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Original Article

IGF-1 and *IGFBP-3* polymorphisms predict risk for non-traumatic osteonecrosis of the femoral head

Wei Wei[1*], Biao-Fang Wei[2*], Xin Liu[2], Wei He[1]

[1]*Department of Orthopedics, First School of Clinical Medicine, Guangzhou University of Chinese Medicine, Guangzhou, P. R. China;* [2]*Department of Orthopedics, Linyi People's Hospital, Linyi, P. R. China.* [*]*Co-first authors.*

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Abstract: Objective: To investigate the roles of *Insulin-Like growth factor-I (IGF-1*) and *Insulin-like growth factor-binding protein-3* (*IGFBP-3*) polymorphisms in non-traumatic osteonecrosis of the femoral head (NONFH). Methods: Case group included 130 NONFH patients who admitted to First School of Clinical Medicine, Guangzhou University of Chinese Medicine from October 2012 to December 2013, and control group enrolled 111 healthy people who had physical examinations during the same period in First School of Clinical Medicine, Guangzhou University of Chinese Medicine. Polymerase Chain Reaction -Restriction Fragment Length Polymorphism (PCR-RFLP) was used to detect the polymorphisms of *IGF-1* rs2946834, *IGF-1* rs2195239, *IGFBP-3* rs924140, and *IGFBP-3* rs2453839. Results: The TT genotype and T allele of *IGF-1* rs2946834 was lower in the case group as compared to the control group (both $P < 0.05$). Meanwhile, the GG, AG+GG and G allele in *IGFBP-3* rs924140 were significantly lower in the case group than those in the control group (all $P < 0.05$). Haplotype CCAT, CGAC, and CGAT were proved to be risk factors for NONFH. *IGF-1* rs2946834 and *IGFBP-3* rs924140 polymorphisms were related to the disease course, clinical staging, and extent of disease of NONFH (all $P < 0.05$). Conclusions: Polymorphisms of *IGF-1* rs2946834 and *IGFBP-3* rs924140 may relate to the risk of NONFH.

Keywords: Insulin-like growth factor-I, Insulin-like growth factor-binding protein-3, non-traumatic osteonecrosis of the femoral head, polymorphism, rs2946834, rs924140, haplotype, clinical features

Introduction

Non-traumatic osteonecrosis of the femoral head (NONFH), also known as nonbacterial necrosis of femoral head or avascular necrosis of the femoral head (ANFH), occurs at the femoral head as a consequence of hypoxia and ischemia of tissue cells in the femoral bone led by blood interruption [1, 2]. According to the severity of the disease, patients with NONFH are likely to have symptoms such as pains, limited hip flexion, limp, bone marrow edema, subchondral cortical fracture [3]. Factors such as congenital genetic factors, the overuse of hormones, alcohol abuse, the use of corticosteroid, systemic lupus erythematosus may contribute to the pathogenesis of NONFH [4]. However, the pathogenesis of NONFH has not been revealed completely [5]. After studying its related risk factors from multiple perspectives, researchers found that both genetic and environmental factors play a key role in the pathogenesis of this disease [6]. In the field of molecular genetics, many susceptible genes related to NONFH have been reported, such as *vascular endothelial growth factor* haplotypes [7], apolipoprotein AI-75G>A polymorphism [8], nitric oxide [9], and cytokine related genes [10], indicating that genes play a role in the occurrence and development of NONFH and providing theoretical basis for its early diagnosis and treatment.

Insulin-like growth factor 1 (*IGF-1*), as a polypeptide containing 70 amino-acid residues and having similar structure with insulin, can be found in almost all kinds of organizations of all mammals [11] and can regulate the growth, survival, proliferation and metastasis of cells [12]. The interaction between IGF-1 signaling pathway and other signaling pathways can regulate the proliferation and differentiation of chondrocytes, maintain the phenotypic stability of chondrocytes and promote the ossification of

cartilage [13]. A Study has indicated that *IGF-1* plays an important role in the development and remodeling of condyle [14]. The main function of *human insulin-like growth factors binding protein 3* (*IGFBP-3*) is connecting and isolating the circulating IGFs, so as to control the biological use of IGFs and prolong their semi retention period. *IGFBP-3* can control the IGF signaling pathway by restricting the combination of IGFs and *IGF* receptors and strengthen the activity of *IGF-1* [15]. It was found that the IGF-1/IGFBP-3 ratio is significantly related to the changes of bone mineral density and related to the increase of bone mass [16]. Obviously *IGF-1* and *IGFBP-3* are closely related to the growth and development of bone, but there is no relevant research about the association of gene polymorphisms of these two genes with the risk of NONFH. Therefore, this paper was conducted aiming to explore how *IGF-1* and *IGFBP-3* polymorphisms are related to NONFH risk.

Materials and methods

Ethics statement

This study was approved by the Ethics Committee of First School of Clinical Medicine, Guangzhou University of Chinese Medicine and in accordance with the standards of the National Research Council. And written informed consent was obtained from each patient prior to study.

Research subjects

This research selected 130 NONFH patients in the Department of Orthopedics, the First School of Clinical Medicine, Guangzhou University of Chinese Medicine from October 2010 to December 2013 (case group, n = 130). There were 88 males and 42 females aging 33~80 years (50.83 ± 8.77 years). According to Ficat staging system [17], 33 patients were at stage II, 50 at stage III, and 47 at stage IV. The average disease course for case group was 59.68 ± 17.69 months (4 months~117 months). In terms of the extent of the disease, 77 patients were unilateral NONFH and 53 were bilateral NONFH. According to the clinical etiological classification of NONFH patients [18], 50 cases were alcohol-induced NONFH with disease course of 62.20 ± 16.57 months, 51 cases were idiopathic NONFH with disease course of 56.92 ± 16.53 months, and 29 cases were steroid-induced NONFH with disease course of 60.21 ± 21.14 months. Diagnostic criteria [19] are as follows: 1. There is femoral head necrosis around the necrotic bone and bone under repair, or there is fracture of cartilage; 2. MRI imaging of necrosis shows low signal on T1W1 or double-line sign on T2W1; 3. Bone biopsy shows that the trabecular bone cells lacuna is over 50% and affects several adjacent trabecular bones, and there is bone marrow necrosis. Inclusion criteria for case group were displayed as follow: 1. Joint pains appear in groin, buttocks and thigh; 2. No serious internal complications; 3. The time between occurrence and treatment of hip joint pain is no more than half a year. Exclusion criteria: 1. Patients with other types of hip joint disease, such as rheumatoid arthritis, ankylosing spondylitis; 2. Patients whose imaging data and clinical information are not complete or whose disease classification was not clear; 3. Patients without informed consent. During the same period, 111 healthy subjects were selected as control group, including 70 males and 41 females, with an average age of 49.62 ± 14.13 years. There was no difference in age and sex between the case group and the control group (both $P > 0.05$).

SNP screening gene polymorphisms

Based on the genomic data of Chinese Han population in HapMap, this study was conducted as follows: literature review was done before searching for Tag-SNP and FAST SNP. Then the functional site of *IGF-1* and *IGFBP-3* gene mutation was found. At last, *IGF-1* rs2946834 and rs2195239 as well as *IGFBP-3* rs924140 and rs2453839 were decided to be the polymorphic loci requiring detection in this study.

Gene polymorphism detection

After fasting for 12 h, all subjects were extracted with 2 ml of venous blood. Blood samples were anti-coagulated with ethylenediaminetetraacetic acid (EDTA) and preserved at low temperature. DNA was extracted using modified potassium iodide method. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) was used to detect the gene polymorphisms of *IGF-1* rs2946834 and *rs2195239* as well as *IGFBP-3* rs924140 and rs2453839. PCR primer was designed with Primer Premier 5.0 and synthesized by the Shanghai Biotechnology Company. The forward

Table 1. Primer design for polymorphic gene

Gene	SNP	Primer sequence
IGF-1	rs2946834	Forward 5'-CATTTGTGACTGACTATTCC-3'
		Reverse 5'-TCTCCAGAAGCCCTGATTCAA-3'
	rs2195239	Forward 5'-CTGAGTGCCTTACTTCTTTA-3'
		Reverse 5'-CAGGGCCCACAGACTGTCTAAC-3'
IGFBP-3	rs924140	Forward 5'-CCTACCAATAAGCGATGAAA-3'
		Reverse 5'-AGCCTGGACTGACCACTGGTTA-3'
	rs2453839	Forward 5'-CTGCTGGTCATGTCCTTGGC-3'
		Reverse 5'-ATGAGGGAACTATTGGGGGA-3'

Note: SNP, single nucleotide polymorphism; *IGF-1*, Insulin-Like growth factor-I; *IGFBP-3, Insulin-like growth factor-binding protein-3*.

and reverse primers for PCR amplification of *IGF-1* and *IGFBP-3* polymorphic genes are shown in **Table 1**. The volume of *IGF-1* rs2946834 and rs2195239 reaction system is 5 μl, containing 2 × TaqMan Master Mix 2.5 μl, 40 × probe 0.1 μl, dH_2O 1.7 μl, template DNA (30 ng/μl) 0.7 μl. Reaction conditions were predenaturation for 10 min at 95°C, then a total of 45 cycles of denaturation for 15 s at 92°C, annealing for 1 min at 60°C, extending for 1 min at 72°C, and at last extending for 3 min at 72°C before preservation at constant 4°C. The volume of *IGFBP-3* rs924140 and rs245383 reaction system is 5 μl, containing 1 μl of genomic DNA (10 ng/μl), 1 μl of 0.5 μM mixed PCR primers, 0.5 μl of 10 × PCR buffer, 0.4 μl of 25 mM $MgCL_2$ solution, 0.1 μl of 25 m MdNTP mixture solution, 0.2 μl of 5 U/μl hot-start PCR enzyme, 1.8 μl of ddH_2O. Reaction conditions were pre-denaturation for 10 min at 95°C, then a total of 40 cycles of denaturation for 0.5 min at 95°C, annealing for 0.5 min at 56°C, extending for 1 min at 72°C, and at last extending for 3 min at 72°C before preservation at constant 4°C. At the end of PCR, the PCR product was added into the 2% agarose gel for 20 min at 250 V voltage electrophoresis. Then the gel imaging system was used to detect electrophoresis results under UV light.

Statistical analysis

The statistical analyses were conducted with SPSS 21.0. Measurement data were presented by mean ± standard deviation (SD). Data consistent with normal distribution was analyzed using *t*-test and variance analysis, and data not conforming to normal distribution was analyzed using rank-sum test. Enumeration data were presented by number or ratio. Differences between groups were analyzed using Chi-Square test. Multiple sets of data were analyzed using partition of chi-square test. Shesis software was used to analyze the differences of genotype, allele frequency and haplotype between the control group and the case group. Risk factors were analyzed using unconditional logistic regression, odds ratios (OR), and 95% confidential interval (CI). $P < 0.05$ was considered statistically different.

Results

Genetic equilibrium test

Hardy-Weinberg equilibrium test was applied to *IGF-1* rs2946834, *IGF-1* rs2195239, *IGFBP-3* rs924140, and *IGFBP-3* rs2453839 respectively. The actual number and the theoretical number of different genotype cases were compared both in case group and control group. Chi-square analysis showed that there was no significant difference between actual distribution and theoretical distribution of each genotype in control group (all $P > 0.05$), indicating they reached genetic equilibrium and had group representation. The results are shown in **Table 2**.

Frequency distribution of IGF-1 and IGFBP-3 genotypes and allele

The allele and genotype distributions of *IGF-1* rs2946834, rs2195239 and *IGFBP-3* rs924140, rs2453839 in case group and control group are shown in **Table 3**. *IGF-1* rs2946834 TT and CT+TT genotypes and T allele can significantly reduce the risk of NONFH (TT vs. CC: OR = 0.363, 95% CI = 0.177~0.745; CT+TT vs. CC: OR = 0.438, 95% CI = 0.256~0.752; T vs. C: OR = 0.549, 95% CI = 0.381~0.793, all $P < 0.05$). There is no significant difference between case group and control group in terms of genotype and allele frequency of *IGF-1* rs2195239 and *IGFBP-3* rs2453839 (all $P > 0.05$). Compared with control group, *IGFBP-3* rs924140 GG and AG+GG genotypes and G allele can significantly reduce the risk of NONFH in case group (AG vs. AA: OR = 0.464, 95% CI = 0.253~0.853; GG vs. AA: OR = 0.425, 95% CI = 0.209~0.865; AG+GG vs. AA: OR = 0.450, 95% CI = 0.255~0.795; G vs. A: OR = 0.619, 95% CI = 0.431~0.889, all $P < 0.05$).

Table 2. Theoretical number and actual number of genotype frequency

Gene	Case group		χ^2	*P*	Control group		χ^2	*P*
	Theoretical number	Actual number			Theoretical number	Actual number		
IGF-1 rs2946834			3.449	0.063			0.432	0.511
CC	47	61			29	31		
CT	38	49			56	52		
TT	15	20			26	28		
IGF-1 rs2195239			2.323	0.135			1.174	0.279
CC	27	31			21	24		
CG	64	56			55	49		
GG	39	43			35	38		
IGFBP-3 rs924140			3.055	0.080			0.014	0.907
AA	46	51			25	25		
AG	63	53			55	56		
GG	21	26			31	30		
IGFBP-3 rs2453839			2.838	0.092			1.925	0.165
CC	38	43			31	35		
CT	65	55			55	48		
TT	27	32			25	28		

Note: *IGF-1*, Insulin-Like growth factor-I; *IGFBP-3*, *Insulin-like growth factor-binding protein-3*.

Table 3. *IGF-1* and *IGFBP-3* genotypes and allele frequency distribution

SNPs		Case group (n = 130)	Control group (n = 111)	χ^2	OR (95% CI)	*P*-value
IGF-1	CC	61 (46.92)	31 (27.93%)		Ref	
rs2946834	CT	49 (37.69%)	52 (46.85%)	6.126	0.479 (0.267-0.858)	0.013
	TT	20 (15.38%)	28 (25.23%)	7.853	0.363 (0.177-0.745)	0.005
	CT+TT	69 (53.08)	80 (72.07%)	9.154	0.438 (0.256-0.752)	0.003
	C	171 (65.77%)	114 (51.35%)		Ref	
	T	89 (34.23%)	108 (48.65%)	10.301	0.549 (0.381~0.793)	0.001
IGF-1	CC	31 (23.85%)	24 (21.62%)		Ref	
rs2195239	CG	56 (43.08%)	49 (44.14%)	0.134	0.885 (0.459~1.706)	0.715
	GG	43 (33.08%)	38 (34.23%)	0.148	0.876 (0.440~1.745)	0.707
	CG+GG	99 (76.15%)	87 (78.38%)	0.139	0.891 (0.486~1.634)	0.710
	C	118 (45.38%)	97 (43.69%)		Ref	
	G	142 (54.62%)	125 (56.31%)	0.139	0.934 (0.651~1.339)	0.710
IGFBP-3	AA	51 (39.23%)	25 (22.52%)		Ref	
rs924140	AG	53 (40.77%)	56 (50.45%)	6.214	0.464 (0.253~0.853)	0.013
	GG	26 (20.00%)	30 (27.03%)	5.671	0.425 (0.209~0.865)	0.017
	AG+GG	79 (60.77%)	86 (77.48%)	7.742	0.450 (0.255~0.795)	0.005
	A	155 (59.62%)	106 (47.75%)		Ref	
	G	105 (40.38%)	116 (52.25%)	6.793	0.619 (0.431~0.889)	0.009
IGFBP-3	CC	43 (33.08%)	35 (31.53%)		Ref	
rs2453839	CT	55 (42.31%)	48 (43.24%)	3.500	1.754 (0.972~3.167)	0.061
	TT	32 (24.62%)	28 (25.23%)	0.535	1.276 (0.664~2.452)	0.465
	CT+TT	87 (66.93%)	76 (68.47%)	0.876	1.278 (0.764~0.137)	0.349
	C	141 (54.23%)	118 (53.15%)		Ref	
	T	119 (45.77%)	104 (46.85%)	0.056	0.958 (0.669~1.372)	0.813

Note: SNP, single nucleotide polymorphism; *IGF-1*, Insulin-Like growth factor-I; *IGFBP-3*, *Insulin-like growth factor-binding protein-3*.

Table 4. Haplotype analysis of *IGF-1* and *IGFBP-3* genotypes

Haplotype				Case group (n = 130)	Control group (n = 111)	*P*	OR	95% CI
rs2946834	rs2195239	rs924140	rs2453839					
C	C	A	C	34 (13.10)	27 (12.00)	0.743	1.095	0.637~1.883
C	C	A	T	26 (10.10)	9 (4.20)	0.014	2.563	1.185~5.553
C	C	G	C	6 (2.10)	20 (9.10)	< 0.001	0.214	0.082~0.558
C	C	G	T	6 (2.30)	15 (6.90)	0.014	0.318	0.122~0.832
C	G	A	C	47 (18.00)	17 (7.70)	0.001	2.579	1.436~4.663
C	G	A	T	39 (15.00)	9 (4.10)	< 0.001	2.579	1.436~4.663
C	G	G	C	10(3.70)	10 (4.30)	0.708	0.839	0.336~2.100
C	G	G	T	4 (1.40)	7 (3.20)	0.175	0.421	0.116~1.527
T	C	A	T	9 (1.90)	10 (4.60)	0.083	0.390	0.130~1.172
T	C	G	C	17 (6.40)	12 (5.20)	0.608	1.224	0.564~2.656
T	G	A	C	3 (0.90)	11 (4.70)	0.009	0.186	0.045~0.762
T	G	G	C	25 (9.50)	18 (8.30)	0.680	1.142	0.606~2.152
T	G	G	T	16 (6.20)	34 (15.40)	< 0.001	0.355	0.190~0.662

Note: *IGF-1*, Insulin-Like growth factor-I; *IGFBP-3*, *Insulin-like growth factor-binding protein-3*.

Haplotype analysis

Haplotype of *IGF-1* rs2946834, rs2195239 and *IGFBP-3* rs924140, rs2453839 (**Table 4**) were analyzed with Shesis software (In case group and control group, the haplotype whose frequency is lower than 0.03 were excluded). The results showed that there is significant difference in the distribution of haplotype CCAT, CCGC, CCGT, CGAC, CGAT, TGAC, and TGGT in two groups (all $P < 0.05$). CCAT, CGAC, and CGAT are risk factors for NONFH, while CCGC, CCGT, TGAC and TGGT are protective factors for NONFH. There is no frequency difference in other haplotype such as CCAC, CGGC, CGGT, TCAT, TCGC, TGGC (all $P > 0.05$).

Gene polymorphism of IGF-1 and IGFBP-3 and clinical feature analysis of NONFH

In terms of *IGF-1* rs2946834, rs2195239 and *IGFBP-3* rs924140, rs2453839 genotypes, there was no significant difference in clinical features such as gender, age, etiology classification (all $P > 0.05$). Patients carrying *IGF-1* rs2946834 CC and CT+TT genotype were significantly different in disease course, clinical staging, and lesion of unilateral hip joints (all $P < 0.05$). Compared with patients carrying AA genotype, patients carrying *IGFBP-3* rs924140 AG+GG genotype were significantly different in disease course, clinical staging, and lesion of unilateral hip joints (all $P < 0.05$) (**Table 5**).

Discussion

Gene therapy, as a new method, has been introduced into the treatment of NONFH and this method is based on the research about the relationship between gene polymorphism and NONFH [20]. This study detected the gene polymorphisms of *IGF-1* rs2946834, rs2195239 and *IGFBP-3* rs924140, rs2453839 in control group and case group, so as to explore the association of *IGF-1* and *IGFBP-3* gene polymorphism with NONFH risk. It was found that *IGF-1* rs2946834 and *IGFBP-3* rs924140 polymorphism is related to the risk of NONFH.

In this study, *IGF-1* rs2946834 TT/CT+TT genotype and T allele can significantly reduce the risk of NONFH. *IGF-1* is an important cell factor involved in the regulation of bone cell function and metabolism [21]. It can not only promote the mitosis of osteoblasts, but also promote the differentiation of osteoblasts by reducing the loss of bone collagen and increasing the rate of bone deposition [22]. *IGF-1* plays an important role in bone remodeling and bone mass obtaining, and it can regulate the differentiation of osteoblasts, thus promoting bone formation and bone growth [23]. *IGF-1* T allele carrying enhancer can significantly reduce or even stop activity compared with C allele, especially in human osteosarcoma cells, liver cancer cells, and differentiated THP-1 macrophages [24], indicating that there was weak or no expression of T allele in those diseases.

Table 5. Gene polymorphism of *IGF-1* and *IGFBP-3* and clinical features of NONFH patients

Indices	*IGF-1 rs2946834*			*IGF-1 rs2195239*			*IGFBP-3 rs924140*			*IGFBP-3 rs2453839*		
	CC (n = 61)	CT+TT (n = 69)	*P*	CC (n = 31)	CG+GG (n = 99)	*P*	AA (n = 51)	AG+GG (n = 79)	*P*	CC (n = 43)	CT+TT (n = 87)	*P*
Gender			0.389			0.995			0.333			0.566
Male/female	39/22	49/20		21/10	67/32		32/19	56/23		28/15	60/27	
Age (years)	50.03 ± 8.77	51.53 ± 8.78	0.332	52.00 ± 8.87	50.46 ± 8.75	0.396	49.95 ± 8.30	51.39 ± 9.07	0.364	51.75 ± 8.10	50.37 ± 9.10	0.620
Course of disease (month)	70.82 ± 14.58	49.84 ± 14.01	< 0.001	57.65 ± 19.73	60.32 ± 17.05	0.465	68.88 ± 17.35	53.75 ± 15.27	< 0.001	63.40 ± 16.53	57.85 ± 18.04	0.096
Etiology			0.650			0.831			0.453			0.400
Alcohol-induced	26	24		12	38		19	31		14	36	
Idiopathic	22	29		11	40		23	28		20	31	
Steroid-induced	13	16		8	21		9	20		9	20	
Clinical staging			0.014			0.905			0.002			0.201
Stage II	13	20		7	26		13	20		7	26	
Stage III	18	32		12	38		11	39		20	30	
Stage IV	30	17		12	35		27	20		16	31	
Lesion range			< 0.001			0.789			< 0.001			0.474
Unilateral	24	53		19	58		17	60		23	54	
Bilateral	37	16		12	41		34	19		20	33	

Note: *IGF-1*, insulin-like growth factor-I; *IGFBP-3*, *insulin-like growth factor-binding protein-3*; NONFH, non-traumatic osteonecrosis of femoral head.

Therefore we speculate that T allele distribution may associate with a decreased risk for NONFH. Consistent with this speculation, the analysis between *IGF-1* rs2946834 TT/CT+TT genotype and baseline characteristics of NONFH showed that carrier with CT+TT genotype were associated with a decreased disease severity, which further confirmed the protective role of *IGF-1* rs2946834 TT/CT+TT genotype in NONFH.

In this study, we found that both GG/AG+GG genotype and G allele of *IGFBP-3* rs924140 can significantly reduce the risk of NONFH. In the development of NONFH, angiogenesis and bone remodeling are of great importance. The IGFBP family can regulate the growth and metabolism of bone cells and is closely related to bone necrosis [23]. *IGFBP-3*, as a part of IGF/IGFBPs system, plays an important role in the regulation of bone cell growth, differentiation and apoptosis, and it can also regulate angiogenesis in the independent IGF pathway [25, 26]. *IGFBP-3* may enhance the effect of *IGF-1* on bone peak mass, and is also considered to be positively regulated by growth hormone or IGF-1 [27, 28]. Research by Jung Min Hong pointed out that *IGFBP-3* rs2453839 was significantly associated with higher risk of NONFH [6]. Another research by Song further showed that the *IGFBP-3* rs2453839 CT+CC genotype is closely related to avascular necrosis of the femoral head [25]. Based on above researches, *IGFBP-3* rs2453839 CT+CC genotype is a risk factor for NONFH. Meanwhile, as an extension based on previous results, our study showed that *IGFBP-3* rs924140GG/AG+GG genotype and G allele are protective factors, which provides a new research direction for a more comprehensive understanding of the relationship between *IGFBP-3* gene polymorphism and NONFH. However, more in-depth study is needed to find out the specific mechanism of rs924140 on NONFH. Moreover, the analysis conducted by Shesis software showed CCAT, CGAC, CGAT were all risk factors for NONFH while CCGC, CCGT, TGAC and TGGT proved to be protective factors for this disease.

In summary, our study proved that the gene polymorphisms of *IGF-1* rs2946834 and *IGFBP-3* rs924140 were related to the risk of NONFH, which is conducive to understanding NONFH etiology and pathology, and early diagnosis. But this research still has some limitations and further study is needed about the signal pathway and mechanism of *IGF-1* rs2946834 and *IGFBP-3* rs924140 polymorphism, and other genotypes should be studied based on multicenter population and correlating validation methods.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Wei He, Department of Orthopedics, First School of Clinical Medicine, Guangzhou University of Chinese Medicine, 16 Jichang Road, Guangzhou 510405, P. R. China. Tel: +86-20-36591211; E-mail: hewei8179@126.com

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