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Original Article

Association between single-nucleotide polymorphisms and haplotype of insulin-like growth factor I and risk of osteoporosis

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Abstract: Osteoporosis is a systemic metabolic disease. Osteoporosis is caused by multiple factors, including environmental and genetic factors. Insulin-like growth factors (IGFs) is reported to be a critical regulator for bone cell function. We investigated the association of IGF-I (rs35767, rs2288377 and rs5742612) polymorphisms with the risk of osteoporosis, and the gene-environmental interaction in the risk of this disease. A total of 320 patients with osteoporosis and 320 controls were selected into this study. The genotyping of IGF-I (rs35767, rs2288377 and rs5742612) was carried out in a 384-well plate format on the sequenom MassARRAY platform. We observed that the AA genotype of rs2288377 was associated with an elevated risk of osteoporosis compared with the TT genotype (OR=3.56, 95% CI=2.04-6.21). IGF-I rs2288377 and rs5742612 showed linkage disequilibrium (D=0.605; r^2=0.032). The T-A-T (rs35767-rs2288377-rs5742612) showed an increased risk of osteoporosis (OR=2.17, 95% CI=1.01-4.69). In conclusion, our study suggests that the IGF-I rs2288377 polymorphism and T-A-T haplotype are associated with risk of osteoporosis.

Keywords: Osteoporosis, IGF-I, polymorphism, haplotype, gene-environmental interaction

Introduction

Osteoporosis is a common systemic metabolic disease characterized by reduced bone mineral density and degeneration of microstructure of bone tissues, and it is a serious health problem worldwide. Osteoporosis is an important risk factor for the occurrence of fracture [1]. In China, it is estimated that the 21% of population at the age of 50-60 years suffers from this disease, and the incidence is about 60% in cases aged 60-70 years [2]. Osteoporosis is caused by multiple factors, including environmental and genetic factors. The environmental factors are involved in lack of vitamins, microelement, minerals, proteins and physical activities, long-term smoking and drinking, and high weight [3, 4]. However, not all individuals exposed to similar environmental factors show the same risk of developing osteoporosis, and thus the genetic factors play an important role in the risk of this disease. Currently, many genome-wide studies have shown that genetic factors are involved in the development of osteoporosis [5-9].

Insulin-like growth factors (IGFs) is an important regulator for bone cell function, owing to the IGFs' anabolic effect on the skeleton [10, 11]. The IGF system plays a critical role in the local regulation of bone formation, and approximately half of the basal bone cell proliferation can be prevented through activity of IGFs intrinsically produced by the bone cells [11]. IGF-I is the target gene of estrogen, which contributes to the bone metabolism through regulating the estrogenic function [12]. Previous experimental studies have shown that IGF-I expression is related to bone formation and bone loss [12, 13]. Genetic polymorphisms in IGF-I could affect the protein function. Currently, several studies have investigated the correlation between IGF-I polymorphisms and osteoporosis risk, but the results are inconsistent as a result of different ethnicities and gene-environmental interactions [14-17]. However, no study reports the interaction between IGF-I haplotype

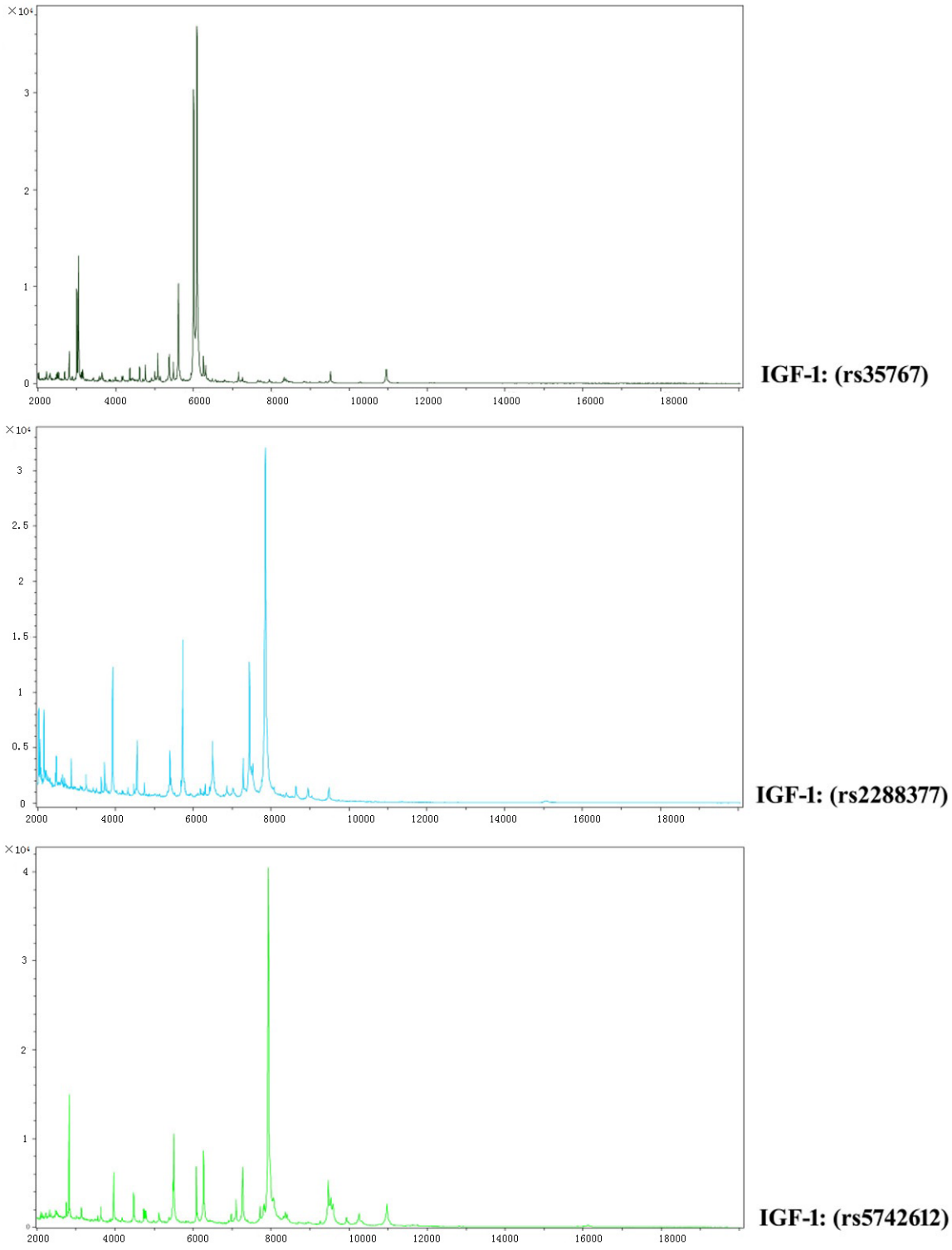

Figure 1. Mass spectrogram of MALDI-TOF for IGF-I rs35767, rs2288377 and rs5742612.

and osteoporosis risk. Therefore, we implemented a case-control study to investigate the association of IGF-I (rs35767, rs2288377 and rs5742612) polymorphisms and haplotype with risk of osteoporosis, and the gene-environmental interaction in the risk of this disease.

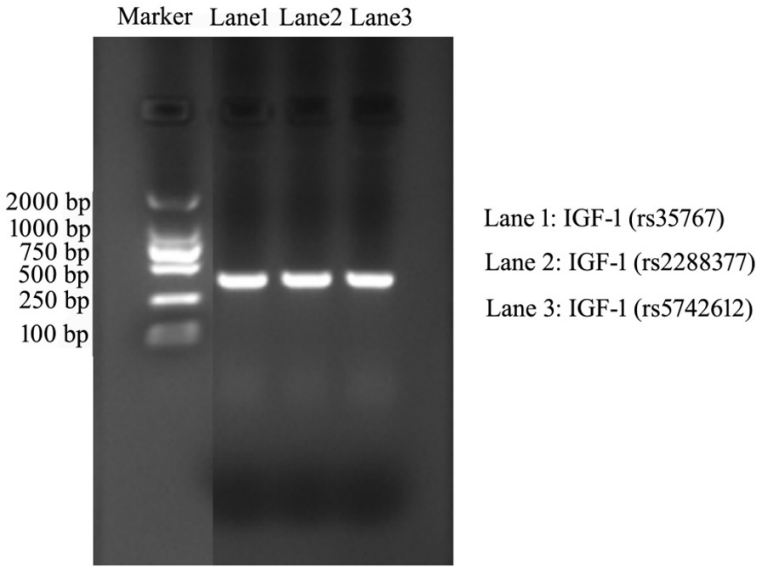

Figure 2. PCR amplification electrophoresis for IGF-I rs35767, rs2288377 and rs5742612.

Methods

Subjects

A total of 320 patients with osteoporosis were included into this study. The patients with osteoporosis were recruited from the department of orthopedics of Zhoukou Central Hospital. The definition of osteoporosis was according to the bone mineral density (BMD). Osteoporosis was diagnosed based on WHO Criteria for Diagnosis of osteoporosis as follows[18]: a T score of BMD≤-2.5 SD at the femoral neck without an evidence of vertebral fractures, or those with a T score of BMD≤-1.5 SD at the femoral neck with an evidence of more than two vertebral fractures. The exclusion criteria for patients with osteoporosis were those without intake of drugs disturbing the balance of bone metabolism; with a chronic kidney disease, rheumatoid arthritis or digestive system diseases affecting the nutrient absorption.

Simultaneously, 320 subjects, designated as controls, were recruited from the health examination center of Zhoukou Central Hospital. These study participants (controls) were confirmed to have no osteoporosis, kidney or liver diseases, rheumatoid arthritis or digestive system diseases.

All the investigated subjects signed informed consents for agreement of participating into our study before enrollment. The protocol of our study was approved by the ethics committee of the Central Hospital of Zhoukou.

Determination of BMD

The BMD of study participants was determined by dual-energy X-ray absorptiometry (Hologic®, Waltham, MA, USA). The L_1-L_4 vertebrae, femoral neck hip, total hip and trochanter were measured. The BMD was calculated by dividing bone mineral content (g) by bone area (cm^2) (g/cm^2). The BMD was measured by radiologists.

Collection of demographic and clinical characteristics

The demographic characteristics were collected through medical records, including sex, age, body mass index (BMI), consumption of tobacco smoking and alcohol drinking. The BMD was also collected from medical records, such as BMD in L_1-L_4 vertebrae, femoral neck, total hip and trochanter.

Genotype analyses

Each participant was asked to provide 5 ml peripheral venous blood before enrollment. The blood samples were kept in tubes with 10.0~12.5 IU/mL EDTA, and stored in 4°C when in use. The DNA was extracted by the TIANamp DNA Blood Mini Kit (QIAGEN GmbH, Germany) following with the instruction. Genotyping of IGF-I (rs35767, rs2288377 and rs5742612) was carried out in a 384-well plate format on the sequenom MassARRAY platform (Sequenom, San Diego, USA). PCR primers for polymerase chain reaction amplification and single base extension assays were designed using Sequenom Assay Design 3.1 software.

The PCR cocktail was prepared using Sequenom PCR reagent set within 5 μL reaction, including 2.8 HPLC grade water, 0.5 μl of 10×PCR buffer with 20 mM $MgCl_2$, 0.4 μl of 25 mM $MgCl_2$, 0.1 μl of 25 mM dNTP Mix, 1.0 μl of 0.5 μM Primer Mix, 0.2 μl Sequenom PCR enzyme (5 U/μl), and 2 μl of genomic DNA (5 ng/ul). Then the extend reaction was carried out by SAP and iPLEX reaction. The PCR samples are then

Table 1. Demographic and clinical characteristics of study participants

Variables	Patients N=320	%	Controls N=320	%	X^2 or t values	*P* values
Sex						
Females	235	73.44	232	72.50		
Males	85	26.56	88	27.50	0.07	0.79
Age, years		68.97±8.79		69.83±8.88	-1.23	0.22
BMI, kg/m^2						
<24	174	54.38	153	47.81		
≥24	146	45.63	167	52.19	2.76	0.1
Tobacco smoking habit						
No	225	70.31	202	63.13		
Yes	95	29.69	118	36.88	3.72	0.05
Alcohol drinking habit						
No	224	70.00	179	55.94		
Yes	96	30.00	141	44.06	13.57	<0.001
BMD, g/cm^2						
L_1-L_4 vertebrae		0.93±0.09		0.97±0.12	-4.02	<0.001
Femoral neck		0.58±0.029		0.66±0.027	-35.60	<0.001
Total hip		0.60±0.035		0.65±0.036	-17.94	<0.001
Trochanter		0.53±0.04		0.61±0.04	-25.34	<0.001

desalted, and dispensed to SpectroCHIP Arrays and analyzed using MALDI-TOF MS (**Figures 1** and **2**).

Statistical analysis

Categorical variables were reported as percentages of the total, and continuous variables were represented as mean ± SD. Student's *t*-test was used to determine differences between means, and Pearson's chi-square (X^2) or Fisher's exact tests were used to evaluate the inter-group significance. Whether the IGF-I (rs35767, rs2288377 and rs5742612) genotypes deviated from Hardy-Weinberg equilibrium (HWE) in patients and controls was assessed by the goodness-of-fit chi-square test, in order to ascertain whether the study sample was representative of the general population. The association of IGF-I (rs35767, rs2288377 and rs5742612) polymorphisms with risk of osteoporosis was assessed using the multiple logistic regression analysis, and the results were expressed using the odds ratios (ORs) and 95% confidence intervals (CI). The gene-environmental interaction was done by Chi-square test. The linkage disequilibrium and haplotype analysis were analyzed by SHEsis software (http://analysis.bio-x.cn/myAnalysis.php) [19]. The results were analyzed by SPSS Statistics for Windows, Version 17.0. (SPSS Inc., Chicago, USA). Statistical significance was set at P<0.05.

Results

The characteristics for patients with osteoporosis and controls were presented in **Table 1**. In comparison with the controls, patients with osteoporosis were more inclined to have a habit of alcohol drinking (X^2=13.57, P<0.001), and had a lower BMD values of L_1-L_4 vertebrae (t=-4.02, P<0.001), femoral neck (t=-35.60, P<0.001), total hip (t=-17.94, P<0.001) and trochanter (t=-25.34, P<0.001). However, no significant differences were observed between the patients and controls in terms of sex, age, BMI and a habit of tobacco smoking.

Comparison the genotype distributions of IGF-I (rs35767, rs2288377 and rs5742612) between the two investigated groups were presented in **Table 2**. Using Chi-square test, we observed that the genotype frequencies of rs2288377 (X^2=20.55, P<0.001) were significantly different between the patients with osteoporosis and controls. However, no significant difference was found between the two groups in terms of the genotype distributions of rs35767 and rs5742612. Moreover, all the SNPs in controls were in line with the Hardy-Weinberg equilibrium (HWE).

Table 2. Genotype distributions of IGF-I rs35767, rs2288377 and rs5742612 genetic polymorphisms between the two investigated groups

SNP of IGF-I		Patients	%	Controls	%	χ^2	*P*	HWE for χ^2 In patients	*P*	HWE for χ^2 In controls	*P*	Crude OR (95% CI)	*P*	Adjusted OR (95% CI)[1]	*P*
rs35767	CC	155	48.44	172	53.75							1.0 (Ref.)	-	1.0 (Ref.)	-
	TC	134	41.88	113	35.31							1.37 (0.98-1.93)	0.07	1.33 (0.94-1.88)	0.11
	TT	31	9.69	35	10.94	2.91	0.23	0.07	0.79	5.84	0.02	1.09 (0.64-1.86)	0.76	1.49 (0.60-1.80)	0.9
rs2288377	TT	114	35.63	144	45							1.0 (Ref.)	-	1.0 (Ref.)	-
	TA	147	45.94	154	48.13							1.23 (0.88-1.72)	0.23	1.25 (0.89-1.75)	0.21
	AA	59	18.44	22	6.88	20.55	<0.001	0.91	0.34	5.1	0.02	3.49 (2.01-6.06)	<0.001	3.56 (2.04-6.21)	<0.001
rs5742612	TT	253	79.06	257	80.31							1.0 (Ref.)	-	1.0 (Ref.)	-
	TG	45	14.06	46	14.38							1.00 (0.64-1.58)	1	0.97 (0.60-1.55)	0.88
	GG	22	6.88	17	5.31	0.68	0.71	54.51	<0.001	37.62	<0.001	1.44 (0.74-2.81)	0.29	1.48 (0.74-2.95)	0.27

[1]Adjusted for tobacco smoking, alcohol drinking and BMD values.

Table 3. Interaction between rs2288377 polymorphism and environmental factors

		TT	TA	AA	X^2 or t values	*P* value
Sex	Females	190	219	58		
	Males	68	82	23	0.14	0.93
Age		69.24±8.83	69.86±8.98	68.18±8.28		
Tobacco smoking habit	No	162	205	60		
	Yes	96	96	21	4.03	0.13
Alcohol drinking habit	No	163	193	47		
	Yes	95	108	34	1.03	0.60
L_1-L_4 vertebrae		0.95±0.12	0.95±0.11	0.93±0.10	1.68	0.19
Femoral neck		0.63±0.05	0.62±0.05	0.60±0.05	8.56	<0.001
Total hip		0.63±0.05	0.63±0.04	0.62±0.04	2.67	0.07
Trochanter		0.58±0.05	0.57±0.06	0.55±0.04	6.21	0.002

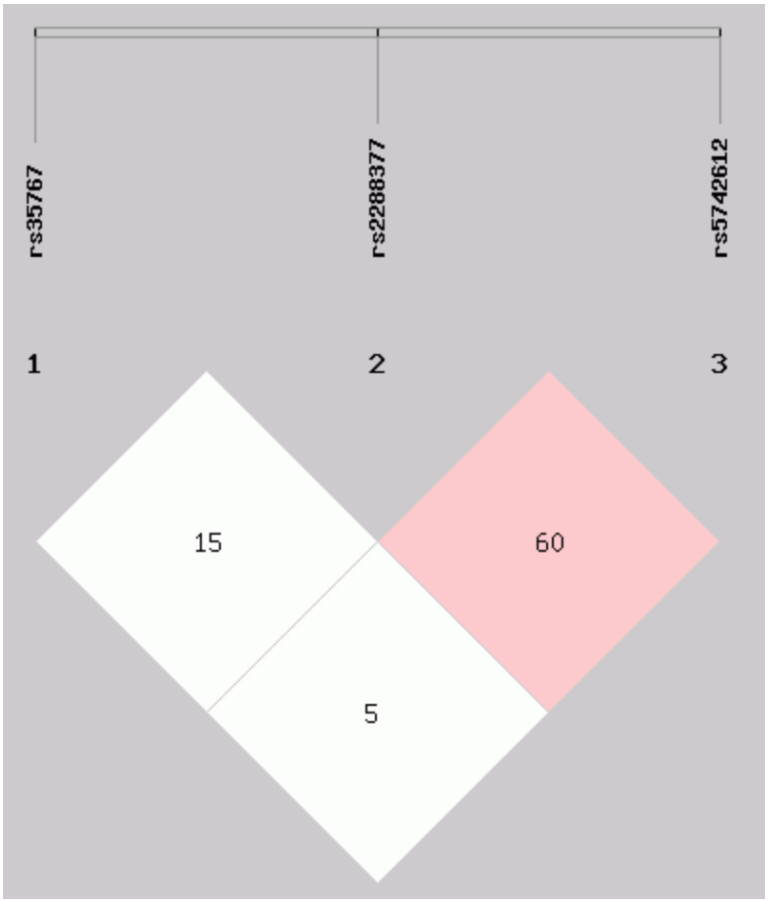

Figure 3. The linkage disequilibrium of IGF-I rs35767, rs2288377 and rs5742612.

A multivariate logistic regression analysis was performed to evaluate the correlation between IGF-I genetic polymorphisms and risk of osteoporosis (**Table 2**). We observed that the AA genotype of rs2288377 was associated with an elevated risk of osteoporosis when compared with the TT genotype, and the association was stronger when adjusting tobacco smoking, alcohol drinking and BMD values (OR=3.56, 95% CI=2.04-6.21). However, we did not observe that the IGF-I rs35767 and rs5742612 polymorphisms were significantly related to the risk of osteoporosis.

We performed an interaction with between the IGF-I rs2288377 polymorphism and sex, age, smoking, drinking and BMD values in the risk of osteoporosis (**Table 3**). However, we did not find any gene-environmental interaction between them (All *P* value >0.05).

The haplotype analysis revealed that IGF-I rs2288377 and rs5742612 showed linkage disequilibrium (D=0.605; r^2=0.032) (**Figure 3**). The T-A-T (rs35767-rs2288377-rs5742612) showed an increased risk of osteoporosis (OR=2.17, 95% CI=1.01-4.69) (**Table 4**). However, the other haplotypes were not associated with risk of this disease.

Discussion

In this study, we hypothesis that the genotype polymorphisms of IGF-I could influence the susceptibility to osteoporosis, and we reported that the IGF-I rs2288377 polymorphism was significantly associated with an increased risk

Table 4. Haplotype analysis of rs35767-rs2288377-rs5742612

Haplotype	Patients	%	Controls	%	*P* value	OR
C-A-G	4	0.012	13	0.035	0.10	0.34 (0.09-1.28)
C-A-T	68	0.189	87	0.234	0.24	0.77 (0.49-1.19)
C-T-G	36	0.1	31	0.084	0.57	1.20 (0.64-2.26)
C-T-T	140	0.388	155	0.418	0.51	0.88 (0.61-1.28)
T-A-T	34	0.094	17	0.046	0.04	2.17 (1.01-4.69)
T-T-G	25	0.07	12	0.033	0.08	2.20 (0.90-5.37)
T-T-T	53	0.147	56	0.15	0.92	0.97 (0.59~1.62)

Global chi^2=11.15 (frequency <0.03 in both control & case has been dropped); Pearson's P=0.084.

of osteoporosis, and T-A-T (rs35767-rs2288377-rs5742612) haplotype was correlated with risk of this disease.

IGF-I gene is located at chromosome 12q22-q24.1 with a length of 83kb, including six introns [20]. IGF-I contributes to the proliferation and differentiation of osteoclasts, and promotes the synthesis and mineralization of bone matrix. IGF-I could reduce the collagen degradation, increase bone deposition, promote osteoblast differentiation and maturation, stimulate bone mineralization and promote bone growth [21, 22]. Kanatani et al. reported that the phosphate could significantly increase cellular DNA synthesis, and high concentrations of alkaline phosphatase (ALP) could enhance the expression of IGF-I and its mRNA; thus, ALP could promote the bone formation through enhancing the activity of IGF-I [23]. Another experimental study reported that IGF-I could significantly promote the proliferation and differentiation of osteoprogenitor cells in normal rats, and enhance the activity of ALP and mineralization in bone [24]. Moreover, experimental studies indicated that the expression of IGF-I in serum was lower in patients with osteoporosis, and the gene expression of IGF-I was associated with bone mineral density [25-28].

Polymorphisms in IGF-I could affect the expression of IGF-I, and thus influence the risk of bone mineral density. Previous studies have reported the correlation between IGF-I polymorphisms and risk of osteoporosis, but the results of these studies are inconsistent [17, 29-33]. Three studies indicated that IGF-I rs35767 polymorphism was significantly associated with the pathogenesis of osteoporosis and bone mineral density in females [17, 31, 32, 34]. Kim et al. (2002) reported that IGF-I 194-base pair allele was correlated with the development of bone mineral density [29]. However, Jiang et al. (2005) performed a large sample size study in a Chinese population, but they did not find an association between IGF-I gene and BMD variation in premenopausal Chinese women [30]. In our study, we only found a correlation between IGF-I rs2288377 polymorphism and risk of osteoporosis. Therefore, results concerning the association between IGF-I rs2288377 polymorphism and risk of osteoporosis are not consistent, principally owing to the interaction between environmental factors, ethnicity, and genetic background.

Moreover, we firstly observed that T-A-T (rs35767-rs2288377-rs5742612) haplotype was correlated with osteoporosis risk, and linkage disequilibrium between rs35767 and rs5742612. The haplotype of T-A-T is a genetic marker for osteoporosis risk, and a mutation may be related to the haplotype of IGF-1, which may alter the activity and function of this protein.

Two limitations of the present study should be considered. Firstly, the included patients with osteoporosis were selected from only one hospital, and this sample may therefore not adequately represent other populations. However, genotype distributions of each of the polymorphisms under investigation were consistent with HWE, indicating that the study group was broadly representative. Secondly, more genes may have synergy effects with IGF-1 in the development of osteoporosis. Thus, further studies with more participants are needed to confirm our findings.

In conclusion, our study suggests that the IGF-I polymorphism and haplotype are associated with the risk of osteoporosis in the Chinese population. Further studies with more participants are required to measure the possible role of the IGF-I polymorphisms in the susceptibility to osteoporosis.

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Disclosure of conflict of interest

None.

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