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GWAS and post-GWAS to identification of genes associated with sheep tail fat deposition

Caiye Zhu , Xiaoyu Huang, Mingna Li, Shizhen Qin, Suli Fang ,Youji Ma*
College of Animal Science and Technology, Gansu Agricultural University, Lanzhou 730070, China; 584806546@qq.com
* Correspondence: yjma@gsau.edu.cn; Tel: +16-0931-7631225 (YJ.M.)

Simple Summary: Chinese indigenous sheep can be classified into three types based on tail morphology: fat-tailed, fat-rumped, and thin-tailed sheep, of which the typical breeds are large-tailed Han sheep, Altay sheep, and Tibetan sheep, respectively. To unravel the genetic mechanisms underlying the phenotypic differences among Chinese indigenous sheep with tails of three different types, we used ovine high-density 600K SNP arrays to detect genome-wide association analysis to identified candidate gene and genotyping technology to validation candidate gene.

Abstract: the type of tail of sheep is an important economic trait. However, the candidate genes associated with the tail type are uncertain. The objective of this study was to identify the genetic region and genotype responsible for the tail type phenotype. Here we perform a genome-wide association study (GWAS) in 40 large tailed Han sheep and 40 Altay sheep as case and 40 Tibetan sheep as control. The results indicated that a total 31 genome-wide significant SNPs associated with type of tail traits were detected. For significant SNPS loci, determine its physical location, and screening of candidate genes within section. By combining information of previously reported and annotated biological functional genes, we identified *SPAG17*, *Tbx15*, *VRTN*, *NPC2*, *BMP2* and *PDGFD* as the most promising candidate genes for type of tail traits. Based on the above identified candidate genes on type of tail traits, we selected *BMP2* and *PDGFD* to conduct the genetic effect analysis in a large Altay sheep and Tibetan sheep population. Rs119 T>C in the exon1 of *BMP2* gene and 1 SNPs in the exon4 (rs69 C>A) of *PDGFD* gene were detected, rs119 that located on exon1 of *BMP2* gene was TT genotype in Altay sheep, while with CC genotype in Tibetan sheep. On rs69 of *PDGFD* gene, Altay sheep with CC genotype, however, Tibetan sheep with AA genotype. These results indicated that the significant associations of SNPs detected in GWAS were indirectly caused by the genetic effects of *BMP2* and *PDGFD* on sheep tail fat deposition.

Keywords: Genome-wide association studies (GWAS); post-GWAS; tail fat deposition; sheep

1. Introduction

According to the shape of tail, Chinese indigenous sheep can be divided into three types, including fat-tailed, fat-rumped and thin-tailed sheep, of which the typical breeds are large-tailed Han sheep, Altay sheep and Tibetan sheep, respectively. Large-tailed Han sheep and Altay sheep are store large amounts of fat in the tail. Worldwide, more than 25% of sheep breeds are fat-tailed or fat-rumped[1]. Fat tail breeds are an important class of sheep breeds and the first time documented was 5000 years ago, The fat that stored in the tail can help sheep migration in winter and through the cold winter; it had additional value to people because it is possible to provide high-energy food during times of drought and famine[2]. In present, customers increasingly improve the quality requirement of meat. Meanwhile, key factors for determine meat quality are distribution and content of animal carcass fat. For fat-tailed type sheep，most fat depositing in tail leaded to decreased fat deposits in other body parts, which affected meat quality. At the same time, with the construction of herdsmen settlements and forage cultivate technologies continue to improve, sheep does not need tail fat to provide heat to make it through the cold winter, therefor, fat deposition in tail is not so important.

Moreover, more feed were need by fat deposition than meat production, and big tail was not conducive to breeding.

A genome-wide association study (GWAS) is one in which a group of genetic markers that are representative of a phenotype were analysed for variation within a set of DNA samples[3]. This method has been used to growth, production and disease susceptibility traits in sheep, for example, zhang et al. [4] were performed a GWAS in purebred sheep for 11 growth and meat production traits, the results indicated that *MEF2B, RFXANK, CAMKMT, TRHDE, RIPK2, GRM1, POL, MBD5, UBR2*, *RPL7* and *SMC2* gens involved in growth and meat production traits in sheep. The mutation of *TMEM154* gene was identified to be associated with Ovine lentivirus[5]. A novel nonsense mutation in the *DMP1* gene identified by GWAS in Corriedale sheep, which is responsible for inherited rickets[6]. Two novel *BMP15* mutations was identified by GWAS that responsible for an atypical hyper prolificacy phenotype in sheep[7].

Mass spectrometry is a medium-throughput SNP typing technique based on primer extension. The extension primers extend one or several bases at the SNP site to be detected, and then depending on the fluorescence of the extension product or the molecular mass determine its genotype. Time-of-flight mass spectrometry (MALDI-TOF) is a SNP typing method based on primer extension method combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Its main advantage lies in the flexible design and high accuracy of the test. The cost performance is best when testing dozens of thousands of samples for dozens of SNPs. Suitable for verification of SNPs found in genome-wide studies and other possible SNPs.

The primary aim of this study is, by Illumina Ovine SNP600 BeadChip and GWAS method, to identify those significant SNPs associated with Chinese indigenous sheep breeds with different types of tails at genome level, and to forecast and explore the major candidate genes associated with fat deposition in tail of sheep, the filtered SNP may be used to genome-wide selection and will guide studies aimed at elucidating the causes of complex traits. In order to validate the GWAS results and screen casual genetic variants as genetic markers that benefit for the sheep of type of tail in an independent sheep population, *BMP2* and *PDGFD* genes were taken to investigate the relationship between SNPs within them and the tail of Altay sheep and Tibetan sheep..

2. Materials and Methods

2.1Ethics Statement

All of the animal procedures were performed in strict accordance with the guidelines proposed by the China Council on Animal Care and the Ministry of Agriculture of the People's Republic of China. All of the animal experiments were approved by the Gansu Agricultural University (Lanzhou, China), approval No. GSAU-AEW-2017-0003.

2.2 DNA sample collection

For this study, 120 individuals from 3 breeds, including 40 large-tailed Han sheep (20 rams and 20 ewes), 40 Altay sheep (20 rams and 20 ewes), and 40 Tibetan sheep (20 rams and 20 ewes), were collected from Liaocheng in Shandong Province, Altay in Xinjiang Province, and Tianzhu in Gansu Province, respectively. All of the specimens were randomly selected.

Genomic DNA samples were extracted from blood using the TIANamp Blood DNA Kit (Tiangen Biotech Co. Ltd., Beijing, China). The purity and concentration of genomic DNA were measured using a NanoVue Spectrophotometer.

2.3 Genotyping and quality control

The genomic DNA of each specimen was genotyped with the Illumina Ovine SNP 600 BeadChip (Illumina Inc., CA, USA), which contains 604,715 SNPs spanning the ovine genome with an average distance of 4.28 Kb.

To increase the accuracy of GWAS inference, stringent quality control criteria were applied: (1) individual call rate >95% and call frequency >90%, (2) Minimum allele frequency (MAF) >3%, (4) Hardy Weinberg equilibrium test P<10-6.

2.4 Genotyping

Illumina Ovine SNP 600 BeadChip (Illumina Inc., CA, USA) were used to genotype, at the time of genotyping, some of the SNP data obtained from the experiment were untyped successfully. Therefore, Beagle [17] software was used to fill in the missing genotype data. Beagle fill command: java -Xmx1000m -jar beagle.jar unphased=beagle-chr.bgl missing=0 out=example.

2.5 Phenotypic trait determination

The phenotypic traits determined by this experiment include: tail length, tail width, tail circumference. Tail length refers to the distance of the fat tail sheep from the leading edge of the first caudal to the tail, Tail width refers to the straight line distance at the widest point of the tail pair, The tail circumference is the length of one week around the tail.

2.6 Genome-wide association analysis

In this study, genome-wide association analysis was performed using a case-control analysis method to compare the frequency of genotypes of large-tailed and thin-tailed individuals (alleles) at each marker locus in the genome to find points that are in linkage disequilibrium with the big tail, in order to correct the possible false-positive effects of genome-wide association analysis by genetic relationship and population stratification, a more general analysis model—General Linear Model (GLM)—was used to perform genome-wide associations using Tassel software. The mathematical expression of this model is:

$Y=G\alpha+P_j \beta+e$

among them

Y is a phenotypic observation vector

α and β are correlation matrices

G is the effect vector of SNP

Pj is the first and second principal component matrix in a generalized linear model

e is the random residual effect vector

2.7 Gene detection and functional analysis

By comparing genomics and bioinformatics, make full use of the UCSC and NCBI databases, the latest sheep 0vis_aries_3.1 genomic information (hnp://www.ncbi.nlm.n.gov/assemblv/457978/), significant SNP locis were aligned to confirm their chromosome and physical location, and gene function annotations were performed in the 100Kb region upstream and downstream of the SNP. The Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/) was used to perform the gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Gene and Genome (KEGG) pathway analysis. To better understand the functions of the genes within the SNP, the Ovis aries Ensembl gene IDs were converted into human orthologue Ensembl gene IDs using BioMart because the annotation of the sheep genome is limited.

2.8 SNP detection and genotyping of candidate gene of BMP2 and PDGFD

385 blood samples collected from Altay sheep in Altay, Xinjiang and Tibetan sheep, At the same time. Genomic DNA extraction of the collected blood samples using the Tiangen blood DNA extraction kit, detection of DNA sample concentration using NANO DROP and detection of DNA quality by 0.8% agarosegel electrophoresis, According to the genealogical information, select 30 distantly related DNA samples and evenly dilute to 50ng•μL-1, take 2 μL of each sample and mix evenly to construct Altay sheep DNA pool.

2.9 SNP detection and genotyping

PRIMER 3 online design software was used to design primers for all exons of the gene. Primer information is shown in Supplementary file. Primers were synthesized by Cybrex Biotech Co., Ltd. The total PCR reaction system was 20 μL: 1μL of 5'primers, 1μL of 3' primers, 10μL for the 2×Taq PCR Master Mix, 2μL for the DNA template, and 6μL for the ddH2O. PCR reaction program: 94°C pre-denaturation 5min; 94 °C denaturation 30s, annealing temperature 30s, 72°C extension 30s, 35cycles; 72°C extension 10min; 4 °C preservation. The PCR product was detected by 0.8% agarosegel electrophoresis and the target band was sent to Bio Miao Biological Technology (Beijing) Co.,Ltd. For sequencing. According to the sequencing peaks, sequence comparisons using DNAMAN 5.2.10 software (Lynnon BioSoft, Quebec, ONT, Canada) and Chromas 2 software performed to screen for SNP sites.

Considering the compatibility of the primers used to extend the SNPs genotyped in the next MALDI-TOF assay process, 2 SNPs were then genotyped in 385 Altay sheep and Tibetan sheep using MALDI-TOF assay (Mass ARRAY; Sequenom Inc, San Diego, CA, US).

2.10 Data Processing and Statistical Analysis

SPSS 22.0 software and case-control analysis method to compare the frequency of genotypes of large-tailed and thin-tailed individuals (alleles) at each marker locus in the genome. The statistical model is as follows:

$$Y = \mu + G + p + m + e \quad (i = 1, 2)$$

In the formula, Y represents the measured value of the trait, μ represents the population mean, G is the *BMP2* and *PDGFD* genotype effect, p is the field effect, m is the gender effect, and e is the random residual.

3. Results

3.1 Markers and group information

After quality control, remaining 119 individuals (Figure 1), of which 39 were large-tail Han sheep (male 16 female 23), 40 Altay sheep (25 female 15), 40 Tibetan sheep (male 21 female 19) and 538762 SNPs distributed among 26 chromosomes.

Figure 1. Test group. (a) large-tail Han sheep; (b) Altay sheep ;(c) Tibetan sheep

3.2 PCA analysis

Principal components analysis (PCA) of genome-wide SNPs was performed using R snpStats Software package and Plink software. The PCA results showed that the samples from the three breeds were clustered two principal components PC1 and PC2. According to PC1, Chinese sheep with different type of tail could be divided into two groups consistent with their fat deposition: fat-tailed sheep (PC1<0，large tailed Han sheep and Altay sheep) and thin-tailed sheep (PC1>0, Tibetan sheep), as showed Figure 2.

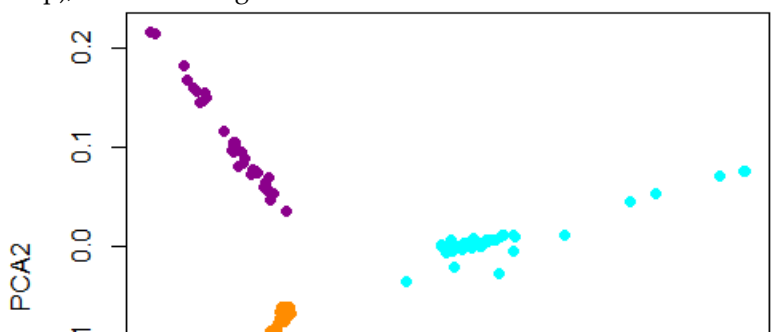
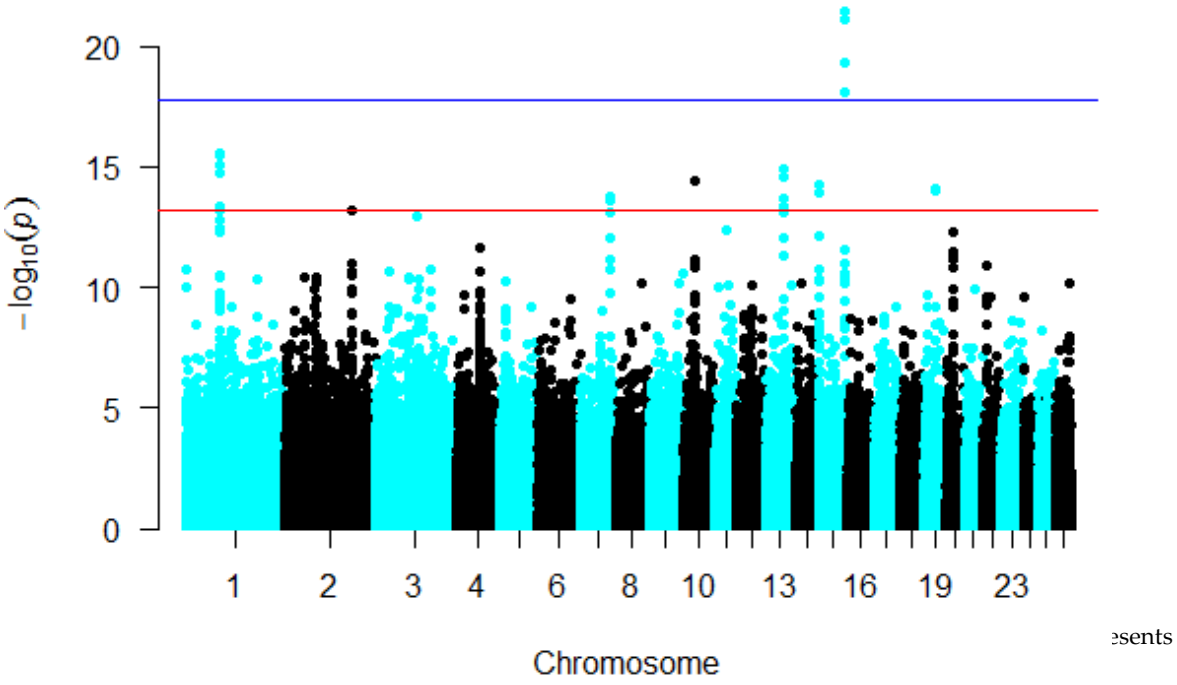


Figure 2. Animals clustered on the basis of principal component (PC) analysis using individual genotypes.

3.3 The results of GWAS

A case-control design GWAS was performed to research the genetic basis of the fat deposition with different type of tails. Tibetan sheep from the three breeds were coded as controls and large-tailed Han sheep and Altay sheep as case. The General Linear Model (GLM) in Tassel 3.1 was used for whole genome-wide association analysis. We defined the whole-genome significance cutoff as top 5%.

The result as show Figure 3, the different color labels on the X-axis represent different chromosomes, the Y-axis represents the -log10 (p) of the SNP, the results indicated that that a total of 31 genome-wide significant SNPs associated with type of tail traits were detected, all of which distributed on chr 1, 2,7, 10, 13, 15 and 19, respectively. Four strong association signal was observed on chromosome 15.



3.4 Identification of candidate genes with significant SNPs

By comparing genomics and bioinformatics, make full use of the USCS, NCBI databases and the latest sheep Ovis_aries_3.1 genomic information (http://www.ncbi.nlm.nih.gov/assembly/457978) to compare significant SNP loci to confirm their chromosomes and physical locations, the significant SNP was extended approximately 100 kb in the upstream and downstream directions. The results showed that 31 SNPs were significantly correlated with the tail type at the genome level, distributed on 7 different chromosomes. Four SNPs were located in the known genes, as shown in Table 1

Table 1. Functional annotation for chromosome-wide significant SNPs associated with TW

SNP name	chr	position(bp)	P value	Gene name
oar3_OAR1_94912049	1	94912049	8.04E-16	
oar3_OAR1_94931158	1	94931158	1.75E-15	
oar3_OAR1_94940531	1	94940531	1.75E-15	
oar3_OAR1_94977426	1	94977426	3.47E-16	*SPAG17*
oar3_OAR1_95037331	1	95037331	6.03E-14	
oar3_OAR1_95069631	1	95069631	4.06E-14	
oar3_OAR1_95596363	1	95596363	2.64E-16	
oar3_OAR1_95597675	1	95597675	2.64E-16	
oar3_OAR1_95600207	1	95600207	2.64E-16	*TBX15*, *WARS2*
oar3_OAR2_183136068	2	183136068	6.28E-14	*EN1*, *MARCO*
s55494.1	7	82545552	1.75E-14	ALDH6A1
oar3_OAR7_82603868	7	82603868	2.30E-14	
oar3_OAR7_82623517	7	82623517	7.33E-14	*LIN52*, *VSX2*, *ABCD4*, *VRTN*, *TMEM90A*, *NPC2*, ISCA2, LTBP2
oar3_OAR10_29373640	10	29373640	3.50E-15	*FRY*, *EEF1A1*, *RXFP2*
oar3_OAR13_48651711	13	48651711	7.82E-14	*BMP2*
oar3_OAR13_48882107	13	48882107	4.01E-14	
oar3_OAR13_48890947	13	48890947	7.18E-14	
oar3_OAR13_48918153	13	48918153	2.40E-15	
oar3_OAR13_48920964	13	48920964	2.40E-15	
oar3_OAR13_48927268	13	48927268	2.40E-15	*PPP1CC*
oar3_OAR13_49290888	13	49290888	1.12E-15	
oar3_OAR13_49330252	13	49330252	2.18E-14	

oar3_OAR13_49354226	13	49354226	2.18E-14	*MUTED*
oar3_OAR15_3870680	15	3870680	5.65E-15	
oar3_OAR15_3871017	15	3871017	1.14E-14	*PDGFD, DDI1,*
oar3_OAR15_72542670	15	72542670	3.63E-22	
oar3_OAR15_72543368	15	72543368	7.62E-22	
oar3_OAR15_72547431	15	72547431	4.50E-20	
oar3_OAR15_72549351	15	72549351	7.83E-19	*ACCSL, EXT2, ALX4*
oar3_OAR19_31653185	19	31653185	7.98E-15	
oar3_OAR19_31657197	19	31657197	9.90E-15	*MITF*

3.5 Enrichment analysis

DAVID v2.1 was used to conduct the GO and KEGG pathway enrichment analyses for a more in-depth understanding of the function of these identified genes.

Table 2. Significant GO terms associated with genes

Biological Process	GO name	Count	P value	Benjamini
GO:0042981	regulation of apoptotic process	2	6.8E-2	9.7E-1

Table 3. Significant KEGG pathway

Catagory	Term	Count	P-Value	Benjamin
KEGG_PATHWAY	Melanoma	2	9.6E-2	9.9E-1

As can be seen from the table, these genes are mainly enriched in biological processes. This GO item mainly involves the regulation of apoptosis process.

3.6 SNP detecting and genotyping of BMP2 and PDGFD

DNA pool sequencing showed 1 SNPs were identified in first exon of *BMP2* gene and 1 SNPs in the fourth exon of *PDGFD* gene, considering the compatibility of the primers used to extend the single site in the MALDI-TOF assay process, rs119 T>C (Figure 4A) in the exon1 of *BMP2* gene and 2 SNPs in the exon4 (rs69 C>A , Figure 4B) of *PDGFD* gene respectively were selected to be genotyped in a new Altay and Tibetan sheep validation group including 385 samples.

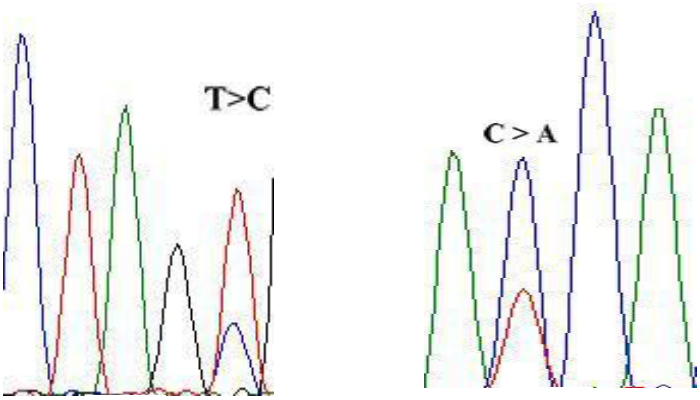


Figure 4. Sequencing profiles of PCR product from DNA pooling

Mass-array spectrometry was used to genotype the screened SNPs (RS1 and RS2). Each locus has 2 genotypes and its genotyping results See Figure 5 and Figure 6. RS1 sites are CC, TT, and RS2 is CC, AA.

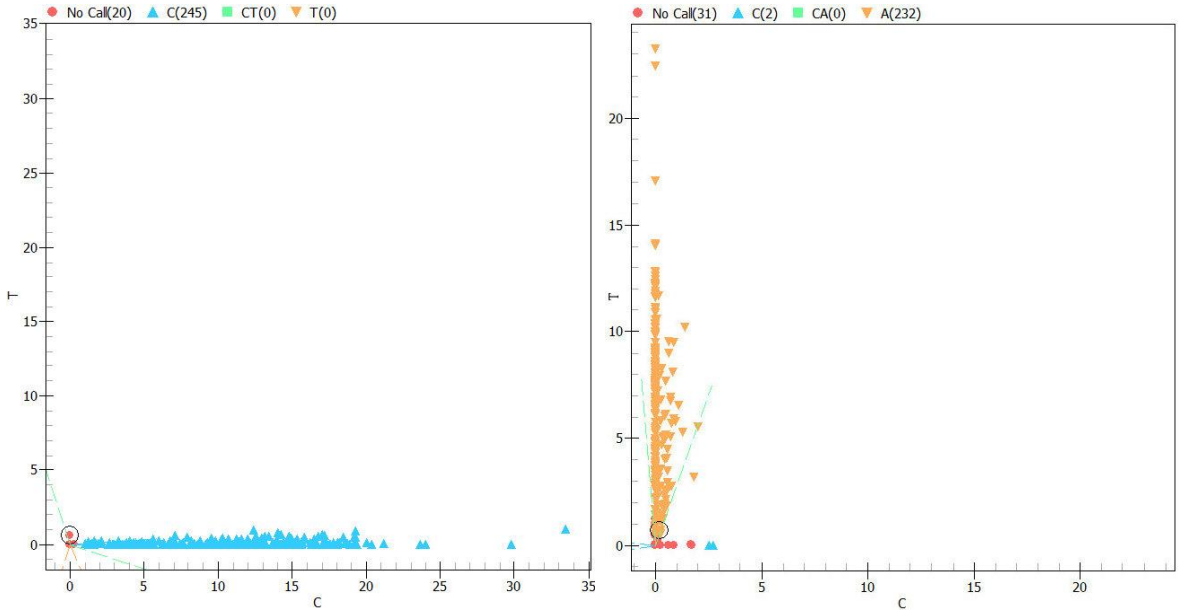


Figure 5. RS1 loci mass spectrometry results. C means the genotype of individuals in the blue region means was CC; T means the genotype of individuals in the yellow region was TT. Numbers in brackets means number of individuals of the two genotypes respectively

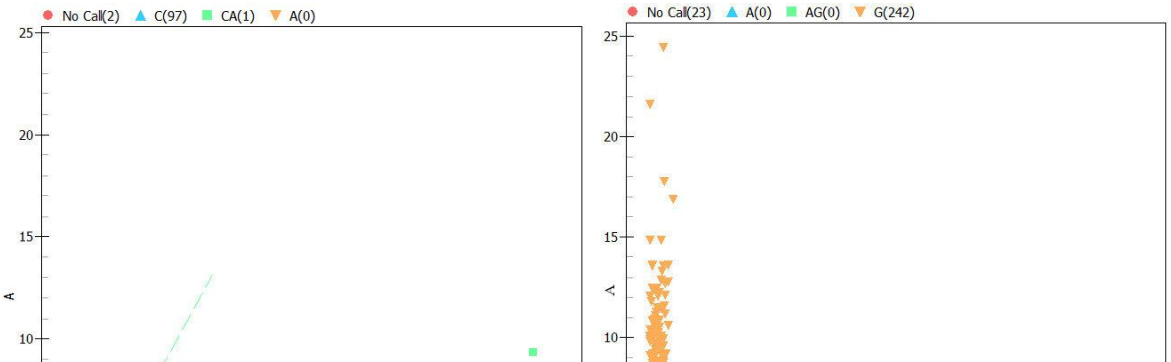


Figure 6. RS2 loci mass spectrometry results. C means the genotype of individuals in the blue region means was CC; A means the genotype of individuals in the yellow region was AA. Numbers in brackets means number of individuals of the two genotypes respectively

3.7 Association of single nucleotide polymorphism with tail in Altay and Tibetan sheep

Single SNP association analysis showed that the two genotypes at the RS1 and RS2 loci were significantly different in Altay sheep and Tibetan sheep($P<0.05$). rs119 that located on exon1 of *BMP2* gene was TT genotype in Altay sheep, while with CC genotype in Tibetan sheep. On rs69 of *PDGFD* gene, Altay sheep with CC genotype , however, Tibetan sheep with AA genotype. The association analysis results were in Table 4.

Table 4. Associations of single marker of the genes with fat-rumped and thin-tailed sheep

Locus	Altay sheep Genotype	Tibetan sheep Genotype
Rs119	TT	CC
Re69	CC	AA

4. Discussion

In this study, large-tailed Han sheep, Altay sheep and Tibetan sheep were used as experimental groups, which are our national sheep genetic resources. These sheep have different tail types and are distributed in different regions of China. The main producing area of the big-tailed Han sheep is the hinterland of the northern plains of China. There are obvious seasonal changes here, the winter is cold and dry, and the summer is humid and rainy, Altay sheep live in the Altay Gobi Desert in Xinjiang, where there is an extreme climate, with an average annual temperature of 4'C and a minimum temperature of -42.7℃, the ground is covered by snow for 200-150 days a year. Tibetan sheep live in the Qinghai-Tibet Plateau at an altitude of 3000-5000 meters, the Tibetan sheep are relatively strong and tall, and the tail is in the shape of a flat cone. They have different genetic backgrounds, and genes that cause differences in tail types are more easily detected.

In our study, a genome-wide study was performed for three different tail types sheep breeds, GWAS have been applied to many species but rarely to sheep tail type. TASSEL software has been commonly used in GWAS, was employed to analyze associations between SNPs and phenotypes [8, 9]. In order to make the GWAS research results more reliable, the quality control of individual and SNP chips can improve the accuracy of data, after the quality control of SNP, the missing genotypes are also filled with BEAGLE software. For statistical methods, we use a generalized linear mixed linear model, which is improved based on the mixed linear model, ignoring the pedigree information of the family, and its statistical effect is higher. From the GWAS results, SNPs that are associated with tail-type traits and are significant at the genome-wide level are located on chromosomes 1, 2, 7, 10, 13, 15, and 19, but most on chromosomes 1, 13 and 15. Some of these SNP

loci are directly located or close to some genes that have been reported in association with bone, fat metabolism, etc. Zhang et al [9] reported that a directed mutation in *SPAG17* (sperm associated antigen17) may cause bone stagnation in mice, such as shortened hind limb length, sternal segment fusion, and bone mineralization defects. Gesta et al[10] reported that *Tbx15* (transcription factor 15) can regulate adipocyte differentiation and mitochondrial respiration, and *Tbx15* gene plays an important role in limb bone development [11]. Fan et al. [12] found that the *VRTN* gene variant was significantly associated with the number of thoracic vertebrae in Chinese and Western pigs. The *VRTN* gene was also found in our results, which may affect the tail type of sheep by affecting the number of sheep's tail vertebrae. When studying the biological function of *BMP2* gene in pluripotent stem cells, Ahrens et al. unexpectedly found that under the action of *BMP2* protein, these cells can not only differentiate into bone cells, but also differentiate into adipocytes [13]. The *BMP2* gene was also detected in this study and may be involved in the formation of fat in the tail of sheep. *PDGF* (Platelet-derived growth factor) is anti-lipogenic and inhibits pre-adipocyte differentiation, but the *PDGF* gene is highly expressed in adipose tissue [14]. The *PDGFD* gene was identified in this study and is estimated to play an important role in the formation of fat in the tail of sheep, leading to the formation of different tail types.

To validate the effect of promising SNPs identified by our previous genome-wide association analysis, We selected *BMP2* and *PDGFD* genes for verification , *BMPs* are important regulators of adipogenesis and may play a role in obesity and *BMP2* expression increases may contribute to partitioning of energy storage into visceral and subcutaneous adipose tissue depots [15]. Zhang et al. findings reveal a notable role of *PDGF-D* in the AA formation during obesity [16]. In the present study, mutations in the rs119 locus C→T affected the sheep tail type, Altay sheep with TT genotype, while with CC genotype in Tibetan sheep, this suggests that this gene TT genotype of *BMP2* is likely to be associated with fat deposition in the tail of sheep. On rs69 of *PDGFD* gene, Altay sheep with CC genotype , however, Tibetan sheep with AA genotype, also this suggests that this gene CC genotype is likely to be associated with fat deposition in the tail of sheep. In general, phenotypic changes are often caused by functional mutations in genes that control the trait, so this mutation can serve as an effective molecular marker for marker-assisted breeding.

5. Conclusions

Chinese indigenous sheep can be classified into three types based on tail morphology, some candidate genes by genome-wide association analysis and genotyping technology discovered that on exon1 of *BMP2* gene was TT genotype in Altay sheep, while with CC genotype in Tibetan sheep. On exon4 of *PDGFD* gene, Altay sheep with CC genotype, however, Tibetan sheep with AA genotype. These genes can be molecular marker for sheep tail type selected.

Author Contributions: Caiye Zhu conceived and designed the experiments; Caiye Zhu performed the experiments; Caiye Zhu, Xiaoyu Huang and Mingna Li analyzed the data; Shizhen Qin , youji Ma and Suli Fang contributed reagents/materials/analysis tools; Caiye Zhu wrote the paper. All authors read and approved the final manuscript.

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