Sequencing, Genomic Structure, Chromosomal Mapping and Association Study of the Porcine *ADAMTS1* Gene with Litter Size

K. Yue, J. Peng, R. Zheng, J. L. Li, J. F. Chen, F. E. Li, L. H. Dai, SH. H. Ding[1]
W. H.Guo[2], N. Y. Xu[3], Y. ZH. Xiong and S. W. Jiang*

Key Lab of Agricultural Animal Genetics, Breeding and Reproduction of Ministry of Education
Huazhong Agriculture University, Wuhan, 430070, China

ABSTRACT : A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif (*ADAMTS1*) plays a critical role in follicular rupture and represents a major advance in the proteolytic events that control ovulation. In this study, a 9,026-bp DNA sequence containing the full coding region, all 8 introns and part of the 5'and 3' untranslated region of the porcine *ADAMTS1* gene was obtained. Analysis of the *ADAMTS1* gene using the porcine radiation hybrid panel indicated that pig *ADAMTS1* is closely linkage with microsatellite marker *S0215*, located on SSC13q49. The open reading frame of its cDNA covered 2,844 bp and encoded 947 amino acids. The coding region of porcine *ADAMTS1* as determined by sequence alignments shared 85% and 81% identity with human and mouse cDNAs, respectively. The deduced protein contained 947 amino acids showing 85% sequence similarity both to the human and mouse proteins, respectively. Comparative sequencing of three pig breeds revealed one single nucleotide polymorphism (SNP) within exon 7 of which a *G-C* substitution at position 6006 changes a codon for arginine into a codon for proline. The substitution was situated within a *Pvu*II recognition site and developed as a PCR-RFLP marker for further use in population variation investigations and association analysis with litter size. Allele frequencies of this SNP were investigated in seven pig breeds/lines. An association analysis in a new Qingping female line suggested that different *ADAMTS1* genotypes have significant differences in litter size (p<0.01). (**Key Words :** Pig, Single Nucleotide Polymorphism, Radiation Hybrid Mapping, Litter Size, *ADAMTS1*)

INTRODUCTION

A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif (*ADAMTS*) is a novel family of extracellular proteases found in both mammals and invertebrates (Tang, 2001). *ADAMTS1* was first cloned in the cachexigenic colon 26 adenocarcinoma subline *in vivo* (Kuno et al., 1996). Later research showed that *ADAMTS1* plays an important role in follicles via progesterone receptor (PR)-dependent pathways (Robker et al., 2000). Female fertility is impaired in *ADAMTS1* knock-out mice, and this is accompanied by obvious abnormalities

of the uterus and ovaries (Shindo et al., 2000). In addition, there is evidence that progesterone- and PR-dependent functions in cumulus cells are essential for expansion of cumulus-oocyte complexes (COC) in the pig, possibly through the action of *ADAMTS1* (Shimada et al., 2005). All these data suggest that *ADAMTS1* plays a critical role in follicular rupture and represents a major advance in understanding of the proteolytic events that control ovulation.

Variations in *ADAMTS1* gene could be used as genetic markers to select animals for breeding. However, there is still no report on how a genetic variant of this gene affects litter size in pigs. The purpose of our research was to characterise the pig *ADAMTS1* gene and determine whether single nucleotide polymorphisms (SNP) of exon 7 in porcine *ADAMTS1* gene are associated with litter size.

MATERIALS AND METHODS

Experimental animals

In this study, 6 pig breeds were used for allele

* Corresponding Author: S. W. Jiang. College of Animal Science & Technology, Huazhong Agriculture University, Wuhan, 430070, P. R. China. Tel: +86-27-87287485, Fax: +86-27-87281378, E-mail: jiangsiwen@mail.hzau.edu.cn
[1] Bureau of Animal Husbandry of Hubei province, Wuhan, 430070, China.
[2] Qingping Breeding Pig Farm of Dangyang City, Dangyang, 440000, China.
[3] Zhejiang University, Hangzhou, 310029, China.

Table 1. Primer pairs used in the analysis of the porcine *ADAMTS1* gene

primers	Primer sequence (5'-3')	Binding region	Tm (°C)	Size (bp)
Pair 1	GCAGCGTCTCCTCTTTAGTGACTCA	5'-UTR	55	503
	CCTACAGTCTGAAGCGTGAAACCG	Exon 1		
Pair 2	AGGCGAAATGGGGAACACG	Exon 1	55	1,070
	GACCAACGAACACACGAACAGT	Intron 1		
Pair 3	ATGTGCGGGGTCGTGGACG	Exon 1	62	2,061
	TCGCTGGGCGGGTTGTGCT	Exon 2		
Pair 4	GTGTCCAGTCACCGCTATG	Exon 2	61	993
	CATCAGCCATCCCAAGAG	Exon 3		
Pair 5	GACCGGGATGCAGAGCAC	Exon 2	63	1,120
	TGGCATGTTAAACACGTGGC	Exon 4		
Pair 6	AGCTGCTCCGTCATAGAAGATG	Exon 3	56	1,132
	TGCCGGTTGGCATCGTAC	Exon 5		
Pair 7	GGGGAATGTTTGATGGACAAG	Exon 5	58	892
	TATTTGGGACTGGGTTGTCAC	Exon 6		
Pair 8	TGGGGAGACTGTTCGAGAAC	Exon 6	59	413
	CTGCAAAACGAAGAAGTAGCC	Exon 7		
Pair 9	CACCAAAGGACAGGTGCAAG	Exon 7	63	1,114
	TTGGAGTCTATGATGCGATCAC	Exon 8		
Pair 10	TAGCCCAGATTCCACCTC	Exon 8	59	703
	CCCACTCTTCAATGACCC	Exon 9		
Pair 11	AGAAGGAATCTTTCAATGC	Exon 9	56	375
	CCACTTAACTGCATTCTGCC	Exon 9 and 3'-UTR		
Pair 12	GCTGCGATCCTCTAAAGAAACCT	Exon 9	60	994
	CAAGTGACTTCAATGTCCCCAAAC	3'-UTR		

genotyping, from which were chosen 24 Meishan pigs, 21 Large White pigs and 20 Landrace pigs from the genetic nucleus station of Huazhong Agricultural University, 74 Qingping and 41 Tongcheng pigs from Hubei province, and 53 Jinhua pigs from Zhejiang province.

The new Qingping female line pigs that was established by mating five Duroc boars to twenty-five Qingping sows were selected from Hubei province in China. 116 new Qingping female line pigs, which were 1 to 2 years old, were selected for association analysis. A total of 247 litter records included total number born (TNB) and the recorded number of piglets born alive (NBA) averaged from 1 to 4 parities.

DNA preparation

Genomic DNA was isolated from white blood cells as described by Xiong (1999). After isolation, the DNA pellet was dissolved in TE buffer and stored at -20°C.

Primer design, polymerase chain reaction (PCR) and sequencing

The sequences of cDNAs of human and mouse *ADAMTS1* genes (GenBank accession numbers: NM_006988 and NM_009621, respectively) were used to search against the porcine EST databases by BLAST (http://www.ncbi.nlm.nih.gov/blast/). Six porcine ESTs (GenBank accession numbers: BP440774, AJ683364, DN127702, BP458319, CJ018555 and BX919340) sharing >80% identity to the corresponding human and mouse

cDNAs were obtained and assembled into a contig. Based on the contig sequence, a set of primers was designed to amplify the DNA sequence of porcine *ADAMTS1* gene (Table 1). The PCR was performed in 25 μl reaction volume containing: 1×PCR buffer, 1.5 mM MgCl$_2$, 250 μmol/L dNTP, 5 ppm of each PCR primer, 2 units *Taq* DNA polymerase (Biostar International, Canada) and 200 ng genomic DNA as template. PCR was run in the GeneAmp PCR system 9600 (Perkin-Elmer Co., USA). The conditions for PCR were as follows: initial denaturation at 94°C for 4 min, 35 cycles of 94°C for 40 sec, annealing temperature for 40 sec, 72°C for 1 min and a final extension for 10 min at 72°C. The purified PCR products were cloned into the pGEM-T vector (Promega, USA) and sequenced. These primers yielded twelve overlapping PCR products that produced a consensus of 9,026-bp DNA sequence of pig *ADAMTS1* gene (Genbank accession number DQ177331).

Chromosomal localization of *ADAMTS1*

The INRA-University of Minnesota porcine radiation hybrid (IMpRH) panel consisting of 118 hybrid clones (Yerle et al., 1998) was employed for localization of *ADAMTS1* to the pig chromosome. Primer pair 10 (Table 1) yielded a 703-bp product designed for mapping. The conditions for PCR were as follows: initial denaturation at 94°C for 4 min, 35 cycles of 94°C for 40 sec, 59°C for 40 sec, 72°C for 1 min and a final extension for 10 min at 72°C. The PCR fragment was sequenced to verify the correctness

Table 2. Exon/intron boundaries of porcine *ADAMTS1* gene

Exon	Coding region size (bp)	5' splice donor	Intron	Intron size (bp)	3' splice donor
1	670	AACAG/**gt**acg	1	1,873	tac**ag**/GAACT
2	347	GACAG/**gt**gag	2	627	ctt**ag**/GACCT
3	133	ATTAG/**gt**aag	3	394	ctt**ag**/GCCAT
4	168	TCACG/**gt**aag	4	427	tgc**ag**/GAGAA
5	287	TTGAT/**gt**gag	5	492	ttt**ag**/ACTCC
6	187	TAATG/**gt**gag	6	92	tcc**ag**/GAAAA
7	176	CCAAG/**gt**agt	7	949	ttc**ag**/GTGGT
8	176	GCAAA/**gt**aag	8	161	tac**ag**/ACCTG
9	700				

Nucleotide positions are numbered according to the first base of each gene as it appears in GenBank.
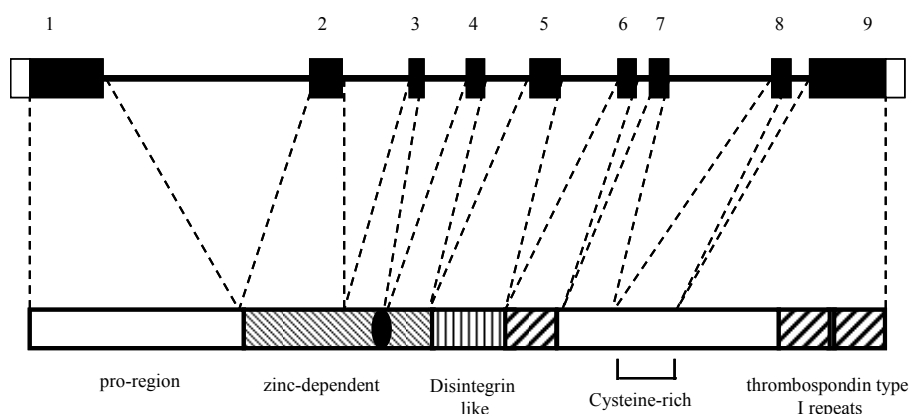
Figure 1. Organization of the porcine ADAMTS1 gene.

of the sequence. PCR products were separated on a 2% agarose gel stained with 0.5 μg/ml ethidium bromide. The radiation hybrid PCR was analyzed with the IMpRH mapping tool (Milan et al., 2000) available from http://imprh.toulouse.inra.fr/.

Detection of PCR-*Pvu*II-RFLP

Comparison of the sequences of *ADAMTS1* in different pig breeds by using BLAST (http://www.ncbi.nlm.nih.gov) revealed one SNP within exon 7 that spanned a *Pvu*II restriction site and comprised a *G-C* substitution at position 6006 which changed the codon for arginine into proline. For the PCR-RFLP assays, 8.5 μl of PCR products were digested with 5 units *Pvu*II (TaKaRa, Tokyo, Japan) in 1×digestion buffer added in a total volume of 10 μl which, following digestion for 4 h at 37°C, was electrophoresed on a 1.5% agarose gel and visualized under UV light.

Statistical analysis

A total of 247 litter records from 116 sows were used for association analyses between different genotypes and litter sizes. TNB and NBA were analyzed with a model including fixed effects of month of farrowing, *ADAMTS1* genotype, random effects of animal, parity effect and residual (Lukovic et al., 2007). The association between genotypes and recorded traits was analyzed using the general linear model (GLM) procedure (SAS Institute Inc.,

Cary, NC, USA).

RESULTS

**Cloning, sequencing and genomic organization of *ADAMTS1***

A 9,026-bp DNA containing the full coding region, all 8 introns and part of the 5'and 3' untranslated region of the porcine *ADAMTS-1* gene was obtained (Genbank accession number DQ177331).

By comparing with human *ADAMTS1* sequence (NT_011512), the exon/intron boundaries of porcine *ADAMTS1* gene have been determined. The sequences of exon/intron boundaries are consistent with the reported consensus sequences for splice donors (GT) and acceptors (AG) (Breathnach and Chambon, 1981) as shown in Table 2. The open reading frame of *ADAMTS1* cDNA covered 2,844 bp and encoded 947 amino acids with a calculated molecular mass of 103 kDa. The coding region of porcine *ADAMTS1* as determined by alignments shared 85% and 81% identity with human and mouse cDNAs, respectively, while the 947 deduced amino acids showed 85% sequence similarity both to the human and mouse proteins, respectively.

The hydropathy plot and the TMAP prediction derived from the World-Wide Web service showed that porcine *ADAMTS1* protein included no transmembrane region and

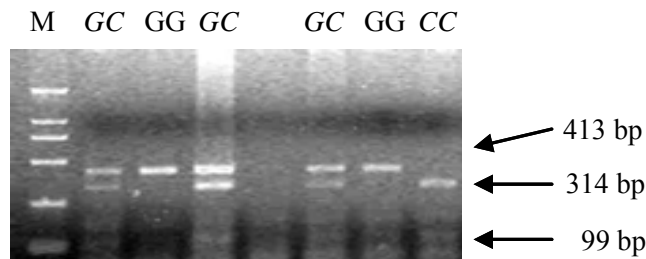**Table 3.** Allele frequencies of the *ADAMTS1* C6006G SNP in ten pig breeds/lines

Breed	Number	Genotype distribution			Allele frequency	
		GG	*GC*	*CC*	*G*	*C*
Large White	21	5	14	2	0.571	0.429
Landrace	20	1	5	13	0.184	0.816
Meishan	24	1	9	14	0.229	0.771
Tongcheng	41	0	0	41	0	1
Qingping	74	0	7	67	0.047	0.953
Jinhua	53	0	10	43	0.094	0.906
New Qingping	116	22	67	27	0.483	0.517
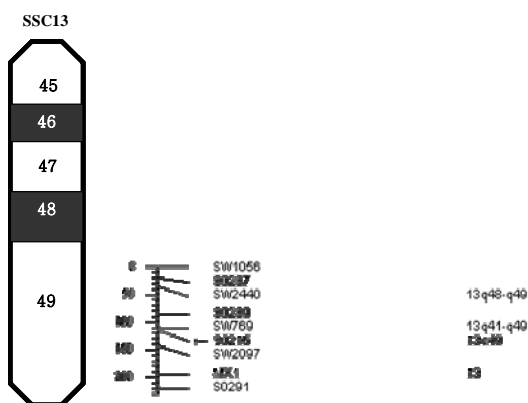
Figure 2. Mapping result of *ADAMTS1*. Pig *ADAMTS1* gene is closely linked with microsatellite marker *S0215* on SSC13q49.

Figure 3. Agarose gel electrophoresis (1.5%) showing the *Pvu*II-RFLP results of *ADAMTS1* gene. The genotypes are indicated on the top of the lanes (M: DNA molecular marker DL2000).

there were many cysteine residues and four putative *N*-glycosylation sites in the protein which indicated that the *ADAMTS1* gene product was a putative cysteine-rich secretory glycoprotein. Sequence analyses of porcine *ADAMTS1* protein by ExPASy (http://au.expasy.org/) revealed that the porcine *ADAMTS1* protein consisted of one disintegrinlike domain, one zinc-dependent metalloproteinase and three thrombospondin type I repeats (TSP1) (Figure 1). All these structural domains have very high similarity (>90%) among mammalian species by ClustalW website (http://www.ebi.ac.uk/clustalw/index.html).

Chromosomal mapping of pig *ADAMTS1*

Using the ImpRH panel, we determined that pig *ADAMTS-1* was closely linked with microsatellite marker S0215 on SSC13q49 (distance of 76cR, LOD = 4.18, retention frequency = 18%) (Figure 2). The *ADAMTS-1* gene has been mapped to chromosome 21q21-q22 in the human (Hurskainen et al., 1999). Our results are in

agreement with comparative mapping data, as human chromosome 21q21-q22 is a syntenic region of porcine chromosome 13q49.

Frequencies of allele and genotype of different pig breeds

The digested products (pair 8) showed allelic fragments of 413 bp (allele *A*) or 314+99 bp (allele *B*) (Figure 3). The allele frequency analysis included 349 unrelated animals from seven breeds/lines (Table 3).

Association analysis

Results of tests for *ADAMTS1* genotypes and litter size traits on the new Qingping female line are given in Table 4. Pigs with *GG* genotype had less TBA and NBA than pigs with *CC* genotype (p<0.05) and *GC* genotype (p<0.01), respectively. The results indicated that *GC* sows showed the highest and *GG* sows showed the lowest litter size values in multiple parities.

DISCUSSION

In this study, the candidate gene approach was used to

Table 4. Effect of the *ADAMTS1* genotypes on reproductive traits in new Qingping female line pigs

Genotype	Trait	Least square means±standardized error		
		GG	*GC*	*CC*
N		22	67	27
Multiple parities	TNB	9.956±0.451[aA]	11.406±0.201[B]	11.267±0.388[b]
	NBA	9.637±0.455[aA]	10.639±0.197[B]	10.040±0.393[b]

N : Total number of pigs observed. Letter denoting significant difference between groups: [a, b], p<0.05; [A, B] p<0.01.

demonstrate associations between specific genes and litter size. Using this approach, polymorphisms in some defined genes such as the estrogen receptor (Li et al., 2004), follicle-stimulating hormone-β (Li et al., 2002) and Oviduct-specific Glycoprotein 1 (Niu et al., 2006) have all been reported to be associated with litter size in swine. So the candidate gene approach is a very efficacious method to find molecular markers.

ADAMTS1 plays an important role in follicles via progesterone receptor (PR)-dependent pathways which control ovulation (Robker et al., 2000). However, there have been no reports of the sequence, mapping and association study of *ADAMTS1* gene in pigs. In our study *ADAMTS1* gene was selected as a candidate gene to be associated with litter size in swine.

The genetic effect on reproductive traits was investigated in the new Qingping female line sows. Based on the data representing litter records from new Qingping female line sows, the NBA and TNB in multiple parities were higher for *GC* sows than for the sows of the other two genotypes. In view of these observations, it is tempting to suggest that *GC* sows have better performance than *GG* sows for litter size traits in Qingping pigs.

Nucleotide mutation can alter gene function, especially changing the coding region of the protein. In the experiments reported here, a polymorphism analyzed in *ADAMTS1* gene was found in exon 7 which changes a codon for arginine into a codon for proline.

In this study, we determined that pig *ADAMTS1* is closely linkage with microsatellite marker S0215 on SSC13q49. So far, two quantitative trait loci (QTLs) have been reported which contributed to the reproductive traits on Pig Chromosome 13. One of these contributed to ovulation rate (Rathje et al., 1997); the other contributed to number of stillborn (Cassady et al., 2001). Although *ADAMTS1* gene was not located on either of these two QTLs, it is possible that *ADAMTS1* is a genetic marker linked with other QTLs which contribute to the reproductive traits.

This is the initial step to consider *ADAMTS1* gene as a candidate gene for litter size. This polymorphism could be a potential genetic marker for litter size. The estimations presented are based on data from the new Qingping female line; however, in this study the populations are too small and the number of animals examined is limited. So, analyzing more animals is necessary to confirm the association between the *ADAMTS1* genotype and reproductive traits in other purebreds and crossbreds.

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