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

X. M. Yan[1], J. Ren, H. S. Ai, N. S. Ding, J. Gao, Y. M. Guo, C. Y. Chen, J. W. Ma, Q. L. Shu and L. S. Huang**
Key Laboratory for Animal Biotechnology of Jiangxi Province and the Ministry of Agriculture of China
Jiangxi Agricultural University, Nanchang, 330045, P. R. China

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-*Hinf*I-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, Leping Spotted and Dongxiang 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 preimmune 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 decompensation (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 1,347 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 (Strauss, 1991; Wang et al., 2002).

Genotyping

Primer pair 1 (forward: 5'-ACC CAG CAC ACC ACT

* This research was financially supported by National Natural Science Foundation of China (30240071).

** Corresponding Author: Lusheng Huang. Tel: +0086-791-3805967, Fax: +0086-791-3818116, E-mail: LushengHuang@hotmail.com

[1] Department of Biotechnology, Nanchang College, Nanchang 330009, P. R. China.

Received December 31, 2003; Accepted May 19, 2004

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		
				AA	*AB*	*BB*	*BC*	*A*	*B*	*C*
Exotic pig breeds	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 Liangyou pig breeding farm, Wanli pig breeding farm, Jiangxi Province	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 Husbandry Insititute, Jiangxi Province	27	0.15	0.07	0.78	0.00	0.19	0.81	0.00
Northern China type	Min pig	Lanxi county pig breeding farm, Heilongjiang Province	41	0.12	0.61	0.27	0.00	0.43	0.57	0.00
	Bamei pig	Huzhu county, Qinghai Province	57	0.05	0.07	0.88	0.00	0.09	0.91	0.00
	Mashen pig	Datong city pig breeding farm, Shanxi Province	39	0.00	0.00	1.00	0.00	0.00	1.00	0.00
Central China type	Dongxiang Spotted pig	Dongxiang county livestock breeding farm, Jiangxi Province	49	0.16	0.29	0.47	0.08	0.31	0.65	0.04
	Leping Spotted pig	Leping city livestock breeding farm, Jiangxi Province	62	0.00	0.37	0.47	0.16	0.19	0.73	0.08
	Guangdong Spotted.	Banling pig breeding farm, Guangdong Province	59	0.66	0.34	0.00	0.00	0.83	0.17	0.00
	Yushan Hei pig	Yushan county pig breeding farm, Jiangxi Province	41	0.68	0.20	0.12	0.00	0.78	0.22	0.00
	Shanggao pig	Shanggao county livestock breeding farm, Jiangxi Province	48	0.85	0.08	0.07	0.00	0.90	0.10	0.00
Southern China type	Luchuan pig	Luchuan county livestock breeding farm, Guangxi Zhuang autonomous region	56	0.66	0.25	0.09	0.00	0.79	0.21	0.00
	Lin'gao pig	Lin'gao county, Hainan Province	58	0.56	0.10	0.34	0.00	0.62	0.38	0.00
	Wuzhishan pig	Wuzhishan 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 Xiang pig	Bama county pig breeding farm, Guangxi Zhuang autonomous region	56	0.84	0.14	0.02	0.00	0.91	0.09	0.00
Southwestern China type	Kele pig	Hezhang county livestock breeding farm, Guizhou Province	49	0.45	0.28	0.27	0.00	0.59	0.41	0.00
	Dahe pig	Fuyuan county pig breeding farm, Yunnan Province	13	0.00	0.23	0.77	0.00	0.12	0.88	0.00
	Rongchang pig	Chongqin municipality pig breeding farm, Chongqin 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
Jianghai type	Erhualian pig	Xishan, Changshu and Jiangyin city livestock breeding farm, Jiangsu Province	50	0.26	0.14	0.60	0.00	0.33	0.67	0.00
Plateau type	Diqing Tibetan pig	Xianggelila county, Yunnan Province	51	0.53	0.12	0.35	0.00	0.59	0.41	0.00
	Xizang Tibetan pig	Gongbujiangda county, Tibet autonomous region	61	0.51	0.38	0.11	0.00	0.70	0.30	0.00
	Hezuo Tibetan pig	Xiahe county, Gansu Province	42	0.14	0.14	0.72	0.00	0.21	0.79	0.00
	Ganzi Tibetan pig	Litang county, Sichuan Province	41	0.05	0.28	0.67	0.00	0.19	0.81	0.00

CAC AC-3'; reverse: 5'-CAG CTT TCG GAG ACT GAA TG-3') 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 30 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; DH: Dahe; DQ: Diqing Tibetan pig; DX: Dongxiang Spotted; ER: Erhualian; GB: Xizang Tibetan pig; GS: Guangdong Spotted; GZ: Ganzi Tibetan pig; HZ: Hezuo Tibetan pig; KL: Kele; LC: Luchuan; LG: Lingao pig; LP: Leping Spotted; MI: Min; MS: Mashen; 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 *Hinf*I n 20 μl reaction volume containing 10 μl PCR product, 1U *Hinf*I (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 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 *Taq* DNA polymerase 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™ Terminators v3.0 Cycle Sequencing Kit

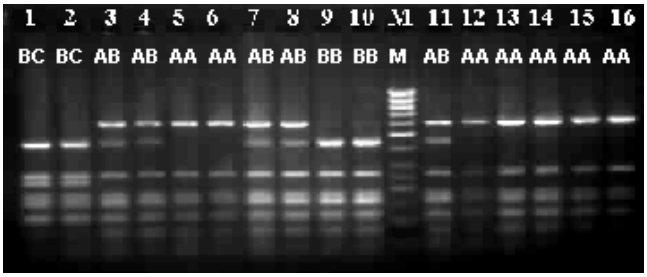

Figure 2. The genotyping results of *NRAMP1* gene by PCR–*Hinf* I-RFLP. Lane 5, 6, 12, 13, 14, 15 and 16: genotype AA; lane 9 and 10: genotype BB; lane 3,4,7,8 and 11: genotype AB; Lane 1 and 2: genotype BC; M was GeneRuler™ 50 bp DNA Ladder marker.

in an ABI PRISM® 3,100 Genetic Analyzer (Applied Biosystems, Foster City, USA) according to standard protocols. The sequence alignments were performed by using the SeqMan software from the DNAStar software package (http://www.lynnon.com) and the homologous sequences were analyzed by using BLAST (http://www.ncbi.nih.gov) and the identity of intron 5 of the *NRAMP1* gene among the different species was calculated by the ClustalX software (downloaded from ftp://ftp-igbmc.u-strasbg.fr).

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 Black 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 *Hinf*I 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

Figure 3. Intron 5 sequence of the porcine *NRAMP1* gene. Nucleotides at the end of exon 5 and the beginning of exon 6 are indicated in bold, the GT-AG consensus sequences are indicated in the box. The length of the isolated intron 5 is 367 bp.

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, Leping Spotted and Dongxiang Spotted pig, and allele *B* was fixed in Hampshire 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 +30 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 +31 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, Lingao 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.* Leping 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 Leping 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 Leping Spotted pig and the Dongxing 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, Diqing in Yunnan provine, 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 Diqing 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-*Hinf*I-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.

REFERENCE

Bellamy, R. 1999. The natural resistance-associated macrophage protein and susceptibility to intracellular pathogens. Microbes and Infection.1: 23-27.

Blackwell, J. M., S. Searle and T. Goswami. 2000. Understanding the multiple functions of *NRAMP1*. Microbes and Infection.2: 317-321.

Cellier, M., G. Govoni and S. Vidal. 1994. Human natural resistance-associated macrophage protein: cDNA cloning, chromosomal mapping, genomic organization, and tissue-specific expression. J. Exp. Med.180:1741-1752.

Forbes, J. R. and P. Gros. 2001. Divalent-metal transport by NRAMP proteins at the interface of host-pathogen interactions. Trends in Microbiology. 9:397-403.

He, L. 2000. Decoding life (In Chinese). Science Press, Beijing.

Lai, Y. B. and F. Y. Huang. 2001. Annals for native livestock and poultry breeds in Jiangxi Province (In Chinese). Jiangxi Science and Technology Press, Nanchang.

Malo, D. and E. Skamene. 1994. Genetic control of host resistance to infection. Trends in Genetics. 10:365-371.

SAS Institue Inc. 1989. SAS/STAT User's Guide: Version 6. 4th edn. SAS Institute Inc, Cary, North Carolina.

Strauss, W. M. 1991. Preparation of genomic DNA from mammalian tissue. In: Current Protocols in molecular Biology (Ed. F. M. Ausubel, R. Brent and R. E. Kingston) Vol. 1, John Wiley and Sons. New York, N.Y., USA. pp. 221-223.

Sun, H. S., L. Wang and M. F. Rothschild. 1998. Mapping of the natural resistance-associated macrophage protein 1 (*NRAMP1*) gene to pig chromosome 15. Anim. Genet. 29:138-140.

Tuggle, C. K., C. B. Schmitz and D. Gingerich-Feil. 1997. Rapid communication: cloning of a pig full-length natural resistance-associated macrophage protein (*NRAMP1*) cDNA. J. Anim. Sci. 75:277.

Vidal, S. M., D. Malo and K. Vogan. 1993. Natural resistant to infection with intracellular parasites: isolation of a candidate for Bcg. Cell. 73:469-485.

Wang, W. J., L. S. Huang and K. F. Chen. 2002. Polymorphism of insulin-like growth factor-I gene in 13 pig breeds and its relationship with pig growth and carcass traits. Asian-Aust. J. Anim. Sci. 15:1391-1394.