Genetic Variations Analysis and Characterization of the Fifth Intron of Porcine NRAMP1 Gene

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ABSTRACT: The natural resistance-associated macrophage protein 1 (NRAMP1) gene was identified as a candidate gene controlling the resistance and susceptibility to a number of intracellular parasites in pigs. The genetic variations in a 1.6 kb region spanning exon 1 and exon 3 of the porcine NRAMP1 gene were investigated by PCR-HinfI-RFLP in samples of 1347 individuals from 21 Chinese indigenous pig populations and 3 western pig breeds. Three alleles (A, B, C) and four genotypes (AA, BB, AB, BC) were detected. Significant differences in genotype and allele frequencies were observed between Chinese indigenous pig populations and exotic pig breeds, while in general the differences in genotype and allele frequencies among Chinese indigenous pig populations were not significant. The allele C was detected only in Duroc, Leipng Spotted and Dongyang Spotted pig, and the two Chinese pig populations showed similar genotype and allele frequencies. Four Chinese Tibetan pig populations displayed genetic differentiation at the NRAMP1 gene locus. In addition, intron 5 of the NRAMP1 gene was isolated and characterized by directly sequencing the PCR products encompassing intron 5. The alignment of intron 5 of the porcine, human, equine and ovine NRAMP1 gene showed a similarity of 45.38% between pig and human, 52.55% between pig and horse, 63.47% between pig and sheep, respectively. (Asian-Aust. J. Anim. Sci. 2004. Vol 17. No. 9: 1183-1187)

Key Words: Pig, NRAMP1 Gene, Genetic Variation, Intron 5

INTRODUCTION

The natural resistance-associated macrophage protein 1 (NRAMP1), the macrophage specific protein encoded by the NRAMP1 gene, acts in the early phagosome phase of infection. NRAMP1 induces lysosomes to fuse with the bacterial phagosome, eventually leading to a mature, acidified and fully bactericidal phagolysosome. On one hand, the hydrolase hydrolyses pathogens in the phagolysosome. The NRAMP1 protein is a divalent-metal transporter and completes for the acquisition of essential divalent metals from the phagolysosome lumen, ultimately resulting in pathogen death for lack of ions and degradation (Vidal et al., 1993; Malo et al., 1994; Blackwell et al., 2000; Forbes et al., 2001). The NRAMP1 gene was therefore identified as a candidate gene controlling the resistance and susceptibility to a number of intracellular parasites such as Salmonella (Sun et al., 1998; Bellamy, 1999).

Porcine NRAMP1 cDNA was isolated (Tuggle et al., 1997) and its chromosomal localization was assigned to SSC15q23-26 and linked with S0088, S0149 and S0284 STS-markers (LOD>3) (Sun et al., 1998). The NRAMP1 gene expressed specifically in reticuloendothelial cells (Cellier et al., 1994), its function was suggested to be associated with resistance to Salmonella (Tuggle et al., 1997) and many other kinds of intracellular pathogens in pigs (Sun et al., 1998). The objective of the present study is to detect genetic variations at the NRAMP1 locus in Chinese indigenous pig populations and exotic pig breeds by PCR-RFLP, and to isolate intron 5 of the NRAMP1 gene in order to study the breed characteristics of Chinese indigenous pig breeds and to take advantage of the valuable genetic resource of Chinese indigenous pigs for pig breeding.

MATERIALS AND METHODS

Animals

The ear notches of 1347 individuals were collected from 3 western commercial pig breeds (Landrace, Large White and Duroc) and 21 Chinese indigenous pig populations (Figure 1). Samples were collected from at least 40 individuals per breed from pig breed conservation farms. Care was taken to avoid sampling full-sib animals and to ensure as board a sampling area as possible in cases where no conservation farm exists. The sampling locations and the sample sizes are shown in Table 1 and Figure 1. Genomic DNA was extracted from the ear notches according to a modified phenol and chloroform method (Straus, 1991; Wang et al., 2002).

Genotyping

Primer pair 1 (forward: 5'-ACC CAG CAC ACC ACT...
Table 1. Sample location, sample size, NRAMP1 genotype and allele frequencies of 24 pig populations used in the study

| Type       | Breed         | Sample location                                                                 | Sample size | Genotype frequency | Allele frequency |
|------------|---------------|---------------------------------------------------------------------------------|-------------|-------------------|-----------------|
| Hawaiian   | Hampshire     | Yunnan Provincial Pig Breeding Farm, Yunnan Province                             | 50          | 0.00 0.00 1.00 0.00 | 0.00 1.00 0.00  |
|            | Duroc         | Pig breeding farm of Jiangxi Agricultural University, Dongxiang Jiangyin pig     | 59          | 0.00 0.00 0.80 0.20 | 0.00 0.90 0.10  |
|            | Landrace      | Pig breeding farm of Jiangxi Agricultural University and Dongxiang Animal        | 27          | 0.15 0.07 0.78 0.00 | 0.19 0.81 0.00  |
|            | Provinces     | Husbandry Institute, Jiangxi Province                                             |             |                   |                 |
|            | Min pig       | Lanzhou county pig breeding farm, Heiloqingning Province                         | 41          | 0.12 0.61 0.27 0.00 | 0.43 0.57 0.00  |
|            | Bantei pig    | Huzhu county, Qinghai Province                                                   | 57          | 0.05 0.07 0.88 0.00 | 0.09 0.91 0.00  |
|            | Mashan pig    |Datong city pig breeding farm, Shanxi Province                                    | 39          | 0.00 0.00 1.00 0.00 | 0.00 1.00 0.00  |
|            | Dongyang pig  | Dongyang county livestock breeding farm, Jiangxi Province                         | 49          | 0.16 0.20 0.47 0.08 | 0.31 0.05 0.04  |
|            | Leping pig    | Leping city livestock breeding farm, Jiangxi Province                             | 62          | 0.00 0.37 0.04 0.16 | 0.19 0.73 0.08  |
|            | Spotted pig   | Changping pig breeding farm, Guangdong Province                                  | 59          | 0.66 0.34 0.01 0.00 | 0.83 0.17 0.00  |
|            | Yushan pig    | Yushan county pig breeding farm, Jiangxi Province                                | 41          | 0.16 0.00 0.12 0.00 | 0.78 0.22 0.00  |
|            | Hei pig       | Shanggao county livestock breeding farm, Jiangxi Province                         | 48          | 0.85 0.00 0.07 0.00 | 0.91 0.19 0.00  |
|            | Lushan pig    | Lushan county livestock breeding farm, Guangxi Zhiang autonomous region           | 56          | 0.66 0.25 0.09 0.00 | 0.79 0.21 0.00  |
|            | Lingao pig    | Lingao county, Hainan Province                                                   | 58          | 0.56 0.10 0.34 0.00 | 0.62 0.58 0.00  |
|            | Wuzishan pig  | Wuzishan pig breeding center of Hainan Provincial Academy of Agricultural Science | 60          | 0.67 0.18 0.15 0.00 | 0.76 0.24 0.00  |
|            | Bama Xing pig | Bama county pig breeding farm, Guangxi Zhiang autonomous region                   | 56          | 0.84 0.14 0.02 0.00 | 0.91 0.09 0.00  |
|            | Kele pig      | Heilongjiang county livestock breeding farm, Guizhou Province                    | 49          | 0.45 0.28 0.27 0.00 | 0.59 0.41 0.00  |
|            | Dahe pig      | Fuyun county pig breeding farm, Yunnan Province                                  | 13          | 0.00 0.23 0.77 0.00 | 0.12 0.88 0.00  |
|            | Rongchang pig | Chongqing municipality pig breeding farm, Chongqing municipality                  | 61          | 0.78 0.15 0.07 0.00 | 0.86 0.14 0.00  |
|            | Neijiang pig  | Neijiang city pig breeding farm, Sichuan Province                                | 51          | 0.27 0.14 0.59 0.00 | 0.34 0.66 0.00  |
|            | Jianghan pig  | Xishan, Chongshu and Jiangyin city livestock breeding farm, Jiangsu Province      | 50          | 0.26 0.14 0.60 0.00 | 0.33 0.67 0.00  |
|            | Plateau pig   | Xianggells county, Yunnan Province                                               | 51          | 0.53 0.12 0.35 0.00 | 0.59 0.41 0.00  |
|            | Tibetan pig   | Gonghujingda county, Tibet autonomous region                                     | 61          | 0.51 0.38 0.11 0.00 | 0.70 0.30 0.00  |
|            | Ganzig Tibetan | Xintze county, Gannen Province                                                   | 42          | 0.14 0.14 0.72 0.00 | 0.21 0.79 0.00  |
|            | Pig          | Linhong county, Sichuan Province                                                 | 41          | 0.05 0.28 0.67 0.00 | 0.19 0.81 0.00  |

CAC AC3'- reverse 5'-CAG CTT TCG GAG ACT GAA TG3') were designed to amplify a 1.6 kb region between exon1 and exon3 of the porcine NRAMP1 gene (Sun et al., 1998). Approximately 100 ng of genomic DNA was amplified in a reaction volume of 50 μl containing 0.25 μM of each primer, 0.2 μM of dNTPs, 2 U Taq DNA polymerase with the provided 10×PCR buffer (MBI, Canada). PCR amplifications were performed on PTC-100 Thermal Cyclers (MJ Research, USA) according to the procedure: 95°C for 3 min; 32 cycles of 95°C for 30 s, 60°C
Figure 1. The geographical distribution of the 21 Chinese indigenous pig populations used in the study. Note: BA: Bamei; BM: Bama Xiang; DB: Dihe; DX: Dqing Tibetan pig; DX: Dongxiang Spotted; ER: Erhualian; GB: Ganzhi Tibetan pig; GS: Guangdong Spotted; GZ: Guizhi Tibetan pig; HZ: Heneo Tibetan pig; KL: Keke; LC: Luchuan; LG: Lingao pig; LP: Leping Spotted; MI: Min; MS: Meishan; NJ: Neijiang; SG: Shanggao, RC: Rongchang; YS: Yushan Hei, WZ: Wuzhishan.

for 1 min, 72°C for 2 min; 72°C for 8 min. PCR products were subsequently digested by *HinfI* n 20 μl reaction volume containing 10 μl PCR product. 1U *HinfI* (Sangon, Shanghai) with the supplemented 10× buffer R' and incubated at 37°C for 3-5 h or overnight. The restriction fragments were separated by 2.5% agarose gel in 1×TAE at a constant current of 50 mA. The gels were stained with ethidium bromide and visualized using an UV transilluminator.

Isolation of the intron 5 of porcine *NRAMP1* gene

Primers pair 2 (forward: 5'-TAC CCC GCA CCC TCC TCT G-3'; reverse: 5'-GGT AGA GGA AGA AAG T-3') were derived from a consensus sequence between porcine *NRAMP1* cDNA (GeneBank accession no. AF132037) and human *NRAMP1* gene (GeneBank accession no. AF229163), the 520 bp amplified region encompassed parts of exon 5 and exon 6 and the complete intron 5.

PCR amplifications were performed in a final volume of 25 μl containing 100 ng of genomic DNA, 0.25 μM of each primer. 0.2 μM of dNTPs, 1 U Tag DNA polymerise with the provided 10×PCR buffer (MBI, Canada). PCR profiles were 34 cycles of 95°C for 45 s, 60°C for 45 s, 72°C for 45 s with an initial denaturation at 95°C for 3 min and a final extension at 72°C for 8 min.

PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) and sequenced directly with two PCR primers using the ABI PRISM® BigDye Termini Kit with sequencing dye Terminator v3.1 Cycle Sequencing Kit.

SNPs screening in the isolated intron 5

Two DNA pools were constructed for screening single nucleotide polymorphisms (SNPs) in the isolated intron 5. Each of the DNA pools comprised of 5 individuals from each of the ten different breeds, namely the Duroc, Landrace, Min, Erhualian, Leping Spotted, Bama Xiang, Lingao, Wuzhishan, Yushan Hei and Xizang Tibetan pig. PCR amplifications were performed with primer pair 2 using the two DNA pools as template, the resulting fragment was sequenced directly with two tagged primers to identify the putative SNPs.

Statistics

Significance between genotypes was assessed by the standard t-test using SAS system (1989).

RESULTS

Genotyping

The 1.6 kb PCR products amplified by primer pair 1 were digested by *HinfI* generating allele A (600 bp fragment), allele B (440 bp fragment) and allele C (230 bp fragment) as shown in Figure 2.

Allele and genotype frequencies

The genotype and allele frequencies of the *NRAMP1* gene in 24 pig breeds are presented in Table 1. Three alleles (A, B, C) and four genotypes (AA, BB, AB, BC) were detected in 1347 animals from 24 pig breeds. The genotype...
BB was the predominant genotype and allele B was the predominant allele in Western pig breeds. There existed three genotypes (AA, BB and AB) in Chinese indigenous pig populations and no predominant allele was observed. Allele C was detected only in Duroc, Lepping Spotted and Dongxiang Spotted pig, and allele B was fixed in Hamphine and Mashen pig. In general, Chinese indigenous pig populations displayed more abundant genetic variations at the NRAMP1 gene locus when compared with Western pig breeds. The results of Chi-square testing showed highly significant difference in genotype frequencies between Chinese indigenous pig populations and Western pig breeds (p<0.01, data not shown) indicating obvious genetic differentiation at the locus between Chinese and Western pigs, whereas the differences of genotype and allele frequencies among Chinese indigenous pig populations were not significant (p>0.05, data not shown).

Isolation of intron 5 of NRAMP1 gene

No SNPs was detected in the amplified region flanking intron 5 of the NRAMP1 gene. Comparative analysis of the novel sequence (Figure 3) with the human NRAMP1 gene and the porcine NRAMP1 cDNA sequence showed that the nucleotides from +1 bp to +20 bp were located at the end of exon 5, while those from +398 bp to +419 bp were at the beginning region of exon 6. The 367 bp region from +51 bp to +397 bp was therefore inferred to be intron 5, which conforms to the GT-AG splicing rule. In addition, the alignment of intron 5 of porcine, human, equine and ovine NRAMP1 gene showed a similarity of 45.38% between pig and human, 52.55% between pig and horse, and 63.47% between pig and sheep, respectively.

DISCUSSION

China is rich in indigenous pig populations, which are traditionally classified into six types according to their geographical locations, breeding history and conformation (Zhang, 1986). Pig breeds in neighboring regions showed similar genotype and allele distributions in this study indicating higher homology at the gene locus. For instance, the B allele frequencies in the Min, Bamei and Mashen pig, from the northern part of China and grouped into the Northern China type, were 0.573, 0.912 and 1.000, respectively. The predominant genotype was BB and the predominant allele was B. The A allele frequencies in the Bama Xiang, Luchuan, Lingqiao and Wuzhishan pig, which were all distributed in the southern part of China and classified into the Southern China type, were 0.911, 0.786, 0.615 and 0.758 respectively, with the predominant genotype being AA and the predominant allele being A.

Allele C was detected only in Duroc and two Chinese indigenous pig populations (i.e. Lepping Spotted and Dongxiang Spotted pig), and the two Chinese populations showed similar genotype and allele frequencies both with the predominant allele being allele B. The localizations of the Lepping Spotted and the Dongxiang Spotted pigs are near, both in the northeast of Jiangxi province. Hence, the possibility of occasional exchanges of sires between the two localities cannot be ruled out. Blood protein markers, RAPD and AFLP analysis coincidently showed that the two populations were closely related and possibly originated from the same pig breed (Lai et al., 2001). It is advisable to combine the Lepping Spotted pig and the Dongxiang Spotted pig into one group for conservation purposes.

The Chinese Tibetan pigs are mainly located in the Qinghai-Tibet Plateau, the highest area above sea level in the world, and characterized for adaptability to the Qinghai-Tibet Plateau. According to the classification of Chinese pig breeds, Tibetan pigs have four populations, Dingqing in Yunnan province, Hezuo in Gansu province, Aba-Ganzi in Sichuan province and Xizang in the Tibetan autonomous region, respectively (Zhang, 1986). The four Chinese Tibetan pig populations displayed genetic differentiation at the NRAMP1 gene locus. The Dingqing and the Xizang Tibetan pigs had similar genotype distributions with the predominant allele being B and the most abundant genotype being BB. This is different from the Hezuo and the Ganzi Tibetan pigs with the predominant allele being A and the most abundant genotype being AA. This may be due to intra-population variability caused by geographical isolation.

Comparing the human NRAMP1 gene and the two pig NRAMP1 cDNA sequences (GeneBank accession No. AF132037 and U55068, respectively), we deduced that there might exist SNPs in intron 5 and the GT box between exon 5 and intron 5 of the pig NRAMP1 gene. In this study the results showed that no SNPs was identified in the isolated intron 5. This might be due to the limited numbers of individuals analyzed and the relatively short sequence used. Intron 5 spans 367 bp, while one SNP is estimated to exists every 1,000 nucleotides in the genome (He, 2000). The homology analysis showed that intron 5 of the NRAMP1 gene was relatively lowly conserved among different mammalian species.

In conclusion, we developed a PCR-Hinfl-RFLP assay...
for the polymorphisms of the porcine NRAMP1 gene, and investigated the genetic variations at the NRAMP1 gene locus among 24 Chinese and Western pig breeds. In addition, intron 5 of the porcine NRAMP1 gene was isolated and characterized. The present study provided a base for further studies on the association of the NRAMP1 gene with disease resistance traits in pigs.

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