTATCTGCGTACTT TTTTTT-FAM-	68/C, 71/T
rs1052717 [A/G]	Sense: TGAAGATTGGTTCAGCAA TAGC Antisense: CTCTGTAGCTTGGCAA AGGCAC	TA: TTTTTTTTTTTTTTGAAGGTATTTATTGGG CATCTTGTA TG: TTTTTTTTTTTTTTTTTTGAAGGTATTTATT GGGCATCTTG TG TR: P-TTGATCACAGCACTGTGAATCCAGAT TTTTTTTTTT-FAM-	75/A, 78/G
rs2267443 [A/G]	Sense: AGTATCCAGTATCAACAC TGGT Antisense: ATAGCACTGTCAGAGT GCCACT	TA: AAGCTGCTTTGAGATTATGAGTA TG: TATAAGCTGCTTTGAGATTATGAGTG TR: P-TGTGTCTATAGAACAGGTTGTTT-FAM-	47/A, 51/G

<sup>a</sup>Primers to amplify the gene fragment of target dbSNP site; <sup>b</sup>Probe to discriminate the target SNPs genotype; <sup>c</sup>Product size indicating the size of the product from a ligase reaction for each SNP genotype. SNP, single nucleotide polymorphism; SREBP2, sterol regulatory element-binding protein 2; LDR, ligase detection reaction.

## Materials and methods

**Patients and ethics statement.** A total of 182 unrelated patients with ONFH (126 men, 56 women; age, 53.50±12.63 years) were consecutively enrolled at the Department of Orthopedics of the Second Clinical College of Jilin University (Changchun, China) from March 2014 to June 2015. Patients with ONFH that was caused by direct trauma and patients with ONFH concurrent with cardiovascular diseases (arterial thrombosis and atherosclerosis etc.), congenital diseases, HIV infection, diabetes mellitus or renal dysfunction were excluded. ONFH diagnoses were established by evidence of osteonecrosis using plain radiographs in stages 2, 3 and 4 of the Ficat Classification system (9). According to the detailed disease history and etiological factors of the patients, the ONFH cases were classified into the following subgroups: alcohol-induced (68 cases, 38.42%), idiopathic (67 cases, 37.85%) and steroid-induced osteonecrosis (45 cases, 25.42%). Steroid-induced osteonecrosis was defined by a history of taking a cumulative 2,000 mg prednisolone or an equivalent over 21 days. Alcohol-induced osteonecrosis was defined by the consumption of >900 ml of pure ethanol per week. The disease course of ONFH ranged from 0.5 to 240 months, with an average of 72.45 months. There were 13 cases of stage II ONFH (7.34%), 52 cases of stage III ONFH (29.38%) and 112 cases of stage IV ONFH (63.28%). There were six cases of ONFH patients who failed to undergo the clinical stages or aetiological classification duo to defect plain radiographs or unclear aetiological factors.

In addition, a total of 179 unrelated control subjects (114 men, 65 women; age, 52.58±11.08 yr.) that were age- and sex-matched with the ONFH group were consecutively enrolled at the Health Examination Center of the Second

Clinical College of Jilin University (Changchun, China) from October 2014 to December 2014. Control subjects were defined in the following way: They had no hip pain, their fasting serum blood glucose, triglyceride (TG) and total cholesterol (TC) levels were in the normal reference value range, abdominal ultrasound examination and chest X-ray radiography were normal, and they did not have a presence of cardiovascular or cerebrovascular diseases. All participants were of a Han Chinese population from northeast China.

The study was approved by the Ethics Committee of the Second Clinical College of Jilin University (Changchun, China), and conformed to the current ethical principles of the Declaration of Helsinki. All individuals provided written informed consent for their participation in the study. The authors declare that they have no competing financial interest.

**Genomic DNA extraction and SNP selection.** Genomic DNA was extracted from 2 ml blood using a genomic DNA Extraction kit (DP318; Tiangen Biotech Co., Ltd., Beijing, China) following the manufacturer's protocols. The HapMap database and related literature were used to select tag SNPs in the SREBP2 gene, and the population distributions of the tag SNPs in different countries, nationalities and regions, especially in Asian populations, were analyzed. Three tag SNPs, rs2267439 [C/T], rs1052717 [A/G] and rs2267443 [A/G], in the intron region of the SREBP2 gene were selected as the research targets.

**Genotyping.** The detection primer, probe sequence and product size following ligase reaction of the rs2267439 [C/T], rs1052717 [A/G] and rs2267443 [A/G] SNPs are presented in Table I. PCR reactions were performed in a buffer containing

1 l DNA, 1.5  $\mu$ l MgCl<sub>2</sub>, 0.3  $\mu$ l dNTPs, 0.15  $\mu$ l primer mix, and 0.3  $\mu$ l Taq DNA ligase in a final reaction volume of 15  $\mu$ l (all reagents from Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The reaction mixture was heated to 94°C for three min for denaturation. Then, the sample was subjected to 35 cycles of 94°C for 15 sec, annealing at 54°C for 15 sec and extension at 72°C for 30 sec, followed by a final extension step at 72°C for 5 min. The specific amplified fragments were used in ligase detection reaction (LDR) to identify the mutations associated with the rs2267439 [C/T], rs1052717 [A/G] and rs2267443 [A/G] SNPs.

The LDR assay was performed in a reaction volume of 10  $\mu$ l that contained 3  $\mu$ l PCR product, 1  $\mu$ l 10X ligase reaction buffer, 0.125  $\mu$ l (40 U/ $\mu$ l) Taq DNA ligase and 0.01  $\mu$ l probe (10 p)/each probe; deionized H<sub>2</sub>O was added to a final volume of 10  $\mu$ l. The ligation reaction was performed using a GeneAmp PCR System 9700 (Applied Biosystems Inc.; Thermo Fisher Scientific, Inc.) with the following temperature program: 2 min at 95°C and 30 cycles of 30 sec at 94°C and 3 min at 56°C. The products were analyzed using an ABI PRISM 3730xl DNA sequencer (Applied Biosystems Inc.; Thermo Fisher Scientific, Inc.). Chromas software version 2.6.4 (Technelysium Pty Ltd., South Brisbane, Australia) was used to analyze the sequencing peak chart.

**Serum SREBP2 protein expression.** Serum SOX9 protein expression was detected using an ELISA kit (DRE12665; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) according to the manufacturer's protocols. Briefly, the assay range of SREBP2 was 5-300 pg/ml. The SREBP2 densities of standards wells on the ELISA plates were 300, 200, 100, 50 and 25 pg/ml, respectively. Blank wells [without sample or horseradish peroxidase (HRP)-conjugate reagent] were kept separately. Then, 40  $\mu$ l sample dilution and 10  $\mu$ l serum were added to the testing sample wells 40  $\mu$ l sample dilution and 10  $\mu$ l serum (final dilution of sample, 5-fold). These was incubated for 30 min at 37°C, before being washed with PBS five times. The HRP-conjugate reagent (50  $\mu$ l) was added and was incubated for 30 min at 37°C. Following this, it was washed five times, and Chromogen Solutions A and B (50  $\mu$ l each) were added separately and kept in a dark place for 15 min at 37°C. The Stop Solution (50  $\mu$ l) was added to each well. Within 15 min, the blank well was taken as zero, and absorbance was read at 450 nm using a multi-wavelength microplate reader (Varioskan; Thermo Fisher Scientific, Inc.).

**Statistical analysis.** Shesis software platform (<http://analysis.bio-x.cn/SHEsisMain.htm>) was used to analyze the Hardy-Weinberg equilibrium and haplotypes between the ONFH and control groups. Logistical regression analyses were used to calculated the odds ratios (OR), 95% confidence intervals (CI), and corresponding p-values of each SNP, controlling for age and sex as covariates. The genetic models of dominant, recessive, and codominant were considered, and the genotypes were given codes of 0, 1, and 2; 0, 1, and 1; or 0, 0, and 1 in the codominant, dominant and recessive models, respectively. SPSS software (version, 10.0; SPSS, Inc., Chicago, IL, USA) was used to analyze the differences in serum SOX9 protein expression between the ONFH and control groups and the

Table II. The genotypes and allele frequencies of SREBP2 between ONFH and control groups.

dbSNP ID	Position	Group	Genotype (n)				HWE <sup>a</sup>	MAF	Co-dominants (11 vs. 12 vs. 22) OR (95%CI) P-value	Dominants 12+22 vs. 11 OR (95%CI) P-value	Recessives 22 vs. 11+12 OR (95%CI) P-value	Allele 2 vs. 1 OR (95%CI) P-value
			11	12	22							
rs1052717 (G>A)	Intron 10	Control	GG	GA	AA							
			83	79	17		0.773	0.316	1.312 (0.806-2.135)	1.123 (0.902-1.399)	0.846 (0.577-1.243)	1.066 (0.780-1.457)
			77	90	15		0.110	0.330	0.275	0.300	0.395	0.686
rs2267439 (T>C)	Intron 1	Control	TT	CT	CC							
			59	83	37		0.299	0.439	1.123 (0.764-1.651)	0.974 (0.770-1.234)	0.978 (0.739-1.294)	0.939 (0.699-1.261)
			64	82	36		0.435	0.396	0.554	0.830	0.875	0.939
rs2267443 (G>A)	Intron 10	Control	GG	GA	AA							
			74	85	20		0.548	0.349	0.629 (0.389-1.016)	0.922 (0.740-1.150)	0.860 (0.593-1.248)	0.828 (0.607-1.131)
			85	82	15		0.437	0.308	0.058	0.472	0.472	0.236

<sup>a</sup>P-values of deviation from Hardy-Weinberg equilibrium between the ONFH group and control group. 11, homozygotes for the major allele; 12, heterozygotes; 22, homozygotes for the minor allele. Logistic regression analyses were used for calculations. MAF, minor allele frequencies; HWE, Hardy-Weinberg equilibrium. SNP, single nucleotide polymorphism; CI, confidence interval; OR, odds ratio; SREBP2, sterol regulatory element-binding protein 2; ONFH, osteonecrosis of the femoral head.

Table III. Haplotype frequencies of SREBP2 SNPs between ONFH and control groups.

			Frequency			
Gene dpSNPs	Haplotype		Controls, n (%)	Patients, n (%)	OR (95%CI)	P-value <sup>a</sup>
SREBP2 rs1052717 (A/G) rs2267439 (C/T) rs2267443 (A/G)	Hap1	A-T-A	93.86 (26.2)	90.83 (25.0)	0.936 (0.670-1.307)	0.697
	Hap2	A-T-G	16.64 (4.6)	29.16 (8.0)	1.787 (0.961-3.324)	0.063
	Hap3	G-C-G	153.22 (42.8)	150.08 (41.2)	0.938 (0.698-1.260)	0.670
	Hap4	G-T-A	28.45 (7.9)	17.25 (4.7)	0.576 (0.311-1.068)	0.076
	Hap5	G-T-G	62.06 (17.3)	72.76 (20.0)	1.191 (0.819-1.734)	0.358
	Hap6	G-C-A	1.28 (0.4)	3.91 (1.1)	-	-
	Hap7	A-C-A	1.41 (0.04)	0.01 (0.00)	-	-
	Hap8	A-C-G	1.09 (0.03)	0.00 (0.00)	-	-

SREBP2, sterol regulatory element-binding protein 2; SNPs, single nucleotide polymorphisms; ONFH, osteonecrosis of the femoral head; OR, odds ratio; CI, confidence interval. <sup>a</sup> $\chi^2$  test. Those with a frequency <0.03 were not analyzed.

associations of protein expression with genotypes and clinical phenotypes using the Student's t-test, ANOVA and  $\chi^2$  test, respectively. Shesis software (haplotype analysis) was also used to analyze the correlation of the haplotype with the clinical phenotypes. Data were presented as mean  $\pm$  standard deviation and  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

*The genotypes, allele frequencies and haplotypes of rs1052717 (A/G), rs2267439 (C/T) and rs2267443 (A/G) of the SREBP2 gene.* SREBP2 genotypes and allele frequencies are presented in Table II. Logistical regression analysis indicated that although possessing no statistical significance, the co-dominant model of rs2267443 exhibited a higher tendency ( $P = 0.058$ ). There are eight haplotypes (A-T-A, A-T-G, G-C-G, G-T-A, G-T-G, G-C-A, A-C-A and A-C-G) among rs1052717, rs2267439 and rs2267443. Although the haplotypes between ONFH and control groups failed to show a statistical difference, the frequencies of the A-T-G (8.0%) and G-T-A (4.7%) haplotypes in the ONFH group were higher and lower tendency, respectively, than those of the control group (4.6, 7.9%;  $P = 0.063$ ,  $P = 0.076$ ), as presented in Table III. The other three haplotypes were not statistically analyzed due to low population frequencies.

*Associations of the genotypes and haplotypes of rs1052717, rs2267439 and rs2267443 with the clinical phenotypes of ONFH.* Correlation analysis of the SREBP2 genotypes with the disease course, etiological classification, unilateral or bilateral hip lesions, and clinical staging of ONFH demonstrated that the rs2267439 genotypes demonstrated a significant association with the clinical stages of ONFH. The proportions of stage IV patients carrying the rs2267439CT (50.9%) or CC (13.4%) genotype were significantly higher and lower, respectively, compared with those of stage III patients (32.7, 26.9%,  $P = 0.039$ ), as indicated in Table IV. The correlation analysis between the SREBP2 haplotypes and the clinical stages or the unilateral and bilateral hip lesions

of ONFH patients showed no significant differences, as presented in Tables V and VI.

*Serum levels of TC, TG, high-density lipoprotein (HDL)-c, low-density lipoprotein (LDL)-c, LDL-C/HDL-C ratios and their association with SREBP2 gene polymorphism.* The serum TG, LDL-C levels and LDL-C/HDL-C ratios of the ONFH group were significantly higher than those of the control group ( $P = 0.01$ ,  $P = 0.005$  and  $P = 0.0001$  respectively), while the HDL-C levels in the ONFH group was statistically lower, compared with that of the control group ( $P = 0.0001$ ), as presented in Fig. 1. The correlation analysis between the rs1052717, rs226743 and, rs2267443 genotypes and serum lipid levels indicated that the serum LDL-C levels of ONFH patients carrying the rs2267443 GG or AG genotypes were significantly increased than those of patients carrying the AA genotype ( $P = 0.039$ ,  $P = 0.05$ ), as presented in Fig. 1.

*Serum protein expression of SREBP2 gene and its association with SREBP2 genotypes and the clinical phenotypes of ONFH.* ELISA analysis revealed that serum SREBP2 protein expression was not significantly different between the ONFH and control groups. The correlation analysis between the genotypes and serum SREBP2 protein expression presented no statistical significance, as demonstrated in Fig. 2. The association analysis of the serum SREBP2 protein level with the etiological classification, clinical stages and the unilateral or bilateral hip lesions of ONFH patients also presented no significant differences, as displayed in Fig. 3.

## Discussion

SREBPs are transcription activation factors that regulate endogenous cholesterol and fatty acid synthesis and are expressed in all tissues and organs of that exhibit lipid synthesis (10,11). A previous study demonstrated that OA is a metabolic disease involving the genetic deregulation of lipid and cholesterol metabolism. A genetic association analysis demonstrated a significant association between the 1784G>C mutation of the SREBP2 gene and OA development in a



Table IV. Association of SREBP2 genotypes with the clinical phenotypes of ONFH.

dbSNP	Genotype	$\chi^2$ test		Disease course (months) ANOWY test	Etiological classification, n (%) <sup>a</sup> $\chi^2$ test			Involved lesions scope n (%) $\chi^2$ test		Clinical stage n (%) $\chi^2$ test		
		Men, n (%)	Women, n (%)		Alc <sup>a</sup>	Ster <sup>a</sup>	Idio <sup>a</sup>	Unilateral	Bilateral	II stage	III stage	IV stage
rs1052717	AA	12 (9.5)	3 (5.4)	61.07+71.21	6 (8.8)	3 (6.9)	6 (9.0)	7 (8.9)	8 (7.8)	1 (7.7)	5 (9.6)	8 (7.1)
	GA	66 (52.4)	24 (42.9)	72.24+69.05	31 (45.6)	26 (57.8)	32 (47.8)	42 (53.2)	48 (47.1)	4 (30.8)	24 (46.2)	62 (55.4)
	GG	48 (38.1)	29 (51.8)	75.59+72.77	31 (45.6)	16 (35.6)	29 (43.2)	30 (38.0)	46 (45.1)	8 (61.5)	23 (44.2)	42 (37.5)
	P-value	0.197				0.782		0.438		0.629		
rs2267439	CC	22 (17.5)	14 (25.0)	78.51+74.16	15 (22.1)	6 (13.3)	14 (20.9)	14 (17.7)	21 (20.6)	2 (15.4)	14 (26.9)	15 (13.4)
	CT	60 (47.6)	22 (39.3)	71.92+67.90	30 (44.1)	21 (46.7)	30 (44.8)	33 (41.8)	49 (48.0)	7 (53.8)	17 (32.7)	57 (50.9)
	TT	44 (34.9)	20 (35.7)	69.82+72.66	23 (33.8)	18 (40.0)	23 (34.3)	32 (40.5)	32 (31.4)	4 (30.8)	21 (40.4)	40 (35.7)
	P-value	0.423				0.817		0.444		0.056		
rs2267443	AA	11 (8.7)	4 (7.1)	79.10+74.43	5 (7.4)	5 (11.1)	4 (6.0)	7 (8.9)	8 (8.5)	1 (7.7)	5 (9.6)	8 (7.1)
	AG	58 (46.0)	24 (42.9)	66.52+66.89	26 (38.2)	25 (55.6)	31 (46.3)	37 (46.8)	45 (44.1)	5 (38.5)	24 (46.2)	53 (47.3)
	GG	57 (45.3)	28 (50.0)	67.63+68.47	37 (54.4)	15 (33.3)	32 (47.8)	35 (44.3)	49 (48.0)	7 (53.8)	23 (44.2)	51 (45.5)
	P-value	0.821				0.254		0.877		0.953		

<sup>a</sup> Alc, alcohol-induced; Ster, steroid-induced; Idio, idiopathic; <sup>b</sup>II vs. III vs. IV, P=0.056, III vs. IV, P=0.039, SREBP2, sterol regulatory element-binding protein 2; ONFH, osteonecrosis of the femoral head; SNP, single nucleotide polymorphism.

Table V. The association of SREBP2 haplotypes with the unilateral and bilateral hips lesions of ONFH.

Haplotypes	Unilateral, n (%)	Bilateral, n (%)	$\chi^2$	P-value	OR (95% CI)
SREBP2	40.58 (25.7)	50.15 (24.3)	0.081	0.775	0.933 (0.577-1.506)
	15.41 (9.8)	13.84 (6.7)	1.106	0.293	0.667 (0.313-1.424)
	58.63 (37.1)	91.60 (44.5)	1.556	0.212	1.312 (0.856-2.010)
rs1052717(A/G)	8.05 (5.1)	9.45 (4.6)	0.050	0.823	0.896 (0.342-2.351)
rs2267439(C/T)	32.95 (20.9)	39.56 (19.2)	0.147	0.701	0.904 (0.538-1.517)
rs2267443(A/G)	2.37 (1.5)	1.41 (0.7)	-	-	-
	0.00 (0.0)	0.01 (0.0)	-	-	-
	1.09 (0.03)	0.00 (0.00)	-	-	-

Those with a frequency <0.03 were not analyzed. SREBP2, sterol regulatory element-binding protein 2; ONFH, osteonecrosis of the femoral head; OR, odds ratio; CI, confidence interval.

Table VI. The association of SREBP2 haplotypes with clinical stages of ONFH patients.

Haplotypes		Stage IV, n (%)	Stage III, n (%)	$\chi^2$	P-value	OR (95% CI)
SREBP2	A-T-A	56.54 (25.2)	27.30 (26.3)	0.037	0.846	0.949 (0.557-1.615)
	A-T-G	21.45 (9.6)	6.70 (6.4)	0.895	0.344	1.541 (0.626-3.793)
rs105271(A/G)	G-CG	84.22 (37.6)	43.76 (42.1)	0.598	0.439	0.829 (0.515-1.334)
rs2267439(C/T)	G-T-A	9.68 (4.3)	5.46 (5.2)	0.137	0.711	0.816 (0.278-2.394)
rs2267443(A/G)	G-T-G	49.32 (22.0)	19.54 (18.8)	0.451	0.502	1.222 (0.680-2.195)
	G-C-A	2.77 (1.2)	1.24 (1.2)	-	-	-
	A-C-A	0.01 (0.0)	0.00 (0.0)	-	-	-
	A-C-G	1.09 (0.03)	0.00 (0.00)	-	-	-

All those with a frequency <0.03 were not analyzed. SREBP2, sterol regulatory element-binding protein 2; ONFH, osteonecrosis of the femoral head; OR, odds ratio; CI, confidence interval.

cohort of 1,410 Greek OA patients and healthy controls (12). A study in a Chinese Han population also demonstrated that the SREBP-2 rs2228314 polymorphism was closely associated with increased risk of knee OA (13).

Few reports have shown an association between SREBP-2 polymorphism and ONFH risk. In 2008, Kim *et al* (14) reported the protective effect of the SREBP-2 rs2267439 C allele against ONFH risk and the significant associations of rs1052717 and rs2267443 with increased ONFH risk. In 2012, the authors confirmed that the rs2267439TT and rs2267443GA genotype of the SREBP2 gene were associated with increased ONFH risk, while rs2267439 C allele frequency was associated with decreased ONFH risk (15). Lipid metabolism disorder has been recognized as a main factor in ONFH pathogenesis. The results of clinical and animal experiments have demonstrated that steroid- and alcohol-induced ONFH are associated with lipid metabolism disorder (16,17). Considering both the critical effects of SREBP2 on lipid homeostasis and the role of lipid metabolism disorder in the development of ONFH, the authors conducted the genotype and haplotype analyses of rs1052717, rs2267439 and rs2267443 of the SREBP2 gene in 182 ONFH patients and 179 controls. The results revealed that, although no statistical significance, the A-T-G haplotype frequency of the ONFH group was lower tendency while the

G-T-A haplotype frequency of the ONFH group was higher tendency than that of the control group, indicating that the A-T-G haplotype may be associated with a lower risk of ONFH and the G-T-A haplotype might be associated with a higher risk of ONFH. In the expanded sample system, the roles of A-T-G and G-T-A haplotypes in the development of ONFH remain to be deeply investigated.

To further explore the association of SREBP2 polymorphism with ONFH risk, conducted correlation analyses were conducted between the genotypes and haplotypes of the SREBP2 gene and the clinical phenotypes of ONFH. The results demonstrated that the SREBP2 rs2267439 genotypes were significantly associated with the clinical stages of ONFH; the frequencies of stage IV patients carrying the homozygous CC genotype and heterozygous CT genotype of rs2267439 were significantly lower and higher, respectively, than those of stage III patients, suggesting that the CC genotype may be a protective genotype against ONFH hip lesion development, while the CT genotype may indicate a higher risk of ONFH hip lesion development. To investigate the effects of SREBP2 polymorphisms on gene expression, the authors examined serum SREBP2 protein expression and no significant difference was identified between the ONFH and control groups. As a key transcription factor, SREBP2 gene expression is

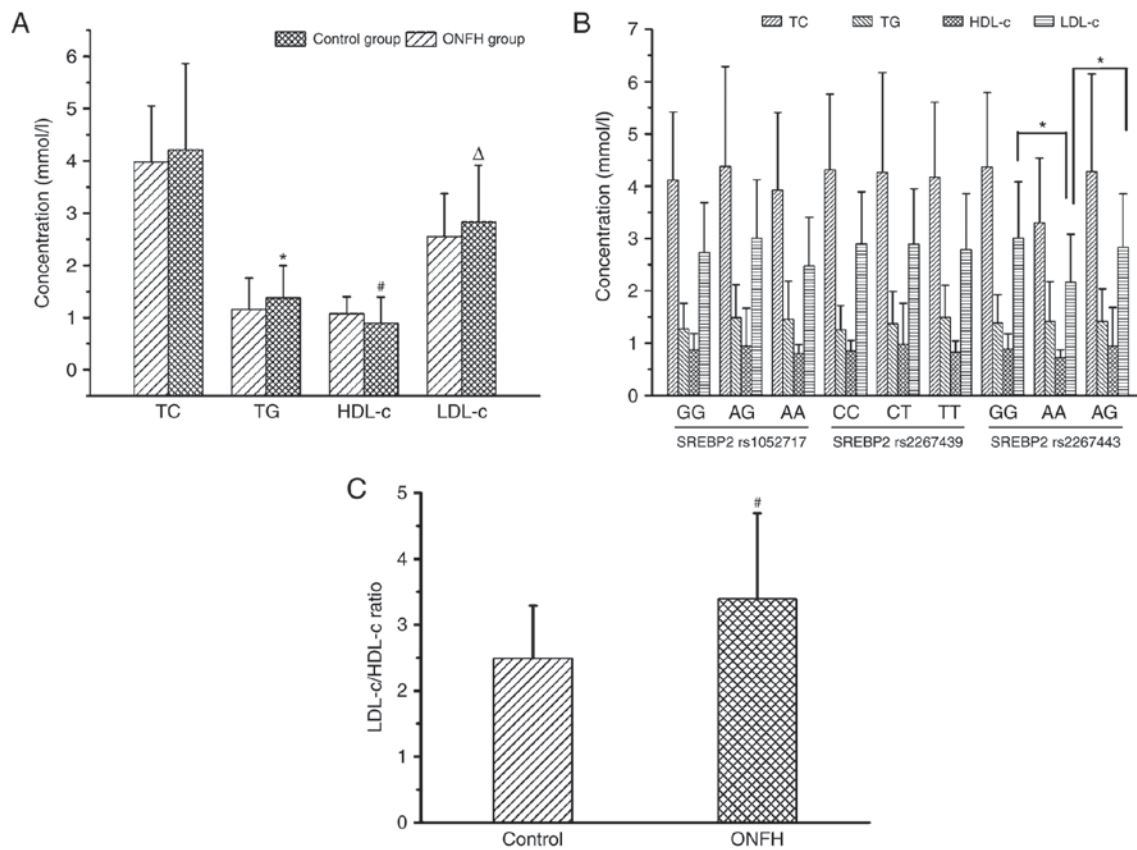


Figure 1. The serum lipid levels between the ONFH and control groups, and the association of SREBP2 genotypes with the serum lipid levels of ONFH patients. (A) The serum levels of TC, TG, HDL-c, and, LDL-c between the ONFH and control groups. \* $P < 0.01$ ,  $^{\Delta}P < 0.005$ ,  $^{\#}P < 0.0001$  vs. control. (B) The association of SREBP2 genotypes with the serum lipid levels of the ONFH group. \* $P < 0.05$  as indicated. (C) The LDL-c/HDL-c ratio between the ONFH and control groups.  $^{\#}P < 0.0001$  vs. control. Data are presented as mean  $\pm$  standard deviation. ONFH, osteonecrosis of the femoral head; SREBP2, sterol regulatory element-binding protein 2; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

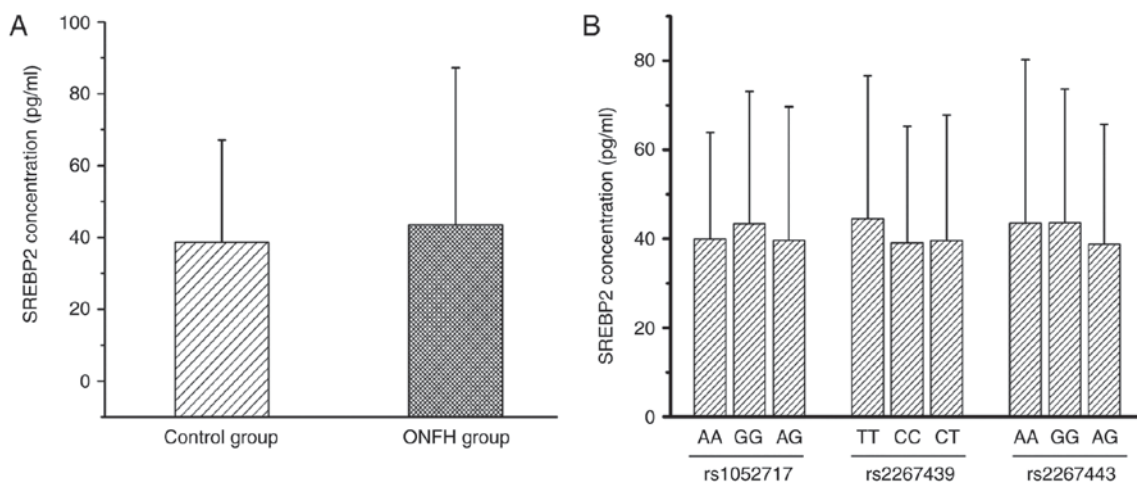


Figure 2. Serum SREBP2 protein expression and the association of protein expression with SREBP2 genotypes in ONFH patients. (A) The serum protein expression of SREBP2 gene between the ONFH and control groups. (B) The association of SREBP2 genotypes with protein expression in ONFH patients. Data are presented as mean  $\pm$  standard deviation. SREBP2, sterol regulatory element-binding protein 2; ONFH, osteonecrosis of the femoral head.

affected by multiple signaling pathways and related genes. The polymorphism of a single minor gene might not be enough to cause obvious changes in serum SREBP2 protein expression. Moreover, the three tag SNPs examined in the present study are located in the intron region of the SREBP2 gene, and the polymorphism effects of SNPs in the intron region on gene

expression are much less than those of SNPs in the promoter, 3'-untranslated region, and open reading frame regions.

Lipid metabolism disorders have been recognized as a primary factor in ONFH pathogenesis. However, the association between SREBP2 polymorphism and the lipid metabolism disorder of ONFH has never been reported. Considering the

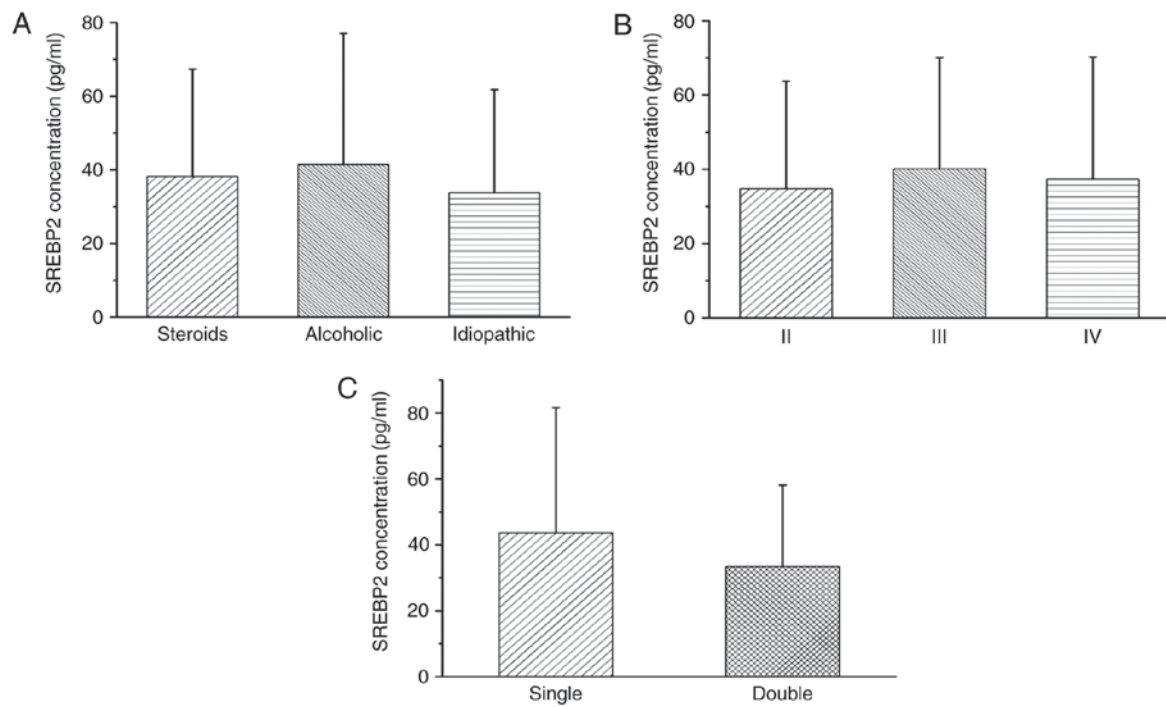


Figure 3. The association of protein expression of the SREBP2 gene with the clinical phenotypes of ONFH. (A) The association of the protein expression of the SREBP2 gene with the clinical etiological classification of ONFH. (B) The association of the protein expression of the SREBP2 gene with the clinical stages of ONFH. (C) The association of the protein expression of the SREBP2 gene with unilateral or bilateral hip lesions of ONFH. Data are presented as mean  $\pm$  standard deviation. SREBP2, sterol regulatory element-binding protein 2; ONFH, osteonecrosis of the femoral head.

important role of SREBP2 in the cholesterol synthesis and lipid metabolism, the authors first conducted the simultaneous detection of SREBP2 gene polymorphism and gene expression and ONFH lipid metabolism disorder. The results showed that ONFH lipid metabolism disorder was characterized by an increase in serum TG and LDL-c levels and the LDL-c/HDL-c ratio and a decrease in HDL-c levels. Furthermore, the serum LDL-c levels of rs2267443 GG or AG genotype carriers were significantly higher than those of AA genotype carriers, suggesting that SREBP2 polymorphism is associated with the lipid metabolism disorder of ONFH.

Excess glucocorticoids lead to an obvious increase of serum lipid levels and the necrosis of the femoral head (18). In an experiment animal model of ONFH induced by excess glucocorticoids, lipid droplets were observed in the bone cells concurrently with increased serum lipids (19). A study of 240 ONFH patient-control systems showed that the serum adiponectin levels of ONFH patients were significantly lower than those of the healthy control subjects. Moreover, serum adiponectin levels positively associated with HDL-c levels and negatively associated with body mass index, TG levels, and the LDL/HDL ratio (20). Therefore, the serum adiponectin level was considered to be an independent predictor of ONFH risk. The pravastatin has been shown to prevent experimental ONFH induced by steroids, but the exact mechanism is unclear. A recent study using a rat ONFH model further demonstrated that pravastatin downregulated the expression of peroxisome proliferator activated receptor  $\gamma$ , a key transcription factors of adipogenic differentiation, and upregulated the expression of wingless-type MMTV integration site family 3A, LDL receptor related protein 5, beta catenin and Runt-related transcription factor 2 genes on the

signaling pathway of osteogenic differentiation at the mRNA and protein levels and prevented ONFH induction by steroids, providing the molecular evidence of lipid metabolism disorders affecting bone metabolism (21).

Bone marrow stromal cells (BMSCs) are a common precursor of osteoblasts and adipocytes. Using an *in vivo* diffusion chamber assay, preadipocytes from BMSCs enter an activated proliferation stage and differentiate into osteoblasts (22). The study using lithium chloride (LiCl) to reverse the abnormal adipogenic and osteoblastic differentiation of ONFH showed that LiCl significantly increased the expression of osteocalcin and Runx2 and enhanced osteogenic mineralization during the osteogenic induction process and also significantly reduced the formation of lipid droplets in the cells and the antagonism of adipogenesis by inhibiting PPAR $\gamma$  and fatty acid binding protein 4 expression in BMSCs during the induced adipogenic differentiation process (23). These results demonstrated that the lipid metabolism disorder serve critical roles in the development of ONFH. The present results further confirmed the lipid metabolism disorder of ONFH as well as the lipid metabolism disorder significantly associated with the SREBP2 genotypes, first suggesting the evidence SREBP2 gene polymorphisms intruding in the lipid metabolism disorder of ONFH. The complicated effects of SREBP2 on lipid homeostasis relate to the molecular functions of multiple signaling pathways. The detailed roles of SREBP2 gene in the lipid metabolism disorder of ONFH need to be elucidated.

In conclusion, the authors simultaneously showed the gene polymorphism and gene expression of SREBP2, as well as the lipid metabolism disorder in 182 ONFH patients and 179 healthy controls and their association with the



clinical phenotypes and development of ONFH. The results demonstrated that SREBP2 gene polymorphisms are closely associated with lipid metabolism disorder and the development of ONFH.

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