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ORIGINAL RESEARCH

PFKP and *GPC6* Variants Were Correlated with Alcohol-Induced Femoral Head Necrosis Risk in the Chinese Han Population

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Background: Osteonecrosis of the femoral head (ONFH) is a common joint disease caused by excessive drinking, genetic factors, etc. The purpose of this study was to investigate the association between *PFKP* and *GPC6* variants and alcohol-induced ONFH (AIONFH) risk in the Chinese Han population.

Methods: This study genotyped 9 selected single nucleotide polymorphisms (SNPs) in 402 males by Agena MassARRAY Assay. By calculating odds ratios (ORs) and 95% confidence intervals (CIs), we assessed the effect of gene polymorphisms on AIONFH occurrence. False-positive report probability (FPRP) analysis and power were also used to evaluate the significant results. Multifactor dimensionality reduction (MDR) software was also utilized to predict the association between the selected SNPs and AIONFH risk.

Results: The overall analysis showed that *PFKP* rs10903966 and *GPC6* rs7320969 were correlated with AIONFH risk. *GPC6* rs4773724 was associated with a reduced risk of AIONFH, while individuals with *GPC6* rs9523981 CC genotype had a higher risk of AIONFH than individuals with the other genotypes among people under 42 years old. Based on stratified analysis of necrotic sites, rs7320969 was related to a decreased risk of AIONFH, while rs10903966 and rs9523981 were related to an increased risk of AIONFH. In addition, rs1008993 and rs7320969 were observed to be linked to AIONFH risk in patients at different clinical stages. Meanwhile, there were significant differences in TC, TG, platelet, ApoA1 and ApoB levels among subjects with different genotypes of rs1008993, rs9523981, rs7320969 and rs59624626. The results of MDR showed that rs11251720 and rs7320969 may play a synergistic role in predicting the risk of AIONFH.

Conclusion: *PFKP* rs10903966 and *GPC6* rs9523981 were associated with an increased risk of AIONFH, while GPC6 (rs7320969 and rs4773724) were correlated with a decreased risk of AIONFH. This result will need further experiments to verify.

Keywords: osteonecrosis of the femoral head, ATP-dependent 6-phosphofructokinase, glypican 6, polymorphism

Introduction

Osteonecrosis of the femoral head (ONFH), also known as avascular necrosis of the femoral head, is a progressive disease, which may result in the collapse of the femoral head, followed by destructive osteoarthritis. In the USA, 20,000 new cases of ONFH are diagnosed each year.[1] In Japan, a survey has reported that about 11,400 ONFH patients needed treatment each year, and about 2200 new cases were reported annually. Approximately 7 million people suffer from osteonecrosis in China, with 100,000–200,000 new cases every year.[2] ONFH usually results from excessive alcohol dependence.[3,4] It is reported that bone marrow mesenchymal stem cells (BMSCs) are regarded as the main precursor cells for bone regeneration with the ability to differentiate into osteoblasts, chondrocytes and adipocytes, which largely affects the proliferation, differentiation and mineralization of BMSCs induced by ethanol.[5,6] Besides, the recovery of reduced osteogenic activity can slow the progression of ONFH.[7]

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At present, there are many effective treatments for ONFH, such as drug interventions, hip replacement therapy and implanted vascular bundle,[8] but the number of ONFH patients seeking treatment is increasing. However, with the rapid development of society, people's pressure from all sides has doubled, leading to changes in people's living habits. Epidemiologic investigations showed that excessive alcohol consumption can increase the morbidity of atraumatic ONFH. Recently, the occurrence and development of AIONFH is a complex dynamic process mediated by a variety of factors and different signaling pathways of some genes. Besides, SNPs of some genes are associated with AIONFH. Variations of nitric oxide synthase 3 (NOS3), Osteoprotegerin (OPG), receptor activator of nuclear factor-kappa B ligand (RANKL) and matrix metalloproteinases (MMPs) genes have been found to be closely related to ONFH.[9–11]

Glypican 6 (also known as GPC6) is a proteoglycan family of proteoglycans that are anchored to the plasma membrane by glycosylphosphatidylinositol. In 2009, it was reported that loss-of-function mutations in *GPC6* gene could arise autosomal-recessive omodysplasia 1 (OMOD1).[12] Ahrens et al have found the expression of GPC6 in the proliferation region of mouse bone growth plate.[13] Capurro et al have also observed that most of GPC6-deficient embryos are abnormal in OMOD1 patients, while Hedgehog signaling is significantly decreased in the long bones of these embryos. And GPC6 accelerated the growth of long bones during development through stimulating the Hedgehog signaling pathway.[14] Therefore, GPC6 is necessary in the development of bones. Besides, *GPC6* variants have been observed to be associated with lumbar disk herniation risk.[15] Hence, *GPC6* SNPs should be paid more attention in bone development.

PFKP (ATP-dependent 6-phosphofructokinase, platelet), a member of the phosphofructokinase A protein family, is an important medium of cell metabolism. PFKP has been reported to take part in the regulation of glycolysis in some cancers, such as lung cancer, oral squamous cell carcinoma.[16,17] Although a meta-analysis of variants related to LDH in Northern Europeans has illustrated that *PFKP* SNPs are not associated with LDH occurrence, they may be involved in LDH development by affecting cell metabolism.

Herein, the case-control study was to discover the association between *PFKP* and *GPC6* SNPs and ONFH risk in the Chinese Han population, contributing to understanding the role of *PFKP* and *GPC6* in the development of alcohol-induced ONFH.

Materials and Methods

Subjects

Totally, 402 males (201 AIONFH patients and 201 controls) were recruited from the Second Hospital of Tangshan. They came from the surrounding areas of Tangshan and belonged to the Han nationality. Among them, 201 AIONFH males with osteonecrosis of the hip and frog position were diagnosed on the basis of X-ray and/or magnetic resonance imaging evidence.[18] Plain radiographs of the stage I, II, III, and IV of the Ficat Classification system were also applied to the diagnosis of ONFH. Patients with hip joint diseases such as traumatic osteonecrosis were excluded. In general, AIONFH is defined as a history of drinking more than 400 mL of alcohol per week.[19] Two hundred and one controls underwent routine physical examinations in the Second Hospital of Tangshan, and no symptoms of osteonecrosis and joint pain were found. In addition, people with chronic diseases such as hypertension and diabetes have been excluded. Informed consent was obtained from all individual participants included in the study. Additionally, the protocol has been approved by the Ethics Committee of the Second Hospital of Tangshan. All participants have signed an informed consent form prior to participating in the study. Meanwhile, our study strictly conformed to the principles of the Declaration of Helsinki.

DNA Extraction, SNP Selection and Genotyping

We extracted genomic DNA from participants' blood samples by GoldMag-Mini Whole Blood Genomic DNA Purification Kit (GoldMag Co. Ltd., Xi'an, China). NanoDrop 2000C spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to detect the DNA concentration and purity. In the present study, we selected a total of 9 variants located in *PFKP* and *GPC6* from the 1000 Genomes Project (https://www.internationalgenome.org/) with minor allele frequency (MAFs) >5% in the global population.[20] Amplification and extension of primers were designed using Agena

MassARRAY Assay Design. Agena MassARRAY RS1000 was utilized to perform SNP genotyping. Finally, we completed data processing and analysis by Agena Bioscience TYPER software 4.0.[10]

Statistical Analysis

Age differences between cases and controls were assessed by Student's *t*-test. In addition, we analyzed whether the genotype distribution of each locus in the control group meets the Hardy-Weinberg equilibrium (HWE) or not, in order to further explain the good representativeness of the study population. Logistic regression analysis was performed to evaluate the association between SNPs and AIONFH risk by PLINK software 1.07. PFRP values were utilized to determine whether the significant results are remarkable under the conditions that the FPRP threshold is 0.2, the power OR is 2.0 and prior probability level is "0.25, 0.1, 0.01, 0.001 and 0.0001". Haploview software 4.2 was used to calculate the degree of linkage between these SNPs based on the linkage disequilibrium (LD) map.[11] Multifactor dimensionality reduction (MDR) software package was utilized to predict the association between the selected SNPs and AIONFH risk. In addition, Geno Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were used to determine the functions of the interacting genes of *PFKP* and *GPC6*, which were analyzed by Fisher's test based on the R packages. $p<0.05$ was considered statistically significant.

Results

Basic Information of Study Subjects and Selected SNPs

The mean age of 201 cases and 201 controls were 42.68 ± 12.88 years and 42.87 ± 13.27 years, respectively. There was no significant difference in age distribution between the two groups ($p = 0.888$). Meanwhile, we also analyzed the clinical information (Clinical indexes: Total cholesterol (TC), Triglycerides (TG), High-density lipoprotein-cholesterol (HDL-C), Low-density lipoprotein-cholesterol (LDL-C), Apolipoproteins A1 (ApoA1), Apolipoproteins B (ApoB), ApoA1/ApoB, Platelet; Clinical characteristics: Necrotic site, Clinical stage) of cases and controls. However, we did not find any significant differences between the two groups (Table 1).

Table 2 showed that the information of 9 selected variants in *PFKP* and *GPC6* genes. The chromosome position, specific locations, minor/major alleles, minor allele frequency in cases and controls, and HWE *p*-value were listed in Table 2. Each locus was in accordance with HWE.

Table 1 Demographic and Clinical Data of Alcohol-Induced ONFH Cases and Healthy Controls

Variables	Case (N = 201)	Control (N = 201)	*P*-value
Age (Mean ± SD)	42.68 ± 12.88	42.87 ± 13.27	0.888
Clinical indexes (Mean ± SD)			
TC (mmol/L)	4.65 ± 0.92	4.54 ± 0.80	0.250
TG (mmol/L)	1.89 ± 1.28	2.01 ± 1.32	0.401
HDL-C (mmol/L)	1.04 ± 0.24	1.09 ± 0.20	0.054
LDL-C (mmol/L)	2.73 ± 0.85	2.65 ± 0.70	0.394
ApoA1 (g/L)	1.22 ± 0.22	1.26 ± 0.14	0.514
ApoB (g/L)	1.62 ± 8.75	1.07 ± 0.20	0.821
Clinical characteristics (N %)			
Hip lesions			
Unilateral	44 (21.9%)		
Bilateral	157 (78.1%)		
Clinical stage			
I + II	54 (26.9%)		
III + IV	147 (73.1%)		

Note: *P*-value was calculated by independent samples *t*-test.
Abbreviations: SD, standard deviation; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; ApoA, Apolipoproteins A; ApoB, Apolipoproteins B.

Table 2 The Information of Selected Variants in *PFKP* and *GPC6* Genes

SNP-ID	Gene	Chr	Position	Allele		MAF		HWE *p*-value
				Ref	Alt	Case	Control	
rs35863365	*PFKP*	10	Intron	C	T	0.149	0.139	0.234
rs8181285	*PFKP*	10	Intron	A	G	0.348	0.393	0.760
rs10903966	*PFKP*	10	Intron	T	C	0.557	0.490	1.000
rs11251720	*PFKP*	10	Intron	T	C	0.067	0.060	0.518
rs4773724	*GPC6*	13	Intron	T	G	0.359	0.405	0.107
rs1008993	*GPC6*	13	Intron	C	T	0.162	0.142	0.036
rs9523981	*GPC6*	13	Intron	C	T	0.384	0.346	0.275
rs7320969	*GPC6*	13	Intron	G	C	0.318	0.341	0.059
rs59624626	*GPC6*	13	Intron	T	G	0.304	0.289	0.864

Note: HWE *p*-value was calculated by Pearson chi-square test.
Abbreviations: SNP, single nucleotide polymorphism; Chr, chromosome; Ref, reference; Alt, alteration; MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium.

Correlation Between *PFKP* and *GPC6* Variants and AIONFH Risk

We also analyzed the association between *PFKP* and *GPC6* variants and AIONFH risk under multiple genetic models (Table 3). In the recessive model showed that individuals with *PFKP* rs10903966 TT genotype had a significantly higher risk than individuals with C/C or T/C genotype (OR = 1.72, 95% CI: 1.11–2.67, $p = 0.015$). After adjustment for gender and age, the significance still existed. *GPC6* rs7320969 was also associated with AIONFH risk in the codominant model (OR = 0.65, 95% CI: 0.43–0.98, $p = 0.041$; adjusted OR = 0.65, 95% CI: 0.43–0.98, $p = 0.041$). The other models (dominant, recessive and log-additive models) were also used to analyze the relationship between rs7320969 and AIONFH risk, while the results were not significant. As shown in Table 4, with a prior probability of 0.25 and 0.1, the power of 0.742, and FPRP values of 0.054 and 0.146, there was a significant association between rs10903966 and AIONFH risk. And the association between rs7320969 and AIONFH risk was noteworthy in the heterozygous model (power = 0.895, FPRP = 0.118, 0.286).

Table 3 Association Analysis of *PFKP* and *GPC6* Polymorphisms with Alcohol-Induced ONFH Susceptibility in Genetic Models

Gene	SNP-ID	Model	Genotype	Frequency		Without Adjustment		With Adjustment	
				Case	Control	OR (95% CI)	p^a-value	OR (95% CI)	p^b-value
PFKP	rs10903966	Codominant	C/C	68	48	1		1	
			T/C	80	101	0.90 (0.55–1.47)	0.661	0.90 (0.55–1.47)	0.667
			T/T	46	52	1.60 (0.93–2.75)	0.089	1.61 (0.94–2.77)	0.086
		Dominant	C/C	68	48	1		1	
			T/C-T/T	126	153	1.12 (0.71–1.77)	0.620	1.13 (0.71–1.78)	0.613
		Recessive	C/C-T/C	148	149	1		1	
			T/T	46	52	1.72 (1.11–2.67)	**0.015**	1.73 (1.11–2.68)	**0.015**
		Log-additive	–	–	–	1.28 (0.98–1.68)	0.072	1.29 (0.98–1.69)	0.070
GPC6	rs7320969	Codominant	C/C	24	17	1		1	
			G/C	80	103	0.65 (0.43–0.98)	**0.041**	0.65 (0.43–0.98)	**0.041**
			G/G	97	81	1.18 (0.59–2.35)	0.639	1.18 (0.59–2.35)	0.639
		Dominant	C/C	24	17	1		1	
			G/C-G/G	177	184	0.72 (0.49–1.07)	0.109	0.72 (0.49–1.08)	0.109
		Recessive	C/C-G/C	104	120	1		1	
			G/G	97	81	1.47 (0.76–2.82)	0.251	1.47 (0.76–2.82)	0.251
		Log-additive	–	–	–	0.90 (0.67–1.22)	0.493	0.90 (0.67–1.22)	0.494

Notes: p^a-value was calculated by logistic regression analysis without adjustment. p^b-value was calculated by logistic regression analysis adjusted for gender and age. Bold indicates that the SNP is statistical significance ($p < 0.05$).
Abbreviations: SNP, single nucleotide polymorphism; OR, odds ratio; 95% CI, 95% confidence interval.

Table 4 FPRP Analysis for the Significant Associations of ARRDC3 SNPs with Glioma Risk

Model	OR(95% CI)	Power	Prior Probability				
			0.25	0.1	0.01	0.001	0.0001
PFKP rs10903966 C/C-T/C vs T/T	1.73 (1.11–2.68)	0.742	0.054	0.146	0.653	0.950	0.995
GPC6 rs7320969 C/C vs G/C	0.65 (0.43–0.98)	0.895	0.118	0.286	0.815	0.978	0.998

Note: Noteworthiness at the 0.2 level of FPRP.

Stratification Analysis of the Association Between *PFKP* and *GPC6* Variants and AIONFH Risk

We further evaluated the correlation between *PFKP* and *GPC6* variants and AIONFH risk in the >42 years old groups and the ≤42 years old groups. However, *GPC6* variants were only found to be associated with AIONFH in subjects aged >42 years (Table 5). Rs4773724-T was correlated with a reduced risk of AIONFH compared to the G allele (adjusted OR = 0.66, 95% CI: 0.44–0.99, $p = 0.045$). Nevertheless, subjects with rs9523981-C allele had no apparent susceptibility to AIONFH compared to the T allele. In the codominant, dominant and log-additive models, rs13177623 did not significantly decreased susceptibility to AIONFH (codominant model: adjusted OR = 0.44, 95% CI: 0.24–0.82, $p = 0.010$; dominant model: adjusted OR = 0.46, 95% CI: 0.26–0.84, $p = 0.011$; log-additive model: adjusted OR = 0.65, 95% CI: 0.43–0.99, $p = 0.045$). Rs9523981 was associated with the risk of AIONFH in codominant (adjusted OR = 3.23, 95% CI: 1.15–9.03, $p = 0.026$) and recessive (adjusted OR = 3.44, 95% CI: 1.30–9.09, $p = 0.013$) models.

Besides, we performed the necrotic sites stratification analysis to evaluate the association between *PFKP* and *GPC6* polymorphisms and AIONFH risk (unilateral ONFH patients vs controls; bilateral ONFH patients vs controls) as shown

Table 5 Association Analysis Between *GPC6* Polymorphisms and Alcohol-Induced ONFH Susceptibility in ≤42 Years Group

SNP-ID	Model	Genotype	Frequency		With Adjustment	
			Case	Control	OR (95% CI)	p^b-value
rs4773724	Allele	G	65	85	1	
		T	129	111	0.66 (0.44–0.99)	**0.045**
	Codominant	G/G	13	15	1	
		T/G	39	55	0.44 (0.24–0.82)	**0.010**
		T/T	45	28	0.54 (0.22–1.30)	0.168
	Dominant	G/G	13	15	1	
		T/G-T/T	84	83	0.46 (0.26–0.84)	**0.011**
	Recessive	G/G-T/G	52	70	1	
		T/T	45	28	0.86 (0.38–1.91)	0.704
	Log-additive	–	–	–	0.65 (0.43–0.99)	**0.045**
rs9523981	Allele	T	80	65	1	
		C	118	133	1.39 (0.92–2.09)	0.118
	Codominant	T/T	18	6	1	
		T/C	44	53	0.90 (0.49–1.64)	0.701
		C/C	37	40	3.23 (1.15–9.03)	**0.026**
	Dominant	T/T	18	6	1	
		T/C-C/C	81	93	1.13 (0.64–2.02)	0.671
	Recessive	T/T-T/C	62	59	1	
		C/C	37	40	3.44 (1.30–9.09)	**0.013**
	Log-additive	–	–	–	1.42 (0.92–2.18)	0.110

Notes: p^a-value was calculated by logistic regression analysis without adjustment. p^b-value was calculated by logistic regression analysis adjusted for gender and age. Bold indicates that the SNP is statistical significance ($p < 0.05$).
Abbreviations: SNP, single nucleotide polymorphism; OR, odds ratio; 95% CI, 95% confidence interval.

in Table 6. Compared to the controls, there was a significant association between *GPC6* rs7320969 and a reduced risk of AIONFH in unilateral ONFH patients in the codominant model (OR = 0.39, 95% CI: 0.19–0.83, $p = 0.015$; adjusted OR = 0.39, 95% CI: 0.19–0.84, $p = 0.015$). We compared controls with bilateral ONFH patients, suggesting that *PFKP* rs10903966 was connected with AIONFH risk in the codominant (OR = 1.89, 95% CI: 1.05–3.41, $p = 0.035$), recessive (OR = 1.77, 95% CI: 1.12–2.82, $p = 0.016$) and log-additive (OR = 1.40, 95% CI: 1.04–1.88, $p = 0.028$) models. After adjustment, the locus was still related to an increased risk of AIONFH risk (adjusted OR = 1.89, 95% CI: 1.05–3.42, $p = 0.035$; adjusted OR = 1.78, 95% CI: 1.12–2.83, $p = 0.015$; adjusted OR = 1.40, 95% CI: 1.04–1.88, $p = 0.027$) in three models. Furthermore, in the codominant model, people with the *GPC6* rs9523981 CC genotype (adjusted OR = 2.02, 95% CI: 1.04–3.93, $p = 0.039$) had an increased risk of AIONFH compared with the TT genotype, up to 2.02-fold. Patients with the rs9523981 T/T or T/C genotype were more likely to develop AIONFH (adjusted OR = 1.98, 95% CI: 1.07–3.67, $p = 0.030$) in contrast with the CC genotype in the recessive model.

Analysis of the Association Between *PFKP* and *GPC6* Variants and AIONFH Risk in Patients with Different Clinical Stages and Clinical Parameters

We also investigated the association between *PFKP* and *GPC6* variants and AIONFH risk in patients at different clinical stages (Table S3). In Table S3, we found that *GPC6* rs1008993 decreased the risk of AIONFH by 0.56-fold in the log-

Table 6 Association Analysis Between *PFKP* and *GPC6* Polymorphisms and Alcohol-Induced ONFH Susceptibility After Necrotic Site Stratification Analysis

SNP-ID	Model	Genotype	Frequency		Without Adjustment		With Adjustment	
			Case	Control	OR (95% CI)	p^a-value	OR (95% CI)	p^b-value
Unilateral necrotic site								
rs7320969	Codominant	C/C	8	17	1		1	
		G/C	12	103	0.39 (0.19–0.83)	**0.015**	0.39 (0.19–0.84)	**0.015**
		G/G	24	81	1.59 (0.61–4.13)	0.343	1.59 (0.61–4.12)	0.346
	Dominant	C/C	8	17	1	–	1	–
		G/C-G/G	36	184	0.56 (0.29–1.09)	0.086	0.56 (0.29–1.09)	0.087
	Recessive	C/C-G/C	20	120	1	–	1	–
		G/G	24	81	2.41 (0.97–5.99)	0.060	2.39 (0.96–5.97)	0.061
	Log-additive	–	–	–	0.90 (0.54–1.49)	0.677	0.90 (0.54–1.49)	0.676
Bilateral necrotic sites								
rs10903966	Codominant	C/C	54	48	1		1	
		T/C	66	101	1.10 (0.64–1.89)	0.740	1.10 (0.64–1.89)	0.735
		T/T	31	52	1.89 (1.05–3.41)	**0.035**	1.89 (1.05–3.42)	**0.035**
	Dominant	C/C	54	48	1		1	
		T/C-T/T	97	153	1.35 (0.81–2.24)	0.244	1.35 (0.82–2.25)	0.241
	Recessive	C/C-T/C	120	149	1		1	
		T/T	31	52	1.77 (1.12–2.82)	**0.016**	1.78 (1.12–2.83)	**0.015**
	Log-additive	–	–	–	1.40 (1.04–1.88)	**0.028**	1.40 (1.04–1.88)	**0.027**
rs9523981	Codominant	T/T	28	20	1		1	
		T/C	71	99	1.03 (0.65–1.63)	0.893	1.03 (0.66–1.63)	0.889
		C/C	57	82	2.01 (1.04–3.92)	**0.039**	2.02 (1.04–3.93)	**0.039**
	Dominant	T/T	28	20	1		1	
		T/C-C/C	128	181	1.20 (0.78–1.84)	0.413	1.20 (0.78–1.84)	0.410
	Recessive	T/T-T/C	99	119	1		1	
		C/C	57	82	1.98 (1.07–3.67)	**0.030**	1.98 (1.07–3.67)	**0.030**
	Log-additive	–	–	–	1.31 (0.96–1.78)	0.091	1.31 (0.96–1.78)	0.090

Notes: p^a-value was calculated by logistic regression analysis without adjustment. p^b-value was calculated by logistic regression analysis adjusted for gender and age. Bold indicates that the SNP is statistical significance ($p < 0.05$).

Abbreviations: SNP, single nucleotide polymorphism; OR, odds ratio; 95% CI, 95% confidence interval.

additive model (adjusted OR = 0.56, 95% CI: 0.32–0.98, p = 0.042). In the codominant model, patients with *GPC6* rs7320969 GG genotype (OR = 2.43, 95% CI: 1.19–4.96, p = 0.015; adjusted OR = 2.49, 95% CI: 1.21–5.11, p = 0.013) had a higher incidence of AIONFH compared with patients with CC genotype, up to 2.43-fold and 2.49-fold, respectively. In the dominant model, subjects with rs7320969 G/C or G/G genotype were more likely to develop AIONFH (OR = 2.04, 95% CI: 1.08–3.85, p = 0.028; adjusted OR = 2.07, 95% CI: 1.09–3.94, p = 0.027) compared with those with the CC genotype.

Moreover, we analyzed the relationship between different genotypes of 9 loci and clinical parameters (TC, TG, HDL-C, LDL-C, ApoA1, ApoB, ApoA1/ApoB and Platelet) in Table S4. We found that the TG level of rs1008993 carriers, the Platelet level of rs9523981 carriers and the TC level of rs59624626 with different genotypes were significantly different (p = 0.000, p = 0.018, p = 0.042), respectively. The contents of ApoA1 and ApoB were also significantly different among individuals with different genotypes of rs7320969 (p = 0.026, p = 0.025).

LD and Haplotype Analysis

Among the nine variants (rs35863365, rs8181285, rs10903966, rs11251720, rs4773724, rs1008993, rs9523981, rs7320969 and rs59624626), we performed the LD analysis (Figures 1 and 2). There was a 0kb LD block 1 between rs35863365 and rs8181285, and another two blocks were formed in *GPC6* (rs4773724 and rs1008993; rs7320969 and rs59624626). However, no haplotype was found to be associated with the risk of AIONFH. Based on age stratification analysis (Table S1), the CG haplotype was related to an increased AIONFH risk in patients aged >42 years (adjusted OR = 1.82, 95% CI: 1.12–2.98, p = 0.016).

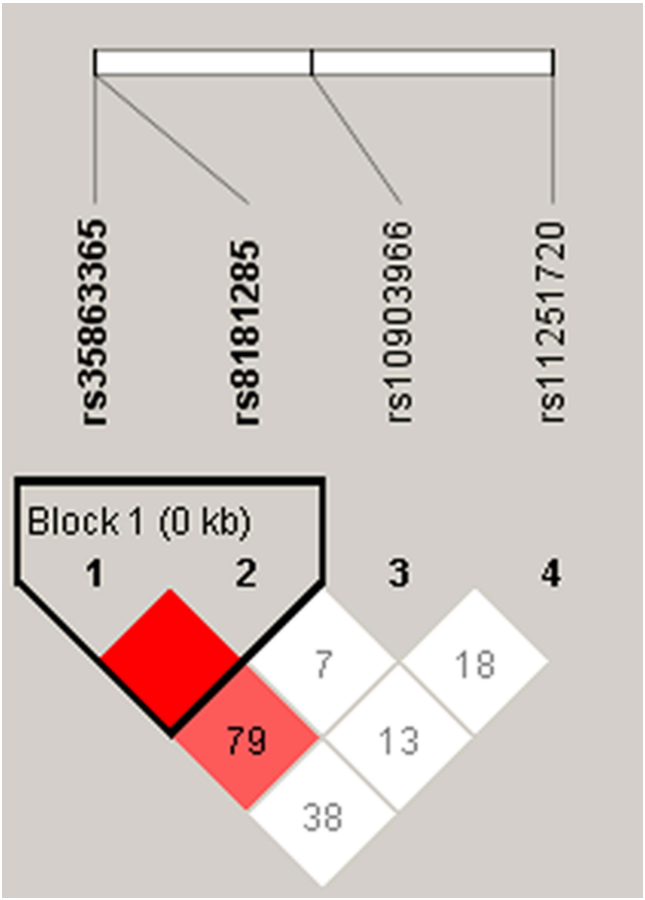

Figure 1 Haplotype block map of *PFKP* variants. The numbers inside the diamonds indicate the D′ for pairwise analyses.

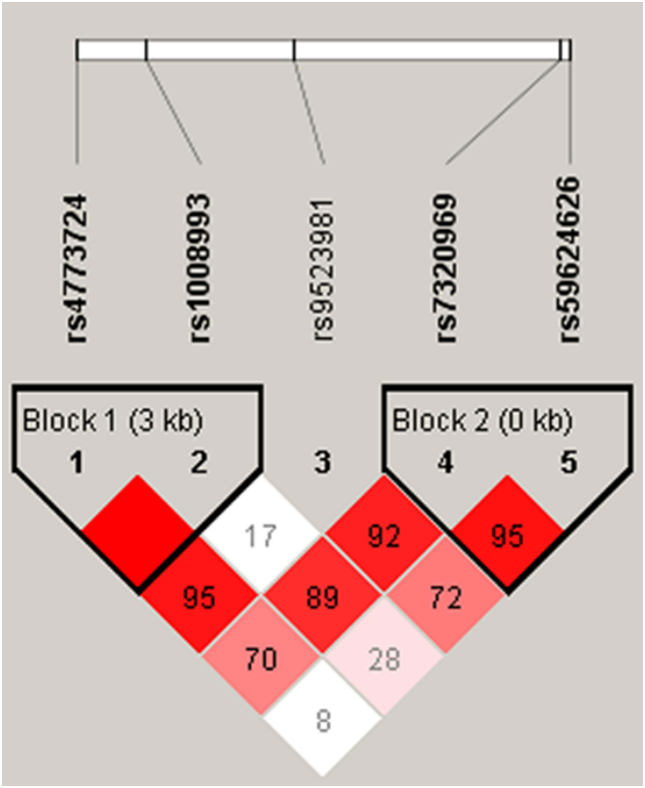

Figure 2 Haplotype block map of *GPC6* variants. The numbers inside the diamonds indicate the D′ for pairwise analyses.

SNP-SNP Interaction and AIONFH Risk

In Table S2, MDR analysis showed that the best model was composed of rs35863365, rs8181285, rs11251720, rs9523981, rs7320969 and rs59624626, with the training accuracy of 82.5%, the testing accuracy of 58.0% and the cross-validation consistency of 10/10. In Figure 3, rs11251720 and rs7320969 may play a synergistic role in predicting the risk of AIONFH.

GO and KEGG Pathway Analyses of the Interacting Genes of *PFKP* and *GPC6*

In order to further study the function of *PFKP* and *GPC6* genes, we used string online software (http://string-db.org/) to screen out the interacting genes of *PFKP* and *GPC6* genes, followed by GO enrichment and KEGG pathway analysis. In Figure 4, we

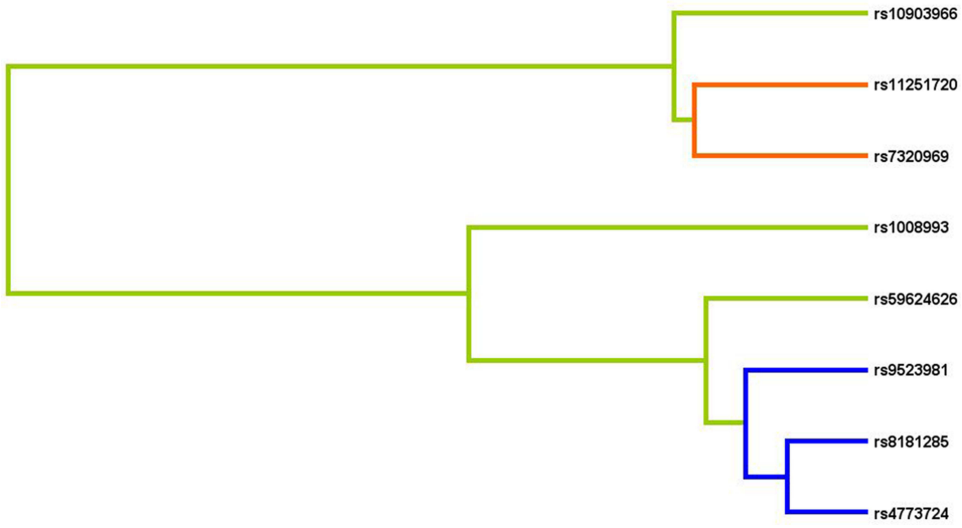

Figure 3 Multifactor dimensionality reduction (MDR) was completed to detect the interaction between SNPs in *PFKP* and *GPC6* genes to predict the alcohol-induced ONFH risk.

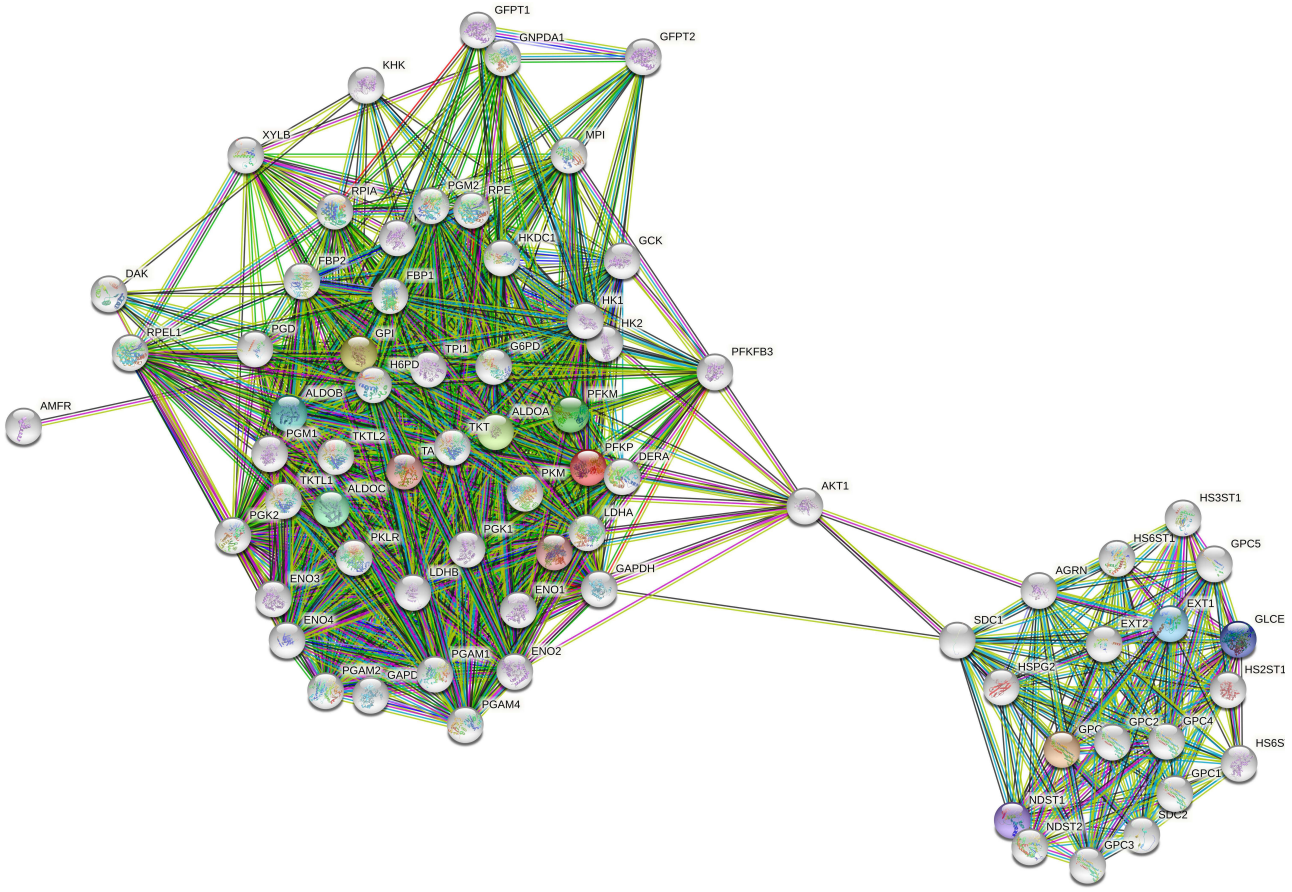

Figure 4 The interacting genes of *PFKP* and *GPC6* were displayed by String software.

displayed the interacting genes of *PFKP* and *GPC6*. In Table S5 and Figure 5, the results of the top 10 GO pathways of the interacting genes of *PFKP* and *GPC6* showed that changes in biological processes (BP) were mainly enriched in monosaccharide metabolic process, pyridine nucleotide metabolic process, nicotinamide nucleotide metabolic process, pyridine-containing compound metabolic process, oxidoreduction coenzyme metabolic process, coenzyme metabolic process, carbohydrate catabolic process, hexose metabolic process, glycolytic process, ATP generation from ADP. Molecular function (MF) changes were mainly distributed in monosaccharide binding, carbohydrate binding, isomerase activity, carbohydrate kinase activity, lyase activity, magnesium ion binding, glucose binding, heparan sulfate sulfotransferase activity, intramolecular transferase activity, sulfotransferase activity. Besides, cell component (CC) changes were mainly concentrated in Golgi lumen, lysosomal lumen, ficolin-1-rich granule lumen, vacuolar lumen, ficolin-1-rich granule, collagen-containing extracellular matrix, secretory granule lumen, cytoplasmic vesicle lumen, vesicle lumen, cytosolic part. The top 10 KEGG pathways of the interacting genes of *PFKP* and *GPC6* were mainly enriched in Metabolic pathways, Carbon metabolism, Glycolysis/

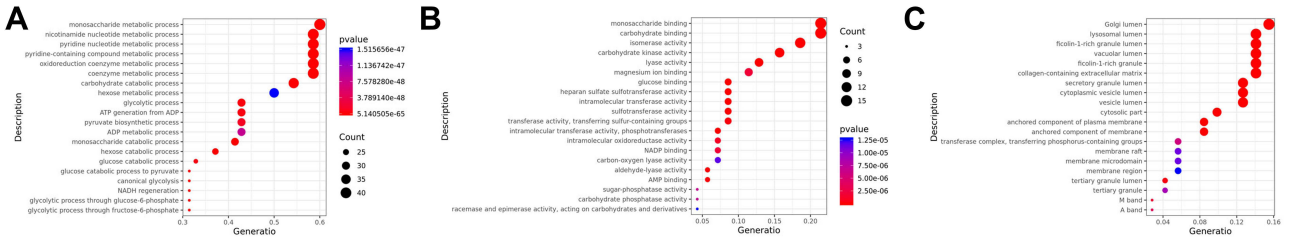

Figure 5 The results of the top 10 GO pathways of the interacting genes of *PFKP* and *GPC6*. (**A**) Bubble plot of BP, (**B**) Bubble plot of MF, (**C**)Bubble plot of CC.

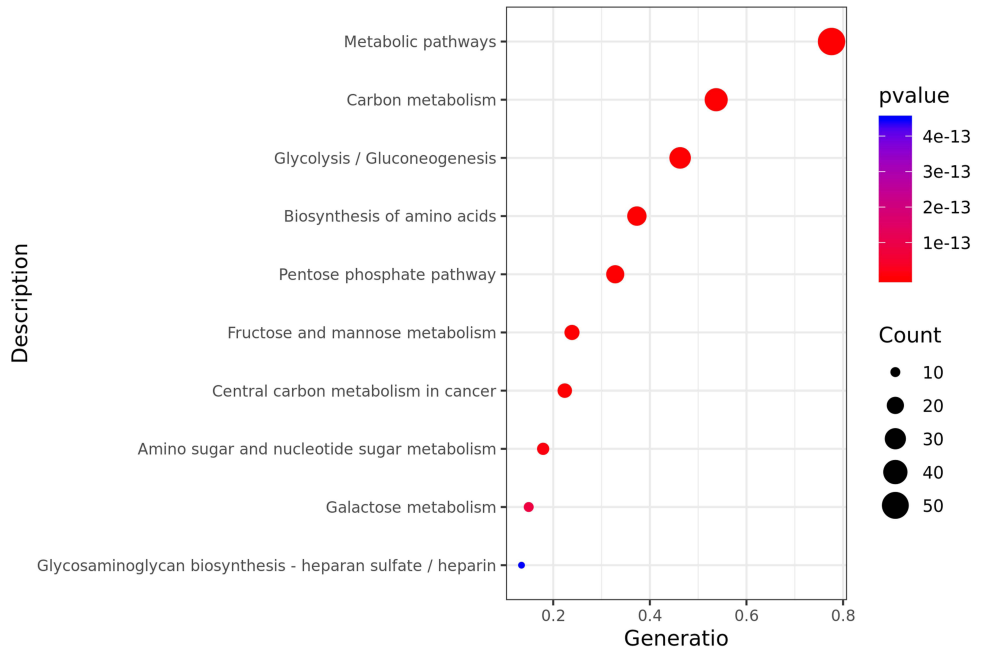

Figure 6 The results of the top 10 KEGG pathways of the interacting genes of *PFKP* and *GPC6*.

Gluconeogenesis, Biosynthesis of amino acids, Pentose phosphate pathway, Fructose and mannose metabolism, Central carbon metabolism in cancer, Amino sugar and nucleotide sugar metabolism, Galactose metabolism, Glycosaminoglycan biosynthesis-heparan sulfate/heparin (Table S6 and Figure 6).

Discussion

In this study, the overall analysis showed that *PFKP* rs10903966 and *GPC6* rs7320969 were correlated with AIONFH risk. Age stratification analysis demonstrated that *GPC6* rs4773724 was related to a reduced risk of AIONFH, while individuals with *GPC6* rs9523981 CC genotype had a higher risk of AIONFH than individuals with the other genotypes. Based on stratified analysis of necrotic sites, rs7320969 was related to a decreased risk of AIONFH, and rs10903966 and rs9523981 were related to an increased risk of AIONFH. In addition, rs1008993 and rs7320969 were observed to be linked with AIONFH risk in patients at different clinical stages. Meanwhile, subjects with different genotypes of rs1008993, rs9523981, rs7320969 and rs59624626 had significantly different levels of TC, TG, Platelet, ApoA1 and ApoB.

Like other proteoglycans, *GPC6* binds to the outer surface of the cell membrane via glycosylphosphatidylinositol junctions.[21] At present, the genomes of members of the GPC family (GPC1-GPC6) have been identified,[22] and their encoded products are related to cell growth regulation and differentiation. Thereinto, most GPCs only carry heparin sulfate chains. Another significant feature of GPCs is that they have no obvious homology with characteristic regions of other proteins, indicating that GPCs have unique functions. Among them, GPC6 has been reported to play a great role in the field of osteoporosis and fracture risk.[23] Kemp et al have suggested that the expression of Gpc6 was found in mouse osteoblasts and osteocytes, and bone mass and strength were increased in adult $Gpc6^{-/-}$ mice compared to the wild-type mice.[24] The phenotype was consistent with that of OMOD1, again suggesting that GPC6 plays an important role in bone development. Furthermore, when the new locus of *GPC6* in osteoporosis was identified by bioinformatics analysis, they have found that *GPC6* rs1933784 and rs72635657 were associated with bone mineral density.[24] Yang et al have found *GPC6* SNPs (rs4773724, rs1008993, rs9523981, rs7320969 and rs59624626) related to LDH risk.[15] In the present study, we firstly found that *GPC6* variants (rs4773724, rs1008993, rs9523981, rs7320969 and rs59624626) were correlated with AIONFH risk. Thus, *GPC6* SNPs can be considered critical in the development of bones.

PFKP, located in the chromosome 10p15.2, has 8 transcript variants, the longest of which is isoform 1 with 784 amino acids. The regulation of PFKP gene expression is closely related to glycolysis. At present, Shen et al have suggested that PFKP was highly expressed in lung cancer tissues and cell lines, and was related to clinical features of patients (tumor size and patient prognosis).[16] Also, the expression level of PFKP had an effect on the glucose metabolism level in lung cancer cells. In addition, PFKP is one of the targets of HIF-1.[25] HIF-1α deletion can increase the expression of PFKP and reduce the glycolysis level to facilitate pancreatic cancer progression.[26] A survey has showed that *PFKP* SNPs were not associated with LDH occurrence, but we found that *PFKP* rs10903966 was associated with AIONFH risk. *PFKP* may take part in the cell metabolism to affect the progression of ONFH.

However, the present study has several limitations. Firstly, insufficient sample size may affect the conclusions of the study. A larger sample size is then required to validate the results. Secondly, the sample contains only one race, which may lead to some uncertainty in the research results, and data from different races are needed to verify the results. Finally, we found that *PFKP* and *GPC6* polymorphisms were associated with the risk of alcohol-induced ONFH, but there was a lack of clear mechanism research. HaploReg v4.1 (https://pubs.broadinstitute.org/mammals/haploreg/haploreg. php) is used to predict the effect of *PFKP* and *GPC6* polymorphisms on their function (Table 2). Then, cellular and molecular experiments were used to further verify the role of *PFKP* and *GPC6* polymorphisms in the process of AIONFH.

Conclusion

In a word, *PFKP* rs10903966 and *GPC6* rs9523981 were associated with an increased risk of AIONFH, while *GPC6* (rs7320969 and rs4773724) were correlated with a decreased risk of AIONFH. The results will lay a foundation for clarifying the mechanism of *PFKP* and *GPC6* in alcoholic osteonecrosis.

Data Sharing Statement

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

The protocol has been approved by the Ethics Committee of the Second Hospital of Tangshan. All participants have signed informed consent form prior to participating in the study. Meanwhile, our study strictly conformed to the principles of the Declaration of Helsinki.

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Disclosure

The authors declare no conflicts of interest in this work.

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