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Full Length Research Paper

Four new single nucleotide polymorphisms (SNPs) of toll-like receptor 7 gene discovered in Chinese ducks

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In order to reveal the single nucleotide polymorphisms (SNPs), genotypes and allelic frequencies of each mutation site of *TLR7* gene in Chinese native duck breeds, SNPs of duck *TLR7* gene were detected by DNA sequencing. The genotypes of 465 native ducks from eight key protected duck breeds were determined by polymerase chain reaction ligase detection reaction (PCR-LDR) method. The results show that four new SNPs (T2606C, T2739C, C2820T and A3284G) were discovered and three genotypes were all found in each mutation site. The first and the fourth SNPs resulted in two amino acid changes of Methionine (M) → Threonine (T) and Glutamine (Q) → Arginine (R), respectively. Allele C frequency of *TLR7* T2606C mutation site in Gaoyou (GY) duck breed was significantly higher than the corresponding allelic frequency in the other duck populations, and allele T frequency of T*LR7* C2820T mutation site in GY duck breed was also significantly higher than the corresponding allelic frequency in the other duck populations except Putian (PT) duck breed. In conclusion, the four new SNPs and the genetic differences among different duck breeds will promote duck disease resistance research.

Key words: *TLR7*, duck, single nucleotide polymorphism (SNP), polymerase chain reaction ligase detection reaction (PCR-LDR).

INTRODUCTION

Toll-like receptors (*TLR*s), a superfamily of pattern-recognition receptors (PRRs) played a pivotal role in host innate immunity against pathogen infection and bridge the innate and adaptive immunity. It had been shown that in mammals, *TLR7* could be an important recognizing receptor of the single-stranded RNA viruses and activated the innate immune responses through a signal transduction pathway (Kadowaki et al., 2001; Jurk et al., 2002; Crozat et al., 2004; Ito et al., 2005).

MacDonald et al. (2008) reported that chicken *TLR7* might exist in functional and non-functional alleles, or a downstream mediator in the pathway was dysfunctional in certain cell or poultry lines. Hulse-Post et al. (2005) reported that avian influenza H5N1 strains, which are highly pathogenic to chickens, rarely harm ducks.

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It is well-known that duck has higher disease resistance than chicken. China is particularly rich in duck genetic resources and 27 domestic duck breeds were identified by the state in 2002. There are some special duck breeds with high disease resistance in China. So, it is necessary to reveal the useful alleles of *TLR7* gene in so huge, a duck gene pool.

As there were no researches about *TLR* 7 SNPs in duck, we chose eight key protected native duck breeds by DNA pooling, sequencing and PCR-LDR methods and investigated the SNPs of *TLR7* gene to reveal the SNPs and difference of *TLR7* allele in the eight Chinese duck breeds.

MATERIALS AND METHODS

Specimen collection and DNA extraction

465 blood samples of eight native duck breeds were collected from

Table 1. The information of primer and *TLR7* SNPs for PCR.

Primer	Forward primer (5'-3')	Reverse primer (5'-3')	PCR length (bp)	SNP information
P1	CTTTTGCATGGCAATCCTTT	ATCACCCATTCATTCACAGC	392	C2606T, C2739T and C2820T
P2	ACCAGCGCCTTCTAGATGAA	TGTTGTAAGCCAGGGTATTGC	204	A3284G

Table 2. The information of probes for LDR.

SNP	Probe (5'-3')	
T2606C	2606C/T_modify	P-TCACAATTGCACTTGAAAGGATTGCTTTTTTTTTTTTTTTT-FAM
	2606C/T_T	TTTTTTTTTTTTTTTTGATCCACCAAACAAACCACACTGCA
	2606C/T_C	TTTTTTTTTTTTTTTTTGATCCACCAAACAAACCACACTGCG
T2739C	2739C/T_modify	P-CTCGCAAGTATACAGATCCAGGAAATTTTTTTTTTTTTTTTT-FAM
	2739C/T_T	TTTTTTTTTTTTTTTTTTACATGATCAAATAGGAGGTGTCCAA
	2739C/T_C	TTTTTTTTTTTTTTTTTTTACATGATCAAATAGGAGGTGTCCAG
C2820T	2820C/Treverse_modify	P-TATATTTCTGGGATGTGTGGTATAGTTTTTTTTTTTTTTTTTT-FAM
	2820C/Treverse_A	TTTTTTTTTTTTTTTTTTTTATGGTGTTTGCTGTTATGAGCCACT
	2820C/Treverse_G	TTTTTTTTTTTTTTTTTTTTTATGGTGTTTGCTGTTATGAGCCACC
A3284G	3284A/G_modify	P-GGCCATTCCAGGACAGAACTTCTGCTTTTTTTTTTTTTTTTTTTT-FAM
	3284A/G_A	TTTTTTTTTTTTTTTTTTTTTTGTAGGGCTGAGATCGAGGGTTAGTT
	3284A/G_G	TTTTTTTTTTTTTTTTTTTTTTTTGTAGGGCTGAGATCGAGGGTTAGTC

conservation farms or zones, respectively; Gaoyou duck (GY, N = 96), Jinding duck (JD, N = 52), Beijing duck (BJ, N = 55), Jianchang duck (JC, N = 57), Liancheng White duck (LC, N = 50), Youxian Sheldrake duck (YX, N = 52), Shaoxing duck (SX, N = 48), and Putian Black duck (PT, N = 55). DNA was isolated from the blood and extracted by phenol/chloroform mixture (Sambrook et al., 1989).

TLR7 SNPs discovering and genotyping

According to the mRNA sequence of duck *TLR7* (DQ888645) and the DNA sequence of chicken *TLR7* (FJ915600), one DNA pooling consisting of ten ducks in each breed were sequenced and 80 duck individuals (ten individuals in each duck breed) were also sequenced. After the SNPs in duck *TLR7* were discovered, all the genotyping experiments were done by the Shanghai BioWing Applied Biotechnology Company (http://www.biowing.com.cn/) using PCR-ligase detection reactions (PCR-LDR).

The target DNA sequences were amplified using a multiplex PCR method. The information of primers and SNPs for PCR is shown in Table 1. After the completion of the amplification, 1 µl of Proteinase K (20 mg/ml) was added, then heated at 70°C for 10 min and quenched at 94°C for 15 min. The ligation reaction for each subject was carried out in a final volume of 20 µl containing 20 mM Tris–HCl (pH 7.6), 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM dithiothreitol(DTT), 1 mM nicotinamide adenine dinucleotide (NAD), 0.1% Triton X-100, 10 µl of Multi-PCR product, 1 pmol of each discriminating oligo,1 pmol of each common probe and 0.5 µl of 40 U/µl Taq DNA ligase (New England Biolabs, USA). The LDR was performed using 30 cycles of 95°C for 4 min and 50°C for 2 min. The fluorescent products of LDR were differentiated by using capillary electrophoresis on ABI sequencer 377. The information of probes for LDR is shown in Table 2.

Sequence and data analysis

The mutation types and the changes of amino acid sequence were analyzed by the software of DNAMAN6.0.3.99. The genotype and allelic frequencies of the SNPs in *TLR7* gene were calculated by EXCEL2003. The assessment of statistical difference among allelic frequencies of the same allele was analyzed by t-test of SPSS12.0.

RESULTS

Four new SNPs (T2606C, T2739C, C2820T and A3284G) in duck *TLR7* gene were firstly discovered by DNA sequencing in this study. Meanwhile, three genotypes of each mutation site in 465 Chinese domestic ducks were identified by PCR-LDR method (Figures 1 and 2). Two SNPs of T2739C and C2820T, which occurred at the third position of DNA codons, were synonymous mutations. The other two SNPs of T2606C and A3284G which occurred at the second position of DNA codons were non-synonymous mutations. The latter two mutations resulted in two amino acid changes of Methionine (M) → Threonine (T) and Glutamine (Q) →

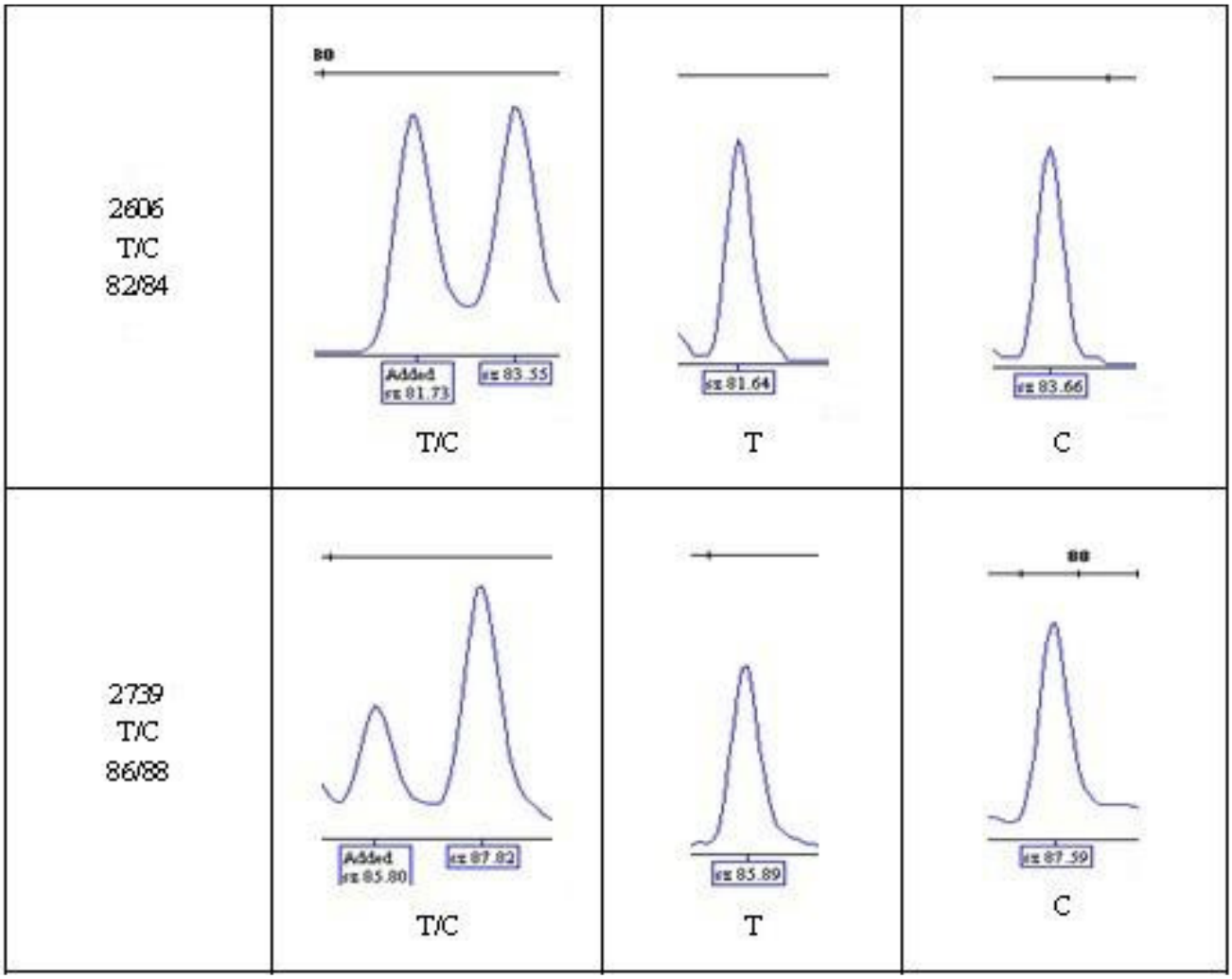

Figure 1. Genotyping by LDR method for two SNPs (T2606C and T2739C) in TLR7 gene (two peaks in capillary electrophoretogram indicated the heterozygote genotype (TC) of the two SNPs (T2606C and T2739C) in TLR7 gene; one peak (size: 82±0.5/86±0.5) in capillary electrophoretogram indicated the homozygous genotype (TT) of the two SNPs (T2606C and T2739C) in TLR7 gene; one peak (size: 84±0.5/88±0.5) in capillary electrophoretogram indicated the homozygous genotype (CC) genotype of the two SNPs (T2606C and T2739C) in TLR7 gene).

Arginine (R), respectively. Allelic frequencies of four sites of T2606C, T2739C, C2820T, and A3284G in each duck population are shown in Table 3. Allele C frequency of *TLR7* T2606C mutation site in GY duck breed was significantly higher than the corresponding allelic frequency in the other duck populations, and allele T frequency T*LR7* C2820T in GY duck breed was significantly higher than the corresponding allelic frequency in the other duck populations except PT duck breed.

DISCUSSION

In this study, PCR-LDR was performed to detect the mutations of *TLR7* gene in duck. It can detect mutations with a sensitivity of approximately one mutant allele in 4000 normal alleles (Khanna et al., 1999). The LDR used two adjacent primers and a thermostable ligase to distinguish all four bases potentially found at any position in a DNA sequence (Barany, 1991a, b; Day et al., 1995; Khanna et al., 1999). Compared to other mutation detecting methods such as DNA sequencing, microarray, Taqman, RFLP and SSCP, which were either too expensive or time consuming, the PCR-LDR method had many advantages, such as being cheap, time saving, highly sensitive and quantitative (Shu et al., 2011).

TLR genes had been recognized in a number of vertebrate genomes, and many partial and full-length sequences were available. Although, duck *TLR7* gene mRNA complete sequence (DQ888645) had already been reported by MacDonald et al. (2008), there was no report on the polymorphisms of duck *TLR7* gene. The four new SNPs (T2606C, T2739C, C2820T and A3284G) in duck *TLR7* gene were first reported in our study. The

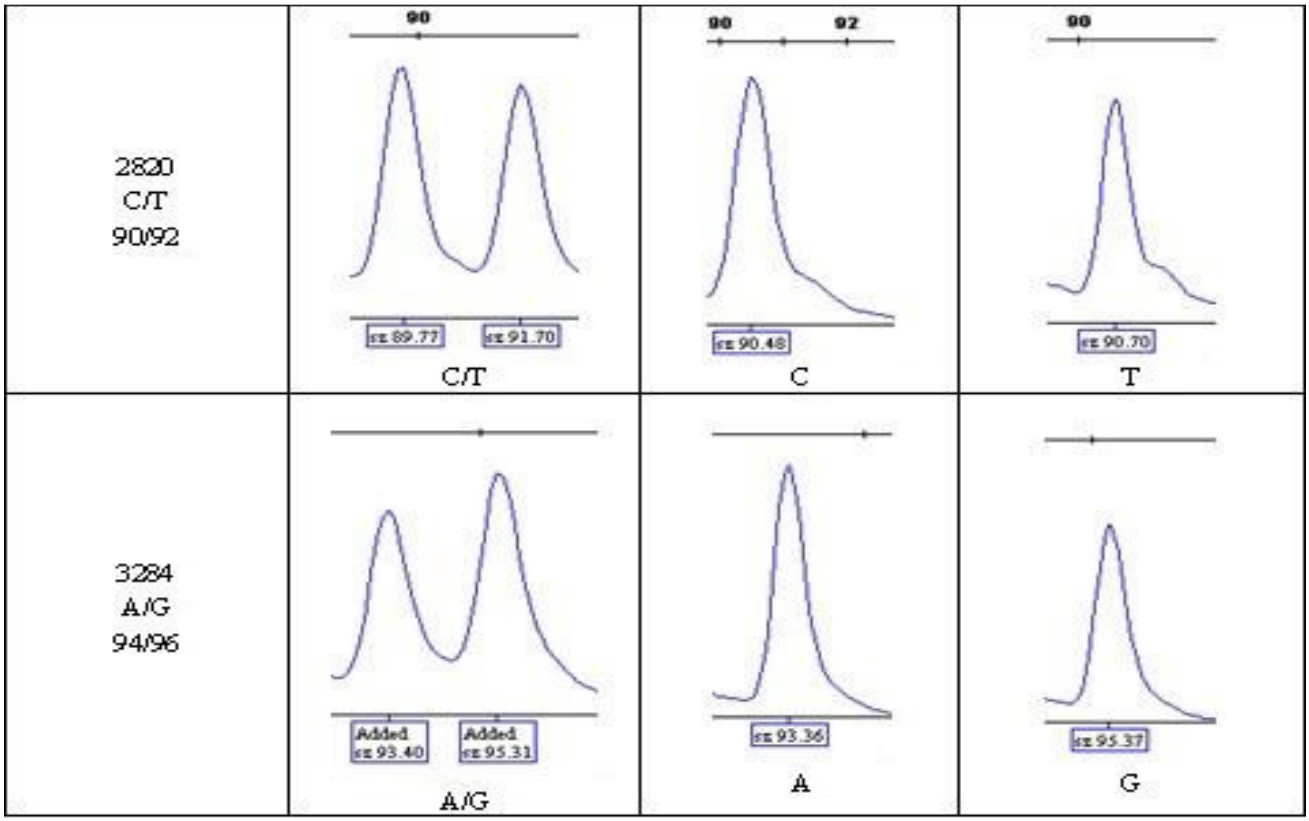

Figure 2. Genotyping by LDR method for two SNPs (C2820T and A3284G) in TLR7 gene (two peaks in capillary electrophoretogram indicated the heterozygote genotype(CT/AG) of the two SNPs (C2820T and A3284G) in TLR7 gene; one peak (size:90±0.5/94±0.5) in capillary electrophoretogram indicated the homozygous genotype(CC/AA) of the two SNPs (C2820T and A3284G) in TLR7 gene; one peak (size: 92±0.5/96±0.5) in capillary electrophoretogram indicated the homozygous genotype(TT/GG) of the two SNPs (C2820T and A3284G) in TLR7 gene).

Table 3. Allelic frequencies of four sites of T2606C, T2739C, C2820T, A3284G in each duck population.

Breed	Sample size	T2606C		T2739C		C2820T		A3284G	
		T	C	T	C	T	C	A	G
GY	96	0.69	0.31*	0.85	0.15	0.69	0.31	0.54	0.46
JD	52	0.97	0.03	0.66	0.34	1	0	0.66	0.34
BJ	55	0.99	0.01	0.58	0.42	0.99	0.01	0.49	0.51
JC	57	0.85	0.15	0.85	0.15	0.99	0.01	0.47	0.53
LC	50	0.99	0.01	0.75	0.28	1	0	0.42	0.58
YX	52	0.97	0.03	0.73	0.27	0.97	0.03	0.37	0.63
SX	48	0.90	0.10	0.72	0.28	0.95	0.05	0.56	0.44
PT	55	0.86	0.14	0.79	0.21	0.83	0.17	0.44	0.56

significant difference of certain allele frequency among different duck breeds may indicate that breed differences in *TLR* polymorphisms may in part reflect the genetic difference and breed diversity of innate immune ability. There were many researches investigating polymorphisms of *TLR7* gene in mammal. Møller-Larsen et al. (2008) analyzed the association between single SNP and haplotype of *TLR7* in 984 individuals between asthma, rhinitis, atopic dermatitis and increased specific IgE. The results provided strong evidence that *TLR7* might confer susceptibility to asthma and related atopic disorders. Sánchez et al. (2009) reported that the tested variations of *TLR5* and *TLR7* genes did not confer a relevant role in the susceptibility or severity to SLE in the

Spanish population. But in poultry, we cannot find any researches in variations of *TLR7* gene. In this research, we found four new SNPs and the genetic differences among different duck breeds. Besides, there were two non-synonymous mutations in duck *TLR7* gene. Along these new meaningful findings, further researches in duck TLR7 protein function and innate immune ability is advised.

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REFERENCES

Barany, F (1991a) Genetic disease detection and DNA amplification using cloned. Proc. Natl Acad. Sci. 88: 189-193.

Barany, F (1991b). Ligase chain reaction in a PCR World. PCR Methods Appl., 1: 5-16.

Crozat K, Beutler B(2004). *TLR7*: a new sensor of viral infection. Proc. Natl.Acad. Sci. 101: 6835-6836.

Day DJ, Speiser PW, White PC, Barany F(1995). Detection of steroid 21-hydroxylase alleles using gene-specific PCR and a multiplexed ligation detection reaction. Genomics. 29:152-62.

Hulse-Post DJ, Sturm-Ramirez KM, Humberd J, Seiler P, Govorkova EA, Krauss S, Scholtissek C, Puthavathana P, Buranathai C, Nguyen TD, Long HT, Naipospos TSP, Chen H, Ellis TM, Guan Y, Peiris JSM, Webster RG(2005). Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. Proc. Natl. Acad. Sci. 102: 10682-10687.

Jurk M, Heil F,Vollmer J, Schetter C, Krieg AM, Wagner H, Lipford G, Bauer S(2002).Human *TLR7* or *TLR8* independently confer responsiveness to the antiviral compound R-848. Nat. Immunol.3: 499.

Kadowaki N, Ho S, Antonenko S, Malefyt RW, Kastelein RA, Bazan F, Liu YJ(2001). Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. J. Exp. Med. 194: 863-869.

Khanna M, Park P, Zirvi M, Cao W, Picon A, Day J, Paty Pand Barany F (1999). Multiplex PCR/LDR for detection of K-ras mutations in primary colon tumors. Oncogene, 18: 27-38.

Ito T, Wang YH, Liu YJ (2005). Plasmacytoid dendritic cell precursors/typeI interferon-producing cells sense viral infection by toll-like receptor (*TLR*)7 and *TLR*9. Springer Semin. Immunopathol. 26: 221-229.

MacDonald MRW, Xia J, Smith AL, Katharine EM (2008). The duck toll like receptor 7: Genomic organization, expression and function. Mol. Immunol. 45: 2055-2061.

Møller-Larsen S, Nyegaard M, Haagerup A, Vestbo J, Kruse T A, Børglum A D (2008). Association analysis identifies *TLR7* and *TLR*8 as novel risk genes in asthma and related disorders. Thorax. 63: 1064-1069.

Sambrook J, Fritsch EF, Maniatis T(1989). Molecular Cloning: a laboratory manual. 2nd ed. N.Y., Cold Spring Harbor Laboratory Press, Cold Spring Harbor Laboratory.

Shu JT, Zhang Y, H W, Yin PA, Li HF (2011). A typing system based on ligase detection reaction for myogenin gene polymorphsim in Chicken.J. Anim. Vet. advances. 10: 29-32.

Sánchez E, Callejas-Rubio JL, Sabio JM, Gónzalez-Gay MA, Jimenez-Alonso J, Micó L, de Ramón E, Camps M, Suarez A, Gutierrez C, Garcia-Portales R, Tolosa C, Ortego-Centeno N, Sánchez-Román J, Garcia-Hernández FJ, Gónzalez-Escribano MF, Martin J (2009). Investigation of *TLR5* and *TLR7* as candidate genes for susceptibility to systemic lupus erythematosus. Clin. Exp. Rheumatol. 27: 267-271.