Molecular cloning, polymorphism and association of porcine WARS2 gene with litter size

Yang Minghua, Zhang Huiling, Zhang Wenyang, Liu Chong, Yang Demin, Zhao Guiying and Liu Yonggang

Yunnan Key Laboratory of Fertility Regulation and Minority Eugenics, Yunnan, Kunming, China; College of Animal Sciences, Yunnan Agricultural University, Yunnan, Kunming, China

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Introduction

Aminoacyl-tRNA synthetase catalyses the aminoacylation of tRNA by their cognate amino acid. There are two forms of tryptophanyl-tRNA synthetase. The cytoplasmic form is named tryptophanyl-tRNA synthetase (WARS) and the mitochondrial form is named mitochondrial tryptophanyl-tRNA synthetase 2 (WARS2). These aminoacyl-tRNA synthetases play a central role in linking amino acids with nucleotide triplets, contained in tRNAs, and are thought to be among the first proteins that appeared in evolution. [1] Recent research works showed that WARS2 is an important gene associated with human fat deposition and obesity. Heid et al. [2] identified that the WARS2 gene was a new locus, which can influence the human waist-hip ratio and body fat distribution. Liu et al. [3,4] studied the body fat distribution in African ancestry populations by genome-wide association analysis and suggested that the WARS2 gene was significantly associated with human fat deposition and obesity. Through fat depot-specific mRNA expression analysis, Schleinitz et al. [5] obtained similar results. WARS2 is also an important reproduction-related gene. Mote et al. [6] indicated that WARS2 is correlated to the sow productive life. Fan et al. [7] reported that WARS2 is significantly associated with both total number of piglets born (TNB) and number of piglets born alive (NBA) of first parity and later parities.

As mentioned above, WARS2 is an important gene, which has many biological functions. The aim of this work was to clone the full-length coding sequence of the porcine WARS2 gene, search for polymorphisms within this gene and perform association analysis between a gene-tagged single nucleotide polymorphism (SNP) and litter size in Large White and Landrace sows.

Materials and methods

Animals and sample preparation

Six adult Large White pigs were killed. Large intestine, spleen, lung, muscle, fat, liver, heart, kidney and ovary samples were collected, frozen in liquid nitrogen, and stored at −80 °C. The total RNA was extracted by using the Total RNA Extraction Kit (Gibco, USA) described by Liu and Xia.[8] These RNA samples were used to perform a reverse transcription-polymerase chain reaction (RT-PCR).

Ear samples were collected from 995 unrelated animals belonging to 8 swine populations presented in Table 1. Genomic DNA isolated from these ear samples by DNA Extraction Kit (SHENGGONG, Shanghai, China) would be used to perform the polymorphism analysis. The 200 purebred Large White sows, shown in Table 1 were all raised in one farm under the same normal feeding conditions. They were all mated with
purebred Large White boars by an artificial fertilization. The 200 purebred Landrace sows, shown in Table 1, were all raised in another farm under the same normal feeding conditions. They were all mated with purebred Landrace boars by an artificial insemination. Both the TNB and the NBA of these Large White sows and Landrace sows were recorded and every sow had a litter size record more than seven parities. The sows and Landrace sows were recorded and every sow had a litter size record more than seven parities. The litter size and genomic DNA of these sows would be used to perform the association analysis.

Isolation of the coding sequences for the porcine \( WARS2 \) gene

RT-PCR was performed to isolate the coding sequence for the porcine \( WARS2 \) gene by using the cDNAs from the different tissues described above. The 25 \( \mu \)L reaction system contained 2.0 \( \mu \)L cDNA (100 ng/\( \mu \)L), 2.5 \( \mu \)L 2 mmol/L mixed dNTPs, 2.5 \( \mu \)L 10 \( \times \) Taq DNA Polymerase buffer (SHENGGONG, Shanghai, China), 2.5 \( \mu \)L 25 mmol/L MgCl\(_2\), 2.0 \( \mu \)L 10 mmol/L forward primer 1, 2.0 \( \mu \)L 10 mmol/L reverse primer 1, 2.0 \( U \) of Taq DNA Polymerase (1 U/\( \mu \)L) and 9.5 \( \mu \)L sterile water. The primers for the porcine \( WARS2 \) gene isolation were designed based on the conserved coding sequences information from human and mouse \( WARS2 \) genes and their highly homologous pig expressed sequence tag (EST) sequences: FS687571 and BE032998. The PCR primers were 5’-ATG GCC GTG CAC TCA ATG-3’ (forward primer 1), 5’-CTA TAG AAA CCC CAC AAA T-3’ (reverse primer 1). The PCR program initially started with a 94 °C denaturation for 4 min, followed by 35 cycles of 94 °C/50 s, 57 °C/50 s, 72 °C/50 s, extension at 72 °C for 10 min and a final termination of the reaction at 4 °C. 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

cDNA sequence analysis was conducted by using GenScan software (http://genes.mit.edu/GENSCAN.html). Protein analysis was performed by using basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/BLAST) and the ClustalW software (http://www.genome.jp/tools/clustalw/). The theoretical isoelectric point (pl) and molecular weight (Mw) of proteins were computed by the Compute pl/Mw tool (http://www.expasy.org/tools/pi_tool.html).[8] Phylogenetic tree was constructed by using the Dendrogram procedure of ClustalW software (http://www.genome.jp/tools/clustalw/) as described by Liu and Gao.[9]

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

DNA samples from the above-mentioned pigs (Table 1) were used as template to perform PCR with primers 5’-AGA AAG ATG CCG TGA AGC-3’ (forward primer 2) and 5’-GTC ACC CTT GAA CAG TTG-3’ (reverse primer 2). The 25 \( \mu \)L reaction mixture contained 2.0 \( \mu \)L DNA (100 ng), 2.5 \( \mu \)L 2 mmol/L mixed dNTPs, 2.5 \( \mu \)L 10 \( \times \) Taq DNA polymerase buffer (SHENGGONG, Shanghai, China), 2.5 \( \mu \)L 25 mmol/L MgCl\(_2\), 1.0 \( \mu \)L 20 mmol/L forward primer, 1.0 \( \mu \)L 20 mmol/L reverse primer, 1.0 \( U \) of Taq DNA Polymerase (1 U / 1 \( \mu \)L) (SHENGGONG, Shanghai, China) and 12.5 \( \mu \)L sterile water. The PCR was run as follows: 94 °C for 4 min, followed by 35 cycles of 94 °C/50 s, 55 °C for 50 s, 72 °C for 1 min, extension at 72 °C for 10 min and a final termination of the reaction at 4 °C. The PCR products were then digested with restriction enzymes (PstI, EcoRI) and separated by electrophoresis on 2.5% agarose gel. The digested DNA fragments were visualized under UV light.

### Table 1. Information of 995 pigs from 8 populations.

| Breed                  | Sampling location                  | Total | Boar | Sow  |
|------------------------|-----------------------------------|-------|------|------|
| 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   |
| Tibetan pig            | Xianggelia 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   |
Statistical analysis

The frequencies of the alleles was calculated as described by Liu and Xia.[8] The association between WARS2 genotypes and the litter size of Large White (n = 200) and Landrace (n = 200) sows were evaluated with the general linear model procedure of SAS version 8.0. Both additive and dominance effects were also estimated by using an REG procedure, where the additive effect was estimated as \(-1, 0, 1\) for AA, GA and GG genotype, respectively and the dominance effect, represented as \(-1, 1\) and \(1\) for AA, GA and GG genotype, respectively.[10] The model,

\[ Yijkl = \mu + Pi + Sj + Fk + Gl + eijkl, \]

where \(Yijkl\) is the observation of the trait, \(\mu\) is the least square means, \(Pi\) is the effect of \(i\)th parity (\(i = 1, 2, 3, 4, 5, 6, 7\) (parity \(\geq 7\), to all parities, \(Pi\) is 0), \(Sj\) 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.[11]

Results and discussion

Isolation of the coding sequence for the porcine WARS2 gene

The full-length open reading frame of the porcine WARS2 gene was cloned through RT-PCR and the PCR product was 1083 bp in length (Figure 1).

Sequence analysis

The coding sequence of the porcine WARS2 gene was analysed by using the BLAST software in the NCBI database. The results revealed that this gene was a novel pig gene and its coding sequence was then deposited into the GenBank database (accession number: EU650788). This 1083 bp coding sequence of the porcine WARS2 gene encoded 360 amino acids (Figure 2). The pl of the pig WARS2 protein was 9.22 and the Mw was 40,086.58 Da.

BLAST analysis revealed that the pig WARS2 protein had high homology with the WARS2 proteins of nine species: cattle (accession number: NP_001029754; 91%), giant panda (accession number: XP_002928305; 85%), mouse (accession number: NP_081738; 83%), rabbit (accession number: XP_00271573; 89%), rhesus monkey (accession number: XP_001113563; 88%), horse (accession number: NP_001162112; 82%), and others.
| Species          | Sequence                                                                 |
|------------------|--------------------------------------------------------------------------|
| Sumatran orangutan | MALHSMRKAERNSFIRALHKGSAAAPAPQDSDSKRVSFGISQPTGLHNLGNVLGAEISWV           |
| Human            | MALHSMRKAERNSFIRALHKGSAAAPAPQDSDSKRVSFGISQPTGLHNLGNVLGAEISWV           |
| Rhesus monkey    | MALHSMRKAERNSFIRALHKGSAAAPAPQDSDSKRVSFGISQPTGLHNLGNVLGAEISWV           |
| Pig              | MALHSMRKAERNSFIRALHKGSAAAPAPQDSDSKRVSFGISQPTGLHNLGNVLGAEISWV           |
| Cattle           | MALHSMRKAERNSFIRALHKGSAAAPAPQDSDSKRVSFGISQPTGLHNLGNVLGAEISWV           |
| Horse            | MALHSMRKAERNSFIRALHKGSAAAPAPQDSDSKRVSFGISQPTGLHNLGNVLGAEISWV           |
| Rabbit           | MALHSMRKAERNSFIRALHKGSAAAPAPQDSDSKRVSFGISQPTGLHNLGNVLGAEISWV           |
| Giant panda      | MALHSMRKAERNSFIRALHKGSAAAPAPQDSDSKRVSFGISQPTGLHNLGNVLGAEISWV           |
| Mouse            | MALHSMRKAERNSFIRALHKGSAAAPAPQDSDSKRVSFGISQPTGLHNLGNVLGAEISWV           |
| Rat              | MALHSMRKAERNSFIRALHKGSAAAPAPQDSDSKRVSFGISQPTGLHNLGNVLGAEISWV           |
| Sumatran orangutan | RLQDEYSVLYS1VLHSDTHVQPFDFQLRQSLDLTDATAVLCINFEEKSFLQYGLVESE            |
| Human            | RLQDEYSVLYS1VLHSDTHVQPFDFQLRQSLDLTDATAVLCINFEEKSFLQYGLVESE            |
| Rhesus monkey    | RLQDEYSVLYS1VLHSDTHVQPFDFQLRQSLDLTDATAVLCINFEEKSFLQYGLVESE            |
| Pig              | RLQDEYSVLYS1VLHSDTHVQPFDFQLRQSLDLTDATAVLCINFEEKSFLQYGLVESE            |
| Cattle           | RLQDEYSVLYS1VLHSDTHVQPFDFQLRQSLDLTDATAVLCINFEEKSFLQYGLVESE            |
| Horse            | RLQDEYSVLYS1VLHSDTHVQPFDFQLRQSLDLTDATAVLCINFEEKSFLQYGLVESE            |
| Rabbit           | RLQDEYSVLYS1VLHSDTHVQPFDFQLRQSLDLTDATAVLCINFEEKSFLQYGLVESE            |
| Giant panda      | RLQDEYSVLYS1VLHSDTHVQPFDFQLRQSLDLTDATAVLCINFEEKSFLQYGLVESE            |
| Mouse            | RLQDEYSVLYS1VLHSDTHVQPFDFQLRQSLDLTDATAVLCINFEEKSFLQYGLVESE            |
| Rat              | RLQDEYSVLYS1VLHSDTHVQPFDFQLRQSLDLTDATAVLCINFEEKSFLQYGLVESE            |
| Sumatran orangutan | TQLSMILCVRMLRLPLQLHMMKATKTVKhDKTGVLLTTYFQVLAQADILLYKSTHVPE            |
| Human            | TQLSMILCVRMLRLPLQLHMMKATKTVKhDKTGVLLTTYFQVLAQADILLYKSTHVPE            |
| Rhesus monkey    | TQLSMILCVRMLRLPLQLHMMKATKTVKhDKTGVLLTTYFQVLAQADILLYKSTHVPE            |
| Pig              | TQLSMILCVRMLRLPLQLHMMKATKTVKhDKTGVLLTTYFQVLAQADILLYKSTHVPE            |
| Cattle           | TQLSMILCVRMLRLPLQLHMMKATKTVKhDKTGVLLTTYFQVLAQADILLYKSTHVPE            |
| Horse            | TQLSMILCVRMLRLPLQLHMMKATKTVKhDKTGVLLTTYFQVLAQADILLYKSTHVPE            |
| Rabbit           | TQLSMILCVRMLRLPLQLHMMKATKTVKhDKTGVLLTTYFQVLAQADILLYKSTHVPE            |
| Giant panda      | TQLSMILCVRMLRLPLQLHMMKATKTVKhDKTGVLLTTYFQVLAQADILLYKSTHVPE            |
| Mouse            | TQLSMILCVRMLRLPLQLHMMKATKTVKhDKTGVLLTTYFQVLAQADILLYKSTHVPE            |
| Rat              | TQLSMILCVRMLRLPLQLHMMKATKTVKhDKTGVLLTTYFQVLAQADILLYKSTHVPE            |
| Sumatran orangutan | DQVHMLVQDLAQSFNKYYEFFFVPEISLTSMKVSKLSLRLPSAKMSKLDSPDKLKATVR           |
| Human            | DQVHMLVQDLAQSFNKYYEFFFVPEISLTSMKVSKLSLRLPSAKMSKLDSPDKLKATVR           |
| Rhesus monkey    | DQVHMLVQDLAQSFNKYYEFFFVPEISLTSMKVSKLSLRLPSAKMSKLDSPDKLKATVR           |
| Pig              | DQVHMLVQDLAQSFNKYYEFFFVPEISLTSMKVSKLSLRLPSAKMSKLDSPDKLKATVR           |
| Cattle           | DQVHMLVQDLAQSFNKYYEFFFVPEISLTSMKVSKLSLRLPSAKMSKLDSPDKLKATVR           |
| Horse            | DQVHMLVQDLAQSFNKYYEFFFVPEISLTSMKVSKLSLRLPSAKMSKLDSPDKLKATVR           |
| Rabbit           | DQVHMLVQDLAQSFNKYYEFFFVPEISLTSMKVSKLSLRLPSAKMSKLDSPDKLKATVR           |
| Giant panda      | DQVHMLVQDLAQSFNKYYEFFFVPEISLTSMKVSKLSLRLPSAKMSKLDSPDKLKATVR           |
| Mouse            | DQVHMLVQDLAQSFNKYYEFFFVPEISLTSMKVSKLSLRLPSAKMSKLDSPDKLKATVR           |
| Rat              | DQVHMLVQDLAQSFNKYYEFFFVPEISLTSMKVSKLSLRLPSAKMSKLDSPDKLKATVR           |
| Sumatran orangutan | TSDESIEIVKFRKAVDTSETVYDEARGVSNIVAVHAATVGLSVVEEVRRSRAGDNTA          |
| Human            | TSDESIEIVKFRKAVDTSETVYDEARGVSNIVAVHAATVGLSVVEEVRRSRAGDNTA          |
| Rhesus monkey    | TSDESIEIVKFRKAVDTSETVYDEARGVSNIVAVHAATVGLSVVEEVRRSRAGDNTA          |
| Pig              | TSDESIEIVKFRKAVDTSETVYDEARGVSNIVAVHAATVGLSVVEEVRRSRAGDNTA          |
| Cattle           | TSDESIEIVKFRKAVDTSETVYDEARGVSNIVAVHAATVGLSVVEEVRRSRAGDNTA          |
| Horse            | TSDESIEIVKFRKAVDTSETVYDEARGVSNIVAVHAATVGLSVVEEVRRSRAGDNTA          |
| Rabbit           | TSDESIEIVKFRKAVDTSETVYDEARGVSNIVAVHAATVGLSVVEEVRRSRAGDNTA          |
| Giant panda      | TSDESIEIVKFRKAVDTSETVYDEARGVSNIVAVHAATVGLSVVEEVRRSRAGDNTA          |
| Mouse            | TSDESIEIVKFRKAVDTSETVYDEARGVSNIVAVHAATVGLSVVEEVRRSRAGDNTA          |
| Rat              | TSDESIEIVKFRKAVDTSETVYDEARGVSNIVAVHAATVGLSVVEEVRRSRAGDNTA          |
| Sumatran orangutan | RYKLAVADAVIEFAPTKREIEKLKLKDKHLEKLQGSIQSAKAKELAYTVCQVWKVLGFL          |
| Human            | RYKLAVADAVIEFAPTKREIEKLKLKDKHLEKLQGSIQSAKAKELAYTVCQVWKVLGFL          |
| Rhesus monkey    | RYKLAVADAVIEFAPTKREIEKLKLKDKHLEKLQGSIQSAKAKELAYTVCQVWKVLGFL          |
| Pig              | RYKLAVADAVIEFAPTKREIEKLKLKDKHLEKLQGSIQSAKAKELAYTVCQVWKVLGFL          |
| Cattle           | RYKLAVADAVIEFAPTKREIEKLKLKDKHLEKLQGSIQSAKAKELAYTVCQVWKVLGFL          |
| Horse            | RYKLAVADAVIEFAPTKREIEKLKLKDKHLEKLQGSIQSAKAKELAYTVCQVWKVLGFL          |
| Rabbit           | RYKLAVADAVIEFAPTKREIEKLKLKDKHLEKLQGSIQSAKAKELAYTVCQVWKVLGFL          |
| Giant panda      | RYKLAVADAVIEFAPTKREIEKLKLKDKHLEKLQGSIQSAKAKELAYTVCQVWKVLGFL          |
| Mouse            | RYKLAVADAVIEFAPTKREIEKLKLKDKHLEKLQGSIQSAKAKELAYTVCQVWKVLGFL          |
| Rat              | RYKLAVADAVIEFAPTKREIEKLKLKDKHLEKLQGSIQSAKAKELAYTVCQVWKVLGFL          |

Figure 3. The alignment of pig WARS2 protein and nine other kinds of WARS2 proteins.
number: XP_001500995; 91%), human (accession number: BAD96917; 89%), Sumatran orangutan (accession number: XP_002810402; 89%) (Figure 3).

The full-length cDNA sequence of the \textit{WARS2} gene was used as a seed to screen the pig genome database at the NCBI Pig Genome Resources (http://www.ncbi.nlm.nih.gov/projects/genome/guide/pig/) by BLASTGen analysis. The results showed that \textit{Sus scrofa} chromosome 4 (GenBank accession number NC_010446.4) encompasses the entire \textit{WARS2} gene. The genomic DNA of the pig \textit{WARS2} gene coding sequence is 148,853 bp in length and it has 6 exons and 5 introns. Exon—intron splice junction conforms to the GT—AG rule (Figure 4).

Based on the results of the alignment of \textit{WARS2} proteins, a phylogenetic tree was constructed and the result revealed that the pig \textit{WARS2} gene has a close genetic relationship with the \textit{WARS2} gene of cattle (Figure 5).

\subsection*{Polymorphism}

Based on the sequencing result of the pig \textit{WARS2} gene, one G-A mutation (EU650788:c.291 G > A) at the position of 291 bp of the coding region was found. This mutation led to one \textit{Pst} I restriction site and it was confirmed by the PCR-\textit{Pst} I-RFLP analysis (Figure 6).

PCR-\textit{Pst} I-RFLP revealed that the frequency of the \textit{A} allele in the two exotic pig breeds: Large White pig (0.520) and Landrace pig (0.540) was higher than that in the other six Yunnan local pig breeds: Saba pig (0.380), Tibetan pig (0.427), Mingguang small-ear pig (0.385), Diannan small-ear pig (0.430), Wujin pig (0.430) and Baoshan pig (0.400). The two exotic pig breeds — Large White pig and Landrace pig had less animals with genotype \textit{GG}, but had more animals with genotype \textit{AA} (Table 2).

For the litter size of the first parity, no significant difference was found among the animals of the three genotypes in the Large White sows and Landrace sows. For the litter size of all parities, Large White sows with the \textit{AA}
genotype had 0.609 more TNB than the GG sows ($P < 0.05$) and had an additional 0.605 TNB, when compared to the GA animals ($P < 0.05$). In addition, for the litter size of all parities, AA Landrace sows had 0.648 more TNB than the GG Landrace sows ($P < 0.05$), and an additional 0.600 TNB of all parities than the GA animals ($P < 0.05$) (Table 3).

Specific pig NCBI EST database, along with different bioinformatics tools made it much easier for the researchers to find the useful ESTs, which were 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 through some modern experimental methods, such as RT-PCR. From the cloning and sequence analysis of the pig WARS2 gene, it could be seen that this is an effective method to isolate some novel pig genes.

In this study, the complete coding sequence of the porcine WARS2 gene was first cloned. Porcine WARS2 gene has been reported to be an important reproduction-related gene, for it has been identified to be associated with the sow productive life and litter size.[6,7] Thus, this work will provide a molecular basis for the associate analysis of DNA polymorphism of this gene with pig litter size.

Based on the association analysis of the SNP and litter size, it could be found that the polymorphism (EU650788:c.291 G > A) of the porcine WARS2 gene can significantly affect the TNB of all parities. The AA genotype animals obviously had better TNB of all parities than the GA and GG, animals both in purebred Large White and in purebred Landrace sows. This indicated that this polymorphic locus of the porcine WARS2 gene is a valuable candidate gene for the selection of increasing TNB of all parities for pigs. Pig industry can select and keep more AA animals to improve the reproductive performance of sows in pig production. Our results also indicated that there were more Large White pigs and Landrace pigs with genotype AA than the other six Yunnan local pig breeds. There were also less animals with genotype GG than with genotype GA and AA among the Large White pigs and Landrace pigs. This implied that Large White pig and Landrace pigs should have better TNB of all parities than the other six Yunnan local pig breeds. Our present experiment did not compare the difference of TNB of all parities between the Large White, Landrace pigs and other six Yunnan local pig breeds. This should be a basis for the conduction of future studies.

Conclusions

In conclusion, we first cloned the pig WARS2 gene and performed the necessary sequence analysis, polymorphic analysis and association analysis. The results indicated that the polymorphism (EU650788:c.291 G > A) of the porcine WARS2 gene was significantly associated with the TNB of all parities in Large White and Landrace sows. Therefore, the porcine WARS2 gene could be a useful candidate gene for increasing the litter size.

Disclosure statement

No potential conflict of interest was reported by the authors.

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