

## Characterization of porcine adiponectin gene (*ADIPOQ*) polymorphisms and their association with litter size

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**Abstract:** Litter size is one of the most important economic traits for pig production as it is directly related to production efficiency. To evaluate the effects of the adiponectin (*ADIPOQ*) gene on porcine litter size, we investigated *ADIPOQ* polymorphisms in three breeds, Wannan Black pigs, Berkshire pigs, and BW pigs (crossbred pigs produced from mating male Berkshire pigs and female Wannan Black pigs), using the PCR-SSCP method. Three SNPs (c. 178G > A, c. 1165A > G, and c. 1138G > A) were identified. In Wannan Black pigs, association analysis indicated that the c. 178G > A SNP genotype was significantly associated with an effect on total number born (TNB;  $P < 0.05$ ) and the number born alive (NBA;  $P < 0.05$ ) in primiparity. The c.1138G > A SNP was significantly associated with an effect on TNB ( $P < 0.05$ ) and NBA ( $P < 0.05$ ) in primiparity and multiparity. In addition, three of the nine possible genotype combinations, AACC, AACD, and AGCD, were found. AGCD resulted in primiparous and multiparous sows with the highest TNB. These results indicated that polymorphisms in the *ADIPOQ* gene could be used for marker-assisted selection programs for the genetic improvement of reproductive characteristics in pigs.

**Key words:** *ADIPOQ* gene, polymorphism, litter size, genotype combinations, pig

### 1. Introduction

Porcine litter size has a close association with economic efficiency and is mainly measured as total number born (TNB) and number born alive (NBA). As a quantitative trait, the heritability of fertility is low and controlled by multiple genes. In recent years, with increased attention on sow fertility, the main objective of breeding has been to improve fecundity (1). Many genes associated with litter size and other reproductive traits have been mapped on the porcine chromosomes (2); previous studies have revealed that adiponectin is related to reproduction and fertility.

The pig *ADIPOQ* gene consists of three exons and two introns, and it was mapped to chromosome 13q36-41 at position 53.6cM on the linkage map (3,4). It shares approximately 88%, 86%, 85%, and 83% homology with dog, human, cow, and mouse adiponectin, respectively (5). Adiponectin seems to be a hormonal link between obesity and reproduction. It was defined as a factor affecting ovarian steroidogenesis, oocyte maturation, and embryo implantation and development (6). Adiponectin acts via two receptors, adiponectin receptor 1 (*ADIPOR1*) and

adiponectin receptor 2 (*ADIPOR2*), and the *ADIPOQ* gene and its receptors are all expressed in the porcine hypothalamic structures responsible for GnRH production (7). Several lines of evidence also suggest that adiponectin influences the reproductive system by exerting central effects on the highest branch of the hypothalamic-pituitary-ovarian axis, inhibiting GnRH (8) and GnRH-induced LH secretion (9,10). Recently, a few articles have referred to the association between polymorphisms in the porcine *ADIPOQ* gene and reproductive traits, suggesting that the single nucleotide polymorphism (SNP) c. 178G > A (rs 335502090) in the coding region was associated with a small number of stillborn and mummified piglets, and shorter weaning-estrus intervals (11). The aim of the present study was to further investigate the association between *ADIPOQ* genotypes and litter size traits within three pig populations, including one Chinese native breed (Wannan Black pig), one lean breed (Berkshire pig), and two-way crossbred pigs (BW pig). The results that c. 178G > A (rs 335502090), c. 1165A > G (rs 321419050), and a new SNP c. 1138G > A were found in this study can provide basic data for marker-assisted selection of pigs.

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## 2. Materials and methods

### 2.1. Animals and sample collection

Three populations of pigs, Wannan Black pigs (n = 93), BW pigs (n = 81), and Berkshire pigs (n = 40), were investigated in the present study. External ear tissues of 214 pigs were collected from the breeding farm in the Jixi county of Anhui Province, China; rearing and feeding conditions were equalized and the external ear tissues were stored at -20 °C after immersion in 75% ethanol. Meanwhile, information that contained the TNB and NBA was collected. Genomic DNA samples were extracted using the phenol-chloroform method (12).

Hybridization, production, and epidemic prevention of the sample groups were organized in strict accordance with the production standards of pig breeding. Experiments were performed according to the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China; revised June 2004) and approved by the ethics committee of Anhui Agricultural University, Anhui, China.

### 2.2. PCR-SSCP and sequencing

Primers were designed using the GenBank *ADOIPOQ* gene sequence (Accession No. AY627882 and No. EF601160). The primer sequences are shown in Table 1, and primers

were synthesized by Shanghai Generay Biotech Co., Ltd. PCR reactions were performed with a Bio-Rad Thermal Cycler (Bio-Rad, Hercules, CA, USA) in a volume of 25 µL, which included 8 µL of 2X Power Taq PCR MasterMix (25 mM MgCl<sub>2</sub> and 2 mM each dNTP), 2 µL of template DNA, 1 µL of each of the upstream and downstream primers (2 µmol/L), and 13 µL of sterile distilled water. The PCR amplification program included an initial denaturation step at 94 °C for 5 min followed by denaturation at 94 °C for 1 min, annealing at 58–61 °C for 30 s, and extension at 72 °C for 30 s for a total of 38 cycles, with a final extension at 72 °C for 10 min before being held at 4 °C.

The PCR products were genotyped using 12% native polyacrylamide gel electrophoresis (PAGE). The SSCP analysis was performed using 3.5 µL of PCR product in 6.5 µL of loading buffer (9 mL of deionized formamide, 1 mL of 10X Tris-borate-EDTA buffer, 0.025% bromophenol blue, and 0.025% xylene cyanol). PCR products were denatured for 10 min at 98 °C and the mixture was immediately cooled on ice for 10 min. Samples were then run on a 12% polyacrylamide gel prerun at 300 V for 20 min followed by electrophoresis at 135 V for 13 h. After that, the gel was shaken lightly for 5 min in 70% ethanol, and then for 18 min in AgNO<sub>3</sub> staining solution after washing with ddH<sub>2</sub>O.

**Table 1.** Sequences of primers for amplification of the adiponectin gene in pigs.

Accession	Primer	Sequences	Annealing temperature (°C)	Product length (bp)
AY627882	ADPN1	F: 5'-AGCTGTTCTACTGCTACTAGCCC-3'	58	218
		R: 5'-AACCAAAGACCTTATTCTTACCTG-3'		
	ADPN2	F: 5'-GATGGATGGACAGAATAAAGGAGGGA-3'	60	210
		R: 5'-CAGAACCAGGAGCCAATGAGA -3'		
EF601160	ADPN3	F: 5'GGTGAGAAAGGAGATACAGGTC-3'	60	208
		R: 5'-TCTTGGTAAAGCGAATG-3'		
	ADPN4	F: 5'-ATCTGAACTGCGTCTGCGA-3'	61	212
		R: 5'-CCAGGGGATGTGCCTTCTTC-3'		
	ADPN5	F: 5'-GTCCCCACATTACTCTGTACTT-3'	60	234
		R: 5'-GGACATCTCACAGACTCAGGA-3'		
	ADPN6	F: 5'-CATTGGCTCCTGGTTCTGTAAAGTCA-3'	60	252
		R: 5'-GAATGCTGAACGGTAGACATAGGCG-3'		
	ADPN7	F: 5'-CTGGAGTGACTGGGGTTGAA-3'	60.5	256
		R: 5'-AAGTAGACCGTGATGTGGAAGGAGA-3'		
	ADPN8	F: 5'- TGGCAAATTCCTACTGCAACA-3'	60	256
		R: 5'-ATTGTCAGCATAGACCCCATTTATTC-3'		

F, upstream primer; R, downstream primer.

The wash step was repeated 3 times after incubation in the staining solution. Development solution was added until bands were clearly visible. PCR products were sequenced by Shanghai Generay Biotech Co., Ltd. Chromas software was used to confirm the *ADOIPOQ* DNA sequence and to detect the polymorphic locus.

**2.3. Data analysis**

Excel 2007 was used to calculate genotype frequencies, allele frequencies, polymorphism information content (PIC), heterozygosity (He), and chi-square values. Associations between genotypes and litter size (TNB and NBA) were analyzed using the general linear model of SPSS 19.0 (13). The SHEsis website was used for linkage disequilibrium analysis and haplotype construction. The least significant difference method was used for multiple comparisons, and the corresponding TNB and NBA of different genotypes were expressed as least squares mean ± standard error (LSM ± SE). The linear model was as follows:

$$Y_{ijkl} = \mu + A_i + B_j + C_k + D_l + E_{ijkl}$$

where  $Y_{ijkl}$  is the phenotypic value of the reproductive traits (TNB or NBA),  $\mu$  is the overall population mean,  $A_i$  is the year and seasonal effect,  $B_j$  is the genotype effect,  $C_k$  is the effect of birth parity,  $D_l$  is the breed effect, and  $E_{ijkl}$  is random error. The significance of difference was tested using Duncan's multiple comparison.

**3. Results**

**3.1. Single nucleotide polymorphism identification and sequencing results**

Polymorphic loci were detected by three pairs of primers (ADPN1, ADPN2, and ADPN4) by comparative sequencing of candidate genes in Wannan Black pigs and BW pigs (Figures 1–3). One of the SNPs was located in the coding region (c. 178G > A), and one SNP was found in intron 2 (c. 1165A > G). The last SNP was located in the 3'-UTR region (c. 1138G > A). Only the c. 178G > A polymorphism encoded for a valine-to-isoleucine amino acid replacement. The SNP c. 1138G > A found in the 3'-UTR region has not been previously described; moreover,

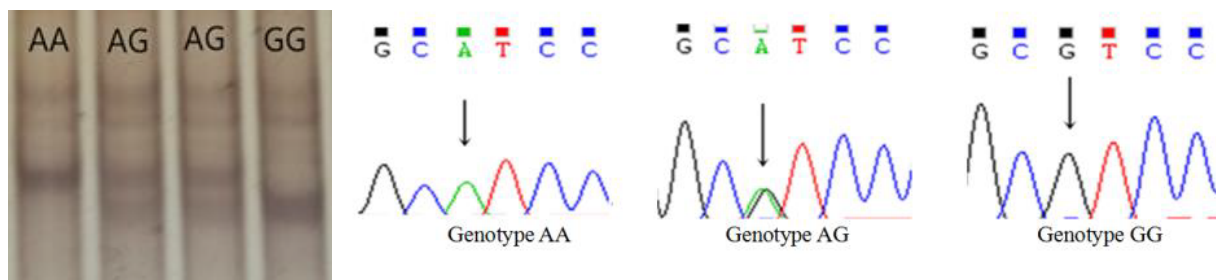


Figure 1. SSCP and sequencing results of c. 178G > A in Wannan Black pigs.

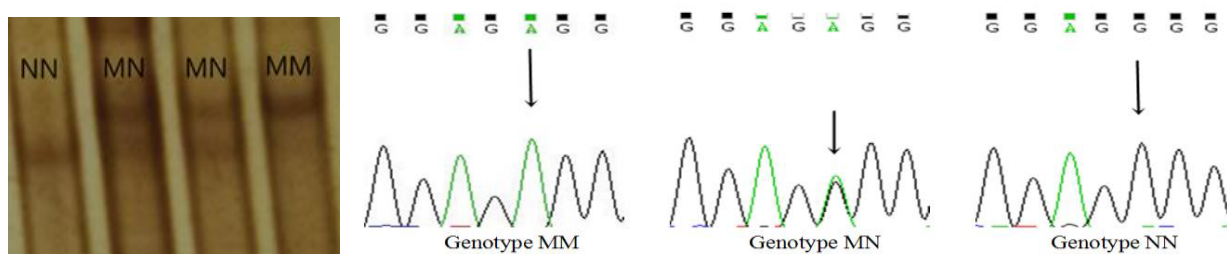


Figure 2. SSCP and sequencing results of c. 1165A > G in Wannan Black pigs.

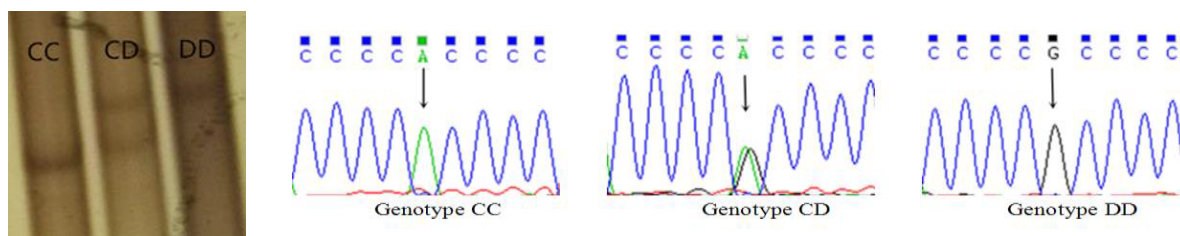


Figure 3. SSCP and sequencing results of c. 1138G > A in Wannan Black pigs.

according to analyses, the SNP c. 1138G > A is significantly associated with litter size.

**3.2. Genetic polymorphism of adiponectin gene**

Three DNA polymorphisms were identified in the porcine *ADIPOQ* gene in Wannan Black pigs and BW pigs (Table 2). In Wannan Black pigs, three genotypes were found: AA, AG, and GG with primer ADPN1; MM, MN, and NN with primer ADPN2; and CC, CD, and DD with primer ADPN4. In BW pigs, only two genotypes were found: GG and AG with primer ADPN1, MM and MN with primer ADPN2, and CD and DD with primer ADPN4. No SNPs were detected in Berkshire pigs; all Berkshire pigs were GG with primer ADPN1, MM with primer ADPN2, and DD with primer ADPN4. Allele frequencies and values of chi-square, genotype frequency, allele frequency, PIC, and He are shown in Table 2. The chi-square test showed that the three SNPs in the population of Wannan Black pigs were in Hardy–Weinberg (H-W) equilibrium ( $\chi^2 = 0.30$ ,  $\chi^2 = 0.30$ , and  $\chi^2 = 0.14$ , respectively). As for BW pigs, the heterozygote was the major genotype (Table 2). The chi-square test showed that the three SNPs in the population

of BW pigs were not in H-W equilibrium ( $\chi^2 = 49.31$ ,  $\chi^2 = 69.084$ , and  $\chi^2 = 29.55$ , respectively). The frequency of MM with primer ADPN2 was less than 5%, so we did not further investigate this polymorphism in BW pigs.

**3.3. Association analysis of adiponectin gene polymorphisms with litter size**

Association analyses involving the records of the TNB and NBA of primiparity and multiparity in Wannan Black pigs and BW pigs were conducted. Significant results obtained in the association analysis between the three SNPs and litter size are shown in Table 3. In Wannan Black pigs (Table 3), at the c. 178G > A locus, genotype AG had a higher TNB and higher NBA than genotype AA ( $P < 0.05$ ) in primiparity, but there was no significant differences between the AA and GG genotypes or between the AG and GG genotypes ( $P > 0.05$  in both cases). For multiparity in Wannan Black pigs, the three genotypes had no significant difference for TNB or NBA. The c. 178G > A locus and the c. 1165A > G locus were not in linkage disequilibrium, so we did not investigate ADPN2 (c. 1165A > G) in further detail.

**Table 2.** Genotype frequency, allele frequency, and polymorphic sites in the adiponectin gene of Wannan Black and Berkshire pigs × Wannan Black pigs (BW pigs).

Breed	Locus	GenBank accession no.	Genotype frequency			Allele frequency		PIC	He	$\chi^2$
			AA	AG	GG	A	G			
Wannan Black	c. 178 G > A	AY667882	AA	AG	GG	A	G	0.24	0.28	0.30
			(63)	(28)	(2)					
			0.68	0.30	0.02					
	c. 1165 A > G	AY667882	MM	MN	NN	M	N	0.24	0.28	0.30
			(2)	(28)	(63)					
			0.02	0.30	0.68					
c. 1138 G > A	EF601160	CC	CD	DD	C	D	0.32	0.40	0.14	
		(49)	(36)	(8)						
		0.53	0.39	0.08						
BW	c. 178 G > A	AY667882	AA	AG	GG	A	G	0.37	0.49	49.31
			(0)	(71)	(10)					
			0	0.88	0.12					
	c. 1165 A > G	AY667882	NN	MN	MM	M	N	0.37	0.49	69.84
			(0)	(78)	(3)					
			0	0.96	0.04					
	c. 1138 G > A	EF601160	CC	CD	DD	C	D	0.36	0.46	29.55
			(0)	(61)	(20)					
			0	0.75	0.25					

PIC: polymorphism information content. He: heterozygosity. The value of  $\chi^2$  is from the test of the distribution of different genotypes for Hardy–Weinberg equilibrium.

**Table 3.** Association of genotypes with litter size in Wannan Black pigs and Berkshire pigs × Wannan Black pigs (BW pigs).

Breed	Locus	Genotype	Primiparity (LSM ± SE)		Multiparity (LSM ± SE)	
			TNB	NBA	TNB	NBA
Wannan Black	c. 178 G > A	AA	8.22 ± 0.27 <sup>a</sup>	8.22 ± 0.27 <sup>a</sup>	12.92 ± 0.26	10.84 ± 0.30
		AG	9.29 ± 0.35 <sup>b</sup>	9.29 ± 0.35 <sup>b</sup>	12.90 ± 0.49	11.09 ± 0.39
		GG	8.33 ± 0.67 <sup>ab</sup>	7.67 ± 0.88 <sup>ab</sup>	10.67 ± 0.33	10.87 ± 0.23
	c. 1138G > A	CC	7.75 ± 0.40 <sup>a</sup>	7.75 ± 0.40 <sup>a</sup>	12.84 ± 0.20 <sup>a</sup>	10.92 ± 0.27 <sup>a</sup>
		CD	9.09 ± 0.29 <sup>b</sup>	9.09 ± 0.29 <sup>b</sup>	13.30 ± 0.63 <sup>a</sup>	11.10 ± 0.46 <sup>a</sup>
		DD	7.20 ± 1.11 <sup>a</sup>	7.20 ± 1.11 <sup>a</sup>	10.50 ± 0.32 <sup>b</sup>	8.50 ± 0.35 <sup>b</sup>
BW	c. 1138G > A	DD			10.86 ± 0.56 <sup>a</sup>	8.64 ± 0.22 <sup>a</sup>
		CD			12.02 ± 0.20 <sup>b</sup>	9.59 ± 0.20 <sup>b</sup>

Data in the table are least squares mean ± standard error (LSM ± SE). Values in each column with different lowercase superscripts are significantly different at  $P < 0.05$ . The same is true for Table 4.

Analysis of the final locus of polymorphism, c. 1138G > A, showed that the heterozygote CD genotype showed a significant tendency toward higher TNB ( $P < 0.05$ ) and NBA ( $P < 0.05$ ; Table 3) in primiparity; in multiparity, the heterozygote CD genotype had a higher TNB and NBA than homozygous genotypes CC or DD ( $P < 0.05$ ).

The relationship between polymorphisms at the c. 1138G > A locus and the litter size of BW pigs was analyzed, and the results are shown in Table 3. CD genotypes had a higher TNB ( $P < 0.05$ ) and NBA ( $P < 0.05$ ) than DD genotypes.

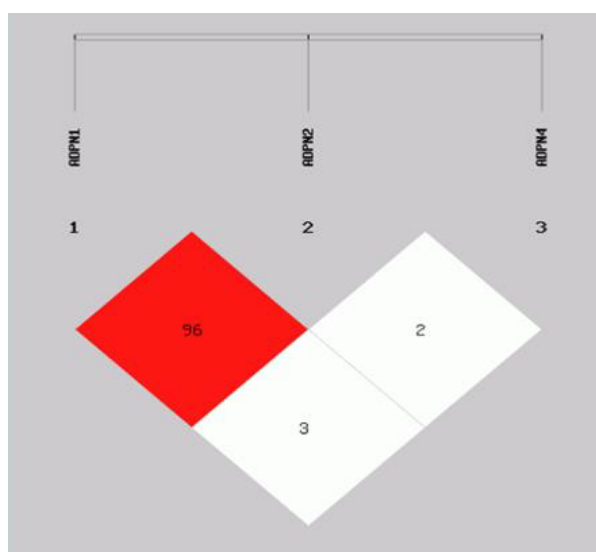
**3.4. Linkage disequilibrium analysis and genotype construction**

To investigate the effects of combined SNPs, haplotypes were constructed by three SNPs of the *ADIPOQ* gene in Wannan Black pigs. According to the results from SHEsis, two polymorphic loci (c. 178G > A and c. 1165A > G) were not in linkage disequilibrium ( $r^2 = 0.96$ ; Figure 4). The c. 178G > A and c. 1138G > A polymorphic loci were in strong linkage disequilibrium ( $r^2 = 0.02$ ; Figure 4). Therefore, we further explored the genotype combinations between c. 178G > A and c. 1138G > A. In Wannan Black pigs, we were able to analyze three of nine possible combinations (AACC, AACD, AGCD) for the two sites (c. 178G > A and c. 1138G > A). Results from the *ADIPOQ* genotype combinations studies are presented in Table 4. Analysis of recorded reproductive data revealed that AGCD is associated with significantly higher TNB and NBA than AACC in primiparity ( $P < 0.05$ ). TNB in animals expressing AACD was 0.56 greater than in animals expressing AACC in primiparity ( $P > 0.05$ ). In multiparity, the TNB of the AGCD genotype was significantly higher than that of the AACD genotype and AACC genotype ( $P < 0.05$ ). The NBA

of the three genotype combinations in multiparity was not significantly different.

**4. Discussion**

According to our results, three SNPs (c. 178G > A, c. 1165A > G, and c. 1138G > A) were detected; one was found in the coding region of the gene (c. 178G > A), one was found in intron 2 (c. 1165A > G), and one was found in the 3'-UTR region (c. 1138 G > A). The two noncoding SNPs did not result in amino acid changes; however,



**Figure 4.** Linkage disequilibrium blocks between the three SNPs found with primers ADPN1, ADPN2, and ADPN4. Upper left region:  $r^2$  value of ADPN1 and ADPN2. Upper right block:  $r^2$  value of ADPN2 and ADPN4. Lower block:  $r^2$  value of ADPN1 and ADPN4.

**Table 4.** Characterization of Wannan Black litter size across adiponectin gene genotype combinations (c. 178G > A and c. 1138G > A).

Genotype combination	Primiparity (LSM ± SE)		Multiparity (LSM ± SE)	
	TNB	NBA	TNB	NBA
AACC	8.00 ± 0.41 <sup>a</sup>	8.00 ± 0.41 <sup>a</sup>	12.80 ± 0.22 <sup>a</sup>	10.80 ± 0.38
AACD	8.56 ± 0.50 <sup>ab</sup>	8.56 ± 0.50 <sup>ab</sup>	12.71 ± 0.78 <sup>a</sup>	10.85 ± 0.63
AGCD	9.33 ± 0.33 <sup>b</sup>	9.33 ± 0.33 <sup>b</sup>	14.67 ± 0.67 <sup>b</sup>	11.67 ± 0.33

numerous studies have demonstrated that SNPs in introns may play a role in regulating gene expression by affecting regulatory elements and some active splice sites (14,15). In addition, many examples exist demonstrating that the 3'-UTR region of a gene can influence the stability of its mRNA (16,17). Based on the current results, it would be interesting to identify a mechanism for the association between these noncoding region SNPs and reproductive traits. The chi-square test showed that the c. 1138G > A locus was in H-W disequilibrium in BW pigs, indicating that there may be directional selection or mating in the two-hybrid breeding process or that the selection pressure on this locus in the population was powerful.

Previous studies have shown that many SNPs exist in the *ADIPOQ* gene. For example, c. 45T > G was found in women with polycystic ovary syndrome (18). According to Guo et al. (19), *ADIPOQ* rs2241766 and rs1501299 SNPs could be associated with colorectal pathogenesis and could have interactions with red meat intake. In cattle, Morsci et al. (20) identified 11 SNPs by sequencing the *ADIPOQ* gene in five registered Angus sires. Another SNP (c. 832T > A) was found in the 3'-UTR region in the goat *ADIPOQ* gene (21).

In pigs, Houde et al. (11) identified five SNPs in the porcine *ADIPOQ* gene of 4 swine breeds; two SNPs, c. 54G > A and c. 178G > A, were located in the coding region, while three other polymorphisms were in the 3'-UTR region (c. 300A > G, c. 1094 \_ 1095insC, and c. 1779A > C). This study also found that the c. 178G > A SNP had a higher frequency of allele A in Chinese indigenous breeds, whereas allele G was only present in the Duroc pig; our results confirm this finding. Moreover, the same SNP (c. 178G > A) was previously identified in pigs (4), and a relationship was found with fat deposition and carcass traits. The c. 1165A > G SNP was previously detected by Cepica et al. (3), but the c. 1138G > A locus has not been described before to the best of our knowledge.

Recently, a few studies demonstrated the relationship between *ADIPOQ* and female reproduction. In rats, adiponectin and its receptors may play an important role in preimplantation embryo development in the uterus (22). The *ADIPOQ* gene was expressed in the rat

hypothalamus and inhibited the GnRH neuronal activity, resulting in regulation of reproduction (22,23). In pigs, the adiponectin gene is expressed in the hypothalamus and the expression level is determined by the estrous cycle. Variations in the expression levels of the *ADIPOQ* gene in the hypothalamus observed during the estrous cycle could be attributed to the influence of ovarian steroids and other hormones controlling reproductive processes (7). According to Houde et al., the c. 178G > A and c. 1094 \_ 1095insC SNPs in the pig *ADIPOQ* gene are significantly associated with a lower number of stillborn piglets (11); our study confirms these results. According to association analyses, genotype AG had a higher TNB and NBA than genotype AA in primiparity. Meanwhile, genotype heterozygote CD showed a tendency toward a high TNB and NBA in primiparity and multiparity. Therefore, our results suggest that the c. 178G > A and c. 1138G > A SNPs may be used as molecular markers for enhanced litter size in Wannan black pigs. Further studies are needed to verify these associations and the results obtained in this study in other breeds.

In conclusion, this is the first time to investigate the association between polymorphisms of the *ADIPOQ* gene and litter size in the Wannan Black pig, a Chinese breed. A new SNP (c. 1138G > A) was identified that has a strong association with litter size. Two other SNPs (c. 178G > A and c. 1165A > G) in Wannan Black pigs were not in linkage disequilibrium and they are significantly associated with litter size. Additionally, genotype combination AGCD of c. 178G > A and c. 1138G > A resulted in a larger litter size in Wannan Black pigs. Therefore, the *ADIPOQ* gene may be a functional candidate gene for enhanced reproductive traits in swine.

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