

Polymorphism in 5' Regulatory Region of the Porcine Fat Mass and Obesity Associated (FTO) Gene is Associated with Intramuscular Fat Content in a Jinhua × Pietrain F₂ Reference Population

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Abstract: As a newly described candidate gene in obesity, FTO gene showed a strong and highly significant association with the obesity-related traits in various human populations. It has been suggested that FTO gene play a key role in the regulation of energy balance and feed intake. Here we sequenced the -2.0 kb of the 5' flanking region of the porcine FTO gene, 7 SNPs were detected in this region and genotyped on the Jinhua × Pietrain F₂ reference population. Using a gene-wide haplotype-tagging approach, three tag SNPs were examined. The SNPs of g -1191 A>G had significant associations with Intramuscular Fat (IMF) content (p<0.05), but not with the Average Backfat Thickness (ABF), Leaf Fat weight (LF) or Average Daily Gain (ADG). Additionally, allele frequencies of the g -1191A>G mutation were studied in 6 pig breeds and the G allele was mainly occurred in Chinese native pig breeds. These results provided the solid evidence that FTO gene was associated with the intramuscular fat deposition in pigs.

Key words: Porcine, regulatory polymorphism, fat mass and obesity associated gene, fat deposition

INTRODUCTION

In China, most of native pig breed exhibited excessive body fat deposition, which was one of the main problems in the pig industry. High content of fat is not only to increase the costs of production, but also lead to decrease the market value of pig meat. The study of genetic variation in candidate gene is an effective way to understand the mechanism of body fat deposition. Recently, studies had shown many genes are associated with the adipose traits in pig, such as SREBF1, TCF7L2, or leptin gene (Chen *et al.*, 2008; Silveira *et al.*, 2008; Du *et al.*, 2009). The FTO (fat mass and obesity associated) gene is exhibited high levels of polymorphism in the noncoding and regulatory region, which most likely contributed to the development of the multiple function of FTO. At present, this gene has become one of the candidate genes for detecting polymorphism associated with the fat deposition in human and pig (Fontanesi *et al.*, 2009; Renstrom *et al.*, 2009; Wing *et al.*, 2009).

FTO gene is the first gene contributing to common forms of human obesity (Loos and Bouchard, 2008). The amino acid sequence of the FTO gene showed high conservation among human, pig and other important domestic animals. FTO gene encodes for 2-oxoglutarate-dependent nucleic acid demethylase and is highly expressed in the hypothalamic-pituitary-adrenal axis,

suggesting this gene may participate in the central control of energy homeostasis or in the development of fat tissue (Dina *et al.*, 2007; Fredriksson *et al.*, 2008). In pigs, the associations between FTO and adipose traits remain not conclusive. The aim of this study was to investigate whether the polymorphic loci in the 5' regulatory region of FTO are associated fat deposition and growth rate in pig. Therefore we detect the SNPs in the 5' regulatory region of FTO and analyze the association of the tag SNPs with the adipose and growth traits in Jinhua × Pietrain F₂ reference population.

MATERIALS AND METHODS

Animals: Animals used in the study included Duroc (n = 30), Yorkshire (n = 55) and Landrace (n = 28) pigs from the Center of Quality Test and Supervision for Breeding Swine Zhejiang and Jinhua pig from Jinhua pig breeding farm (n = 46), Jiaxing Black pig from Jiaxing pig breeding farm (n = 38) and Chalu Black pig from Ningbo pig breeding farm (n = 30) in Zhejiang, China.

Reference population and phenotypic traits: A Jinhua × Pietrain resource population was developed, including 6 F₁ boars and 30 F₁ sows and ~250 F₂ progeny. The Jinhua pig has high backfat thickness, whereas the Pietrain has a heavy muscle, which leads to low backfat thickness. A

Table 1: Primer sets designed for porcine FTO gene

Set	Position	Sense/antisense	Function	PCR (Tm)	Size (bp)
1	-750~ +94	F: 5'-AACTGAGCAAACAAAATGTCACTC-3' R: 5'-CCCATTACAAGTGCATGAAAGAT-3'	Sequencing	63	843
2	-1461~ -654	F: 5'-GAAGAACTGAGACAAGGCTTAGG-3' R: 5'-AAGTAGGTCCCAGAAACAGTATGC-3'	Sequencing	63	808
3	-2051~ -1255	F: 5'-TGAGAACAACCTGCATTTTACTGTG-3' R: 5'-GGTTTCTCAACCTTGGCTCTATT-3'	Sequencing	65	797
4	-1191	F: 5'-CCAAGTAGCTGTCTGGTCTATTT-3' R: 5'-ATAAAACAATGTAGGTGCCCACTC-3'	RFLP for SNP	65	560

sample of longissimus dorsi muscle between the 13th and 14th rib was taken from each animal and used for the determination of intramuscular fat content. The Average Backfat (ABF) thickness was measured on the left carcass at four locations (shoulder, 6-7 ribs, last rib and gluteus medius). The Leaf Fat (LF) weight was weighted by electronic scale. Intramuscular fat content were determined by a Meat Analyzer, Antaris (Thermo Electron, USA), working in the wavelength range 780-2500 nm by the Near Infrared Transmission (NIT) principle. Average daily gain was calculated as the weight gain divided by the number of days elapsed between the 2 measurements.

Amplification and sequencing of 5' regulatory region:

Genomic Blast programs at NCBI and Sanger institute were used for homology searches. The three primer pairs were used to amplify the 2 kb of 5' regulatory regions (Table 1). Genomic DNA was extracted from tissue using standard phenol protocol. All PCR were performed by 36 cycles of 94°C for 30 sec, annealing at 55-68°C for 30 sec and extension at 72°C for 1 min and last extension at 72°C for 10 min. All products were ligated into the pGEM-T easy vector system (Takara, Japan) and then transformed into competent DH5a cells. Plasmid DNA was purified and sequenced on ABI 3730 sequencer (ABI, USA).

Polymorphism detection and genotype:

All primers were shown in Table 1. Approximately, 50 ng of genomic DNA for each animal were amplified in a final volume of 20 µL that contained 12.5 ng of each primer, 2.5 mM dNTPs, 1.5 mM MgCl₂ and 0.5 U of Taq polymerases. The PCR conditions were carried out as follows: 94°C for 3 min, 36 cycles at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 50 sec and last extension at 72°C for 10 min. SNP discovery was implemented by sequencing the pooled PCR products. The PCR products of primer 1 and 2 direct sequencing approach was used to genotype the polymorphisms on all F₂ animals. Additionally, because the SNPs of g -1191A>G creates a restriction site for enzyme Hha I (GCG/C), then lead to PCR-RFLP method for genotyping the pigs.

Statistical analysis: The Hardy-Weinberg equilibrium of each mutation and linkage disequilibrium among mutations in porcine FTO gene was estimated using the HAPLOVIEW program (Barrett *et al.*, 2005). Association analysis of the SNPs with the traits in reference population was performed using the GLM (General Linear Model) procedure of SAS ver. 9.0 (SAS institute Inc., Cary, NC) with the following model:

$$Y_{ijklmn} = \mu + bW_i + B_j + S_k + D_l + G_m + e_{ijklmn}$$

Where,

- Y_{ijklmn} = The dependent variable (traits)
- µ = The general mean
- W_i = Live weight (kg) as a covariate
- B_j = The birth year
- S_k = The sex
- D_l = The age (days)
- G_m = The genotype of SNPs
- e_{ijklmn} = The random error

RESULTS AND DISCUSSION

Single and multiple nucleotide polymorphisms:

The regulatory sequence of porcine FTO gene obtained from three PCR fragments have been deposited in GenBank (GenBank ACC No.: GQ179639). The partial genomic DNA sequence is similar to the FJ668 708. A regulatory sequence (spaning -2051 to + 94 bp) was amplified and 7 SNPs were detected in this region (Fig. 1).

Haplotype analysis:

All markers were in Hardy-Weinberg equilibrium. HAPLOVIEW indicated that g -1336T>C, g -1271A>T, g -1191A>G and g -1034T>C have no-historical recombination by forming two haplotypes of TAAT and CTGC. The SNPs of g -449C>G and g -115G>T form two haplotypes CG and GT and the complete linkage status between these two SNPs was confirmed by HAPLOVIEW. Using the tagging analysis, three tagging SNPs, g -115G>T, g -1015G>A and g -1191A>G, were used in the association analysis.

Association analysis of FTO gene with adipose and growth traits:

GLM analysis revealed that the SNPs of g -1191A>G was significantly associated with IMF in the

Table 2: The associations of three tag SNPs in FTO gene 5' regulatory region with adipose and growth traits in Jinhua × Pietrain F₂ resource family

Trait*	Least squares mean±SE of genotype			P _{GLM}
	GG (n = 70)	GT (n = 129)	TT (n = 53)	
SNP g -115G>T				
ABF	3.230±0.105	3.311±0.089	3.395±0.102	0.2997
Shoulder BF	4.540±0.145	4.616±0.124	4.685±0.141	0.6075
6-7 rib BF	3.263±0.121 ^a	3.402±0.104 ^{ab}	3.536±0.118 ^b	0.0820
Last rib BF	2.495±0.107	2.659±0.091	2.638±0.104	0.1659
Gluteus-medius BF	2.544±0.121	2.611±0.104	2.724±0.118	0.3309
LF	0.820±0.036	0.842±0.031	0.839±0.035	0.7664
IMF	2.604±0.153	2.792±0.131	2.869±0.149	0.1896
ADG1	166.452±10.382	173.560±8.950	175.813±10.199	0.6234
ADG2	388.287±20.304	375.125±17.543	374.668±20.143	0.7039
ADG3	480.255±17.646	483.036±15.212	477.456±17.334	0.9299
	GG (n = 221)	GA (n = 31)	AA	
SNP g -1015 G>A**				
ABF	3.268±0.082	3.418±0.134	-	0.2237
Shoulder BF	4.554±0.112	4.674±0.182	-	0.4783
6-7 rib BF	3.359±0.097	3.540±0.157	-	0.2128
Last rib BF	2.580±0.086	2.705±0.140	-	0.3315
Gluteus medius BF	2.576±0.097	2.790±0.158	-	0.1436
LF	0.829±0.028	0.828±0.046	-	0.9883
IMF	2.764±0.125	2.824±0.202	-	0.7467
ADG1	173.105±8.362	169.343±13.579	-	0.7643
ADG2	378.633±16.509	374.158±26.057	-	0.8530
ADG3	482.678±14.177	468.791±23.023	-	0.5140
	AA (n = 66)	AG (n = 132)	GG (n = 54)	
SNP g -1191 A>G				
ABF	3.210±0.102	3.299±0.088	3.331±0.100	0.4488
Shoulder BF	4.525±0.139	4.572±0.121	4.603±0.136	0.8516
6-7 rib BF	3.269±0.119	3.384±0.103	3.471±0.117	0.2384
Last rib BF	2.450±0.106	2.641±0.092	2.601±0.104	0.2692
Gluteus medius BF	2.546±0.121	2.606±0.105	2.649±0.118	0.6908
LF	0.822±0.035	0.834±0.031	0.824±0.035	0.8933
IMF	2.525±0.152 ^a	2.797±0.132 ^b	2.920±0.149 ^b	0.0258
ADG1	165.192±9.705	174.515±8.420	173.393±9.506	0.4914
ADG2	388.281±20.342	372.734±17.649	378.395±19.925	0.6442
ADG3	485.964±17.576	479.626±15.248	478.713±17.214	0.8906

*ABF: Average Back Fat thickness on 4 sites (mm); Shoulder BF, Backfat of shoulder (mm); 6-7 rib BF, Backfat of 6-7 ribs (mm); Last rib BF, Backfat of the last rib (mm); Gluteus medius BF, Backfat of gluteus medius (mm); LF: Leaf Fat weight (kg); IMF: Intramuscular Fat content (%); ADG₁: Average Daily Gain from birth to 45 days (g); ADG₂: Average Daily Gain from 45-90 days (g); ADG₃: Average Daily Gain from 90-150 days (g); The values bearing a or b differ significantly (p<0.05) from each other; **The homozygous genotype AA was not identified for g -1015 G>A

population (p = 0.0258), but not with ABF (p = 0.4488) or LF (p = 0.8933) (Table 2). The AA animals had 0.272 and 0.395% of IMF content less than the AG and GG animals. No any significance level was found to associate the SNPs of g -1015G>A and g -115G>T with ABF, LF or IMF. Three tag SNPs were also no significantly associated with ADG1, ADG2 or ADG3.

Allele frequencies of g -1191A>G in porcine FTO gene in different pig breeds: The different band patterns of digested PCR product with g -1191A>G mutation was as follows: genotype AA (560 bp), genotype GA (560+339+221 bp) and genotype GG (339+221 bp) (Fig. 2). Allele distribution of g -1191A>G in porcine FTO gene indicated that the polymorphisms in this site occur in three Chinese indigenous breeds, while the western breeds of Large White, Landrace and

Duroc pigs have only the A allele (Table 3). Jinhua and Chalu Black pigs had higher frequency of the G allele.

In the present study, we detected the SNPs in the 5' regulatory region in porcine FTO gene. A total of 7 mutations were found. The SNP of g -1191A>G yielded strong association with IMF (p<0.05). In human, FTO gene had confirmed that a strong and highly significant association with obesity-related traits in different populations (Scuteri *et al.*, 2007; Do *et al.*, 2008; Hennig *et al.*, 2009; Legry *et al.*, 2009; Renstrom *et al.*, 2009).

The porcine FTO gene was linked to markers already mapped to SSC6 between S0297 and S0502: S0297-FTO-7.1cM-S0502. In this region, many studies have reported the presence of important QTL for carcass and meat quality and growth traits, including QTL for backfat thickness at 10th rib, abdominal fat weight, fat thickness

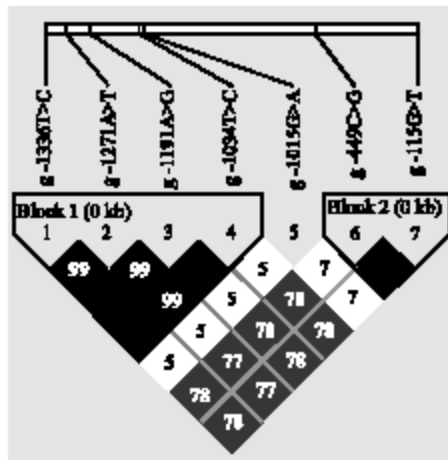


Fig. 1: Haplotype analysis in the 5' regulatory region of porcine FTO gene. Pairwise linkage disequilibrium relationship for 7 mutations is noted based on r^2 measurements. LD is ranging from 0 ($D' = 0$, white) to 100 ($D' = 1$, dark gray (if $LOD \geq 2$) or black (if $LOD < 2$))

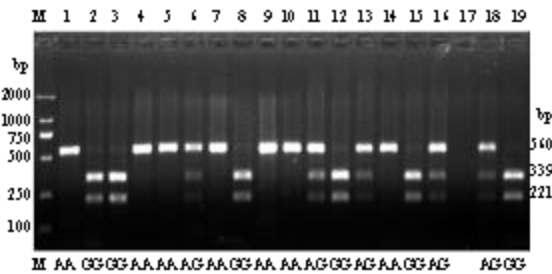


Fig. 2: PCR-RFLP electrophoresis of g-1191A>G mutation

Table 3: Allele frequencies of the FTO g-1191A>G mutation in different pig breeds

Breeds	No. of pigs	Allele frequency	
		g-1191A	g-1191G
Yorkshire	55	1.000	0.000
Landrace	28	1.000	0.000
Duroc	30	1.000	0.000
Jiaying black	38	0.434	0.566
Chahu black	30	0.267	0.733
Jinhua	46	0.163	0.837

For these animals, phenotypic traits were not available

at shoulder, average backfat thickness, intramuscular fat content and average daily gain (Knott *et al.*, 1998; Gerbens *et al.*, 2001; Paszek *et al.*, 2001; Yue *et al.*, 2003; Harmegnies *et al.*, 2006). The SNPs of FTO gene have also shown association with various obesity-related traits in human population such as body weight (Scuteri *et al.*, 2007; Peeters *et al.*, 2008), subcutaneous fatness (Frayling *et al.*, 2007; Peeters *et al.*, 2008), abdominal adipose mass (Renstrom *et al.*, 2009), fat mass

(Andreasen *et al.*, 2008; Peeters *et al.*, 2008) and waist circumference (Andreasen *et al.*, 2008). All previous studies indicated that the porcine FTO gene may play an important role for fat deposition.

In pigs, Back Fat Thickness (BFT), Leaf Fat (LF) weight and Intramuscular Fat (IMF) are major contributors to whole body fat deposition. Fontanesi *et al.* (2009) reported that the 3'-untranslated region of the FTO gene is associated with intramuscular fat deposition and not with backfat thickness in Italian Duroc pigs. In the ISU Berkshire \times Yorkshire pig resource family, the SNPs of c0.594A>G within exon 3 of FTO gene is also association with IMF and ADG, but not with ABF (Fan *et al.*, 2009). The results also confirmed that this gene is associated with IMF and not ABF, LF and ADG of the SNPs in the 5' regulatory region. This was not surprising because Back Fat (BF) thickness and Intramuscular Fat (IMF) percentage are poorly correlated to each other (Sellier, 1998). All studies suggested that the porcine FTO gene might play an important function in intramuscular fat and not directly in subcutaneous or abdominal fat deposition. Additionally, the G allele of -1191 locus was to distribute the different Chinese native pig breeds while the other pig breeds including Duroc, large white and Landrace pig breeds were observed the only A allele. We speculated that highly selection tension for commercial order in these three pig breeds compared to Chinese native pig breeds might be a major reason. Certainly, it may also be the genetic difference in these pig breeds. In general, the Chinese pig breed has higher IMF content than other Western pig breeds.

The recent studies shown that FTO is a member of the non-heme dioxygenase nucleic acid demethylase and demethylates single-stranded DNA *in vitro* in the presence of Fe (II) and ascorbate, suggesting a possible role for FTO in regulation of gene transcription or DNA damage repair (Gerken *et al.*, 2007). The expression of FTO gene is increased by ~60% in the hypothalamus of mice in the fed state compared with mice in the fasting state. Furthermore, its mRNA in the arcuate nucleus of mice was shown to be up-regulated by feeding and down-regulated by fasting (Gerken *et al.*, 2007; Stratiqopoulos *et al.*, 2008). Although, few research of the role of porcine FTO gene was reported until now, the research provided a foundation for further investigation on function of porcine FTO gene.

CONCLUSION

We firstly cloned the 2 kb 5' regulatory region of the porcine FTO gene and found the SNP of g-1191A>G associated with the fat deposition in intramuscular adipose and not in subcutaneous or abdominal fat

deposition and growth rate. Therefore, we propose FTO as a good candidate gene for intramuscular fat deposition and further studies are needed to evaluate the effects of this locus in other pig breeds and investigate in more detail this gene in pig.

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