



## 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) in exon1 and one SNP (ENSSSCG00000025058: g.657 A>G) in intron1 region of porcine *NRAMP1* gene were demonstrated by DNA sequencing and PCR-RFLP analysis. A further analysis of SNP genotypes associated with immune traits including contain of white blood cell (WBC), granulocyte, lymphocyte, monocyte (MO), rate of cytotoxin in monocyte (MC) and CD4/CD8 T lymphocyte subpopulations in blood was carried out in four pig populations including Large White and three Chinese indigenous breeds (Wannan Black, Huai pig and Wei pig). The results showed that the SNP (ENSSSCG00000025058: g.130 C>T) was significantly associated with level of WBC % ( $p = 0.031$ ), MO% ( $p = 0.024$ ), MC% ( $p = 0.013$ ) and CD4<sup>+</sup>CD8<sup>+</sup> 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<sup>+</sup>CD8<sup>+</sup> 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 maintain 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*

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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 choleraesuis* (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  $-80^{\circ}\text{C}$  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  $\mu\text{g}/\text{mL}$  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  $70^{\circ}\text{C}$  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 (moloney 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:  $42^{\circ}\text{C}$  1 h,  $95^{\circ}\text{C}$  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 cytotoxin 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 cytotoxin 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  $\mu\text{L}$  of mouse anti porcine CD4-FITC (Serotec, Oxford, UK) and 10  $\mu\text{L}$  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 (ENSEMBL 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  $\mu\text{g}$  total RNA, 0.5  $\mu\text{M}$  oligo(dT)18, 200  $\mu\text{M}$  dNTPs, 10 U RNAsin (Promega, Madison, WI, USA), 1 $\times$ M-MLV RT buffer, and 300 U M-MLV reverse transcriptase (Promega, USA) in a volume of 50  $\mu\text{L}$  at  $40^{\circ}\text{C}$  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'-TGAGACACGATGGTGAAGGT-3' and GAPDH-R1 5'-GGCATTGCTGATGATCTTGA-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

**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	CGGGCTTGGGAAGGTCTTTTC CTCCTGATGCTCTCCTCCTG	560	59.0	SNP identification
NRAMP1-2	AGGCTCTGCTTCTCACACAT TTCGGGATGGGGATCTTCTC	260	59.0	SNP detection
NRAMP1-3	TGCATGCTCCACTCACCC GAGCCATGTATGCCACCTTG	430	59.5	SNP identification
NRAMP1-41	GGAGACCGCCAGTTTGCC AGGCTAGGGGTCAAATTGGA	620	60.0	SNP identification
NRAMP1-42	CTCTGGTGTAGGCTGGCG CCAGGCTAGAGGTCAATCA	420	59.5	SNP identification
NRAMP1-Q	ACTTCTTAGGGCCTGTTC GGGCGATGGTGGATTCAATC	210	56.5	mRNA expression

SNP, single nucleotide polymorphism; NRAMP1, natural resistance-associated macrophage protein 1; PCR, polymerase chain reaction.

protocol. In each assay, 1  $\mu$ L of cDNA was added to 19  $\mu$ L of mix containing 10  $\mu$ L 2 $\times$ SYBR green SuperReal PreMix (Tiangen, Beijing, China), 0.4  $\mu$ L of each primer and 8.2  $\mu$ L RNase free H<sub>2</sub>O. The reaction was amplified at 95°C for 15 min, followed by 40 cycles of 95°C 10 s and 56.5°C 30 s. For each miRNA, three replicates were performed, and all of the reactions were run in triplicate. The cycle threshold was collected from each reaction, and the relative expression level of *NRAMP1* gene mRNA to GAPDH mRNA was evaluated using the 2<sup>- $\Delta\Delta$ CT</sup> method (Hellemans et al., 2007).

### SNP identification and genotyping of the porcine *NRAMP1* gene

The porcine *NRAMP1* genes consist of four exons and three introns (ENSEMBLE gene ID: ENSSSCG00000025058), 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  $\mu$ L) were digested with 10 U of restriction enzyme, mixed with 2  $\mu$ L of 10 $\times$ T buffer and 2  $\mu$ L 0.01% bovine serum albumin, and added water up to 20  $\mu$ L. 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:

$$Y_{ijklmno} = \mu + S_i + \text{Sex}_j + B_k + G_l + p_{km} + q_{kmn} + e_{ijklmno}$$

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;  $\text{Sex}_j$  is effect of the sex;  $B_k$  is the effect of the breed;  $G_l$  is the effect of genotypes of the SNP;  $p_{km}$  is the random effect of the sire within breed;  $q_{kmn}$  is the random effect of the dam within breed and sire;  $e_{ijklmnop}$  is the random residual effect.

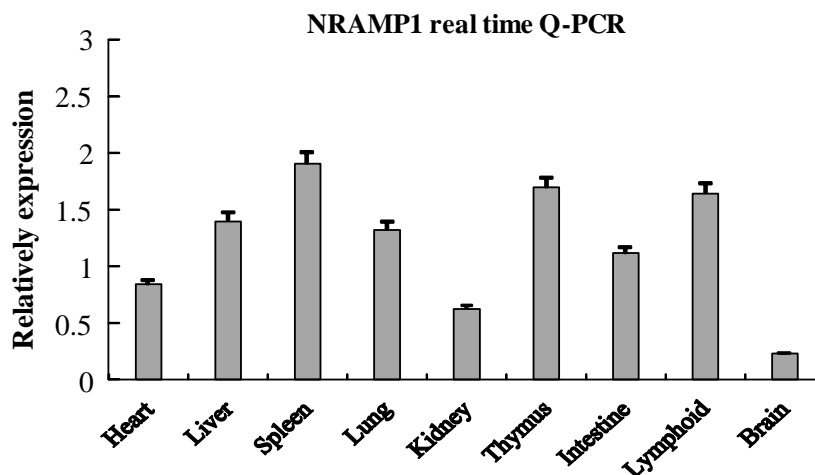
## RESULTS AND DISCUSSION

### 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 liver, 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



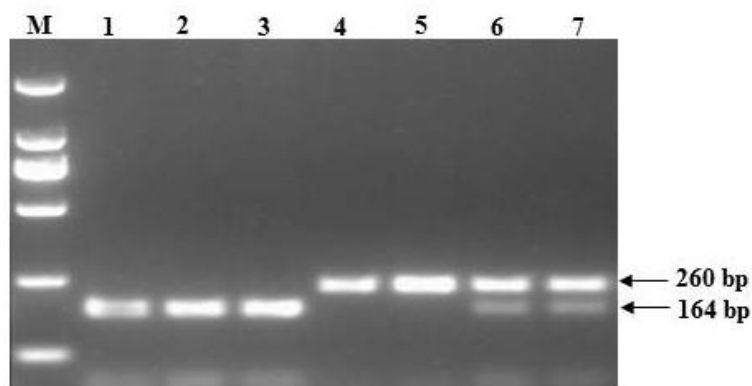
**Figure 1.** Expression of porcine NRAMP1 mRNA detected by real time Q-PCR in different porcine tissues. The relative expression level of NRAMP1 mRNA to GAPDH mRNA was evaluated using the  $2^{-\Delta\Delta CT}$  method. NRAMP1, natural resistance-associated macrophage protein 1; Q-PCR, real-time quantitative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

(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<sup>-</sup>CD8<sup>-</sup> 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<sup>+</sup>CD8<sup>-</sup>, CD4<sup>+</sup>CD8<sup>+</sup> 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<sup>-</sup>CD8<sup>+</sup> 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 2.** PCR-SmaI-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-SmaI-RFLP, polymerase chain reaction-SmaI-Restriction Fragment Length Polymorphism; SNP, single nucleotide polymorphism; NRAMP1, natural resistance-associated macrophage protein 1.

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

Breed	(ENSSSCG00000025058:g.130 C>T)					(ENSSSCG00000025058:g. 657 A>G)				
	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. The number of animals in each breed is indicated between parentheses.

factors that influence the immune capacity (Liu et al., 2011).

The results of association analysis between SNPs and immune traits also showed that the SNP (ENSSSCG00000025058: g.130 C>T) of *NRAMP1* gene was significantly associated with level of WBC%, MO%, MC% and CD4<sup>-</sup>CD8<sup>+</sup> T lymphocyte (p<0.05) (Table 4). The other SNP (ENSSSCG00000025058: g.657 A>G) was highly significantly associated with the level of MO%, MC% and CD4<sup>-</sup>CD8<sup>+</sup> T lymphocyte (p<0.05) (Table 4). In further multiple tests for the SNP (ENSSSCG00000025058: g.130 C>T), the levels of MO% and MC% of pigs associated with genotype CC were significantly higher than with genotype TT (p<0.05), but the levels of WBC% and CD4<sup>-</sup>CD8<sup>+</sup> T lymphocyte with genotype CC were significantly lower than those with genotype TT (p<0.05). For the other SNP (ENSSSCG00000025058: g.657 A>G), the levels of MO%, MC%, and CD4<sup>-</sup>CD8<sup>+</sup> T lymphocyte of pigs associated with genotype GG were significantly higher than those with genotype AA (p<0.01).

The *NRAMP1* gene was firstly isolated by positional cloning the host resistance locus *Bcg/Ity/Lsh*, and mutations at this locus impair the resistance of mice to infections with intracellular parasites, such as *Salmonella*, *Leishmania* and *Mycobacterium* (Vidal et al., 1993). *NRAMP1* gene is expressed in monocyte/macrophages and has key roles in phagosome maturation and cell activation. Moreover,

*NRAMP1* gene has an important role in activation of a large subset of innate lymphocytes that are critical sources of interferon- $\gamma$  (Hedges et al., 2013). In humans, mutations in *NRAMP1* gene are associated with T1D (Yang et al., 2011), tuberculosis risk (Meilang et al., 2012) and pulmonary *Mycobacterium avium* complex infection (Sapkota et al., 2012). In pigs, a single nucleotide polymorphism in the sixth intron of the *NRAMP1* gene has been found to be significantly associated with polymorphonuclear leukocyte levels, cytotoxin on monocytes and 180-day-old body weight (Wu et al., 2008). In our study, the SNPs of *NRAMP1* gene were significantly associated with monocytes traits and CD4<sup>-</sup>CD8<sup>+</sup> T lymphocyte. Monocytes play critical roles in immunopathology. *NRAMP1* mediates the iron homeostasis in monocytes/macrophages and hence modulates their response to acute inflammatory stimuli (Wyllie et al., 2002). So the higher percentage of monocytes in animals with CC genotype in our study may imply that the CC genotype enhances the activity of the monocytes by mediating iron homeostasis. On the other hand, the CD4<sup>-</sup>CD8<sup>+</sup> T lymphocytes are also important traits in disease resistance and could mediate the activity of the CSFV-specific cytotoxic T lymphocyte (Pauly et al., 1995). The differentiation of the T lymphocyte into CD4/CD8 subpopulations is induced by the antigen-presenting cells (APCs) such as macrophages, which in turn are regulated

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

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

**Table 4.** Association analysis and multiple tests of the SNPs of *NRAMP1* gene in four pig populations

Immune traits	p-value		Polymorphism genotypes		
	( ENSSSCG00000025058 )		(Least square mean±SE)		
	g.130 C>T	g.657 A>G			
WBC (g/L)	0.031*	0.724	g.130 C>T		
			CC	CT	TT
			18.73±0.84 <sup>a</sup>	19.94±0.91 <sup>b</sup>	20.46±0.96 <sup>b</sup>
LY % (%)	0.472	0.641			
MO % (%)	0.024*	0.012*	g.130 C>T		
			CC	CT	TT
			15.12±0.41 <sup>a</sup>	14.74±0.39 <sup>a</sup>	14.28±0.32 <sup>b</sup>
MC (%)	0.013*	0.019*	g.657 A>G		
			AA	AG	GG
			13.64±0.34 <sup>A</sup>	14.72±0.39 <sup>B</sup>	15.33±0.42 <sup>B</sup>
CD4 <sup>-</sup> CD8 <sup>-</sup> (%)	0.051	0.062	g.130 C>T		
			CC	CT	TT
			84.89±6.45 <sup>A</sup>	84.31±6.26 <sup>A</sup>	81.43±6.11 <sup>B</sup>
CD4 <sup>+</sup> CD8 <sup>-</sup> (%)	0.871	0.693	g.657 A>G		
			AA	AG	GG
			80.81±6.03 <sup>A</sup>	83.24±6.27 <sup>B</sup>	84.92±6.42 <sup>B</sup>
CD4 <sup>-</sup> CD8 <sup>+</sup> (%)	0.023*	0.037*	g.130 C>T		
			CC	CT	TT
			40.46±0.81 <sup>A</sup>	42.17±0.91 <sup>A</sup>	50.23±1.03 <sup>B</sup>
CD4 <sup>+</sup> CD8 <sup>+</sup> (%)	0.962	0.874	g.657 A>G		
			AA	AG	GG
			41.07±0.84 <sup>A</sup>	48.34±0.93 <sup>B</sup>	51.13±1.08 <sup>B</sup>

SNP, single nucleotide polymorphism; NRAMP1, natural resistance-associated macrophage protein 1; SE, standard error; WBC, white blood cell count; LY%, lymphocyte count percentage; MO%, monocytes count percentage; MC (%), rate of cytotoxin in monocyte; CD4<sup>+</sup>CD8<sup>+</sup> T lymphocyte subsets. p<0.05; <sup>a,b</sup> Statistically different of least square means (p<0.05). <sup>A,B</sup> Statistically different of least square means (p<0.01).

by the *NRAMP1* gene (Smit et al., 2004). Therefore, the higher CD4<sup>-</sup>CD8<sup>+</sup> percentage in animals with GG genotype in our study indicate that GG genotype may enhance the differentiation of the T lymphocyte into CD4<sup>-</sup>CD8<sup>+</sup> by regulating the activity of APCs.

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 for selection (Crawley et al., 2005). 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<sup>-</sup>CD8<sup>+</sup> 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. One SNP (ENSSSCG00000025058: g.130 C>T) in exon1 and one SNP (ENSSSCG00000025058: g.657 A>G) in intron1 region of porcine NRAMP1 gene were found. The further association analysis showed that the two SNPs were significantly associated with level of WBC%, MO%, MC% and CD4<sup>-</sup>CD8<sup>+</sup> T lymphocytes respectively. The results indicated that *NRAMP1* gene could be regarded as a genetic marker for these immune traits in porcine disease resistance breeding.

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## REFERENCES

- Ates, O., L. Dalyan, B. Musellim, G. Hatemi, H. Türker, G. Ongen, V. Hamuryudan, and A. Topal-Sarikaya. 2009. NRAMP1 (SLC11A1) gene polymorphisms that correlate with autoimmune versus infectious disease susceptibility in tuberculosis and rheumatoid arthritis. *Int. J. Immunogenet.* 36: 15-19.
- Blackwell, J. M., S. Searle, T. Goswami, and E. N. Miller. 2000. Understanding the multiple functions of Nramp1. *Microbes Infect.* 2:317-321.
- Blackwell, J. M., S. Searle, H. Mohamed, and J. K. White. 2003. Divalent cation transport and susceptibility to infectious and autoimmune disease: Continuation of the *Ity/Lsh/Bcg/Nramp1/Slc11a1* gene story. *Immunol. Lett.* 85:197-203.
- Canonne-Hergaux, F., S. Gruenheid, P. Ponka, and P. Gros. 1999. Cellular and subcellular localization of the Nramp2 iron transporter in the intestinal brush border and regulation by dietary iron. *Blood* 93:4406-4417.
- Capon, F., M. H. Allen, M. Ameen, A. D. Burden, D. Tillman, J. N. Barkerand, and R. C. Trembath. 2004. A synonymous SNP of the corneodesmosin gene leads to increased mRNA stability and demonstrates association with psoriasis across diverse ethnic groups. *Hum. Mol. Genet.* 13:2361-2368.
- Courville, P., R. Chaloupka, and M. F. Cellier. 2006. Recent progress in structure-function analyses of Nramp proton-dependent metal-ion transporters. *Biochem. Cell Biol.* 84:960-978.
- Crawley, A. M., B. Mallard, and B. N. Wilkie. 2005. Genetic selection for high and low immune response in pigs: Effects on immunoglobulin isotype expression. *Vet. Immunol. Immunopathol.* 108:71-76.
- Gazouli, M., V. Atsaves, G. Mantzaris, M. Economou, G. Nasioulas, K. Evangelou, A. J. Archimandritis, and N. P. Anagnou. 2008. Role of functional polymorphisms of NRAMP1 gene for the development of Crohn's disease. *Inflamm. Bowel. Dis.* 14:1323-1330.
- Hellemans, J., G. Mortier, A. De Paepe, F. Speleman, and J. Vandesompele. 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol.* 8:R19.
- He, X. M., M. X. Fang, Z. T. Zhang, Y. S. Hu, X. Z. Jia, D. L. He, S. D. Liang, Q. H. Nie, and X. Q. Zhang. 2013. Characterization of chicken natural resistance-associated macrophage protein encoding genes (*Nramp1* and *Nramp2*) and association with salmonellosis resistance. *Genet. Mol. Res.* 12:618-630.
- Hedges, J. F., E. Kimmel, D. T. Snyder, M. Jerome, and M. A. Jutila. 2013. Solute carrier 11A1 is expressed by innate lymphocytes and augments their activation. *J. Immunol.* 190:4263-4273.
- Kimchi-Sarfaty, C., J. M. Oh, I. W. Kim, Z. E. Sauna, A. M. Calcagno, S. V. Ambudkar, and M. M. Gottesman. 2007. A "silent" polymorphism in the *MDR1* gene changes substrate specificity. *Science* 315(5811):525-528.
- Liu, Y., X. T. Qiu, J. J. Xu, F. Hu, Y. H. Li, H. J. Li, Y. F. Gong, and Q. Zhang. 2011. Association analysis between the polymorphism of the SLC11A1 gene and immune response traits in pigs. *Asian J. Anim. Vet. Adv.* 6:580-586.
- Meilang, Q., Y. Zhang, J. Zhang, Y. Zhao, C. Tian, J. Huang, and H. Fan. 2012. Polymorphisms in the *SLC11A1* gene and tuberculosis risk: a meta-analysis update. *Int. J. Tuberc. Lung Dis.* 16:437-446.
- Pauly, T., K. Elbers, M. König, T. Lengsfeld, A. Saalmüller, and H. J. Thiel. 1995. Classical swine fever virus-specific cytotoxic T lymphocytes and identification of a T cell epitope. *J. Gen. Virol.* 76:3039-3049.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York, USA.
- Sapkota, B. R., M. Hijikata, I. Matsushita, G. Tanaka, R. Ieki, N. Kobayashi, E. Toyota, H. Nagai, A. Kurashima, K. Tokunaga, and N. Keicho. 2012. Association of *SLC11A1* (*NRAMP1*) polymorphisms with pulmonary *Mycobacterium avium* complex infection. *Hum. Immunol.* 73:529-536.
- Smit, J. J., G. Folkerts, and F. P. Nijkamp. 2004. Ramp-ing up allergies: *Nramp1* (*Slc11a1*), macrophages and the hygiene hypothesis. *Trends Immunol.* 25:342-347.
- Tanaka, G., J. Shojima, I. Matsushita, H. Nagai, A. Kurashima, K. Nakata, E. Toyota, N. Kobayashi, K. Kudo, and N. Keicho. 2007. Pulmonary *Mycobacterium avium* complex infection: Association with *NRAMP1* polymorphisms. *Eur. Respir. J.* 30: 90-96.
- Tuggle, C. K., C. B. Schmitz, and D. Gingerich-Feil. 1997. Rapid communication: cloning of a pig full-length NRAMP1 cDNA. *J. Anim. Sci.* 75:277.
- Tuggle, C. K., L. Marklund, T. J. Stabel, M. A. Mellencamp, and A. Stumbaugh. 2005. Genetic markers for screening animals for improved disease resistance (NRAMP). United States Patent, Patent No. :US 6,844,159 B2.
- Vidal, S. M., D. Malo, K. Vogan, E. Skamene, and P. Gros. 1993. Natural resistance to infection with intracellular parasites: isolation of a candidate for *Bcg*. *Cell* 73:469-485.
- Wu, H., D. Cheng, and L. Wang. 2008. Association of polymorphisms of *Nramp1* gene with immune function and production performance of Large White pig. *J. Genet. Genomics* 35:91-95.
- Wyllie, S., P. Seu, and J. A. Goss. 2002. The natural resistance-associated macrophage protein 1 NRAMP1 (formerly Nramp1) and iron metabolism in macrophages. *Microbes Infect.* 4:351-359.
- Yang, J. H., K. Downes, J. M. Howson, S. Nutland, H. E. Stevens, N. M. Walker, and J. A. Todd. 2011. Evidence of association with type 1 diabetes in the SLC11A1 gene region. *BMC Med. Genet.* 12:59.