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Original Article

Association of *ALOX15* gene polymorphisms with obesity-related phenotypes in Chinese nuclear families with male offspring

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Aim: Genetic variation in ALOX12, which encoded human 12-lipoxygenase, was found to be associated with fat mass in young Chinese men. The objective of this study was to investigate the relationship between single nucleotide polymorphisms (SNPs) and haplotypes in the ALOX15 gene and obesity-related phenotypes in Chinese nuclear families with male offspring.
Methods: We recruited 1,296 subjects from 427 nuclear families with male offspring and genotyped five SNPs (rs9894225, rs748694, rs2619112, rs2619118, and rs916055) in the ALOX15 gene locus. The total fat mass (TFM), trunk fat mass (tFM), leg fat mass (LFM) and arm fat mass (AFM) were measured using dual-energy X-ray absorptiometry (DXA). The percentage of fat mass (PFM) was the ratio of TFM and body weight. The association between SNPs and haplotypes of ALOX15 and obesity-related phenotypic variation was measured using quantitative transmission disequilibrium test (QTDT).
Results: Using QTDT to measure family-based genetic association, we found that rs916055 had a statistically significant association with PFM (P=0.038), whereas rs916055 had a marginal but statistically insignificant association with tFM (P=0.093). The multiple-parameter 1000 permutations test agreed with the family-based association results: both showed that rs916055 had a statistically significant association with PFM (P=0.033).
Conclusion: rs916055 in ALOX15 gene was significantly associated with the percentage of fat mass in Chinese nuclear families with male offspring in the family-based association study using QTDT approach.

Keywords: obesity; fat mass; *ALOX15*; lipoxygenase; single nucleotide polymorphism; obesity-related phenotypes; family-based association study; quantitative transmission disequilibrium test

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Introduction

There has been an alarming increase in the number of patients with metabolic syndrome, a disorder with a constellation of conditions that includes glucose intolerance, obesity, dyslipidemia and hypertension. Obesity is the central and causal component of this syndrome[1], but the underlying mechanisms have not been fully elucidated. It is now widely accepted that the activation of inflammatory and oxidative stress is one of the common causes of obesity and largely contributes to the related pathological outcomes[2–4]. Several factors are responsible for inflammation in obesity, such as elevated nuclear-factor kappaB (NF-κB) activity, the presence of free fatty acids, and increased levels of adipokines including tumor necrosis factor-alpha (TNFα), interleukins (ILs), resistin and leptin[2, 5, 6]. Oxidative stress activates kinases such as c-Jun N-terminal kinase (JNK) and mitogen-activated protein kinase (MAPK) and inhibits NF-κB kinase (IKK). These kinases may directly interfere insulin signaling or indirectly enhance inflammatory processes via common biochemical pathways (*ie*, NF-κB)[7–9]. Exploring the interface between inflammation and oxidative stress at a molecular and genetic level will enhance our understanding of obesity and the complications associated with it.

The lipoxygenase pathway, which generates proinflammatory metabolites from arachidonic acid, has recently attracted a great deal of attention for its potential role in obesity and atherosclerosis disease. Expression of 12/15-lipoxygenase is elevated in adipocytes isolated from insulin resistant obese

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Zucker rats relative to lean rats[10]. A diet high in fat induced macrophage infiltration into the adipose tissue of wild-type but not 12/15-lipoxygenase gene knock-out mice[11]. The addition of the 12/15-lipoxygenase metabolic products, 12(*S*)-HETE and 15(*S*)-HETE, directly to 3T3-LI adipose cells significantly upregulated the expression of key proinflammatory genes (*TNF-a, IL-6,* and *IL-12*) and downregulatesd an important anti-inflammatory gene (*adiponectin*)[12]. These findings support the hypothesis that 12/15-lipoxygenase is important for obesity. The human 12/15 lipoxygenase is encoded by arachidonate lipoxygenase 12/15 (*ALOX12/15*), both of which are located on chromosome 17p13, a region that is linked to obesity-related traits in several independent studies[13]. More recently, we have shown that genetic variation in *ALOX12* is associated with total fat mass (TFM) in young Chinese men[14]. An analysis of tar *ALOX12* cDNA showed that *ALOX12* raised 12-HETE and 15-HETE levels but had a greater effect on 12-HETE[15]. This finding suggests that two *ALOX* genes may, at least to some extent, be similar in function. Furthermore, UV-irradiation suppressed *ALOX12* expression, whereas it up-regulated *ALOX15* expression. Treatment with *ALOX15* metabolites significantly suppressed insulin-like growth factor II induced-*ALOX12* expression in human keratinocyte cells[16]. Together, these findings have raised our interest in assessing whether the *ALOX15* pathway is involved in the etiology of obesity. In the present study, we performed family-based association studies of *ALOX15* using the quantitative transmission disequilibrium test (QTDT) to determine whether SNPs in *ALOX15* are associated with obesity-related phenotypic variation in a sample of Chinese nuclear families used in a previous study.

Materials and methods

Subjects

We recruited 1296 individuals from 427 nuclear families whose offspring were sons from 2004 to 2007. The average family size was 3.03. Four hundred two families had one child, and 25 families had two children. Each study subject completed a questionnaire concerning his or her age, sex, medical history, family history, marital status, physical activity, alcohol use, dietary habits and smoking history. All of the male offspring were healthy. Exclusion criteria were the same as previously reported[14, 17] .

All the study subjects belonged to the Chinese Han ethnic group and were residents of Shanghai which is located approximately halfway along the coast of China. The study was approved by the Ethics Committee of the Sixth People's Hospital affiliated with Shanghai Jiao Tong University. All of the subjects involved in the study signed informed consent documents before entering the project.

Body composition measurements

A total-body dual-energy X-ray absorptiometry (DXA) scan was performed using pan-beam technology (GE-LUNAR Prodigy, USA; enhanced whole-body detector, software version 5.71). A standard soft-tissue examination to analyze body composition consisted of TFM measurements, regional measurements of trunk fat mass (tFM) and arm (AFM) and leg fat mass (LFM) measurement. Height was measured using a stadiometer. Arm and leg fat mass together constitute extremity fat mass. Trunk fat mass (tFM) is an indicator of the tendency of adipose to accumulate in the central trunk region. The tFM has been found to correlate well with abdominal fat. All male progeny were measured for body composition, as described above. The machine was calibrated daily, and the coefficient of variability (*CV*) values of the DXA measurements (which were obtained from fifteen individuals repeatedly measured three times) were calculated to be 3.72% for AFM, 3.28% for LFM, 2.52% for tFM and 1.69% for TFM. The long-term reproducibility of our DXA data during the trial, based on repeated weekly phantom measurements using standardized equipment, was 0.45%. The body mass index (BMI) was defined as weight/height^2 (units of kilogram/meter^2). The percentage of fat mass (PFM) is the TFM divided by body weight.

Genotyping

SNPs were selected using the following criteria: (1) validation status (validated experimentally in human populations). (2) degree of heterozygosity (*ie*, minor allele frequency (MAF)>0.1) and (3) reported to the dbSNP (SNP database) by multiple sources. A total of five SNPs within *ALOX15* were selected: rs9894225, rs748694, rs2619112, rs2619118, and rs916055. Three of the five SNPs (rs2619112, rs2619118, and rs916055) are tagging SNPs (tagSNPs). They were selected from HapMap (hapmap.org). The algorithm removes SNPs with pairwise linkage disequilibrium (LD) values that exceed a certain threshold (r^2=0.8). The other two SNPs (rs9894225 and rs748694) were located in the *ALOX15* promoter region, which is known to contain multiple regulatory elements[18, 19]. Study subjects were genotyped for all five SNPs. The TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA, USA) was used for genotyping, while primer and probe sequences were optimized with the SNP assay-by-design service from Applied Biosystems. Amplification reactions were performed on the Mx3000P Real-Time PCR System (Stratagene, Santa Clara, CA). The probe for one allele in the two-allele PCR system was labeled with 6-carboxyfluorescein (FAM) dye and the other probe was labeled with hexachlorofluorescein phosphoramidite (HEX) dye. Twenty nanograms of genomic DNA was amplified in 96-well plates in the presence of the 1×TaqMan probe and primer mix and the 1×TaqMan Universal PCR Master Mix (Applied Biosystems). The PCR program consisted of an initial cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

Linkage disequilibrium (LD) and haplotype analyses

Haplotypes were constructed from the population genotype data using the algorithm constructed by Stephens *et al* (2001) and the PHASE program (version 2.0.2)[20]. The significance threshold for linkage disequilibrium (LD) between the gene markers was based on the haplotypes and allele frequencies determined by the Haploview (version 3.2)[21]. We examined

the LD coefficients, D′ and r^2 , between all pairs of biallelic loci. The frequencies of genotypes and haplotypes were calculated with genotypic data from the unrelated parents of nuclear families.

Statistical analyses

Allele frequencies were estimated by gene counting. The Hardy-Weinberg equilibrium was measured by a χ^2 goodness-of-fit test. The orthogonal model in the QTDT program was used to test for population stratification, linkage, and family-based association between SNPs and haplotypes and obesity-related phenotypes. (The QTDT software package is available online at http://www.sph.umich.edu/csg/abecasis/QTDT/index.html.) This method of statistical analysis, as defined by the QTDT software, extends the trio-based transmission disequilibrium test (TDT) to quantitative trait data and uses genotypic data from available siblings and parents. Because all of the children in the nuclear families were sons and the effects of the parent's phenotypes were excluded in the QTDT analyses, sex was not used as a covariate to adjust for the sons' phenotypic variations. However, raw obesity phenotypic measurements, such as BMI, TFM, tFM, AFM, LFM, and percentage of fat mass (PFM), which are covariates, were adjusted by age. Because false-positive results can confound conclusions, the reliability of our results was assessed by performing a permutation procedure (1000 simulations) to generate empirical *P* values[22, 23]. The statistical power was estimated with Piface (version 1.65) (http://www.math.uiowa.edu/~rlenth/Power/) taking into account the sample size, minor allele frequency of every genotype and variation in obesity phenotypes. The distribution of the obesity-related phenotypic data was calculated by performing the Shapiro-Wilk test. A *P*-value of less than 0.05 was considered significant for all the analyses.

Results

Characteristics of the study subjects

The DNA of 15 individuals could not be subjected to genotypic analysis due to the poor quality of DNA obtained after amplification. Twelve sons were removed from the study when initial analysis showed that they deviated from Mendelian inheritance. This left a total of 1215 individuals from 400 nuclear families in the study. The basic characteristics of the study subjects are shown in Table 1. Because the effects of the parents' phenotypes were excluded from the statistical analysis (QTDT), we only made use of the body composition measurements of the sons.

Table 1. Basic characteristics of the subjects (Mean±SD).

Variation	Father (*n*=400)	Mother (*n*=400)	Son (*n*=415)
Age (years)	61.10±7.07	58.39±6.37	30.14±6.09
Height (m)	1.68±6.04	1.56±5.45	1.73±5.91
Weight (kg)	69.63±9.48	58.28±8.22	70.55±10.57
BMI (kg/m^2)	24.76±3.10	24.01±3.12	23.55±3.24
Arms fat mass (kg)	–	–	1.27±0.73
Legs fat mass (kg)	–	–	4.66±1.87
Trunk fat mass (kg)	–	–	9.38±4.37
Total fat mass (kg)	–	–	15.89±6.86
Percentage of fat mass	–	–	0.22±0.07

SNP genotyping and linkage disequilibrium

Five SNPs in *ALOX15* were examined initially; however, rs9894225 was excluded from further analysis when only one SNP (GG) was found after genotypic analysis, despite the use of multiple strategies to identify additional SNPs. The remaining four SNPs were in Hardy-Weinberg equilibrium (Table 2).

To gain further insight into the pattern of LD between alleles at polymorphic loci, pairwise disequilibrium measures (D′) were calculated. As shown in Figure 1, three SNPs (rs2619112, rs2619118, and rs916055) constituted one block. The SNP rs748694 was an "orphan", independent of the block. LD was observed for each SNP, with D′ values ranging from 0.69 to 0.72. Using the likelihood method implemented by PHASE, we inferred that 8 different haplotypes were presented in our population, using a likelihood method based on a PHASE. The most common haplotype (TGT) had a frequency of 42.4% (haplotype1), and four common haplotypes (TGT, CAC, TGC, and TAC) accounted for 86.6% of the haplotypes identified within our sample population of total unrelated parents (Table 3).

Association between SNPs and haplotypes and obesity-related phenotypes

All 400 families were included in the following analyses because the effects of the parents' phenotypes were excluded

Table 2. Information of the analyzed *ALOX15* SNPs in this study.

SNP	Physical position	Location and function	Allele change	Amino acid change	HWE	MAF in dbSNP	MAF in this study
rs9894225	4149933	Promoter	A>G	NA	0.22	0.26	1.00
rs748694	4496938	Promoter	A>G	NA	0.83	0.47	0.32
rs2619112	4482134	Intron 12	A>G	NA	0.90	0.49	0.40
rs2619118	4487026	Intron	C>T	NA	0.55	0.48	0.49
rs916055	4481583	3′-UTR	C>T	NA	0.32	0.42	0.39

NA=not applicable; MAF: minor allele frequency; HWE: Hardy-Weinberg equilibrium.

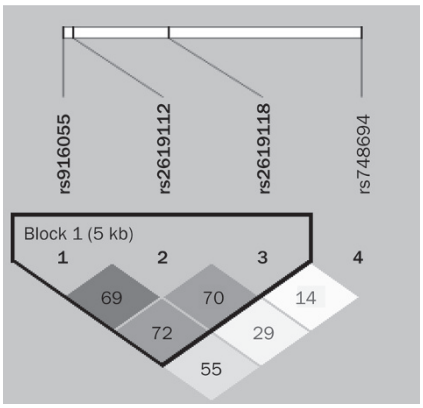

Figure 1. LD patterns for the ALOX15 gene. The increasing degree of darkness of the cells from white to black represents the increasing strength of LD. There is a strong LD (0.6<D′<0.8) across rs2619112, rs2619118, and rs916055. Rs748694 is not included for LD analysis.

Table 3. Frequency of *ALOX15* haplotypes for all available SNPs.

Index	rs916055	rs2619112	rs2619118	Frequency
1	T	G	T	0.424
2	C	A	C	0.266
3	T	G	C	0.103
4	T	A	C	0.073
5	C	G	C	0.067
6	C	A	T	0.055
7	T	A	T	0.007
8	C	G	T	0.006

Table 4. *P* values of tests for population stratification, total association, and within-family association using QTDT.

	rs748694	rs2619112	rs2619118	rs916055
Tests of population stratification				
BMI	0.894	0.355	0.919	0.755
Arm fat mass	0.222	0.329	0.814	0.687
Leg fat mass	0.099	0.364	0.576	0.441
Trunk fat mass	0.478	0.256	0.653	0.491
Total fat mass	0.142	0.132	0.680	0.312
Percentage of fat mass	0.108	0.220	0.294	0.361
Tests of total association				
BMI	0.934	0.575	0.845	0.589
Arm fat mass	0.740	0.564	0.354	0.372
Leg fat mass	0.862	0.543	0.569	0.198
Trunk fat mass	0.585	0.776	0.478	0.437
Total fat mass	0.928	0.558	0.402	0.173
Percentage of fat mass	0.775	0.844	0.075	**0.042**
Tests of within-family association				
BMI	0.939	0.588	0.850	0.992
Arm fat mass	0.541	0.767	0.589	0.360
Leg fat mass	0.202	0.822	0.954	0.146
Trunk fat mass	0.587	0.662	0.732	0.511
Total fat mass	0.275	0.499	0.723	0.093
Percentage of fat mass	0.186	0.309	0.520	**0.038**
P 1000 permutation of within-family association				
BMI	0.938	0.612	0.850	0.985
Arm fat mass	0.580	0.775	0.634	0.331
Leg fat mass	0.180	0.817	0.942	0.170
Trunk fat mass	0.834	0.223	0.297	0.135
Total fat mass	0.388	0.617	0.797	0.170
Percentage of fat mass	0.194	0.353	0.540	**0.033**

All body compositon values are adjusted for age. Bold indicates significant *P* values ($P<0.05$).

from the QTDT test. The results of the QTDT are summarized in Table 4. The analysis showed that there were 258, 278, 302, and 284 informative families at SNPs rs748694, rs2619112, rs2619118, and rs916055, respectively. We did not find significant population stratification in our samples. For the total and the family-based association analyses performed on the 400 nuclear families, we found that only rs916055 was significantly associated with PFM ($P=0.042$, and $P=0.038$, respectively), rs916055 had a marginal but insignificant association with tFM ($P=0.093$) in the family-based association results. No other SNP showed significant evidence of association (in either family-based association or the total family association) with any body composition parameters. Results of the multiple-parameter test involving 1000 permutations, greed with family-based association results. In this case, rs916055 also had a significant association with PFM ($P=0.033$).

Figure 1 is a graphical representation of pairwise LD as measured by D′. The results showed a region of substantial LD between rs2619112, rs2619118, and rs916055. Four common haplotypes accounted for 86.6% of the haplotypes in the sample population of unrelated parents (Table 3). The haplotype analysis is an important source of information that complements the LD analysis of SNPs and was generated using genotypic data from the three SNPs. We also investigated the association between obesity phenotypes and the four common haplotypes identified here. The TDT analysis showed that there were 288, 245, 105, and 84 informative families at haplotypes TGT, CAC, TGC and TAC, respectively. Significant population stratification was found for haplotype TGC with regard to the PFM ($P=0.027$). For total family association analysis, we detected a significant association between the most common haplotype (TGT) and PFM ($P=0.032$). However, we failed to find significant evidence of any link between a haplotype and obesity-related phenotypes by family-based association or the 1000 permutations test ($P>0.05$) (data not shown).

Discussion

It has become evident that the activation of inflammation and oxidative stress is involved in the development of obesity and its complications[2, 5–7]. The chronic inflammation caused by the recruitment of macrophages and the secretion of chemok-

Table 5. *P* values of association of haplotypes of *ALOX15* with obesity related phenotypes using QTDT.

	TGT	CAC	TGC	TAC
Tests of population stratification				
BMI	0.942	0.278	0.910	0.824
Arm fat mass	0.711	0.590	0.393	0.534
Leg fat mass	0.986	0.394	0.076	0.964
Trunk fat mass	0.546	0.510	0.218	0.450
Total fat mass	0.556	0.348	0.077	0.481
Percentage of fat mass	0.871	0.364	**0.027**	0.754
Tests of total association				
BMI	0.766	0.773	0.884	0.858
Arm fat mass	0.160	0.387	0.410	0.737
Leg fat mass	0.259	0.494	0.978	0.468
Trunk fat mass	0.378	0.591	0.892	0.959
Total fat mass	0.179	0.502	0.964	0.706
Percentage of fat mass	**0.032**	0.812	0.656	0.671
Tests of within-family association				
BMI	0.834	0.433	0.869	0.902
Arm fat mass	0.209	0.802	0.896	0.797
Leg fat mass	0.415	0.931	0.174	0.672
Trunk fat mass	0.291	0.955	0.301	0.587
Total fat mass	0.171	0.878	0.169	0.772
Percentage of fat mass	0.157	0.430	0.163	0.971
P 1000 permutation of within-family association				
BMI	0.843	0.447	0.871	0.912
Arm fat mass	0.225	0.814	0.888	0.768
Leg fat mass	0.428	0.935	0.199	0.624
Trunk fat mass	0.269	0.911	0.267	0.536
Total fat mass	0.282	0.923	0.261	0.776
Percentage of fat mass	0.180	0.459	0.147	0.961

All body compositon values are adjusted for age. Bold indicates significant *P* values ($P<0.05$).

ines and adipocytokines, affects adipose function[5, 6]. The products of lipoxygenases raise the expression of MCP-1, IL-6, TNF, and adhesion molecules in macrophages and vascular cells[24, 25]. Moreover, 12/15-lipoxygenases exacerbate oxidative stress by attacking mitochondria, leading to the production of reactive oxygen species (ROS)[26]. Recently, Almeida *et al*[27] demonstrated that the lipoxygenase-oxidized polyunsaturated fatty acids (PUFA) activates the ROS/FoxO/PPARγ catenin cascade, which results in elevated oxidative stress and PPARγ expression and reduced canonical Wnt signaling (the latter is the pathway linked to adipose inflammation). Therefore, lipoxygenase pathways may play an important role in the development of obesity. Indeed, previous studies have identified *ALOX5*[28, 29] and *ALOX12*[14] as susceptibility genes for obesity. *ALOX15*, another member of the lipoxygenases family, has been linked to obesity risk in several studies[13]. In the present study, we investigated the relationship between *ALOX15* gene polymorphisms and obesity-related phenotypes in a large group of Chinese nuclear families. Significant association was found between SNP rs916055 and PFM through QTDT analysis. The 1000 permutation test confirmed the results of the family-based association study.

The SNP rs916055 is located in the 3′-UTR of *ALOX15*. This region is important for the translational regulation of gene expression, particularly during embryonic development and differentiation[30]. Studies shown that the presence of an alternative differentiation control element (DICE) in the 3′-UTR of the *ALOX15* gene alters *ALOX15* mRNA and protein expression[24, 31]. Another SNP (rs11568131) within the *ALOX15* locus was shown to be significantly associated with the expression of key proinflammatory genes, such as those coding for IL-6, TNF, and IL-1, all of which influence obesity[32]. According to Hapmap, rs11568131 is in high LD with rs916055 (D′=1) (HapMap Data Phase III/Rel#2, Feb09, on NCBIB36 assembly, dbSNP b126), although rs11568131 displays a modest heterozygosity (MAF=0.155). Furthermore, one SNP (rs916055) has been shown to have significant association with bone mineral density (BMD) within the lumbar portion of the spine in postmenopausal women[33]. Because the genomic organization of mammalian ALOX is highly conserved[34], we speculated that SNP rs916055, which is located within the 3′-UTR of ALOX15 may affect the binding of the transcriptional machinery, which might, in turn, generate potentially functional RNA variants. However, no other SNPs or haplotypes were found to have a significant association with any obesity-related phenotype. All of the SNPs in this study had high heterozygosity (MAF>0.2), which increased our power to detect associations. This does not exclude the existence of common variants with low penetrance or rare variants with high penetrance, which make a contribution to the susceptibility to complex diseases[35, 36]. For example, a rare nonsynonymous SNP in *ALOX15*, rs34210653, was recently found to be associated with a higher risk of coronary heart disease[37]. To our knowledge, this is the first study to investigate the possible influence of *ALOX15* genetic variation on obesity in humans. Further studies should be conducted with a larger sample population and high density SNP genotyping among different ethnic groups to confirm our results.

This study has several strengths. First, more obesity-related phenotypes were examined in this study than are normally employed in such studies. The phenotypes, include TFM, tFM, LFM, and AFM, but not BMI. BMI has been widely used as a surrogate phenotype; however, it alone may not be sufficiently accurate to be used to indicate the proportion of fat in the body or the relative contributions of body muscle and fat[38]. DXA is a reliable and convenient method of assessing obesity and is considered the golden standard for anthropometric measures[39]. Although CT and MRI scans can also provide these measurements, those methods are limited by costs, radiation exposure and other factors[40]. DXA analyses can yield information on the fat composition of body segments, such as the hip, trunk and limbs. It is well known that abdominal or visceral fat is strongly associated with metabolic disturbances and cardiovascular disease[41–43]. Studies indicate

that DXA-based measurements of the fat mass in the trunk region strongly correlate with CT and MRI scans of abdominal fat[44–46]. In the present study, we measured the total, central (trunk) and peripheral (leg and arm) fat mass in young men aged 20–40 years. Another strength of this study is the use of QTDT analysis. Family-based associations are unaffected by population stratification. Thus, QTDT avoids the false-positive and false-negative results that occur more than with other association analyses[47]. With 400 nuclear families in our sample, the test has a power of >80% to identify a candidate gene as a quantitative trait locus (QTL), which can explain ~10% of the variation in the obesity phenotypes. We also performed permutations (1000 simulations) to generate empirical *P*-values[15, 16], which helps to eliminate false-positive results generated by multiple QTDT tests.

Our study is limited by several factors. First, we selected only five SNPs of *ALOX15* for the study. Second, the aim of this study was to explore the impact of *ALOX15* gene on obesity-related phenotypes; however, only 9% of the sons were obese (a BMI of 30 or higher is considered obese). A larger obese population should be recruited for the next study.

In conclusion, we found that the SNP rs916055 had a significant family-based association with PFM in nuclear families with young males using the QTDT approach to demonstrate linkage. Further studies to validate our results should be conducted with larger samples, a great proportion of obese individuals and rare SNPs from different ethnic groups.

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Author contribution

Zhen-lin ZHANG designed the research; Yao-hua KE, Wen-jin XIAO, Jin-wei HE, Jin-bo YU, Gao GAO, Hua YUE, Chun WANG, and Wen-zhen FU performed the research; Hao ZHANG, Jin-bo YU, Wei-wei HU, Jie-mei GU, and Yu-juan LIU recruited research subjects; Yun-qiu HU and Miao LI took fat mass and lean mass measurements. Zhen-lin ZHANG contributed new analytical tools; Wen-jin XIAO and Zhen-lin ZHANG analyzed the data; Wen-jin XIAO wrote the paper.

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