Short Communication

Molecular cloning and expression pattern of the porcine 5-aminolevulinate synthase 1 (*ALAS1*) gene and its association with reproductive traits

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ABSTRACT. 5-Aminolevulinate synthase 1 (*ALAS1*) is the first enzyme in the heme biosynthetic pathway and is upregulated in follicular tissue during the early stages of ovulation. In this study, we isolated the complete coding sequence of the porcine *ALAS1* gene and its 2-9 intron sequence, identified a single nucleotide polymorphism (SNP; T/C) in intron 9, and developed a PCR-*Msp*I-restriction fragment length polymorphism genotyping assay. Association of the SNP with litter size was assessed in two populations [purebred Large White and the experimental synthetic (DIV) line]. Statistical analysis demonstrated that for total number of piglets born (TNB) in all parities, pigs with the CC genotype had an additional 0.68

and 0.74 piglets compared to the TC and TT animals (P < 0.05) in the DIV line, respectively. Purebred Large White sows inheriting the CC and TC genotypes gave birth to an additional 0.96 and 0.70 piglets compared to the TT animals (P < 0.05) in all parities, respectively. In addition, for TNB in all parities, a significant additive effect of 0.48 ± 0.23 and 0.37 ± 0.17 piglets/litter was detected in sows of both populations (P < 0.05), respectively. The highest levels of *ALAS1* gene expression were observed in isolated ovarian granulosa cells 2 and 12 h after stimulation with pregnant mare serum gonadotropin human chorionic gonadotropin, which represents the time of follicular development and ovulation, respectively. Therefore, the *ALAS1* gene was significantly associated with litter size in two populations and could be a useful molecular marker for the selection of increasing litter size in pigs.

Key words: *ALAS1*; Porcine; Litter size; Polymorphism

INTRODUCTION

5-Aminolevulinate synthase 1 (*ALAS1*), the first and rate-limiting enzyme of the heme pathway, is a member of the a-oxoamine synthase family of pyridoxal 5'-phosphate-dependent enzymes. *ALAS1* is best known for its role in catalyzing the condensation of glycine and succinyl-CoA to yield 5-aminolevulinate, a universal precursor of tetrapyrrole compounds, which is involved in a variety of reactions, including heme biosynthesis, single electron transport, and the catalysis of redox reactions (May et al., 1990; Ferreira and Gong, 1995; Sassa and Nagai, 1996; Oh-Hama, 1997; Thunell, 2000). *ALAS1* expression is regulated by heme and by a number of regulatory regions that respond to cellular signals, including those associated with drug responses and nutrition (Fraser et al., 2003; Guberman et al., 2003; Kakizaki et al., 2003; Kaasik and Lee, 2004; Handschin et al., 2005). It is particularly relevant to note that *ALAS1* has been associated with mitochondrial P450 cytochromes, and with steroid metabolism and steroid hormone production (Aragonés et al., 1985; May et al., 1990; Thunell, 2000; Ju et al., 2012), i.e., with factors known to be involved in ovulation. In addition, possible functional variants within the 5'-region of the *ALAS1* gene mediate an increased transcriptional response in the presence of estrogen and estrogen receptor (du Plessis et al., 2009). In addition, there is limited information to suggest that *ALAS1* expression is associated with acute inflammatory reactions (Iwasa et al., 1989; Suhasini et al., 1995). It is possible that *ALAS1* expression is an early event in the so-called acute phase response, which is a cascade of overlapping inflammatory pathways activated by tissue injury and/or infection (Suffredini et al., 1999; Lentsch and Ward, 2000). Espey and Richards (2002) reported that *ALAS1* is among the first genes to be upregulated in follicular tissue during the early stages of ovulation and is downregulated when the mature ovarian follicles begin to rupture. Hence, *ALAS1* might be a functional candidate gene for reproductive traits.

The objective of our study was to examine *ALAS1* as a candidate gene for porcine reproductive traits. We isolated the porcine *ALAS1* gene, and its 2-9 intron sequences, identified polymorphisms in this gene sequence, investigated allele frequencies, and examined their associations with litter size. Furthermore, the expression pattern of the *ALAS1* gene was analyzed by RT-PCR following treatment of ovarian granulosa cells with PMSG/HCG.

MATERIAL AND METHODS

Animals and data collection

All animal procedures were performed according to protocols approved by the Biological Studies of Animal Care and Use Committee of Hubei Province, China. Ovarian tissues were sampled from three 4-month-old Landrace x Large White sows and then ovarian follicle fluid was collected and used to isolate ovarian granulosa cells. Total DNA and RNA were extracted using phenol chloroform and TRIzol (Invitrogen, Carlsbad, CA, USA), respectively. RNA was reverse transcribed to cDNA using an oligo-dT primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA).

To investigate allelic and genotypic frequencies, 42 Chinese Meishan pigs, 39 Tongcheng pigs, 128 experimental synthetic line (DIV) pigs, 106 Large White^{I} pigs, and 161 Large White^{II} pigs were used.

The association analyses were conducted in two populations with different genetic backgrounds. One population included 267 purebred Large White (106 Large White^{I} and 161 Large White^{II}) sows kept at the Hubei Academy of Agricultural Sciences research farm and the Experimental Pig Station of Huazhong Agricultural University (HAU), respectively. Another population (DIV line) included 128 sows (101 $\geq$2 litters per sow in 2005; 82 $\geq$2 litters per sow in 2006) from a synthetic line originating from Landrace, Large White, Tongcheng, and/or Meishan. These animals were reared by HAU. During 2004-2006, both the total number of piglets born (TNB) and the number of piglets born alive (NBA) were recorded in 422 litters of the DIV line sows and 384 litters in purebred Large White sows (140 and 244 litters for Large White^{I} and Large White^{II}, respectively). Genomic DNA was extracted from white blood cells according to the methods described by Xiong (1999).

Isolation and culture of ovarian granulosa cells

Ovarian granulosa cells were cultured as previously described (Yue, 2008). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 containing 10% new calf serum in a 5% CO_2, 37°C incubator. After 24 h, the culture medium was replaced with 2 mL DMEM/F12 + 2 IU PMSG + 1 IU HCG. The cells were harvested at 0, 2, 4, 8, 12, 16, 20, and 24 h, respectively. Total RNA was extracted and reverse-transcribed to cDNA for use in RT-PCR analysis.

Expression profile of the porcine *ALAS1* gene

Total RNA from the cultured cells (0, 2, 4, 8, 12, 16, 20, and 24 h) was extracted and reverse transcription was performed as described above. RT-PCR analysis of the cultured cells was carried out with primer pair 1 (F: 5'-AGGTGTCCGTCTGGTGTA-3', R: 5'-AGAGTGAAGAGGGTTGAG-3'), which amplified a fragment spanning the complete coding sequence of the *ALAS1* gene. *GAPDH* was used as an internal control. Primers used for *GAPDH* amplification were F: 5'-ACCACAGTCCATGCCATCAC-3', R: 5'-TCCACCACCCTGTTGCTGTA-3', which amplified a fragment spanning intron 8 of the *GAPDH* gene, were applied to exclude the possibility of DNA contamination during all RT-PCRs. PCR conditions were as follows: 94°C for 4 min; 30 cycles at 94°C for 45 s; 60°C for 40 s, 72°C for 45 s; and a final extension at 72°C for 10 min.

Genomic sequence amplification and sequence analysis

A porcine expressed sequence tag homologous to human *ALAS1* mRNA (GenBank accession No. NM_199166) was used to design primers. PCRs were all performed in a 25-μL reaction volume consisting of 100 ng porcine genomic DNA, 1X buffer, 0.5 μM each primer, 100 μM each dNTP, and 1 U *Taq* DNA polymerase (Promega). The PCR parameters were 4 min at 94°C, followed by 45 s at 94°C, 50 s at the optimal temperature, and 1 min at 72°C for 35 cycles followed by a final extension of 10 min at 72°C. The purified PCR products were cloned into the pMD18-T vector (TaKaRa, Tokyo, Japan) and sequenced commercially (Sangon, Shanghai, China). The sequencing results for two Large White and two Meishan pig breeds were compared using BLAST (http://www.ncbi.nlm.nih.gov).

Genetic variation identification and PCR-RFLP analysis

Based on the BLAST results, a PCR-*Msp*I-RFLP assay was established to detect the single nucleotide polymorphism (SNP) T/C in intron 9 of the *ALAS1* gene. For the PCR-RFLP assay, 8.8 μL PCR products amplified by primer pair 2 (F: 5'-CACACCCCGCAGATGATGAC-3', R: 5'-AAATAGAAGTGCAGAGCCCAGC-3') were digested with 6 U *Msp*I (TaKaRa) for 4 h at 37°C, and then separated by electrophoresis on a 2% agarose gel (containing ethidium bromide) in 1X TAE buffer.

Statistical analysis

The relationship between genotypes and reproductive traits was evaluated using the general linear model procedure in SAS 8.0. Both additive and dominance effects were also estimated using the regression procedure, where the additive effect was estimated as -1, 0, and 1 for TT, TC, and CC, respectively, and the dominance effect was represented as 1, -1, and 1 for TT, TC, and CC, respectively (Liu, 1998). The model used was as follows:

$$Y_{ijkl} = \mu + P_i + S_j + F_k + G_l + e_{ijkl} \quad \text{(Equation 1)}$$

where, Y_{ijkl} is the observed trait, μ is the least square means, P_i is the effect of the *i*th parity [i = 1, 2, 3, 4 (parity ≥ 4)], S_j is the effect of the *j*th year (j = 1 for 2004, 2 for 2005, and 3 for 2006), F_k is the effect of the *k*th farm (k = *1, 2*), G_l is the effect of the *l*th genotype (l = *1-3)*, and e_{ijkl} is the random residual.

RESULTS

Sequence obtained and characterization of porcine *ALAS1*

A 9137-bp DNA fragment was obtained (GenBank accession No. FJ548763), which contained 54 bp of the 5'-untranslated sequence, 2-9 intron sequences, and the complete open reading fragment encoding a protein of 641 amino acids. The molecular mass of the putative *ALAS1* protein was 38.0 kDa and the predicted isoelectric point was 9.07. Sequence analysis revealed that it contained the complete coding sequence of *ALAS1*, which showed 89 and 85% identity in the nucleotide sequence or 100% identity in the amino acid sequence compared to their counterparts in human (NM_199166.1) and rat (NM_024484.2), respectively.

Expression analysis of the porcine *ALAS1* gene

Expression analysis using RT-PCR showed that the porcine *ALAS1* gene was highly expressed 2 and 12 h after treatment with PMSG/HCG, which represents the time of follicular development and ovulation, respectively, and the lowest levels were observed 16-24 h, which represents the time of follicle luteinization (Figure 1).

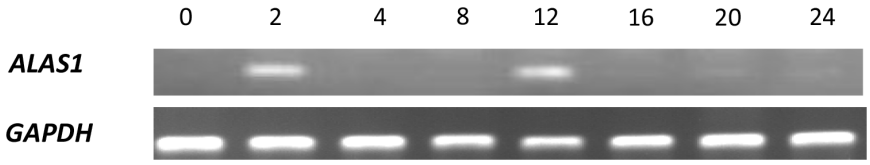

Figure 1. Expression pattern of the porcine *ALAS1* gene in granular cell by RT-PCR.

Genetic variation

Using comparative sequencing of DNA and its unique intron in Large White and Meishan pigs, a T/C mutation in intron 9 was found, which could be detected using the restriction enzyme *Msp*I (TaKaRa). A 359-bp fragment was amplified with primer pair 2. Allelic forms of porcine *ALAS1* were identified as CC (digested by *Msp*I into 277-, 35-, and 47-bp fragments), TT (324- and 35-bp fragments digested by *Msp*I), and TC (digested by *Mva*I into 324-, 277-, 35-, and 47-bp fragments) (Figure 2).

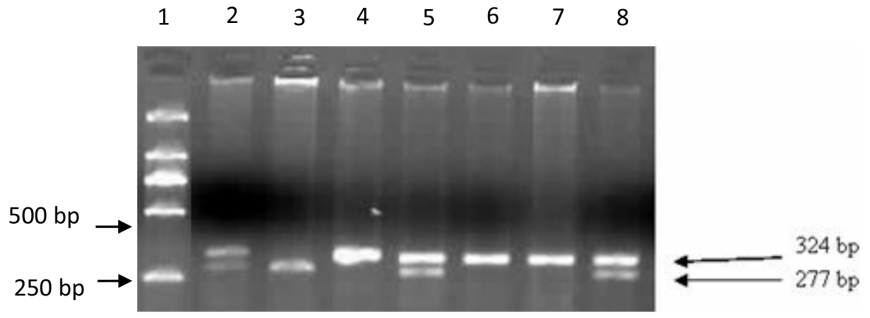

Figure 2. PCR-*Msp*I-RFLP results of intron 9 with primer pair 2. *Lane 1* = DNA molecular marker DL 2000; *lanes 2*, *5*, *8* = genotype TC; *lane 3* = genotype CC; *lanes 4*, *6*, *7* = genotype TT.

Frequency distribution of *ALAS1* in different breeds

Genotypic and allelic frequencies of the SNP were examined in five different populations using PCR-*Msp*I-RFLP (Table 1). From Table 1, we can conclude that allele frequencies of this polymorphism differ significantly among Chinese and Western pig breeds. Allelic distribution revealed that the Western breeds (Large White[I] and Large White[II]) had higher frequencies of the T allele, sharing 64 and 65%, respectively. In comparison, allele C was found at higher frequencies of 66 and 95% in Chinese indigenous breeds (Meishan, Tongcheng), respectively.

Table 1. Distribution of genotypic and allelic frequencies of the porcine *ALAS1* gene PCR-*Msp*I-RFLP.

Breed	No. of animals	Genotypic frequency			Allelic frequency	
		TT	TC	CC	T	C
Tongcheng	39	2 (0.05)	0 (0)	38 (0.95)	0.05	0.95
Meishan	42	6 (0.14)	17 (0.40)	9 (0.46)	0.34	0.66
Large white[I]	106	46 (0.43)	43 (0.41)	17 (0.16)	0.64	0.36
Large white[II]	161	64 (0.40)	79 (0.49)	18 (0.11)	0.65	0.35
Experimental synthetic line (DIV)	128	74 (0.58)	33 (0.26)	21 (0.16)	0.71	0.29

Association of the polymorphism with porcine reproductive traits

The results of association analyses between *ALAS1* genotypes and litter size traits in Large White and the DIV line sows are given in Table 2. In the first parity, animals of the TC genotype in the DIV sows had 1.39 more piglets born alive than the animals of the TT genotype ($P < 0.05$). For TNB in all parities, pigs with the CC genotype had 0.68 and 0.74 more piglets born than the TC and TT genotype animals ($P < 0.05$) in the DIV line, respectively. Purebred Large White sows inheriting the CC and TC genotypes had 0.96 and 0.70 more piglets born than the TT animals ($P < 0.05$) in all parities, respectively. For NBA in all parities, sows with the CC genotype in the Purebred Large White lines had 1.63 more piglets born than the TT animals ($P < 0.01$), and TC animals had 0.89 more piglets born than the TT animals ($P < 0.05$). In addition, for TNB in all parities, a significant additive effect of 0.48 ± 0.23 and 0.37 ± 0.17 piglets/litter was detected in both the DIV sows and purebred Large White lines ($P < 0.05$), respectively.

Table 2. Association between *ALAS1* genotypes and reproductive traits.

Traits	*ALAS1* genotype (means ± SE)			Effect (means ± SE)	
	TT	TC	CC	Additive	Dominance
ALAS1 effects for first parity in purebred Large White pigs					
N	21	20	7		
TNB	10.190 ± 0.606	10.400 ± 0.621	8.857 ± 1.049	-0.667 ± 0.606	-0.438 ± 0.434
NBA	8.619 ± 0.661	8.900 ± 0.677	8.000 ± 1.145	-0.310 ± 0.661	-0.295 ± 0.473
ALAS1 effects for all parities in purebred Large White pigs					
N	156	193	66		
TNB	9.711 ± 0.257^{a}	10.414 ± 0.206^{b}	10.667 ± 0.391^{b}	0.478 ± 0.234*	-0.113 ± 0.1156
NBA	8.198 ± 0.287^{aA}	9.083 ± 0.186^{b}	9.828 ± 0.479 ^{bB}	0.210 ± 0.213	-0.045 ± 0.148
ALAS1 effects for first parity in experimental synthetic (DIV) line pigs					
N	26	16	10		
TNB	10.385 ± 0.399	10.750 ± 0.509	11.200 ± 0.643	0.408 ± 0.378	0.021 ± 0.317
NBA	8.731 ± 0.415^{a}	10.125 ± 0.528^{b}	9.300 ± 0.668^{ab}	0.285 ± 0.393	-0.555 ± 0.329
ALAS1 effects for all parities in experimental synthetic (DIV) line pigs					
N	225	96	64		
TNB	11.678 ± 0.157^{a}	11.737 ± 0.241^{a}	12.417 ± 0.297^{b}	0.369 ± 0.168*	0.205 ± 0.147
NBA	10.839 ± 0.162	10.618 ± 0.249	10.892 ± 0.308	0.027 ± 0.174	0.124 ± 0.152

$^{A,B}P < 0.01$; $^{a,b}P < 0.05$; *$P < 0.05$; N = number of litters investigated.

DISCUSSION

Ovarian granulosa cells play pivotal roles in many aspects of ovarian function, including folliculogenesis and steroidogenesis. The spatial and temporal expression of *ALAS1* during HCG-stimulated ovulation has been demonstrated (Espey and Richards, 2002) and is associated with mitochondrial P450 cytochromes, steroid metabolism, and steroid hormone production (Aragones et al., 1985; May et al., 1990; Thunell, 2000; Ju et al., 2012). Therefore, we selected *ALAS1* as a candidate gene for porcine reproductive traits.

In this study, we isolated the complete coding region of the porcine *ALAS1* gene. It is well known that the gene sequence, especially that of the coding region, is very important for research on gene expression and function. The high amino acid sequence similarity between the porcine *ALAS1* gene and its counterparts in mammals reveals the significance and conservation of their biological functions during evolution. Expression pattern analysis revealed that treatment with PMSG/HCG caused a rapid induction of *ALAS1* transcription in granulosa cells isolated from preovulatory follicles, which is consistent with the observation. *ALAS1* was one of the first in which transcription was upregulated in follicular tissue during the early stages of the ovulatory process and was downregulated when the mature ovarian follicles are beginning to rupture (Espey and Richards, 2002).

In this study, we identified an SNP (T/C) in intron 9 and the polymorphism was significantly associated with TNB and NBA. For TNB in all parities, a significant additive effect (0.48 ± 0.23 and 0.37 ± 0.17 piglets/litter; $P < 0.05$) was detected in both the DIV line and in purebred Large White animals, respectively. Pigs with the CC genotype had more TNB than pigs with the TC and TT genotypes. Thus, increasing the frequency of the C allele could be beneficial in the DIV line and Large White animals to accelerate the genetic improvement of reproductive traits.

In this study, the polymorphism analyzed in the *ALAS1* gene was found in intron 9. Although SNPs in introns do not directly alter the amino acid sequence, they may contain an enhancer or another cis-acting element; thus, constituent SNPs might regulate gene transcription (Bachl et al., 1998) and be directly related to functional variation (Padma et al., 2004). In addition, Nobuyoshi et al. (1997) reported that SNPs in introns may play a role in mRNA splicing and stability. Therefore, variations in *ALAS1* sequences may have important regulatory roles and also be directly related to functional variations. A statistical model with additive and dominance effects only is not ideal. We could not assume the effect of sow because of the insufficient data.

This presumable association is restricted to all parity in the purebred Large White pig group. This may be due to two reasons, one is that the number of animals was not sufficient to demonstrate the true event; the other is that this SNP has no effect on reproductive trait components. Therefore, there is a need for further verification.

Although the SNP (T/C) in intron 9 was found to be associated with TNB and NBA, our results were based on a limited number of animals, which suggests that this preliminary result should be confirmed in more pig populations with a large sample size, and the role of *ALAS1* in porcine reproductive traits or its linkage disequilibrium with other causative mutations should be investigated. If the effect is steady and functional in other populations, this SNP may be a useful molecular marker for reproductive traits in porcine breeding.

Conflicts of interest

The authors declare no conflict of interest.

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