Original Article

Molecular Cloning of the Porcine *HTRA3* Gene and Association of a SNP with Litter Size Traits

(pig / HTRA3 / polymorphism / reproductive trait)

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Abstract. HtrA serine peptidase 3 (*HTRA3*) is an important reproduction-related gene. In this study, we cloned the full-length coding sequence of the porcine *HTRA3* gene using RT-PCR. Sequence analysis of this gene revealed that the pig *HTRA3* gene encodes a protein of 453 amino acids, which has high homology with the HTRA3 protein of five species: water buffalo (96 %), killer whale (96 %), sperm whale (96 %), Yangtze River dolphin (96 %) and small-eared galago (93 %). Phylogenetic analysis revealed that the pig *HTRA3* gene has a closer genetic relationship with the *HTRA3* gene of cattle. PCR-*Hpa*II-RFLP was established to detect the GU373693:c.1140 G>A substitution of the porcine *HTRA3* gene coding sequence, and eight pig breeds displayed obvious genotype and allele frequency differences at this mutation locus. Association of this SNP with litter size traits was assessed in Large White (N = 200) and Landrace (N = 200) pig populations, and the results demonstrated that this polymorphic locus was significantly associated with the litter size of all parities in Large White and Landrace sows (P < 0.05). Therefore, the *HTRA3* gene could be a useful candidate for increasing the litter size in pigs. These data serve as a foundation for further insight into this novel porcine gene.

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Abbreviations: GLM – general linear model, HTRA – high-temperature requirement factor A, MAS – marker-assisted selection, Mw – molecular weight, NBA – number of piglets born alive, TNB – total number of piglets born.

Introduction

HtrA serine peptidase 3 (HTRA3) is a member of serine peptidases of the mammalian HTRA (high-temperature requirement factor A) family. *HTRA3* is an important reproduction-related gene. In mammals, *HTRA3* is upregulated dramatically during mouse placental development. In humans, upregulation of *HTRA3* expression in association with placental development was revealed by a significant elevation of this protein in the maternal serum during the first trimester (Nie et al., 2006). Singh et al. (2010) reported that inhibition of HTRA3 stimulates trophoblast invasion during human placental development. HTRA3 is now known to regulate ovarian development, granulosa cell differentiation and luteinization (Bowden et al., 2008, 2009; Dynon et al., 2012). Studies in mice have also suggested that HtrA3 inhibits TGF-β signalling during embryo development (Tocharus et al., 2004; Dynon et al., 2012).

As mentioned above, the *HTRA3* gene is an important gene fulfilling many biological functions. Until today, the *HTRA3* gene has been reported in the human, mouse, cattle and other animals. The aim of this work was to clone the full-length porcine *HTRA3* coding sequence, search for polymorphisms within this gene, and perform association analyses between a gene-tagged SNP and litter size traits in the Large White and Landrace pig breeds.

Material and Methods

Animals and sample preparation

Six adult Large White pigs were slaughtered. Large intestine, spleen, lung, muscle, fat, liver, heart, kidney and ovary samples were collected, frozen in liquid nitrogen, and then stored at –80 °C. Total RNA was extracted using the Total RNA Extraction Kit (Gibco, Gaithersburg,

Table 1. Information on 795 unrelated pigs from eight populations

Breed	Sampling location	Sample size		
		Total	Male	Female
Large White pig	Hubei Province	200	0	200
Landrace pig	Hubei Province	200	0	200
Saba pig	Dongchuan county of Yunnan Province	100	50	50
Zang pig	Xianggelila county of Yunnan Province	95	50	45
Mingguang small-ear pig	Tengchong county of Yunnan Province	100	50	50
Diannan small-ear pig	Banna state of Yunnan Province	100	50	50
Wujin pig	Qujing city of Yunnan Province	100	50	50
Baoshan pig	Baoshan city of Yunnan Province	100	50	50

MD). These RNA samples were used to perform the real-time polymerase chain reaction (RT-PCR).

Ear samples were collected from 995 unrelated animals belonging to eight swine populations presented in Table 1. Genomic DNA isolated from these ear samples was to be used to perform the polymorphism analyses.

Both the total number of piglets born (TNB) and the number of piglets born alive (NBA) of 200 Large White sows and 200 Landrace sows listed in Table 1 were recorded for 700 litters. The litter size traits data and the genomic DNA of these pigs were to be used to perform association analyses.

Isolation of the coding sequences for the porcine HTRA3 gene

RT-PCR was performed to isolate the coding sequence for the porcine *HTRA3* gene using the cDNAs from different tissues mentioned above. The 25 μl reaction system included: 2.0 μl cDNA (100 ng/μl), 2.5 μl 2 mM mixed dNTPs, 2.5 μl 10×Taq DNA polymerase buffer, 2.5 μl 25 mM MgCl2, 2.0 μl 10 μM forward primer1, 2.0 μl 10 μM reverse primer1, 2.0 units of Taq DNA polymerase (1 U/1 μl), and 9.5 μl sterile water. The primers for the porcine *HTRA3* gene isolation were designed based on the conserved coding sequence information from human and mouse *HTRA3* genes and their highly homologous pig EST sequences: FS689332 and CJ026294. PCR primers were: 5'- ATG CAG GCA CGG GCG CTG-3' (forward primer1), 5'- TCA CGG GAC CAC CTC GGG-3' (reverse primer1). The PCR programme initially started with 94 °C denaturation for 4 min, followed by 35 cycles of 94 °C/50 s, 64 °C/50 s, 72°C/50 s, then 72 °C extension for 10 min, finally 4 °C to terminate the reaction.

The PCR products were then cloned into pMD18-T vector (TaKaRa, Dalian, China) and sequenced bidirectionally with the commercial fluorometric method (SHENGGONG, Shanghai, China). At least five independent clones were sequenced for each PCR product.

Sequence analysis

Gene analysis for the cDNA sequence was conducted using GenScan software (http://genes.mit.edu/GENSCAN.html). Protein prediction and analysis were performed using the Conserved Domain Architecture Retrieval Tool of BLAST at the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/BLAST) and the ClustalW software (http://align.genome.jp/). The theoretical isoelectric point (pI) and molecular weight (Mw) of the proteins was computed using the Compute pI/Mw Tool (http://www.expasy.org/tools/pitool.html).

Polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP)

DNA from the above-mentioned pigs (Table 1) was used as a template to perform PCR with primers: 5'-CTA CCT CTG AGA AGG TGA-3' (forward primer2), 5'- GAC ACA GAC AAG AAC CAG -3' (reverse primer2). The PCR product size was 273 bp. The 25 μl reaction system included: 2.0 μl DNA (100 ng), 2.5 μl 2 mM mixed dNTPs, 2.5 μl 10× Taq DNA polymerase buffer, 2.5 μl 25 mM $MgCl_2$, 1.0 μl 20 μM forward primer, 1.0 μl 20 μM reverse primer, 1.0 units of Taq DNA polymerase (1 U/1 μl) (Sangon Biotech, Shanghai, China), and 12.5 μl sterile water. PCR was run as follows: 94°C for 4 min, followed by 35 cycles of 94 °C for 50 s, 55 °C for 50 s, 72 °C for 1 min, then 72 °C extension for 10 min, finally 4 °C to terminate the reaction. The 31 μl PCR-RFLP reaction volume included: 10 μl PCR product, 18 μl sterile water, 1 μl *Hpa*II (10 U), 2 μl 10 × buffer. The mixture was incubated in an air incubator at 37 °C for 4 h, and then the genotypes were analysed in agarose gel (2.5%) containing ethidium bromide.

Statistical analysis

The relationships between the *HTRA3* genotypes and the litter size traits of Large White (N = 200) and Landrace (N = 200) sows were evaluated with the general linear model (GLM) procedure of SAS version 8.0. Both additive and dominance effects were also estimated using the REG procedure, where the additive effect was estimated as –1, 0 and 1 for the AA, GA and GG genotype, respectively; and the dominance effect represented as 1, –1 and 1 for the AA, GA and GG genotype, respectively (Zhang et al., 2009). The model is repre-

sented by: $Yijkl = \mu + Pi + Sj + Fk + Gl + eijkl$, where *Yijkl* is the observation of the trait, μ is the least square means, *Pi* is the effect of i^{th} parity (i = 1, 2, 3, 4, 5, 6, 7 (parity ≥ 7)), *Sj* is the effect of j^{th} season, *Fk* is the effect of k^{th} farm (k = 1, 2), *Gl* is the effect of l^{th} genotype (l = 1–3) and *eijkl* is the random residual (Niu et al., 2009).

Results

Isolation of the coding sequences for the porcine HTRA3 gene

For the pig *HTRA3* gene, one PCR product of 1362 bp was obtained using RT-PCR (Fig. 1).

Sequence analysis

The cDNA nucleotide sequence analysis using the BLAST software revealed that this gene was not homologous to any of the known pig genes, and it was then deposited into the GenBank database (accession number: GU373693). The sequence prediction was carried out using the GenScan software and the results showed that the 1362-bp cDNA sequence represented one single gene encoding 453 amino acids. The theoretical isoelectric point (pI) and molecular weight (Mw) of the deduced proteins of this pig gene were computed using the Compute pI/Mw Tool. The pI of pig HTRA3 is 7.85. The molecular weight of this putative protein is 48441.55.

BLAST analysis revealed that the pig HTRA3 protein has high homology with the HtrA serine peptidase 3 (HTRA3) of nine species: water buffalo (accession number: XP_006075913; 96 %), killer whale (accession number: XP_004265202; 96 %), sperm whale (accession number: XP_007109316; 96 %), Yangtze River dolphin (accession number: XP_007456927; 96 %) and small-eared galago (accession number: XP_003799192; 93 %) (Fig. 2).

Based on the results of the alignment of HTRA3 proteins, phylogenetic trees were constructed using the dendrogram procedure of ClustalW software (http://align.genome.jp/), as shown in Fig. 3.

The phylogenetic analysis revealed that the pig *HTRA3* gene has a closer genetic relationship with the *HTRA3* gene of cattle.

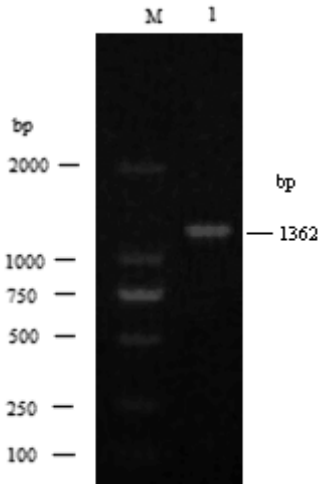

Fig. 1. RT-PCR result for the pig *HTRA3* gene. M, DL2000 DNA markers; 1, PCR product for the pig *HTRA3* gene

Fig. 2. Alignment of the protein encoded by the pig *HTRA3* gene and five other types of HTRA3 proteins

Fig. 3. Phylogenetic analysis of the *HTRA3* gene

Polymorphism

Based on the sequence of the pig *HTRA3* gene, primers (forward primer2 and reverse primer2) were used to amplify the DNA of Large white and Landrace pigs, and the products were then cloned into PMD18-T vector and sequenced bidirectionally with the commercial fluorometric method. One G-A mutation (GU373693:c.1140 G>A) was found at the position of 1140 bp of the coding region. This led to mutation of one *Hpa*II restriction site. This was confirmed by PCR-*Hpa*II-RFLP (Fig. 4).

Subsequently, PCR-*Pst*I-RFLP were performed using the DNA from 995 unrelated animals belonging to eight pig populations including Large White pig, Landrace pig, Saba pig, Zang pig, Mingguang small-ear pig, Diannan small-ear pig, Wujin pig and Baoshan pig (Table 2). The results revealed that the frequency of A allele in the two exotic pig breeds: Large White pig (0.638) and Landrace pig (0.665) was higher than that in the other six Yunnan local pig breeds: Saba pig (0.380), Zang pig (0.495), Mingguang small-ear pig (0.485), Diannan small-ear pig (0.480), Wujin pig (0.490) and Baoshan pig (0.520). The two exotic pig breeds, Large White pig and Landrace pig, had less animals of genotype GG, but more animals of genotype AA. This indicated that the Yunnan local pig breeds and exotic pig breeds displayed obvious genotype and allele frequency differences at this G-A mutation locus.

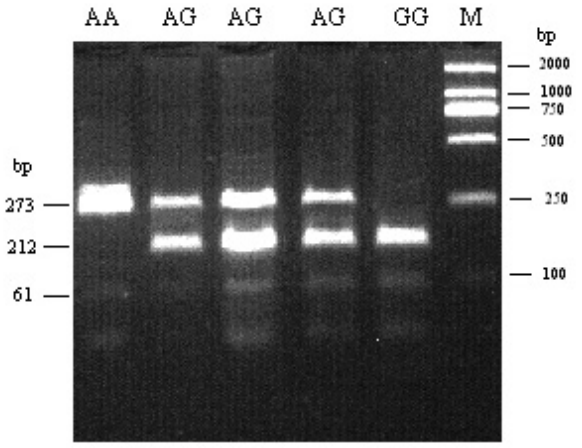

Fig. 4. Polymorphism analysis of the pig *HTRA3* gene by PCR-*Hpa*II-RFLP. AA: 273 bp; AG: 273 bp + 212 bp + 61 bp; GG: 212 bp + 61 bp

Association of the SNP and litter size was assessed in two populations (purebred Large White and purebred Landrace sows). Statistical analysis demonstrated that, for the first parity, no significant difference was found between animals of the three genotypes in the experimental purebred Large White sows and in the experimental purebred Landrace sows.

For all parities, in the purebred Large White sows, those with the AA genotype had an additional 0.526 TNB ($P < 0.05$) and an additional 0.495 NBA ($P < 0.05$) compared to the GG animals. In addition, for all parities, in the purebred Landrace sows, AA animals had an additional 0.520 TNB ($P < 0.05$) and an additional 0.640 NBA ($P < 0.05$) compared to the GG animals (Table 3).

Discussion

With the development of modern bioinformatics and establishment of a specific pig NCBI EST database along with various convenient analysis tools, it is much easier for researchers to find the useful ESTs that are highly homologous to the coding sequence of human genes. Based on these swine EST sequences, we can obtain the complete coding sequences of some novel pig genes using modern experimental methods such as the RT-PCR method. The cloning and sequence analysis of the pig *HTRA3* gene confirmed the effectiveness of this method in isolating novel pig genes.

In the current study, we were the first to obtain the complete coding sequence of the porcine *HTRA3* gene. It has been reported that HtrA3 inhibits TGF-β signalling during the embryo development (Tocharus et al., 2004; Dynon et al., 2012). Our work will thus provide the molecular basis for association analysis of the DNA polymorphisms of this gene with the pig litter size traits. Based on the association analysis, we were able to found that the polymorphism (EU650788:c.291 G>A) of the

Table 2. Allele frequency and genotype of the HpaII polymorphic locus in different pig breeds

Breed	Number of pigs	Genotype			Allele frequency	
		GG	AG	AA	G	A
Large White pig	200	23	99	78	0.362	0.638
Landrace pig	200	22	90	88	0.335	0.665
Saba pig	100	30	44	16	0.620	0.380
Zang pig	95	19	58	18	0.505	0.495
Mingguang small-ear pig	100	18	67	15	0.515	0.485
Diannan small-ear pig	100	22	60	18	0.520	0.480
Wujin pig	100	18	66	16	0.510	0.490
Baoshan pig	100	20	56	24	0.480	0.520

Table 3. Association between pig HTRA3 gene PCR-HpaII-RFLP genotypes and litter size

Breed	Traits		Genotype (mean ± S.E.)			Genetic effects (mean ± S.E.)	
			GG	AG	AA	Additive	Dominant
Large White	1st parity	N	23	99	78		
		TNB	10.319 ± 0.302	10.320 ± 0.315	10.373 ± 0.351	0.027 ± 0.012	–0.026 ± 0.020
		NBA	9.336 ± 0.244	9.254 ± 0.235	9.317 ± 0.320	–0.009 ± 0.004	–0.072 ± 0.022
	All parities	N	23	99	78		
		TNB	11.524 ± 0.410[a]	11.733 ± 0.312[ab]	12.050 ± 0.343[b]	0.263 ± 0.025	–0.054 ± 0.029
		NBA	10.510 ± 0.342[a]	10.700 ± 0.228[ab]	11.005 ± 0.306[b]	0.248 ± 0.018	–0.057 ± 0.034
Landrace	1st parity	N	22	90	88		
		TNB	10.356 ± 0.308	10.300 ± 0.344	10.413 ± 0.328	0.029 ± 0.021	–0.084 ± 0.021
		NBA	9.351 ± 0.317	9.322 ± 0.226	9.375 ± 0.311	0.012 ± 0.002	–0.041 ± 0.008
	All parities	N	22	90	88		
		TNB	11.580 ± 0.340[a]	11.735 ± 0.355[ab]	12.100 ± 0.318[b]	0.260 ± 0.111	–0.105 ± 0.103
		NBA	10.495 ± 0.222[a]	10.735 ± 0.284[ab]	11.135 ± 0.246[b]	0.320 ± 0.153	–0.080 ± 0.012

N – number of investigated litters. Least square mean values are significantly different with: [a] $P < 0.05$, [b] $P < 0.01$.

porcine *HTRA3* gene can significantly affect the litter size. The AA genotype animals obviously had better litter size of all parities than the GA and GG animals both in purebred Large White and purebred Landrace sows. This indicates that this polymorphic locus of the porcine *HTRA3* gene is a valuable marker deserving to be applied to marker-assisted selection (MAS) in the pig breeding. Therefore, the *HTRA3* gene could be a useful candidate in selection for increasing the litter size in pigs. Pig industry can select and retain more AA animals to improve the reproductive performance of sows in the pig production.

In conclusion, we were the first to isolate the pig *HTRA3* gene and perform the necessary sequence analysis, polymorphic analysis and association analysis. This established the primary foundation for further insight into this novel pig gene.

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