Replication of FTO Gene associated with lean mass in a Meta-Analysis of Genome-Wide Association Studies

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Sarcopenia is characterized by low skeletal muscle, a complex trait with high heritability. With the dramatically increasing prevalence of obesity, obesity and sarcopenia occur simultaneously, a condition known as sarcopenic obesity. Fat mass and obesity-associated (FTO) gene is a candidate gene of obesity. To identify associations between lean mass and FTO gene, we performed a genome-wide association study (GWAS) of lean mass index (LMI) in 2207 unrelated Caucasian subjects and replicated major findings in two replication samples including 6,004 unrelated Caucasian and 38,292 unrelated Caucasian. We found 29 single nucleotide polymorphisms (SNPs) in FTO significantly associated with sarcopenia (combined $p$-values ranging from $5.92 \times 10^{-12}$ to $1.69 \times 10^{-9}$). Potential biological functions of SNPs were analyzed by HaploReg v4.1, RegulomeDB, GTEx, IMPC and STRING. Our results provide suggestive evidence that FTO gene is associated with lean mass.

Sarcopenia is a complex disease described as the age-associated loss of skeletal muscle mass, strength and function impairment1,2. The low skeletal muscle mass will lead to many public health problems such as sarcopenia, osteoporosis and increased mortality3,4, especially in the elderly. Skeletal muscle is heritable with heritability estimates of 30–85% for muscle strength and 45–90% for muscle mass5. Although there are many genetic researches have shown some SNPs and copy number variants (CNVs) associated with lean mass6–14, the majority of specific genes underlying the variations in low lean body mass (LBM) are still unknown. And sarcopenia can be predicted by LMI15.

FTO gene is proved the association with fat mass, which contributes to human obesity16–21. According to many vivo studies using FTO overexpression or knockout mouse models, FTO gene can cause abnormal adipose tissues and body mass, implying a pivotal role of FTO in adipogenesis and energy homeostasis22–25. But the exact biological functions of this gene are unknown yet. In recent researches, FTO gene is proved the association with lean mass22,23,26–31. Zillikens et al. reported a series of SNPs of FTO associated with LBM and appendicular lean mass (ALM)30. In our study, we performed a GWAS to identify the associations between FTO and LMI in 2,207 unrelated Caucasians (516 men and 1,691 women). Then we replicated our findings in two replication samples, including 6,004 unrelated Caucasians and 38,292 unrelated Caucasians subjects30.
Methods
Ethic statement. This study was approved by institutional review boards of Creighton University and the University of Missouri-Kansas City. Before entering the study, all subjects provide written informed consent documents. The methods carried out in accordance with the approved study protocol.

Discovery sample. The discovery sample consisted of 2,207 unrelated Caucasian subjects of European ancestry that were recruited in Midwestern U.S. (Kansas City, Missouri and Omaha, Nebraska). All discovery subjects completed a structured questionnaire covering lifestyle, diet, family information, medical history, etc. The inclusion and exclusion criteria for cases were described in our previous publication32.

Replication sample. There were two replication samples which were performed association studies with other anthropometric phenotypes.

Replication sample 1 contains 6,004 unrelated Caucasian of European ancestry from Framingham heart study (FHS) which is a longitudinal and prospective cohort comprising >16,000 pedigree participants spanning three generations of European ancestry. Details about the FHS have reported previously33.

Replication sample 2 contains 38,292 unrelated Caucasian of European ancestry from 20 cohorts30. The details and GWAS results are from the genetic factors for osteoporosis (GEFOS) (http://www.gefos.org).

Phenotyping. In present study, LBM and fat body mass (FBM) were measured using a dual-energy X-ray absorptiometry (DXA) scanner Hologic QDR 4500W machine (Hologic Inc., Bedford, MA, USA) that was calibrated daily. Height was obtained by using a calibrated stadiometer and weight was measured in light indoor clothing by a calibrated balance beam scale. LMI was calculated as the ratio of the sum of lean soft tissue (nonfat, non-bone) mass in whole body to square of height34.

Genotyping and quality control. Genomic DNA was extracted from peripheral blood leukocytes using Puregene DNA Isolation Kit (Gentra systems, Minneapolis, MN, USA). For discovery sample, SNP genotyping with Affymetrix Genome-Wide Human SNP Array 6.0 was performed using the standard protocol recommended by the manufacturer. Fluorescence intensities were quantified using an Affymetrix array scanner 30007G. Data management and analyses were conducted using the Genotyping Command Console Software. We conducted strict quality control (QC) procedure. All subjects (n = 2,283) had a minimum call rate 95% and the final mean call rate reached a high level of 98.93%. We discarded SNPs that deviated from Hardy-Weinberg equilibrium (p < 0.01) and those containing a minor allele frequency (MAF) less than 0.01. Then we found 21,247 SNPs allele frequencies deviated from Hardy-Weinberg equilibrium, and additional 141,666 SNPs had MAF < 0.01. After QC, 746,709 SNPs remained in the discovery sample.

For replication sample 1, SNP genotyped using approximately 550,000 SNPs (Affymetrix 500 K mapping array plus Affymetrix 50 K supplemental array). For details of the genotyping method, please refer to FHS SHARE at NCBI dbGaP website (http://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000007.v3.p2).

|                          | Discovery sample | Replication sample 1* |
|--------------------------|------------------|-----------------------|
|                          | Male | Female | Male | Female |
| Number                   | 516  | 1,691 | 2,525 | 3,479 |
| Age (years)              | 51.2 (16.1) | 51.7 (12.9) | 54.0 (13.1) | 55.9 (13.7) |
| Height (cm)              | 175.9 (7.3) | 163.3 (6.3) | 176.0 (7.1) | 162.0 (6.8) |
| Weight (kg)              | 86.8 (16.3) | 71.4 (16.0) | 84.4 (13.3) | 68.0 (13.8) |
| FBM (kg)                 | 20.6 (9.1) | 25.3 (10.8) | 24.9 (9.0) | 27.8 (10.5) |
| LBM (kg)                 | 66.3 (9.5) | 46.8 (7.0) | 57.3 (7.1) | 38.3 (5.2) |
| LMI (g/cm²)              | 2.2 (1.0) | 1.8 (1.3) | 1.8 (0.2) | 1.5 (0.2) |

Table 1. Basic characters of study subjects. Note: The numbers within parentheses are standard deviation (SD). *The replication sample 1 includes 6004 unrelated Caucasian from FHS.

Figure 1. QQ plot. Logarithmic quantile–quantile (QQ) plot of individual SNP-based association for fat-adjusted LMI in the discovery sample.
Genotype imputation. Genotype imputation was applied to both the discovery and replication samples, with the 1000 Genomes projects sequence variants as reference panel (as of August 2010). Reference sample included 283 individuals of European ancestry. The details of genotype imputation process had been described earlier\(^3^5\). Briefly, strand orientations between reference panel and test sample were checked before imputation, and inconsistencies were resolved by changing the test sample to reverse strand or removing the SNP from the test sample. Imputation was performed with MINIMAC\(^3^6\). Quality control was applied to impute SNPs with the following criteria: imputation \(r^2 > 0.5\) and MAF > 0.01. SNPs failing the QC criteria were excluded from subsequent association analyses.

Statistical analyses. GWAS analysis. In discovery sample, we used the first five principal components, gender, age, age\(^2\) and FBM as covariates to screen for significance with the step-wise linear regression model implemented in R function stepAIC. Raw LMI values of discovery sample were adjusted by significant covariates (age, gender and FBM), and the residuals were normalized by inverse quantiles of standard normal distribution. MACH2QTL was used to perform genetic association analyses between SNPs and normalized residuals of LMI with an additive mode of inheritance.

Meta-analysis. Meta-analyses were performed by METAL software \((https://genome.sph.umich.edu/wiki/METAL_Documentation)\) using the weighted fixed -effects model, which takes into account effect size and their standard errors.

The linkage disequilibrium (LD) patterns of the interested SNPs were analyzed and plotted using the Haplovie program\(^3^7\) \((http://www.broad.tamit.edu/mpg/haplovie/)\).

Functional annotation. We used HaploReg v4.1 \((https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php)\) to search for significant SNPs with functional annotations and the RegulomeDB\(^3^8\) \((http://www.regulomedb.org/)\) program to rank potential functional roles.
| SNP          | position | region | Allele* | Discovery sample (LMI) | Replication sample 1 (LBM) | Replication sample 2 (LBM) | Combined p |
|--------------|----------|--------|---------|------------------------|-----------------------------|-----------------------------|-------------|
| rs17817964   | 53787154 | Inttron| C/T     | 0.60 −0.10             | 9.28 × 10⁻⁴                | 2.207                       | 0.60        |
| rs7185735    | 53787339 | Inttron| A/G     | 0.60 −0.10             | 9.62 × 10⁻⁴                | 2.207                       | 0.59        |
| rs9936385    | 53785257 | Inttron| C/T     | 0.40 0.10              | 9.86 × 10⁻⁴                | 2.207                       | 0.40 0.08   |
| rs12149832   | 53808996 | Inttron| A/G     | 0.41 0.12              | 1.22 × 10⁻⁴                | 2.207                       | 0.41 0.08   |
| rs993609     | 53786615 | Inttron| A/T     | 0.40 0.10              | 7.21 × 10⁻⁴                | 2.207                       | 0.40 0.08   |
| rs11075899   | 53785965 | Inttron| C/T     | 0.60 −0.10             | 9.76 × 10⁻⁴                | 2.207                       | 0.59 −0.08  |
| rs9930501    | 53782926 | Inttron| A/G     | 0.60 −0.10             | 1.01 × 10⁻⁴                | 2.207                       | 0.59 −0.08  |
| rs1787449    | 53779455 | Inttron| C/T     | 0.40 0.10              | 1.01 × 10⁻⁴                | 2.207                       | 0.41 0.08   |
| rs9843757    | 53779538 | Inttron| A/T     | 0.60 −0.10             | 9.16 × 10⁻⁴                | 2.207                       | 0.59 −0.08  |
| rs9932333    | 53785286 | Inttron| C/G     | 0.40 0.10              | 9.86 × 10⁻⁴                | 2.207                       | 0.41 0.08   |
| rs17817288   | 53773852 | Inttron| A/G     | 0.51 −0.09             | 2.44 × 10⁻⁵                | 2.207                       | 0.50 −0.08  |
| rs1358902    | 53769662 | Inttron| A/T     | 0.41 0.09              | 2.40 × 10⁻⁵                | 2.207                       | 0.42 0.08   |
| rs7202116    | 53787703 | Inttron| A/G     | 0.60 −0.10             | 9.63 × 10⁻⁴                | 2.207                       | 0.59 −0.08  |
| rs1421085    | 53764042 | Inttron| C/T     | 0.41 0.09              | 2.30 × 10⁻⁵                | 2.207                       | 0.42 0.08   |
| rs9930506    | 53769553 | Inttron| A/G     | 0.56 −0.10             | 7.99 × 10⁻⁴                | 2.207                       | 0.57 −0.07  |
| rs9922619    | 53797859 | Inttron| G/T     | 0.56 −0.10             | 8.76 × 10⁻⁴                | 2.207                       | 0.57 −0.07  |
| rs9922708    | 53797234 | Inttron| C/T     | 0.56 −0.10             | 8.90 × 10⁻⁴                | 2.207                       | 0.57 −0.07  |
| rs9932754    | 53766579 | Inttron| G/T     | 0.44 0.10              | 1.04 × 10⁻⁴                | 2.207                       | 0.43 0.07   |
| rs9930501    | 53769540 | Inttron| A/G     | 0.56 −0.10             | 1.04 × 10⁻⁴                | 2.207                       | 0.57 −0.07  |
| rs9931494    | 53793267 | Inttron| C/G     | 0.58 −0.09             | 3.38 × 10⁻⁵                | 2.207                       | 0.58 −0.07  |
| rs7201850    | 53787950 | Inttron| A/G     | 0.58 −0.09             | 3.37 × 10⁻⁵                | 2.207                       | 0.58 −0.07  |
| rs9941349    | 53791576 | Inttron| C/T     | 0.58 −0.09             | 3.41 × 10⁻⁵                | 2.207                       | 0.58 −0.07  |
| rs8044769    | 53805223 | Inttron| C/G     | 0.52 0.10              | 5.37 × 10⁻⁴                | 2.207                       | 0.52 0.07   |
| rs9922047    | 53772368 | Inttron| C/G     | 0.49 −0.08             | 5.85 × 10⁻⁵                | 2.207                       | 0.48 −0.08  |
| rs11075987   | 53781249 | Inttron| G/T     | 0.51 0.09              | 3.09 × 10⁻³                | 2.207                       | 0.51 0.07   |

*The allele represents the minor allele of each marker.

**Figure 4.** Forest plot of SNPs with combined p-value less than 1 × 10⁻¹¹. Regression coefficient (beta) and its 95% confidence interval (CI) are presented in untransformed estimates from individual studies. “Total” refers to the combined meta-analysis.
To investigate the association between the identified SNP polymorphisms and the nearby gene expressions, we performed cis-eQTL analysis. We used the GTEx (https://gtexportal.org) project dataset for analysis. The GTEx project was designed to establish a sample and data resource to enable studies of the relationship among genetic variation, gene expression, and other molecular phenotypes in multiple human tissues.

We annotated gene by constructing gene interaction networks with STRING v.10 online platform (https://string-db.org/). STRING uses information based on gene co-expression, text-mining and others, to construct gene interactive networks.

Results

Table 1 is the basic characteristics of the subjects used in discovery sample and replication sample 1. The basic characteristics of replication sample 2 are summarized in the previous research. Genomic control inflation factor of discovery sample is 0.976. In order to avoid potential population stratification, we used the inflation factor to adjust individual \( p \)-values. Figure 1 shows the logarithmic quantile–quantile (QQ) plot of SNP-based association results. After adjustment by the genomic control approach there is no evidence of population stratification is observed. Figure 2 is Manhattan plot of the discovery sample.

We identified 29 SNPs located in the FTO gene demonstrated associations with LMI in the discovery sample \( (p < 10^{-2}) \). LD analysis showed that these 29 SNPs were in LD \( (r^2 > 0.91) \) and were located within two LD blocks (Figure 3). These SNPs were replicated in independent Caucasian replication samples (Table 2). Meta-analysis \( p \)-values ranging from \( 5.92 \times 10^{-12} \) to \( 1.69 \times 10^{-9} \). SNP rs17817964 is the most significant SNP with combined \( p = 5.92 \times 10^{-12} \) in discovery sample and two replication samples of Caucasian. There are 6 SNPs with \( p \) value less than \( 1 \times 10^{-11} \). Forest plot of SNPs with combined \( p < 1 \times 10^{-11} \) was drawn in Figure 4. Regional plot of the gene FTO was drawn by LocusZoom in Figure 5.

The results of biological functional annotation using HaploReg v4.1, Regulome DB and GTEx are performed in Table 3. 25 SNPs may locate in a strong enhancer region marked by peaks of several active histone methylation modifications (H3K27ac, H3K9ac, H3K4me1 and H3K4me3). SNP rs17817288 (discovery \( p = 2.44 \times 10^{-3} \), combined \( p = 5.44 \times 10^{-11} \)) occupies promoter histone marks in muscle satellite cultured cells. It was predicted to have enhancer activity by chromatin states, H3K4me1 and H3K27ac marks in skeletal muscle myoblasts cells and H3K4me1 marks in muscle satellite cultured cells. Besides it has promoter activity, implied by H3K4me3 and H3K9ac in muscle satellite cultured cells and H3K9ac in HSMM skeletal muscle myoblasts cells. Among the 29 SNPs evaluated with Regulome DB, 7 had no data. Of the 22 SNPs for which Regulome DB provided a score, 2 had a score of \(< 3 \) (likely to affect the binding) including rs17817964 and rs7202116 with Regulome DB score = 2b respectively. Analyses using GTEx data reveal 11 SNPs of our GWAS results have strong signals of cis-eQTL for FTO gene in skeletal muscle tissue \( (p < 1 \times 10^{-4}) \). SNPs rs7201850 and rs8044769 were deposited in the GTEx eQTL database as a cis-eQTL for FTO in skeletal muscle with the same direction of effect \( (p = 1 \times 10^{-3}) \), Figure 6. Gene-gene interaction networks shows there are some connections between FTO and IGF-1, myogenic regulatory factors (MRFs: MYF5, MYOD1, MYOG, and MYF6) and IRX3, implying that FTO may play an important role in muscle development (Figure 7).

Discussion

In this study, we have performed a GWAS in 2,207 Caucasian subjects and replicated this result in three replication samples including 6,004 unrelated Caucasian from FHS and 38,292 unrelated Caucasian. We identified 29 SNPs in FTO gene associated with LMI then we performed the potential biological function annotation of SNPs. In this study, FTO is suggested to be associated with lean mass.
FTO gene encodes a 2-oxoglutarate (2-OG) Fe(II)-dependent nucleic acid demethylase belonging to the AlkB-related non-heme dioxygenase (Fe(II)- and 2-oxoglutarate-dependent dioxygenases) superfamily of proteins. In the previous studies, FTO was identified to be related to increased risk of obesity and a T2D incidence\(^1\). Studies have shown that the expression of FTO protein in lean mass and adipose tissue is related to the oxidation rate of whole body substrate. With the increase of age, the body's carbohydrate oxidation rate decreases, the fat oxidation rate increases, and at the same time FTO protein expression increases in adipose but decreases in skeletal muscle mass\(^4\). Loos et al. have shown that homozygous Fto\(^−/−\) mice have postnatal growth retardation, obviously decreasing in adipose tissue, and LBM\(^4\). According to the studies of athletes the A-allele is related with decreased slow-twitch muscle fibers\(^2\). AMPK (AMP-activated protein kinase) is an essential part of skeletal muscle lipid metabolism and is the major cellular energy sensor. In skeletal muscle cells AMPK reduces mRNA m6A methylation and lipid accumulation by FTO-dependent demethylation\(^4\). FTO-deficient mice showed skeletal muscle development was damaged\(^2\)\(^8\). Some in vitro and vivo experiments have shown during myoblasts differentiation FTO expression increased and FTO silencing inhibited myoblasts...
Homozygote FTO deficiency mice have decreased body weight including decreased body size, abnormal body weight and decreased total tissue weight in the IMPC database. Because there is a greater browning of white adipose tissues, IRX3 knockout mice need more energy to expend, particularly at night. Recent findings show brown fat is associated with muscle developmental precursor Myf5. Homozygote IRX3 deficiency mice have decreased LBM and increased total body fat mass in the IMPC database.

**Conclusion**

In summary, we identified the FTO gene were significantly association with lean mass in the Caucasian subjects. However, the clear function between FTO gene and lean mass is still unknown that needs more researches to reveal.
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Author contributions

Conceived and designed the experiments: H.W.D. Performed the experiments: S.R., L.Z., Y.F.P. and Z.X.J. Analyzed the data: Y.X.Z., J.Y.W., B.L.L. and Y.L. Wrote the paper: S.R., Z.X.J. and H.W.D. All authors reviewed and approved the manuscript.

Competing interests

The authors declare no competing interests.

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