

Combination Analysis of *NR3C1*, *MTHFR* and *IGFBP3* Gene Polymorphisms and DNA Methylation With Steroid-induced Osteonecrosis of the Femoral Head Risk in Chinese Han Population

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Abstract

Background: Although the precise etiology of osteonecrosis of the femoral head (ONFH) has yet to be fully elucidated, it is known that nuclear receptor subfamily3, group C, member 1 (*NR3C1*), 5, 10-methylenetetrahydrofolate reductase (*MTHFR*) and insulin-like growth factor-binding protein 3 (*IGFBP3*) are related to the pathophysiology of steroid-induced osteonecrosis of the femoral head (SONFH). The expression of *NR3C1*, *MTHFR* and *IGFBP3* are regulated by epigenetics and genetic profiles.

Objective: The primary objective of this study was to investigated the association between *NR3C1*, *MTHFR* and *IGFBP3* gene polymorphisms and DNA methylation status and SONFH.

Methods: This case-control study included 79 patients with SONFH and 114 patients who took steroids but did not develop SONFH. We evaluated 5 single-nucleotide polymorphisms (SNPs) out of 3 genes in Chinese Han population. These SNPs were genotyped by improved multiplex ligation detection reaction (iMLDR). Methyltarget was used to test the methylation level of positive sites, the interaction between SNPs and DNA methylation level was analyzed using eQTLD technique.

Results: We identified rs3110697 in the *IGFBP3* gene that was potentially associated with a reduced risk of SONFH in the genotype ($P=0.008$; odds ratio [OR]: 0.741; 95% confidence intervals [CI]: 0.456–1.205) and in the recessive model ($P=0.003$; OR: NA; 95% CI: NA–NA).

Furthermore, CpG sites with significant differences in methylation levels were screened as follows: *IGFBP3_2-143*, *MTHFR_1-36*, *MTHFR_1-77*, *MTHFR_1-139*, *MTHFR_2-42*, *NR3C1_2-163*, *NR3C1_4-47*, and the differences were statistically significant compared with the control group ($p<0.05$). A total of 10 pairs of linear regression tests of SNP and methylation sites were statistically significant ($p<0.05$).

Conclusions: SONFH is a polygenic disorder in which a wide range of interactions between SNPs and DNA methylation levels may dominate the course of the disease.

Introduction

Although osteonecrosis of the femoral head (ONFH) is an orthopedic disease, it is also a complication of multiple disciplines. Due to the interruption of blood supply to the femoral head, bone cell apoptosis and changes in the microstructure of the femoral head were induced, resulting in the presence of femoral head pain and progressive aggravation. Clinical and epidemiological studies have identified excessive alcohol consumption and the use of high-dose corticosteroids as the two most important causes of non-traumatic ONFH (Cui et al. 2016). In recent years, association studies based on candidate genes have successfully located susceptibility to many complex diseases (Uitto 2019; Wan 2018). Some genetic background has been shown to be associated with ONFH in previous studies. In clinical practice, we can find that not all patients with steroid hormones use develop SONFH, suggesting that genetic factors may influence SONFH susceptibility or drug resistance (Hadjigeorgiou et al. 2008). In addition to gene sequence differences, gene expression and function are also regulated by epigenetic regulation, and DNA methylation is one of the most in-depth epigenetic modifications. Studying the genetic etiology of SONFH will help us to understand the biological and physiological mechanisms of SONFH and provide a basis for individualized treatment of SONFH.

Current evidence suggests that 5 SNPs of the 3 genes we studied are associated with SONFH and can be identified by genome-wide association studies. Nuclear receptor subfamily3, group C, member 1 (*NR3C1*) is an important transcriptional regulator that mediates the role of glucocorticoids. There are many mutations and polymorphisms, which affect the sensitivity of individuals to exogenous glucocorticoids (Nordkap et al. 2017). BCI1 (rs41423247) polymorphism of *NR3C1* gene is a relatively common polymorphism variant and has been reported to be associated with hypertension and increased glucocorticoid sensitivity (Ukkola et al. 2001; Rosmond et al. 2000). *NR3C1* rs6196 A allele carriers had a higher risk of steroid resistance than G allele carriers. Compared with G allele, the presence of rs10052957 and rs258751 A alleles reduced the incidence of steroid resistance (Liu et al. 2018). In humans, *NR3C1* DNA methylation has been linked to depression, anxiety, post-traumatic stress disorder, bulimia nervosa, and borderline personality disorder (Monk et al. 2016; Yehuda et al. 2014; Steiger et al. 2013; Dammann et al. 2011). 5, 10-methylenetetrahydrofolate reductase (*MTHFR*) gene is located on chromosome 1 (1p36.3). *MTHFR* is an enzyme that provides the methyl group to homocysteine and converts it into methionine. Mutations in the *MTHFR* gene significantly reduce the activity of the enzyme, resulting in elevated plasma homocysteine levels. Homocysteine can lead to vascular endothelial injury, thereby proliferating vascular endothelial cells, enhancing the coagulation activity of vascular walls, and leading to the formation of microvascular thrombosis (Chai et al. 2015). In addition, homocysteine directly damages bone tissue by enhancing osteoclast production and activity (Koh et al. 2006). The 677C > T (rs1801133) variant of the *MTHFR* gene is closely associated with elevated plasma homocysteine levels (Wang et al. 2018). It has been reported that plasma homocysteine levels in patients with TT genotype were significantly higher in patients with rs1801133 CC genotype than in patients with rs1801133 CC genotype. Higher homocysteine concentrations may hinder collagen synthesis, leading to decreased bone mass and an increased risk of ONFH (Frosst et al. 1995). The results of most studies confirmed the association between elevated homocysteine and rs1801133 polymorphism and hypo-methylation (Mandaviya et al. 2014). Insulin-like growth factor-binding protein 3 (*IGFBP3*) is the main carrier of serum insulin-like growth factor-1 (IGF1). *IGFBP3* inhibits IGF1 by

competitive binding with ligands. IGF1 regulates the proliferation and differentiation of osteoblast through autocrine and paracrine (Feng et al. 2014). Norihiro Kato et al. (2015) proved that the SNPs of *IGFBP3* gene affecting blood pressure were related to the methylation of multiple CpG sites. It further suggests that DNA methylation may play a broader role in associating common genetic variations with multiple phenotypes.

A few studies have investigated *NR3C1*, *MTHFR* and *IGFBP3* gene polymorphisms and DNA methylation with the pathogenesis of SONFH. We hypothesized that *NR3C1*, *MTHFR* and *IGFBP3* genes play a key role in the occurrence of SONFH, and explored the relationship between the polymorphism and DNA methylation of these genes and SONFH in Chinese Han population.

Materials And Methods

Study participants

The 79 SONFH patients were derived from the Affiliated Hospital of the Institute of Neurology of Anhui University of Chinese Medicine and the First Affiliated Hospital of the Anhui University of Traditional Chinese Medicine from July 2017 to February 2019. These SONFH patients were treated with standard steroids after being diagnosed with Devic's disease, chronic inflammatory demyelinating polyneuropathy (CIDP), myasthenia gravis, or rheumatoid arthritis. Patients with traumatic ONFH, other hip joint disorders, and patients not conforming to the diagnosis of SONFH were excluded.

According to the medical examination of the Affiliated Hospital of the Institute of Neurology of Anhui University of Chinese Medicine, we recruited 114 patients who had not suffered from femoral head necrosis for more than 3 years after 3 months of oral steroid hormone (prednisone > 30 mg/d) treatment as the control group from June 2017 to February 2019. All participants were limited to the Han Chinese population living in and around Hefei. This study protocol was approved by the ethics committee of the Affiliated Hospital of the Institute of Neurology of Anhui University of Chinese Medicine and obtained the written informed consent of all participants or their guardians.

Genotyping

The genomic DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin, USA) as per the product instruction. The SNP genotyping work was performed using an improved multiplex ligation detection reaction (iMLDR) technique developed by Genesky Biotechnologies Inc. (Shanghai, China). A multiplex PCR-ligase detection reaction method was used in the iMLDR. For each SNP, the alleles were distinguished by different fluorescent labels of allele-specific oligonucleotide probe pairs.

CpG Islands Selected

CpG islands located in the proximal promoter of *NR3C1*, *MTHFR* and *IGFBP3* gene were selected for measurement according to the following criteria: (1) 200 bp minimum length; (2) 50% or higher GC content; (3) 0.60 or higher ratio of observed/expected dinucleotides CpG. Finally, six CpG islands regions of *NR3C1*, *MTHFR* and *IGFBP3* gene were selected and sequencing.

Dna Methylation Detection

Bisulfite conversion of 400 ng genomic DNA was conducted using the EZ DNA Methylation™-GOLD Kit (ZYMO RESEARCH, CA, USA). Firstly, the samples with the rate of DNA bisulfite conversion < 98% were filtered out. PCR amplicons of target CpG regions were separated by agarose electrophoresis and purified using QIAquick Gel Extraction kit (QIAGEN, Hilden, Germany), and CpG sites methylation assay was performed using Illumina Hiseq 2500 according to the manufacturer's protocol. The methylation level of the IL12B gene was analyzed by MethylITarget™ (Genesky Biotechnologies Inc., Shanghai, China). The details of the CpG regions are listed in Table 5.

Statistical analysis

SPSS 24.0 (SPSS, Chicago, IL) was used for statistical analysis. The allele distribution was tested for Hardy-Weinberg equilibrium (HWE) by χ^2 . The ORs and 95% CI was determined by unconditional logistic regression analysis. Significant results were defined if p value was less than 0.05. Haplovview (Chicago, IL) (<https://sourceforge.net/projects/haplovview/>) were used to analyze the linkage imbalance of different SNPs within the same genus.

Results

The analysis included 79 cases (34 males, 45 females; mean age 50.65 ± 13.95 years) who were SONFH patients and 114 controls (49 males, 65 females; mean age 42.63 ± 19.27 years) who received steroid therapy but did not develop SONFH. The demographic characteristics of patients and control subjects are illustrated in Table 1.

Table 1
Demographic characteristics of 79 patients and 114 controls

Characteristics	SONFH (n = 79) (mean \pm SD)	Controls (n = 114) (mean \pm SD)	t value	P value
Age	50.65 ± 13.95	42.63 ± 19.27	3.3305	0.001
Gender	Female 45, Male 34	Female 65, Male 49	0.1273	0.8989

We selected 5 SNPs (rs10052957 and rs41423247 of *NR3C1* gene, rs1801133 of *MTHFR* gene, rs2453839 and rs3110697 of *IGFBP3* gene) were genotyped by iMLDR. SNP ID, gene, location, allele, minor allele frequency (MAF) and P value of HWE for 5 SNPs are shown in Table 2. All 5 selected SNPs passed the HWE test. As listed in Table 3. We identified one significant SNP, rs3110697 ($P = 0.008$; OR: 0.7411; 95% CI: 0.456–1.205), as being associated with the disease status of SONFH. The genotype “A/G” of rs3110697 is a protective genotype. Meanwhile, we found that rs3110697 in the *IGFBP3* gene was associated with SONFH as a protective factor in the allele model ($P = 0.003$; OR: NA; 95% CI: NA–NA) (Table 4).

Table 2
Single nucleotide polymorphism information and Hardy Weinberg equilibrium test

SNP ID	Gene	Location(GRCh37)	Allele	MAF	P value of HWE (CHBS_1000 g)
rs10052957	<i>NR3C1</i>	chr5:142786701	A/G	0.115385	1
rs41423247	<i>NR3C1</i>	chr5:142778575	C/G	0.237981	0.2351
rs1801133	<i>MTHFR</i>	chr1:11856378	G/A	0.375	0.5665
rs2453839	<i>IGFBP3</i>	chr7:45953573	C/T	0.216346	0.4604
rs3110697	<i>IGFBP3</i>	chr7:45955029	A/G	0.747596	0.2785

Table 3
Analyses of association of allele and genotype frequency with the risk of SONFH between controls and patients with SONFH

SNP ID	Allele	SONFH	Controls	OR (95%CI)	P value	genotype	SONFH	Controls	OR (95%CI)	P value
rs10052957	A	25	27	1.443(0.802–2.595)	0.221	AA	2	1	1.458(0.802–2.652)	0.377
	G	129	201			AG	21	25		
						GG	54	88		
rs41423247	C	36	56	0.906(0.5616–1.463)	0.687	CC	2	9	0.913(0.577–1.446)	0.946
	G	122	172			CG	26	38		
						GG	48	67		
rs1801133	G	83	108	1.23(0.8191–1.846)	0.319	GG	20	25	1.243(0.818–1.888)	0.524
	A	75	120			GA	43	58		
						AA	16	31		
rs2453839	C	36	65	0.731(0.4567–1.17)	0.191	CC	3	8	0.715(0.439–1.165)	0.428
	T	122	161			CT	30	49		
						TT	46	56		
rs3110697	A	38	67	0.761(0.479–1.209)	0.247	AA	0	11	0.741(0.456–1.205)	0.008*
	G	120	161			AG	38	45		
						GG	41	58		

Table 4
Genetic model analyses of association between controls and patients with SONFH

SNP ID	Recessive Model	SONFH	Controls	OR(95%CI)	P-value	Dominant Model	SONFH	Controls	OR(95%CI)	P-value
rs10052957	AA	2	1	3.013(0.268–33.820)	0.566	AA + AG	22	26	1.281(0.645–2.543)	0.480
	AG + GG	75	113			GG	52	82		
rs41423247	CC	5	9	0.788(0.254–2.448)	0.783	CC + CG	29	46	0.745(0.400–1.388)	0.354
	CG + GG	74	105			GG	47	62		
rs1801133	GG	20	25	1.207(0.615–2.367)	0.607	GG + GA	60	79	1.420(0.692–2.914)	0.339
	GA + AA	59	89			AA	16	29		
rs2453839	CC	3	8	0.518(0.133–2.017)	0.530	CC + CT	32	54	0.717(0.39–1.319)	0.285
	CT + TT	76	105			TT	44	53		
rs3110697	AA	0	11	NA(NA-NA)	0.003*	AA + AG	36	52	1.056(0.576–1.936)	0.861
	AG + GG	79	103			GG	40	56		

Table 5
The position of the two CpG regions and primer sequences

Target	Location(GRCh37)	Length	Primer sequences
IGFBP3_1	chr7::45961336 - 45961138	199	GGAATTAAATTTAGAAAGTTTAYGAGGT
			CACTCCAAACCACCTAACAC
IGFBP3_2	chr7::45960153-45960353	201	TYGGTTGGGGAGGGTTTT
			AACRCCRCCAACTCAAATAAAC
IGFBP3_3	chr7::45960753-45960920	168	GGGGTAYGTTGTTGGTAGGTT
			CCTAAACCACCCRACTTCT
MTHFR_1	chr1::11866211 - 11865947	265	GGATTGAGATTAGGAGTGGTTGT
			TCCAATCCCRAATAACTCAAAC
MTHFR_2	chr1::11865629 - 11865376	254	ATTTAGTGATTGGTGATTGGATTT
			TCTCTCAAAAATAACCTCTACCACT
NR3C1_1	chr5::142785050 - 142784881	170	GYGAATTTTGTAAAGATGGTGGT
			CCACAACCACCTCTCACCTC
NR3C1_2	chr5::142783157 - 142782953	205	TTTTTATTTGYGAGTTYGTTGTG
			AATCTCCCATTACCCAACTAACAAAC
NR3C1_3	chr5::142783652-142783828	177	ATTYGGGAGTTYGTTGTGTTTT
			CAACCCRTAACCCCTTC
NR3C1_4	chr5::142782574-142782730	157	GGAGGGAGAGGAAGAGGTTAG
			AAATACCRCTAAACCRAAAACAAC

Haplovew software was used to analyze the linkage imbalance of NR3C1 gene SNPs (rs41423247 and rs10052957) and IGFBP3 gene SNPs (rs2453839 and rs3110697), and it was found that there was no strong linkage between SNPs.

In addition, we screened the CpG islands of *NR3C1*, *MTHFR* and *IGFBP3* genes, looking for the CpG islands in the region from upstream 2K to downstream 1K of the first exon (including all shear modes) of the gene transcription initiation site. Primer3(<http://primer3.ut.ee/>)software was also used to design primers for Bisulfite treated sequences (Table 5). In this study, a total of 164 CpG sites were detected, and the differences in methylation levels at each CpG site were tested for significance. The CpG sites with significant differences in methylation levels were screened out as follows: *IGFBP3_2*-143, *MTHFR_1*-36, *MTHFR_1*-77, *MTHFR_1*-139, *MTHFR_2*-42, *NR3C1_2*-163, *NR3C1_4*-47(Table 6). Further, quantitative trait loci (QTL) mapping method was used to jointly analyze the SNP typing results of all samples and the methylation level of the corresponding methylation sites, and a total of 10 pairs of linear regression tests between SNPs and methylation sites were conducted with P < 0.05, which was statistically significant (Table 7).

Table 6
Differences of methylation levels of sites, targets and gene

Target	Position	Type	SONFH (mean ± SD)	Controls (mean ± SD)	OR(L95-U95)	P-value
<i>IGFBP3_2</i>	143	CG	6.2 ± 2.5	5.3 ± 1.8	1.207(1.024-1.423)	0.025
<i>MTHFR_1</i>	36	CG	0.5 ± 0.3	0.6 ± 0.3	0.260(0.077-0.879)	0.030
<i>MTHFR_1</i>	77	CG	0.4 ± 0.3	0.5 ± 0.3	0.257(0.068-0.963)	0.044
<i>MTHFR_1</i>	139	CG	0.7 ± 0.4	0.9 ± 0.4	0.286(0.108-0.759)	0.012
<i>MTHFR_2</i>	42	CG	0.9 ± 0.3	0.8 ± 0.3	3.679(1.00-13.511)	0.049
<i>NR3C1_2</i>	163	CG	1.0 ± 0.3	1.1 ± 0.3	0.265(0.078-0.895)	0.032
<i>NR3C1_4</i>	47	CG	0.6 ± 0.2	0.6 ± 0.2	0.140(0.024-0.813)	0.028

Table 7
meQTL analysis between SNP and CpG loci

SNP	gene	beta	t-stat	p-value
rs3110697	<i>IGFBP3_2-106</i>	0.003685037	2.691716501	0.008
rs2453839	<i>IGFBP3_2-171</i>	0.007416736	2.601485177	0.010
rs10052957	<i>NR3C1_1-68</i>	-0.000705172	-2.220407393	0.028
rs10052957	<i>NR3C1_1-144</i>	-0.000943779	-2.241099197	0.027
rs10052957	<i>NR3C1_3-65</i>	-0.000817371	-2.916176834	0.004
rs10052957	<i>NR3C1_3-51</i>	0.00057689	2.47573142	0.014
rs10052957	<i>NR3C1_4-32</i>	-0.001172311	-2.38633154	0.018
rs10052957	<i>NR3C1_4-91</i>	0.000697272	2.100263333	0.037
rs1801133	<i>MTHFR_1-202</i>	0.000965155	2.077630625	0.040
rs1801133	<i>MTHFR_2-85</i>	-0.000579351	-2.660524526	0.009

Discussion

ONFH is a disease characterized by necrosis of one or more parts of the femoral head. ONFH can cause severe joint pain and limited physical activity, and some severe cases even require surgical intervention to restore normal function (Werner et al. 2003). Steroids are widely used in clinical practice. However, in recent years, steroids have become the leading cause of non-traumatic ONFH (Kerachian et al. 2009). We hope to find the genetic and epigenetic links between several important genes related to SONFH through the study of gene polymorphisms and DNA methylation.

In our case-to-case study, we did not find a relationship between rs10052957 and rs41423247 in *NR3C1* gene and SONFH risk. However, in the further study of DNA methylation, compared with the control group, we screened for the first time the CpG sites of *NR3C1_2-163* and *NR3C1_4-47* with significant differences in methylation levels, and found that there was a correlation between *NR3C1* gene SNPs and DNA methylation. According to previous researches, glucocorticoid is an important regulator of energy homeostasis. Most effects of glucocorticoids are mediated by the glucocorticoid receptor (Nicolaides et al. 2010). Glucocorticoid receptor is composed of DNA-binding domain, hormone binding domain and amino terminal region, and plays a decisive role in glucocorticoid sensitivity (Bamberger et al. 1995). Any of these changes in the molecular mechanisms of glucocorticoid receptor action can alter tissue sensitivity to glucocorticoid, possibly leading to drug resistance or allergy, and serious complications. *NR3C1* polymorphisms are associated with changes in glucocorticoid sensitivity, body composition, and metabolic parameters. The polymorphism of rs41423247 is the change of C → G nucleotide downstream of the connection region of intron 2 of exon 2 of glucocorticoid receptor gene (van Rossum and Lamberts 2004). Rs41423247 polymorphism is associated with changes in susceptibility to exogenous glucocorticoid in rheumatoid arthritis and healthy individuals (van Rossum et al. 2003; Derijk et al. 2001). Yan et al. (2013) reported that in rs10052957, A allele carriers had significantly lower systolic blood pressure, total cholesterol and Low-density lipoprotein than GG carriers. People with GG, a homozygote of rs41423247, had a lower body mass index (BMI) and a lower triglyceride than those with the C allele. Although no polymorphism of *NR3C1* has been reported on SONFH, the lower risk of blood lipids, systolic blood pressure, and other risk factors for the disease could be studied. In this study, we did not find the relationship between the SNPs allele frequency and genotype frequency of *NR3C1* gene and the susceptibility to SONFH, which may be related to the study subjects we selected. The control group we recruited was from the same hospital, but the blood samples of SONFH patients were obtained from different regions and hospitals. It is well known that population confounding is a confounding factor in association analysis, which affects the results of association analysis. Further research is needed to control the interference of regional differences on the research results. Interestingly, this study showed that epigenetic modification of *NR3C1* gene can interact with gene polymorphism, providing a new perspective for the pathogenesis of SONFH.

MTHFR is a key enzyme in homocysteine metabolism and folic acid metabolism. It can catalyze 5, 10-Methylenetetrahydrofolate to methylate homocysteine in blood and maintain its level in blood. The rs1801133 polymorphism of the *MTHFR* gene is caused by a point mutation at position 677, resulting in a decrease in enzyme activity that can cause hyperhomocysteinemia (Zhu et al. 2015). High concentration of homocysteine in the blood will interfere with the function of vascular endothelial cells, resulting in abnormal coagulation and fibrinolysis systems, making the body in a pre-thrombotic state. It can also act on endothelial cells to constrict blood vessels, increase blood pressure, and promote the formation of atherosclerosis. The growth of new blood vessels is the basis of blood supply, and angiogenesis is crucial for wound healing and bone remodeling in most tissues. Therefore, this study included *MTHFR* as a candidate gene.

A genetic link between ONFH and a tendency to low fibrinolysis or thrombosis has been reported (Gagala et al. 2013). Clotting factors such as MTHFR and plasma plasminogen activation inhibitor – 1(PAI-1) are related to the occurrence of ONFH. Therefore, many studies have proposed that pathogenesis such as coagulation and angiogenesis may also be major risk factors for ONFH. Several case-control studies examined the relationship between the rs1801133 polymorphism of *MTHFR* gene and ONFH. However, there are inconsistent views on the role of this polymorphisms in the pathogenesis of ONFH, which is also an important factor for us to include rs1801133 in the study. Zalavras et al. (2002) reported a significant statistical difference between the incidence of non-traumatic femoral head necrosis and rs1801133 gene mutation. But there are also some research does not support this result, a south Korean contains 443 ONFH patients and 273 healthy subjects case-control study was carried out on 15 SNPs genetic analysis, found that rs1801133 polymorphism of *MTHFR* gene and non-traumatic ONFH and no obvious relationship between genetic susceptibility, the author thinks that different regions and ethnic may affect the *MTHFR* gene polymorphisms between individuals (Kim et al. 2010). Through further research, Shang et al. (2012) conducted a meta-analysis of the association between rs1801133 polymorphism and ONFH, and found in the Asian population cannot find rs1801133 polymorphism and the correlation of ONFH, but can be found in Africa, the crowd and the relative importance of ONFH, suggesting that rs1801133 polymorphism of *MTHFR* gene may not be a major risk factor for Asian populations ONFH, but a major risk factor for African people ONFH. When ethnicity was stratified, this meta-analysis provided no evidence of an association between rs1801133 polymorphism and ONFH. Twelve studies were included in the meta-analysis of Chai et al. (2015). The results showed that there was no direct association between ONFH susceptibility and rs1801133 polymorphism. Based on previous studies, we independently analyzed SONFH, hoping to explain the various associations between the *MTHFR* gene and steroid-induced ONFH reported in the literature. However, our results are also consistent with the results of most studies. We did not find a significant correlation between rs1801133 polymorphism of *MTHFR* gene and SONFH, and rs1801133 polymorphism may not be an explicit risk factor for thrombosis. Our study did not include an assessment of the mechanisms of hemostasis and, in addition, we did not measure homocysteine levels. The hypothesis that rs1801133 polymorphism may cause SONFH by promoting intravascular coagulation has yet to be confirmed. In addition, in order to study the interaction between SNPs and the methylation level at the site, the methylation level on the CpG island of the *MTHFR* gene amplicon was detected, and the methylation levels at the site, fragment and gene were statistically analyzed. The results showed that there were statistically significant differences in methylation levels at the sites of *MTHFR*_1–36, *MTHFR*_1–77, *MTHFR*_1-139 and *MTHFR*_2–42 compared with the control group. In the development of SONFH, epigenetics may be an important bridge connecting the interaction between environmental factors and genes.

Meanwhile, in the current case-control study, we investigated the relationship between rs2453839, rs3110697 of *IGFBP3* gene and the risk of ONFH. We confirmed that rs3110697 polymorphism of *IGFBP3* gene is related to the occurrence of SONFH in Chinese Han population. Compared with the control group, rs3110697 carriers with A/G genotype had A reduced risk of disease. In the recessive genetic model, the risk of rs3110697 A allele carriers was also reduced. We found that CpG locus *IGFBP3*_2-143 may interact with *IGFBP3* gene polymorphisms, thus affecting the occurrence of SONFH.

IGFBP3 is the main carrier of IGF-1 in blood circulation and can prolong the half-life of IGF-1 in blood circulation. IGF-1 can promote the proliferation of bone cells, increase bone length, participate in the regulation of protein metabolism, induce vitamin D activation (Duchén et al. 2017). *IGFBP3* plays a role in hypoxia-induced angiogenesis (Granata et al. 2007). Among IGFBP members, *IGFBP3* is the most abundant in the circulatory system and is associated with arteriosclerosis (Jones and Clemmons 1995). Therefore, we believe that *IGFBP3* gene polymorphisms may affect blood circulation, damage the femoral head, and further lead to SONFH. Multiple polymorphisms in the *IGFBP3* gene are also associated with cancer risk. Previous studies have found a negative association between *IGFBP3* rs2453839 and endometrial cancer risk, and a genetic variant of *IGFBP3* may influence endometrial cancer risk in Caucasians (Mcgrath et al. 2001). Two *IGFBP3* polymorphisms (rs3110697 and rs2854744) were significantly associated with premenopausal women with breast cancer survival (Deming et al. 2007). The relative specificity of *IGFBP3* to ONFH has also aroused widespread concern. Jung et al. (2010) used Affymetrix targeted genotyping 3K microarray array to study the SNPs of *IGFBP3* gene in 460 patients with ONFH and 300 control subjects. Rs2453839 was significantly associated with alcohol-induced and idiopathic ONFH in a recessive model. Subjects carrying the small homozygous allele CC of rs2453839 had a higher risk of ONFH. Song et al. (2012) reported that *IGFBP3* rs2453839 TT and CT frequency of ONFH group increased and decreased compared with normal control group. The association analysis of polymorphism and clinical phenotype showed that the course of disease in patients with *IGFBP3* gene rs2453839 TT ONFH was significantly shorter than that in patients with CT + CC. The frequency of CT + CC genotype in stage III / IV bilateral hip lesions group was significantly higher than that in stage III / Iv unilateral hip lesions group and stage II / III bilateral hip lesions group. However, we did not find a correlation between rs2453839 polymorphism and SONFH in this statistical analysis. The major models of *IGFBP3* gene rs3110697 and rs2453839 were significantly associated with an increased risk of femoral head failure in 182 patients and 179 healthy controls. The genotype of rs2453839 was also associated with clinical stage. Compared with control group, IGF1 in ONFH group increased. Serum triglyceride and Low-density Lipoprotein levels in the ONFH group were significantly higher than those in the control group, but serum high-density Lipoprotein cholesterol levels were significantly lower than those in the control group (Song et al. 2016).

To sum up, our study proved for the first time that epigenetic modifications of *NR3C1*, *MTHFR* and *IGFBP3* genes can interact with genetic polymorphism to influence SONFH in Chinese Han population. Based on the study of genetic structural characteristics of the disease, we can accurately grasp the development law of the disease. While there are some important findings in our study, there are some limitations. First, our study did not include an analysis of biological function, which is essential to elucidate the role of *NR3C1*, *MTHFR*, and *IGFBP3* genes in SONFH. Secondly, SONFH needs to be further analyzed by different clinical stages. Thirdly, the control group in our study were all Han recruited from the affiliated hospital of neurology institute of Anhui university of traditional Chinese medicine, which may be a selection bias. Further functional studies and larger case-control studies are expected to avoid these issues and strengthen our conclusions.

Declarations

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Author Contributions

RIH, QHZ, WBH conceived and designed the experiments. RIH, QHZ, RMY, NC, YSH performed the experiments. RIH analyzed the data and wrote the manuscript. All the authors reviewed the manuscript.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

Ethics committee approval was received for this study from the Institute of Neurology of Anhui University of Chinese Medicine.

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