Polymorphism, Expression of Natural Resistance-associated Macrophage Protein 1 Encoding Gene (NRAMP1) and Its Association with Immune Traits in Pigs

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ABSTRACT: Natural resistance-associated macrophage protein 1 encoding gene (NRAMP1) plays an important role in immune response against intracellular pathogens. To evaluate the effects of NRAMP1 gene on immune capacity in pigs, tissue expression of NRAMP1 mRNA was observed by real time quantitative polymerase chain reaction (PCR), and the results revealed NRAMP1 expressed widely in nine tissues. One single nucleotide polymorphism (SNP) (ENSSSCG00000025058: g.130 C>T) was significantly associated with WBC% (p = 0.031), MO% (p = 0.024), MC% (p = 0.013) and CD4⁺CD8⁻ T lymphocyte (%p = 0.023). The other SNP (ENSSSCG00000025058: g.657 A>G) was significantly associated with the level of MO% (p = 0.012), MC% (p = 0.019) and CD4⁺CD8⁻ T lymphocyte (%p = 0.037). These results indicate that the NRAMP1 gene can be regarded as a molecular marker for genetic selection of disease susceptibility in pig breeding. (Key Words: Pig, Natural Resistance-associated Macrophage Protein 1 (NRAMP1), Polymorphism, Expression, Association)

INTRODUCTION

The natural resistance-associated macrophage protein 1 encoding gene (NRAMP1) belongs to a large gene family encoding divalent cation transporters that are localized to late endosomes/lysosomes (Blackwell et al., 2000). The NRAMP1 protein is targeted to the membrane of the microbe-containing phagosome, where it may modify the intraphagosomal milieu to affect microbial replication (Canonne-Hergaux et al., 1999). The NRAMP1 gene also called solute carrier family 11 member 1 (SLC11A1) gene and is the first positional cloned gene related to infectious disease susceptibility in mouse (Vidal et al., 1993). In mammals, NRAMP1 gene is associated with the transport of iron and other divalent cations, and the transition metal ions are essential for maintaining divalent metal homeostasis, including regulation of transcription through DNA binding proteins and metal response elements (Blackwell, 2003; Courville et al., 2006).

The NRAMP1 protein has 12 conserved transmembrane domains, two N-linked glycosylation sites, and a transport motif. These conserved domains perform various important functions. Mutations of these conserved domains are associated with various diseases in human. Polymorphisms of the human Nramp1 gene are significantly associated with pulmonary Mycobacterium avium complex infection (Tanaka et al., 2007), Crohn’s disease (Gazouli et al., 2008), rheumatoid arthritis (Ates et al., 2009), type 1 diabetes (T1D) (Yang et al., 2011). In pig, the cDNA of NRAMP1...
gene has been reported and several single nucleotide polymorphism (SNPs) were also detected and some were found to be associated with the fecal bacteria counts of piglets after challenging with Salmonella cholevasuis (Tuggle et al., 1997; 2005).

Considering the important role of NRAMP1 gene in defending bacterial and viral infection, in this study, we investigated its mRNA expression in different tissues, identified the polymorphisms of porcine NRAMP1 gene, and then analyzed their association with some immune traits to evaluate its potential effects in four pig populations including Large White and three Chinese native breeds (Wannan Black, Huai pig, and Wei pig).

MATERIALS AND METHODS

Animals
The animals used in this study were 466 pigs, including Large White (140) and three Chinese indigenous breeds, Wannan Black pig (118), Huai pig (105), Wei pig (103). All pigs were raised in 2012 and 2013 under standard indoor conditions at the experimental farm of Academy of Agricultural Sciences of Anhui province, China. After the vaccination with classical swine fever live vaccine, blood samples were collected (day 35). All blood samples were directly injected into VACUETTE Serum Clot Activator tubes. Ear tissue samples of all pigs were also collected for DNA extraction. Eight different tissues, including heart, liver, spleen, lung, kidney, lymph node, brain and thymus, were collected from three 35-day-old Huai pigs. For each tissue, three samples were collected, then immediately frozen in liquid nitrogen and stored at –80°C for spatial expression analysis.

Total RNA extraction and genomic DNA isolation
The total RNA was extracted from different tissues with a TRIZol reagent (Invitrogen, USA). In order to prevent serious contamination with genomic DNA, DNase I treatment of the total RNA was carried out before first-strand cDNA synthesis. The RNA quality was checked by 0.8% agarose gel electrophoresis, stained with 0.5 μg/mL ethidium bromide, showing the 28S and 18S rRNAs clearly. Then reverse transcription was performed for the first-strand cDNA synthesis. All supplies and reagents were RNase-free. First, we incubated the total RNA at 70°C for 5 min to destabilize the secondary structure of RNA, and then immediately put it on the ice for at least 2 min; second, thoroughly mix various reagents (molyneux murine leukemia virus [M-MLV] Buffer, dNTP, M-MLV reverse transcriptase, Oligo(dT) and RNAsin) (Promega, Madison, WI, USA) with the total RNA on the ice; finally, the mixture was placed in the thermal cycler to accomplish the first-strand cDNA synthesis followed by the program: 42°C 1 h, 95°C 10 min. Genomic DNA was isolated from the ear tissue samples of Large White, Wannan Black, Huai pig and Wei pig using phenol/chloroform extraction and ethanol precipitation (Sambrook et al., 1989).

Measurement of immune traits in blood
Immune traits were contain of white blood cell (WBC), granulocyte, lymphocyte, monocyte (MO), rate of cytotoxicity in monocyte (MC) and CD4/CD8 T lymphocyte subpopulations. All blood samples were directly injected into VACUETTE Serum Clot Activator tubes for detection of blood parameters and T lymphocyte subpopulations; All these hematological parameters were measured by MEK-6318K type full automatic Hematology Analyzer (Nihon Kohden, Tokyo, Japan). Rate of cytotoxicity in monocytes was detected by rapid colorimetric assay of a multiwall scanning spectrophotometer (Mosmann, 1983). The percentages of various CD4/CD8 T lymphocyte subpopulations were obtained by the double cytofluorometric analysis. The blood cells were incubated with 10 μL of mouse anti porcine CD4-FITC (Serotec, Oxford, UK) and 10 μL of mouse anti porcine CD8-RPE (Serotec, UK) for 30 min, and then washed with 0.1 M phosphate buffer solution (pH7.2), containing 0.3% bovine serum albumin). The stained cells were detected by EPICS Flow Cytometer (Beckman-Coulter Company, Brea, CA, USA).

mRNA expression by real time quantitative polymerase chain reaction
Based on the NRAMP1 gene mRNA sequence (ENSEMBLE Transcript ID: ENSSSCT00000024413), the reverse transcriptase-polymerase chain reaction (PCR) amplification method was used to detect mRNA expression of the NRAMP1 gene. The first strand cDNA was synthesized in the presence of 2 μg total RNA, 0.5 μM oligo(dT)18, 200 μM dNTPs, 10 U RNAsin (Promega, Madison, WI, USA), 1×M-MLV RT buffer, and 300 U M-MLV reverse transcriptase (Promega, USA) in a volume of 50 μL at 40°C for 1 h. A specific primer setting for NRAMP1 gene mRNA expression analysis was designed to eliminate potential confounding results from genomic DNA contamination (Table 1). Another specific primer set (GAPDH-F1 5′-TGAGACACGATGGTGAAAGT-3′ and GAPDH-R1 5′-GGCATTTGCTGATCTTGA-3′) was designed to amplify the porcine house-keeping gene glyceraldehyde3-phosphate dehydrogenase (GAPDH) mRNA (GenBank accession number: AF017079) as a positive control. The PCR experiments were performed with an ABI Prism 7900 HT sequence-detection system (Applied Biosystems, Foster, CA, USA), using SYBR Green PCR Master Mix according to the manufacturer’s.
SNP identification and genotyping of the porcine NRAMP1 gene

The porcine NRAMP1 genes consist of four exons and three introns (ENSEMBLE gene ID: ENSSSCG000000025058), and using porcine genomic DNA, we designs five primer pairs for the NRAMP1 gene including all exon regions (Table 1). All PCR fragments were purified with a Gel Extraction Mini Kit (Beijing Tiangen Biotechnology, Beijing, China) and then sequenced directly. Polymorphisms of NRAMP1 gene were identified through sequence comparison of the obtained same PCR fragments from two Large White, two Wannan Black and two Huai pigs using DNAMAN (version 5.2.10, Lyn-non Biosoft, Quebec, Canada). In order to detect the SNPs, direct sequencing of the PCR fragments and PCR-RFLP methods were used. For the PCR-RFLP method, at first, the PCR products (12 μL) were digested with 10 μL of restriction enzyme, mixed with 2 μL of 10xT buffer and 2 μL 0.01% bovine serum albumin, and added water up to 20 μL. The mixtures were incubated at 37°C overnight, then the digested PCR products were analyzed by 3% to 4% agarose gel electrophoresis and ethidium bromide staining. The gel was visualized by Gel Doc XR system (Bio-Rad, Hercules, CA, USA). The genotypes were identified according to the restriction fragment patterns with Sma I restriction enzyme and later confirmed by sequencing in ABI 377 DNA sequencer (Applied Biosystems, USA).

Association analysis

Statistical analysis between the polymorphisms of the NRAMP1 gene and the immune traits were performed using PROC MIXED of SAS9.13 (SAS Institute, Cary, NC, USA). The model was as below following:

\[ Y_{ijklmno} = \mu + S_i + Sex_j + B_k + G_l + p_{km} + q_{lm} + e_{ijklmno} \]

where \( Y_{ijklmno} \) is the observation on the 35th day immune traits; \( \mu \) is the overall mean; \( S_i \) is the effect of the season; \( Sex_j \) is the effect of the sex; \( B_k \) is the effect of the breed; \( G_l \) is the effect of the genotype of the SNP; \( p_{km} \) is the random effect of the sire within breed; \( q_{lm} \) is the random effect of the dam within breed and sire; \( e_{ijklmno} \) is the random residual effect.

RESULTS AND DISCUSSION

Tissue expression of porcine NRAMP1 gene

To determine tissue distribution of the porcine NRAMP1 gene, reverse transcription quantitative PCR (RT-PCR) was applied on nine different tissues of samples. Results showed that the mRNA of NRAMP1 was expressed in all analyzed tissues (Figure 1). The levels of NRAMP1 expressed in brain, spleen and lymphoid were higher, comparatively, while the levels in brain and kidney were lower. NRAMP1 gene expression has been observed by RT-PCR in different tissues from chickens where higher expression levels were found in liver, spleen, thymus and much lower expression levels in brain, kidney, which is consistent with our results in pigs (He et al., 2013).

Polymorphisms detection of porcine NRAMP1 gene

Sequence comparisons of the same PCR fragment among four pig populations detected one SNP

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Table 1. Primers used for mRNA expression and SNPs detection of the porcine NRAMP1 gene

| Fragments | Primers sequence (5′-3′) | Product size (bp) | PCR (Tm) | Used for |
|-----------|--------------------------|------------------|----------|---------|
| NRAMP1-1  | CGGGCTTGGAGGCTTTTTC      | 560              | 59.0     | SNP identification |
| NRAMP1-2  | AGGCTCTGTCTCTCAGC       | 260              | 59.0     | SNP detection |
| NRAMP1-3  | TGCATGCTCCTACTACCC      | 430              | 59.5     | SNP identification |
| NRAMP1-4  | CATTGTTGGAGGCTTTTC      | 620              | 60.0     | SNP identification |
| NRAMP1-Q  | GGGCGATGTTGGAGGATTATTTAC | 420               | 59.5    | SNP identification |

**SNP, single nucleotide polymorphism; NRAMP1, natural resistance-associated macrophage protein 1; PCR, polymerase chain reaction.**
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(ENSSSCG00000025058: g.130 C>T) in exon1 and one SNP (ENSSSCG00000025058: g.657 A>G) in intron1 region of the porcine NRAMP1 gene. The two SNPs were both synonymous mutations, did not induce substitution of an amino acid. The SNP (ENSSSCG00000025058: g.130 C>T) was genotyped by directly sequencing the PCR products and the other SNP (ENSSSCG00000025058: g.657 A>G) was genotyped by PCR-RFLP method in all 466 pigs (Figure 2). The genotypes and allele frequencies of the identified SNPs in four different pig populations are presented in Table 2. For the SNP (ENSSSCG00000025058: g.130 C>T), the allele T has higher frequencies than allele C in Wannan Black, Huai and Wei pig populations, but have lower allele frequencies in Large White population. In addition, for the SNP (ENSSSCG00000025058: g.657 A>G), the allele G was obviously with high allele frequencies, the allele A has lower frequencies and AA genotype is not detected in four pig populations.

Association analysis of porcine NRAMP1 gene in four pig populations

Base on the aforementioned work, we performed association analysis to confirm whether the identified SNPs from the four breeds have relationship with immune traits. Results showed that all the traits among four breeds were significantly different except ratio of CD4−CD8− T lymphocyte (p<0.05) (Table 3). The MO% levels of Large White was significantly higher than those of Huai and Wei pig (Table 3); The CD4+CD8−, CD4+CD8+ T lymphocyte levels of Large White was significantly higher than those of Wannan Black, Huai and Wei pig (Table 3); In contrast, Wannan Black, Huai and Wei pigs had a significantly higher WBC, CD4−CD8+ T lymphocyte level than Large White (p<0.05) (Table 3). As reported in a previous study, the results implied that genetic background is one of important

Figure 1. Expression of porcine NRAMP1 mRNA detected by real time Q-PCR in different porcine tissues. The relative expression level of NRAMP1 mRNA to GAPDH mRNA was evaluated using the $2^{-\Delta\Delta CT}$ method. NRAMP1, natural resistance-associated macrophage protein 1; Q-PCR, real-time quantitative polymerase chain reaction; GAPDH, glyceraldehyde3-phosphate dehydrogenase.

Figure 2. PCR-Smal-RFLP analysis of the SNP (ENSSSCG00000025058: g. 657A>G) in porcine NRAMP1 gene. The lane M is Marker DL2000; The lane 1-3 are AA genotype, and 4-5 are GG genotype, and 6-7 are AG heterozygote. PCR-Smal-RFLP, polymerase chain reaction-Smal-Restriction Fragment Length Polymorphism; SNP, single nucleotide polymorphism; NRAMP1, natural resistance-associated macrophage protein 1.
The number of animals in each breed is indicated between parentheses (Table 2).

### Table 2. Genotype and allele frequencies of the SNPs detected in the porcine NRAMP1 gene

| Breed                | Genotype frequencies | Allele frequencies | Genotype frequencies | Allele frequencies |
|----------------------|----------------------|--------------------|----------------------|--------------------|
|                      | CC       | CT      | TT      | C   | T   | AA   | AG   | GG  | A  | G  |
| Large White (140)    | 0.54     | 0.18    | 0.28    | 0.63 | 0.37 | 0    | 0.32 | 0.68 | 0.16 | 0.84 |
| Wannan Black (118)   | 0.17     | 0.21    | 0.62    | 0.28 | 0.72 | 0    | 0.28 | 0.72 | 0.14 | 0.86 |
| Huai (105)           | 0.23     | 0.31    | 0.46    | 0.39 | 0.61 | 0    | 0.25 | 0.75 | 0.12 | 0.88 |
| Wei (103)            | 0.21     | 0.22    | 0.57    | 0.32 | 0.68 | 0    | 0.22 | 0.78 | 0.11 | 0.89 |

SNP, single nucleotide polymorphism; NRAMP1, natural resistance-associated macrophage protein 1.

### Table 3. Association analysis of immune traits in four pig breeds

| Traits          | Large White (n = 140) | Wannan Black (n = 118) | Huai (n = 105) | Wei (103) | p value |
|-----------------|-----------------------|------------------------|---------------|-----------|---------|
| WBC (g/L)       | 18.81±0.81<sup>a</sup> | 19.92±0.93<sup>b</sup> | 20.25±1.02<sup>b</sup> | 21.35±1.16<sup>b</sup> | 0.043*  |
| LY (%)          | 59.22±1.28<sup>a</sup> | 60.60±1.70<sup>a</sup> | 61.84±1.82<sup>a</sup> | 63.84±1.86<sup>b</sup> | 0.048*  |
| MO (%)          | 15.31±0.43<sup>a</sup> | 14.97±0.72<sup>a</sup> | 13.18±0.36<sup>b</sup> | 14.18±0.42<sup>b</sup> | 0.024*  |
| MC (%)          | 83.21±5.21<sup>a</sup> | 83.32±6.54<sup>a</sup> | 85.41±7.63<sup>a</sup> | 81.29±5.82<sup>b</sup> | 0.013*  |
| CD4⁺CD8⁺ (%)    | 11.41±0.21            | 10.84±0.24            | 10.33±0.23     | 10.27±0.18     | 0.574   |
| CD4⁺CD8⁻ (%)    | 19.70±0.73<sup>b</sup> | 13.62±0.59<sup>b</sup> | 11.46±0.57<sup>b</sup> | 13.38±0.64<sup>b</sup> | 0.019*  |
| CD4⁻CD8⁺ (%)    | 39.64±0.72<sup>a</sup> | 44.38±0.93<sup>b</sup> | 51.17±1.05<sup>b</sup> | 48.25±1.06<sup>b</sup> | 0.026*  |
| CD4⁻CD8⁻ (%)    | 35.41±0.67<sup>a</sup> | 30.43±0.55<sup>b</sup> | 29.65±0.64<sup>b</sup> | 28.42±0.63<sup>b</sup> | 0.032*  |

SE, standard error; WBC, white blood cell count; LY%, lymphocyte count percentage; MO%, monocytes count percentage; MC (%), rate of cytotoxic in monocyte; CD4⁺CD8⁺ T lymphocyte subsets.

<sup>a,b</sup> Signed by small letters differ significantly at p<0.05.

<sup>a,b</sup> Means signed by capital letters differ significantly at p<0.01.
The expression by alteration or increasing the stability of mRNA. However, the synonymous SNPs can also affect protein function or induce amino acid substitutions.

As the immune system plays an essential role in disease resistance of animals, genes involved in the response of immune system could be regarded as the candidate genes (Smit et al., 2004). Therefore, the higher CD4^+ CD8^+ percentage in animals with GG genotype may enhance the resistance of animals, genes involved in the response of immune system could be regarded as the candidate genes. But the number of pigs analyzed in our study was restricted, further association analysis showed that the two SNPs were not missense mutations or induced amino acid substitutions. However, the synonymous SNPs can also affect protein expression by alteration or increasing the stability of mRNA (Capon et al., 2004). Additionally, a “silent” polymorphism can change substrate specificity (Kimchi-Sarfaty et al., 2007). Our results provided a straightforward insight that NRAMP1 gene which has effects on levels of WBC, MO%, MC%, CD4^+ CD8^+ T lymphocytes and it could serve as a genetic marker for immune traits. But the number of pigs analyzed in our study was restricted, further investigations are needed to confirm the relationships between the SNPs with immune traits among other pig populations.

In summary, the mRNA expression results revealed that the porcine NRAMP1 gene was expressed widely in liver, spleen, lymphoid, kidney, thymus, lung, brain and heart. The two SNPs were not missense mutations or induced amino acid substitutions. However, the synonymous SNPs can also affect protein expression by alteration or increasing the stability of mRNA (Capon et al., 2004). Additionally, a “silent” polymorphism can change substrate specificity (Kimchi-Sarfaty et al., 2007). Our results provided a straightforward insight that NRAMP1 gene which has effects on levels of WBC, MO%, MC%, CD4^+ CD8^+ T lymphocytes and it could serve as a genetic marker for immune traits. But the number of pigs analyzed in our study was restricted, further investigations are needed to confirm the relationships between the SNPs with immune traits among other pig populations.

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