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Characterization of chicken natural resistance-associated macrophage protein encoding genes (*Nramp1* and *Nramp2*) and association with salmonellosis resistance

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ABSTRACT. Natural resistance-associated macrophage protein 1 and 2 encoding genes (*Nramp1* and *Nramp2*) are related to many diseases. We cloned the cDNA of chicken *Nramp1* and *Nramp2* genes, characterized their expression and polymorphisms, and investigated the association of some SNPs with resistance to salmonellosis. The *Nramp1* cDNA was 1746 bp long and the *Nramp2* cDNA was 1938 bp long. These cDNAs are similar to previously reported cDNAs, varying by two and one amino acids, respectively. The chicken *Nramp1* gene expressed predominantly in liver, thymus and spleen in both females and males. The *Nramp2* gene expressed in almost all tissues, but

predominantly in breast muscle, leg muscle, cerebrum, cerebellum, lung, kidney, and heart in both females and males. We identified 45 SNPs and 2 indels in the chicken *Nramp1* gene; three of 13 SNPs in the exons were missense mutations (Arg223Gln, Ala273Glu and Arg497Gln). Association analysis indicated that A24101991G is significantly associated with chicken salmonellosis resistance. These results will be useful for functional investigation of chicken *Nramp1* and *Nramp2* genes.

Key words: Chicken; *Nramp1* gene; *Nramp2* gene; Cloning; Expression; Polymorphism

INTRODUCTION

Homologs of natural resistance-associated macrophage protein (Nramp) or solute carrier 11 (SLC11), conserved in eukaryotes and bacteria, form a family of proton-coupled transporters that maintain divalent metal (Me^{2+}, including Mn^{2+}, Fe^{2+}, Co^{2+}, and Cd^{2+}) homeostasis (Mackenzie and Hediger, 2004; Courville et al., 2006). There are two *Nramp* genes that are associated with diseases in vertebrates. *Nramp1* (also called *SLC11A1*) restricts microbial access to essential micronutrients such as Fe^{2+}, Mn^{2+}, Co^{2+}, and Zn^{2+} within professional phagosomes (Boyer et al., 2002; Jabado et al., 2000, 2003; Peracino et al., 2006; Cellier et al., 2007). It defends against various intracellular pathogens including *Salmonella typhimurim*, *Leishmania donovani* and *Mycobacterium bovis* (Vidal et al., 1993; Blackwell et al., 1999, 2000, 2003). *Nramp2* (also known as *SLC11A2*, *DMT1* or *DCT1*), expresses specifically at the apical membrane of epithelial cells or ubiquitously in recycling endosomes, plays an important role in regulating body iron levels and transports a broad range of divalent metal ions (Cu^{2+}, Mn^{2+}, Co^{2+}, and Pb^{2+}) (Gunshin et al., 1997; Gruenheid et al., 1999; Tandy et al., 2000; Trinder et al., 2000; Canonne-Hergaux et al., 1999, 2002; Touret et al., 2003). Recently, it was reported that Nramp are cellular receptors for Sindbis virus in both insect and mammalian hosts (Rose et al., 2011), but this issue was elusive according to other report (Stiles and Kielian, 2011).

Both Nramp1 and Nramp2 proteins have 12 conserved transmembrane domains (TM), two N-linked glycosylation sites, and a transport motif. These conserved domains perform various important functions. Mutations within TMs are likely to be of vital consequence to organisms. In Nramp1, mice with mutation G169D within TM4 renders them susceptible to intracellular pathogens (White et al., 2004). The deletion of the residue Thr178 impairs intermolecular interactions and alters the cooperativity in proton binding of Asp14 and Asp22 (Xue et al., 2009). In Nramp2, many variations are reported to decrease Me^{2+} absorption, to cause protein dysfunction, and to alter functional expression (Cohen et al., 2003; Lam-Yuk-Tseung et al., 2003, 2006; Courville et al., 2006; Mackenzie et al., 2006).

Polymorphisms of *Nramp* genes are associated with various diseases. Polymorphisms of the human *Nramp1* gene [5'(GT)n, D543N, and INT4G/C (rs3731865)] are significantly associated with Crohn's disease and rheumatoid arthritis (Gazouli et al., 2008; Ates et al., 2009). A 4-bp deletion polymorphism in the 3'-UTR is related to the incidence of post-transplant lymphoproliferative disease after liver transplantation (Barshes et al.,

2006). Some polymorphisms were further identified to be associated with type 1 diabetes (T1D) (Yang et al., 2011). The association between D543N polymorphism and tuberculosis is sex- and age-dependent in Chinese (Leung et al., 2007). The $(GT)_{13}$ allele of 3'-UTR was significantly associated with increased production of H_2O_2 and NO (Ganguly et al., 2008). In pig, a single nucleotide polymorphism (SNP) in the sixth intron of the *Nramp1* gene is associated with polymorphonuclear leukocyte levels, effect of cytotoxin on monocytes and 180-day-old body weight (Wu et al., 2008). In humans, the C allele of rs407135 in the *Nramp2* gene is related to shorter disease duration in sporadic amyotrophic lateral sclerosis patients (Blasco et al., 2011).

Until now, the variations of *Nramp* genes and their effects on disease have been investigated far less either in chicken or other birds. In this study, we aimed to clone *Nramp1* and *Nramp2* cDNA in Chinese local chickens, to identify the expression and variations of these two genes, as well as to investigate the associations of the *Nramp1* gene with salmonellosis resistance.

MATERIAL AND METHODS

Animals

Three male and three female Partridge chickens were purchased from the Institute of Animal Science, Guangdong Academy of Agricultural Science (Guangdong Province, China). Each bird was slaughtered to collect 20 tissues, including cerebrum, cerebellum, hypothalamus, pituitary, breast muscle, leg muscle, subcutaneous fat, abdominal fat, thymus, heart, liver, spleen, lung, kidney, gizzard, stomachus glandularis, small intestine, cecum, cloacal bursa, testis, or ovary. All tissues were stored in a -80°C freezer (Thermo Forma, USA) for long-term preservation. A total of 25 DNA samples were used to identify the polymorphisms across the whole genome of the chicken *Nramp1* gene. These samples were from five Chinese local populations including Qingyuan partridge, Wens partridge (WP), Guangdong partridge, Xinguang Tiejiao partridge, and Xinhua chicken with five random chickens representing each population. Two hundred WP chickens with phenotypic record of salmonellosis infection (or resistance) were used in association analysis. These samples were collected from Guangdong Wens Foodstuffs Group Co., Ltd. (Guangdong, China).

Primers

Based on the *Gallus gallus* mRNA sequence of the *Nramp1* gene on NCBI (GenBank: NM_204964.1), the *Nramp2* gene (GenBank: EF635922), the beta-actin gene (*β-actin*) (GenBank: NM_205518.1), and the genomic sequence of chicken on UCSC (http://genome.ucsc.edu/cgi-bin/hgBlat), 19 primer pairs were designed using the GeneTool Lite Launcher software (http://www.BioTools.com/) and then synthesized by Biosune Co. Ltd. (Shanghai, China) (Table 1).

cDNA and DNA preparation

Total RNA of all tissues was isolated by Trizol (Takara Co., Japan), and then used to amplify the cDNA of chicken *Nramp1* and *Nramp2* genes. Total RNA of all tissues was

reverse transcribed with the use of the PrimeScript® RT reagent kit with gDNA Eraser (Takara Co.) for real-time quantitative PCR analysis. Genomic DNA was extracted from the chicken blood with the standard phenol-chloroform method.

Table 1. Description of 19 primers used in this study.

Gene	Primer	Sequence	Annealing temperature (°C)	Product size (bp)	Purpose
Nramp1	P1	F: 5'-ggggggatgggggctataa-3' R: 5'-ccccacggcccagatgtaga-3'	61	1183	cDNA cloning
	P2	F: 5'-ttcggcgaggctttctac-3' R: 5'-gacggaacccatcagtgc-3'	59	749	
	P3	F: 5'-cccccacatcaccccgtcc-3' R: 5'-ggccccacactgcaggtctgac-3'	63	180	Real-time PCR
β-actin	P4	F: 5'-tggcattgctgacaggat-3' R: 5'-ctgcttgctgatccacat-3'	63	160	
Nramp2	P5	F: 5'-cggcccggctgctctctacat-3' R: 5'-gcagcacgcgggcaaagc-3'	63	140	
	P6	F: 5'-tggggagcagagcacgga-3' R: 5'-gccgggccgaagtaacag-3'	58	1188	cDNA cloning
	P7	F: 5'-tgcgctcttcgtctccttcatc-3' R: 5'-tgccgtcccacctccatctc-3'	60	828	
	P8	F: 5'-ggcggtgcggtcatcctc-3' R: 5'-cccagtgccatccccgtgt-3'	62	422	
Nramp1	P9	F: 5'-cccccaaccccatcctcttt-3' R: 5'-gcggggaacacgggcag-3'	64	628	Identification of variations
	P10	F: 5'-ccggcagggtcagcaactc-3' R: 5'-tcccagcttcatcccagagac-3'	57	707	
	P11	F: 5'-gggctttgagtgacacctgtg-3' R: 5'-ccgtttgggcatagggttagat-3'	57	759	
	P12	F: 5'-cccagaaagaggcgaccagta-3' R: 5'-cctgggatgcttgggagag-3'	64	726	
	P13	F: 5'-cccatcccctccaggtgcc-3' R: 5'-ggcctccttcacctcctcctte-3'	65	858	
	P14	F: 5'-catcgttggtgccatcatcat-3' R: 5'-cccaaaatagcagcccagga-3'	64	737	
	P15	F: 5'-cccaaacccaaccccctcgcta-3' R: 5'-ggtcgttcatgcccgtcagct-3'	64	701	
	P16	F: 5'-gccccgtcttttccccgcac-3' R: 5'-cggcggtgacatcgaagctga-3'	65	844	
Nramp1	P17	F: 5'-gggctttgagtgacacctgtg-3' R: 5'-tgccctgtgccttggtttt-3'	63	490	PCR-RFLP
	P18	F: 5'-gccagcatcttccccatcaac-3' R: 5'-accccaagtgaaccccctctgc-3'	63	461	
	P19	F: 5'-cccaaacccaaccccctcgcta-3' R: 5'-gggggtgcggggaaaagac-3'	64	575	

F and R indicate forward and reverse primers, respectively.

Cloning and sequencing

The total cDNA from liver, kidney, spleen, thymus, cecum, and cloacal bursa tissues was pooled to amplify the *Nramp1* and *Nramp2* genes by PCR. All required reagents were mixed according to instructions of KOD FX DNA polymerase (Toyobo, Japan). The PCR procedure was run at 94°C pre-denaturing for 2 min, followed by 32 cycles of 98°C for 10 s, the specific annealing temperature for 30 s (Table 1) and 68°C for 2 min, and a final extension of 10 min at 68°C. PCR products were purified with the E.Z.N.A® Gel reaction kit (Omega Bio-Tek, GA, USA). After using the DNA A-Tailing kit (Takara, Co.) to add poly(A) to the

3'-terminal at 72°C for 20 min, PCR products were cloned into PMD18-T vector (Takara Co.) and subsequently sent to Invitrogen Co. Ltd. (Shanghai, China) for sequencing.

Homology analysis

The cDNA and protein sequences of the *Nramp1* and *Nramp2* genes in various species were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/) (Table S1). The protein sequences were analyzed to indicate homology and phylogenetic relationship. In this study, MegAlign of the DNASTAR software package (http://www.dnastar.com) was used to calculate the percent identities among species and to construct the phylogenetic tree.

Variation analysis

According to the genomic sequence of the chicken *Nramp1* gene released by the UCSC database (http://mgc.ucsc.edu/cgi-bin/hgBlat), PCR was performed with 8 primer pairs (P9-P16). PCR products amplified from 25 DNA samples were sequenced by Invitrogen Co. Ltd. All sequences were blasted thoroughly to identify variations across the whole chicken *Nramp1* gene. Partial SNPs were genotyped with the PCR-RFLP method to analyze their associations with salmonellosis resistance in chicken.

Association of some SNPs with salmonellosis resistance

Using the MapDraw program of the DNASTAR software package (http://www.dnastar.com), restriction enzymes of *Bsu*36I, *Hha*I, *Bst*UIL, and *Ava*I specifically for C24103249G, A24101991G, C24098493T, and C24098282G, respectively, were used in PCR-RFLP analysis. Minor allele frequency and Hardy-Weinberg equilibrium were determined by the Plink 1.07 software (http://www.softpedia.com). Marker-trait association analysis was performed by the SAS 8.0 GLM procedure and the genetic effects were analyzed using the following model:

$$Y = \mu + G + Pi + e$$

where Y is the observation on the trait, μ is the overall population mean, G is the fixed effect of genotype, Pi is the fixed effect of parity, and e is the residual random error. Multiple comparisons were analyzed with least squares means. Significance was considered at $P \leq 0.05$.

Real-time (RT) quantitative PCR analysis

In this study, *β-actin* was used as internal control and SYBR green as fluorescent dye to quantify the mRNA level of chicken *Nramp1* and *Nramp2* genes by RT-PCR analysis. Each sample was repeated three times. The reaction mixture contained 10 µL 2X Q-PCR SYBR Green Mix (Toyobo), 0.2 µL 10 µM of each primer, 8.6 µL ultrapure RNase-free water, and 1 µL cDNA in a final volume of 20 µL. Running program was performed in a BIO-RAD CFX96 system (Bio-Rad, USA) as follows: 95°C for 1 min; 40 cycles of 95°C for 5 s and 63°C for 30 s; 65°C for 5 s, and 95°C for 5 s, and sample values were displayed when the run was over. Melting curve analysis was performed to confirm the specificity of the product. Chicken *Nramp1* and *Nramp2* mRNA relative

expression was indicated by $2^{-\Delta\Delta Ct}$, where ΔΔCt corresponded to the difference between the Ct measured for the mRNA level of each tissue and the Ct measured for the mRNA level of the reference tissue (the gizzard in this study), ΔCt = Ct (target gene) - Ct (β-actin).

RESULTS

Chicken *Nramp1* and *Nramp2* cDNA sequences

In this study, we obtained a 1746-bp cDNA for the chicken *Nramp1* gene, including 67-bp 5'-UTR, 11-bp 3'-UTR and 1668-bp open reading frame encoding 555 amino acids (Figure 1). We also obtained a 1938-bp cDNA for the chicken *Nramp2* gene, including 152-bp 3'-UTR and 1786-bp incomplete coding sequence encoding 594 amino acids (Figure 2). Moreover, we found that there were two single amino acid variations in the *Nramp1* gene (Met8Thr and Gly81Ser) and one amino acid variation (Ala537Val) in the *Nramp2* gene compared with the amino acid sequence of NP_990295.1 and ABV00877.1, respectively.

```
  gggggatgggggctataagagctgtgcgctgggtgcagcacccagaggtagaggttccccagcaccATGTCTGGATCCGGCCCTGCT
                                                                    M  S  G  S  G  P  A
ACGGCATCATTGGAGCCAGGCCTCGCTGGGTCCCTGAACCGAGGCCAAACTGACGCCAGCAATGTCCCTGTTCCCCCACATCACCCCGTC
[T] A  S  L  E  P  G  L  A  G  S  L  N  R  G  Q  T  D  A  S  N  V  P  V  P  P  H  H  P  V
CCCCATGCTCAGACCTACCTGGATGAGCTCATCAGCATCCCCAAAGGCAGCACGCCCGGCTTCAGCTTCAGGAAGCTGTGGGCTTTCACC
 P  H  A  Q  T  Y  L  D  E  L  I  S  I  P  K  G  S  T  P  G  F  S  F  R  K  L  W  A  F  T
GGCCCTGGCTTCCTGATGAGCATCGCCTACCTGGACCCGAGCAACGTGGAGTCAGACCTGCAGTGTGGGGCCGTGGCTGGCTTCAAGCTG
 G  P  G  F  L  M  S  I  A  Y  L  D  P [S] N  V  E  S  D  L  Q  C  G  A  V  A  G  F  K  L
CTGTGGGTGCTGCTGTGGGCCACAGTGCTGGGACTGCTGTGCCAGAGGTTGGCCATCCGCCTGGGCGTGGTGACGGGGAAGGACCTGGCA
 L  W  V  L  L  W  A  T  V  L  G  L  L  C  Q  R  L  A  I  R  L  G  V  V  T  G  K  D  L  A
GAGATCTGCTACCTCTATTACCCCAGGGTGCCCCGTGTGCTGCTGTGGCTCATGATGGAAATCGCCATCATCGGCTCAGACATGCAGGAG
 E  I  C  Y  L  Y  Y  P  R  V  P  R  V  L  L  W  L  M  M  E  I  A  I  I  G  S  D  M  Q  E
GTGATTGGGACGGCCATTGCCTTCAGCCTGCTCTCAGCCGGACGCATCCCTCTCTGGGGTGGTGTCCTCATCACCATCACAGACACCCTC
 V  I  G  T  A  I  A  F  S  L  L  S  A  G  R  I  P  L  W  G  G  V  L  I  T  I  T  D  T  L
TTCTTCCTCTTCCTTGACAAGTACGGGCTCCGCAAGCTGGAGGCCTTCTTCGGCTTCCTCATCACCATCATGGCTCTGACCTTTGGCTAC
 F  F  L  F  L  D  K  Y  G  L  R  K  L  E  A  F  F  G  F  L  I  T  I  M  A  L  T  F  G  Y
GAGTATGTGATGGTGCGACCGGCGCAGACGGAGGTGCTGAAGGGCATTTTCCTGCCCTACTGCCCGGGCTGCGGGCGAGAGGAGCTGCTC
 E  Y  V  M  V  R  P  A  Q  T  E  V  L  K  G  I  F  L  P  Y  C  P  G  C  G  R  E  E  L  L
CAGGCTGTGGGCATTGTTGGTGCCATCATCATGCCCCATAACATCTTCCTGCACTCCTCCTTGGTGAAGACCCGGGCGATCGACCGCTCC
 Q  A  V  G  I  V  G  A  I  I  M  P  H  N  I  F  L  H  S  S  L  V  K  T  R  A  I  D  R  S
AAGAAGGAGGAGGTGAAGGAGGCCAACATGTACTTCCTGACCGAGTCCTGCCTGGCTCTCTTCGTCTCCTTCCTCATCAACCTCTTCGTC
 K  K  E  E  V  K  E  A  N  M  Y  F  L  T  E  S  C  L  A  L  F  V  S  F  L  I  N  L  F  V
ATGGCCGTCTTCGGCGAGGCTTTCTACCACCAGCGCAATGAGGACGTGCATAACAAGTGCGTCAACAGCAGCGTCAGTCGCTATGCCAGC
 M  A  V  F  G  E  A  F  Y  H  Q  R  N  E  D  V  H  N  K  C  V  N  S  S  V  S  R  Y  A  S
ATCTTCCCCATCAACAATGAGACGGTGTCTGTGGATATCTACCAGGGGGGCGTCATCCTGGGCTGCTATTTTGGGGCTGCAGCGCTCTAC
 I  F  P  I  N  N  E  T  V  S  V  D  I  Y  Q  G  G  V  I  L  G  C  Y  F  G  A  A  A  L  Y
ATCTGGGCCGTGGGGATCCTGGCAGCAGGGCAGAGCTCCACCATGACAGGCACCTATGCGGGACAGTTTGTCATGGAGGGCTTCCTGCAG
 I  W  A  V  G  I  L  A  A  G  Q  S  S  T  M  T  G  T  Y  A  G  Q  F  V  M  E  G  F  L  Q
CTGCGCTGGTCTCGCTTCACCCGGGTGCTGTTCACTCGCTCCCTGGCCATCCTGCCCACCCTCTTCGTGGCCGCCTTTAGGGACGTGAGC
 L  R  W  S  R  F  T  R  V  L  F  T  R  S  L  A  I  L  P  T  L  F  V  A  A  F  R  D  V  S
CAGCTGACGGGCATGAACGACCTGCTCAACGTGCTGCAGAGCATCCTGCTGCCCTTCGCCGTGCTGCCCGTCCTCACCTTCACCAGCCTG
 Q  L  T  G  M  N  D  L  L  N  V  L  Q  S  I  L  L  P  F  A  V  L  P  V  L  T  F  T  S  L
CGCCCGCTCATGCATGACTTCGCCAACGGTCTCCTGGGGCAGGTGCTGATGTCCCTCATCACGGGCTTGGTGTGTGCCATCAATGTGTAC
 R  P  L  M  H  D  F  A  N  G  L  L  G  Q  V  L  M  S  L  I  T  G  L  V  C  A  I  N  V  Y
TTCGTGGTGGATTTCCTGCCCACGCTGCGGGGCCTGGGCTACCTCATCCCCCTGGGGCTGCTGCTGGTGGCCTACGTGGCCTTTGTCACA
 F  V  V  D  F  L  P  T  L  R  G  L  G  Y  L  I  P  L  G  L  L  L  V  A  Y  V  A  F  V  T
TACCTGCTCTGGACGTGCAGCATTGCTCACGGGGCACGCTTCCTGGCCAGGGGACGCTACAACCGGTTCAGCTTCGATGTCACCGCCGAC
 Y  L  L  W  T  C  S  I  A  H  G  A  R  F  L  A  R  G  R  Y  N  R  F  S  F  D  V  T  A  D
GTGCCGGGGCTGGCAGGGCCGCACTGAtgggttccgtc
 V  P  G  L  A  G  P  H  .
```

Figure 1. cDNA sequence and encoded amino acids of the chicken *Nramp1* gene. Capital letters show open reading frame (ORF) and lower case letters show 5'- and 3'-UTR. Capital letters below ORF indicate amino acids for each codon upside, and "." refers to the stop codon. Underlined nucleotides indicate a "TATA" box and the boxed amino acids indicate amino acid variation compared with reported database.

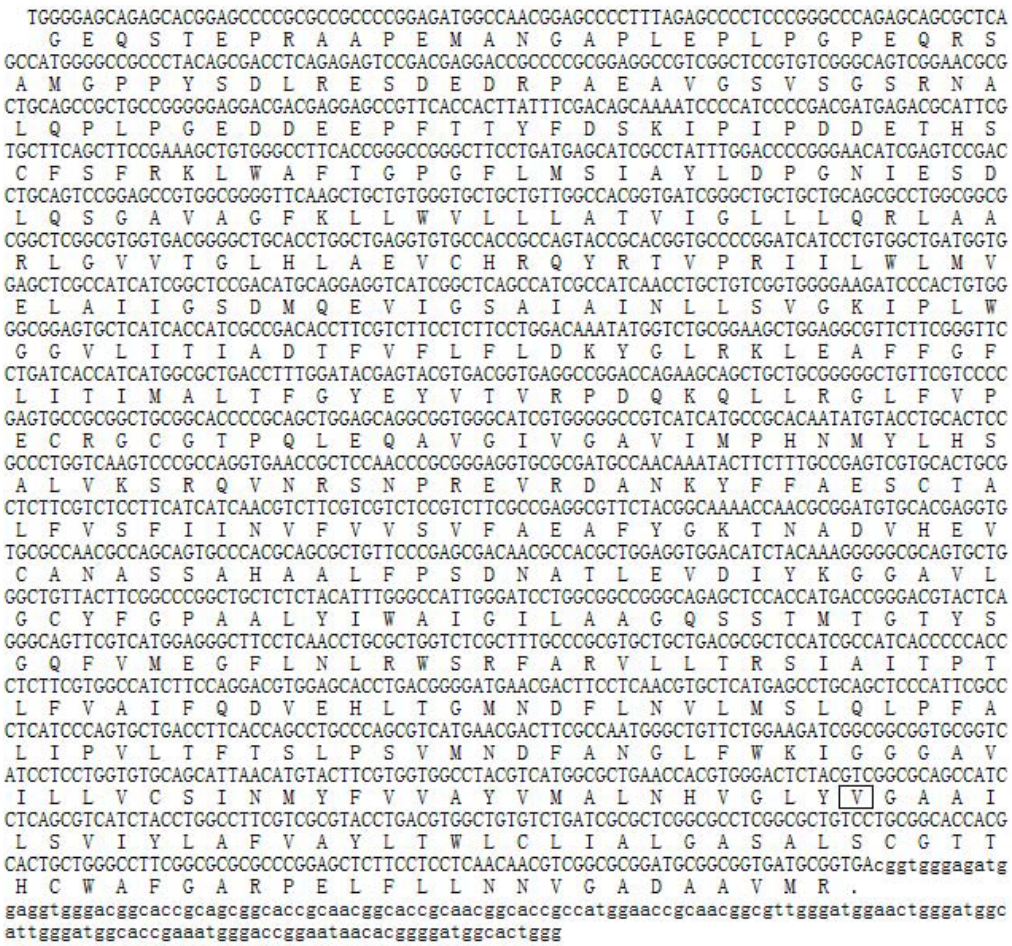

Figure 2. cDNA sequence and encoded amino acids of the *Nramp2* gene. Capital letters show CDS and lower case letters show 3'-UTR. Capital letters below CDS indicate amino acids for each codon upside, and "." refers to the stop codon. The boxed amino acid indicates amino acid variation compared with reported database.

Sequence analysis

Sequence analysis revealed that the chicken Nramp1 protein shared 67.3 to 69.7% identities with mammals (human, mouse, rat, pig, dog, cattle, sheep, deer, and horse), which are lower than that with quail (96.6%), but higher than that with fish (62.0%) (Figure S1A). The chicken Nramp2 protein shared 77.5 to 79.6% identities with mammals (human, mouse, rat, pig, dog, cattle, and horse), and 75.2 and 76.1% identities with *Danio rerio* and *Xenopus*, respectively (Figure S1B).

The constructed phylogenetic tree of the Nramp1 protein displayed three distinct groups of mammals, avian species (quail and chicken) and fish (Figure 3A), and similar results were shown by Nramp2 homology (Figure 3B).

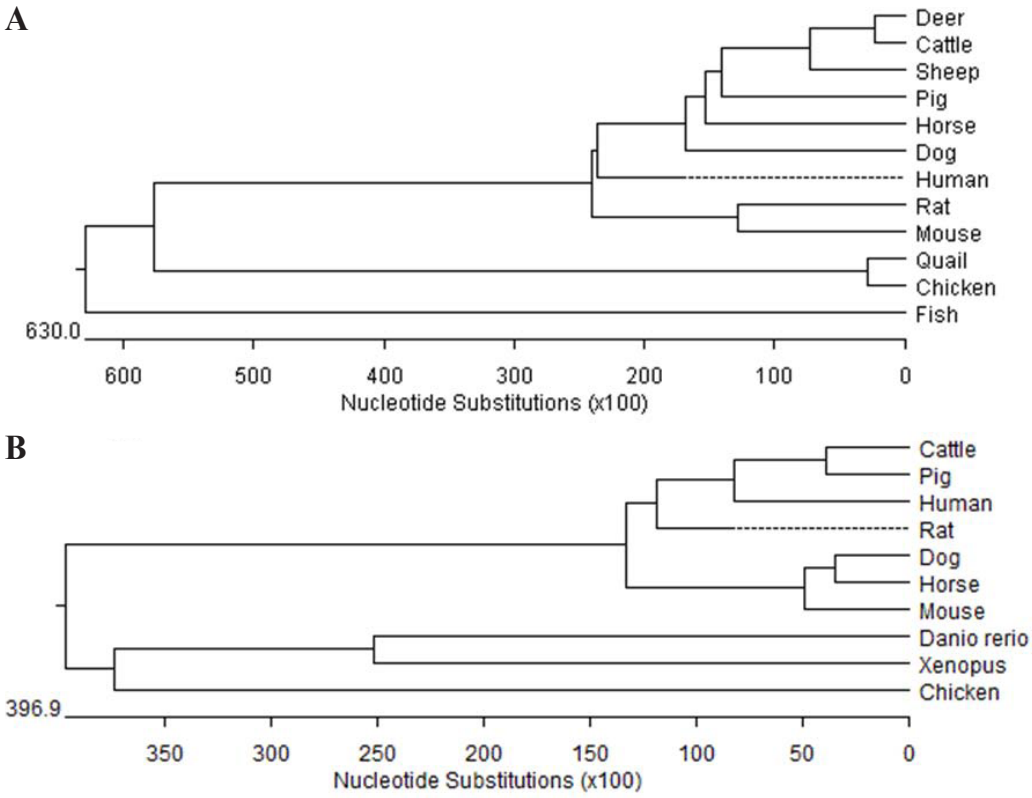

Figure 3. Phylogenetic tree among species based on *Nramp1* (**A**) and *Nramp2* (**B**) homologies.

SNPs

We identified in total 45 SNPs and 2 indels across the whole *Nramp1* gene (Table 2). Among these SNPs and indels, 11 were located in the 5'-flanking region or 5'-UTR, 13 in the coding region (3 led to amino acid substitutions: Arg223Gln, Ala273Glu and Arg497Gln) and the rest in the intron. Moreover, 42 SNPs were PCR-RFLP markers, as they could be recognized by restriction enzymes.

Association study

Four SNPs of C24103249G, A24101991G, C24098493T, and C24098282G were used in association analysis. C24103249G was located in the 5'-flanking region, whereas A24101991G and C24098493T were located in exons and were both synonymous mutations. In this study, the minor allele frequency for each of four SNPs was higher than 0.05. Association analysis showed that only A24101991G was significantly associated with salmonellosis resistance ($P < 0.05$), and that the A allele was advantageous for salmonellosis resistance (Table 3).

Table 2. Forty-five SNPs and two indels identified in the chicken *Nramp1* gene.

No.	Site	Region	SNP	No.	Site	Region	SNP
1	24103249	5'-flanking	C>G	25	24099597	In6	G>A
2	24103055	5'-flanking	G>A	26	24099590	In6	T>C
3	24102989	5'-flanking	T>A	27	24099570	In6	A>T
4	24102956	5'-flanking	T>G	28	24099386	Ex8	G>A
5	24102928	5'-flanking	C>T	29	24099253	Ex8	A>C
6	24102891	5'-flanking	G>A	30	24099202	In8	C>T
7	24102873	5'-flanking	T>C	31	24099187	In8	G>A
8	24102844	5'-flanking	T>C	32	24099155	In8	T>C
9	24102650	5'-UTR	C>T	33	24099122	Ex9	C>A
10	24102558-63	5'-UTR	TCCTCC>del.	34	24099076	Ex9	C>T
11	24102514	5'-UTR	G>A	35	24099049	Ex9	G>C
12	24101991	Ex2	A>G	36	24099007	Ex9	C>T
13	24101943	In2	T>A	37	24099004	Ex9	T>C
14	24101903	In2	C>G	38	24098949	In9	T>G
15	24101826	In2	A>G	39	24098937	In9	C>T
16	24101821	In2	G>A	40	24098916	In9	A>G
17	24100620	In2	T>C	41	24098903-02	In9	Insert TGGATGG
18	24100562	Ex3	C>T	42	24098514	Ex11	C>T
19	24100248	In4	G>A	43	24098493	Ex11	C>T
20	24100162	In4	G>A	44	24098394	In11	G>A
21	24100076	In4	G>A	45	24098282	In11	C>G
22	24099838	Ex5	C>T	46	24097633	In13	T>G
23	24099784	In5	T>C	47	24097516	Ex14	G>A
24	24099652	Ex6	G>A				

In and Ex indicate intron and exon regions, respectively.

Table 3. Associations of *Nramp1* SNPs with chicken salmonellosis resistance.

SNP	Case (DD/Dd/dd)	Control (DD/Dd/dd)	Minor allele frequency	P
C24103249G	167/21/11	164/22/13	C = 0.1143	0.897
A24101991G	127/66/6	153/43/4	G = 0.1617	0.0215*
C24098493T	121/69/9	131/62/7	T = 0.2043	0.603
C24098282G	117/66/14	131/61/8	G = 0.2154	0.274

Case = susceptible to salmonellosis; control = resistant to salmonellosis; d = minor allele; D = major allele; DD/Dd/dd show three genotypes for each locus. *P < 0.05.

Tissue-specific expression

RT-PCR indicated that the chicken *Nramp1* gene predominantly expressed in liver, thymus and spleen in both males and females, and in male subcutaneous fat and female small intestine, whereas little in gizzard, stomachus glandularis, cloacal bursa, and gonadal tissues in both males and females (Figure 4A). The chicken *Nramp2* gene expressed in almost all tissues, but predominantly in breast muscle, leg muscle, cerebrum, cerebellum, lung, kidney, and heart, and relatively less in gizzard, cecum and cloacal bursa in both males and females (Figure 4B). Moreover, the total *Nramp2* mRNA level of female chicken was higher than that of males (Figure 4B).

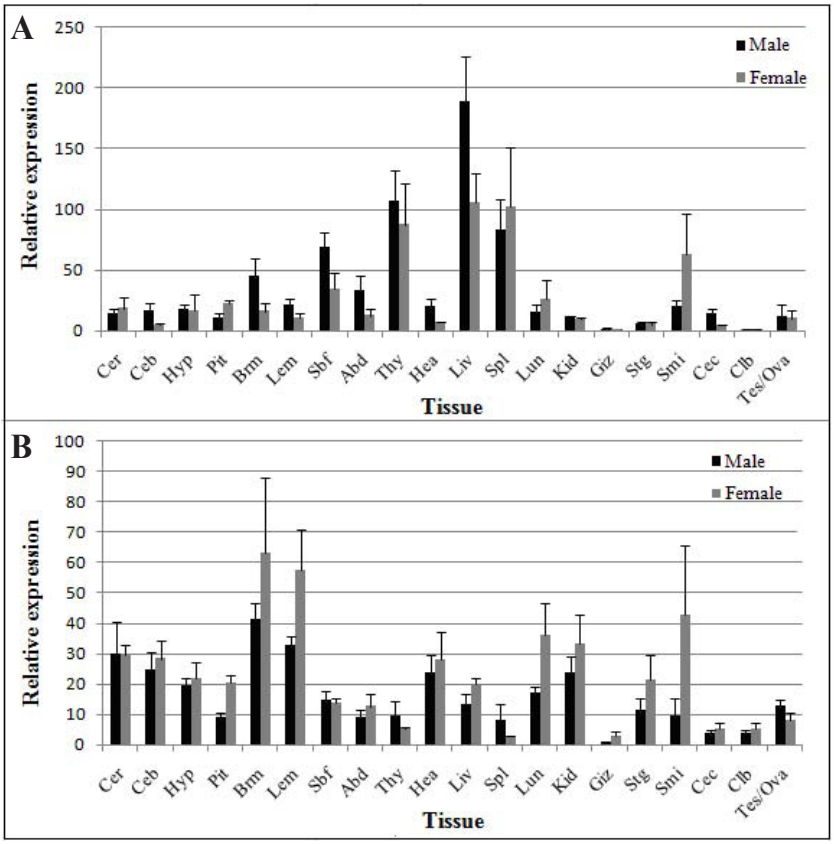

Figure 4. mRNA level of chicken *Nramp1* (**A**) and *Nramp2* (**B**) genes in different tissues. Cer = cerebrum; Ceb = cerebellum; Hyp = hypothalamus; Pit = pituitary; Brm = breast muscle; Lem = leg muscle; Sbf = subcutaneous fat; Abd = abdominal fat; thy = thymus; Hea = heart; Liv = liver; Spl = spleen; Lun = lung; Kid = kidney; Giz = gizzard; Stg = stomachus glandularis; Smi = small intestine; Cec = cecum; Clb = cloacal bursa; Tes = testis, and Ova = ovary.

DISCUSSION

In this study, we cloned the cDNA of the *Nramp1* and *Nramp2* genes in Chinese local chickens. We found two amino acid variations in the *Nramp1* gene (Met8Thr and Gly81Ser) and one amino acid variation in the *Nramp2* gene (Ala537Val) compared to the reported databases (NP_990295.1 and ABV00877.1). Another study also detected three amino acid variants in six chicken lines with *Salmonella*-resistant or *Salmonella*-susceptible phenotypes, namely Thr55Ala, Arg223Gln and Val307Ile (Hu et al., 1997).

Sequence analysis revealed that both Nramp1 and Nramp2 have high homology among all studied species (Figure S1). Similar to that in mammals, the chicken Nramp proteins (both Nramp1 and Nramp2) have 12 conserved TMs and a transport motif between TM8 and TM9, and the functional motifs are very conserved in various species. Moreover, these motifs are also conserved between Nramp1 and Nramp2, especially the first TM (Figure S2). This result is con-

sistent with previously reported studies (Courville et al., 2006; Lam-Yuk-Tseung et al., 2006).

The expression patterns of the chicken *Nramp1* and *Nramp2* genes are well studied in different tissues of males and females. The *Nramp1* gene expresses mainly in the phagosomal membrane of macrophages, where inflammatory stimuli, iron, and sodium nitroprusside increase expression (Baker et al., 2000). In this study, we revealed that the chicken *Nramp1* gene expressed mainly in liver, thymus and spleen by RT-PCR analysis, which was consistent with the reported results by the Northern blot method (Hu et al., 1996). It was previously reported that the levels of the *Nramp1* gene in resistant chickens were similar to those in susceptible ones (Hu et al., 1997). The *Nramp2* gene widely expresses in a majority of mammalian tissues (Gruenheid et al., 1995; Kishi et al., 1996). Our studies showed that the chicken *Nramp2* gene displayed wide expression in almost all tissues, and predominantly in breast muscle, leg muscle, cerebrum, cerebellum, lung, kidney, and heart.

We identified a total of 45 SNPs and 2 indels across the whole chicken *Nramp1* gene. Three SNPs lead to amino acid substitutions, namely Arg223Gln, Ala273Glu and Arg497Gln. The mutation Arg223Gln within the predicted TM5-6 region was also detected in chicken, and this allelic variant was specific to the susceptible line C and not observed in any of the resistant strains (Hu et al., 1997). Another study identified 37 SNPs in 3.1-kb genome region and found that the SNP in male chickens was significantly associated with the antibody level after vaccination and spleen *Salmonella* load (Liu et al., 2003). In this study, only A24101991G was significantly associated with salmonellosis resistance, and the A allele was advantageous for salmonellosis resistance. This SNP is therefore a potential marker for salmonellosis resistance in poultry production.

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Supplementary material

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