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Full Length Research Paper

Genetic variation at Exon2 of TLR4 gene and its association with resistant traits in chicken

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This study was conducted to analyze the polymorphisms of chicken Toll-like receptors 4(TLR4) gene and aimed to provide a theoretical foundation for a further research on correlation between chicken TLR4 gene and disease resistance. Genetic variations at exon 2 of TLR4 gene in 14 chicken breeds and the red jungle fowl were detected by PCR-SSCP method and two alleles and three genotypes were found, Tibetan chicken and red jungle fowl only had BB genotype, while the others presented three genotypes of AA, BB and AB. Sequencing results showed two mutations, G114A and G142A, located at exon 2 of TLR4 gene. The results of Chi square test showed that all populations, except Xianju chicken, were in accordance with Hardy-Weinberg equilibrium at this locus (P > 0.05). According to analysis of population genetic variation, all the populations were at moderate polymorphism (0.25 < PIC < 0.5) except red jungle fowl and Tibetan chicken (PIC = 0). The study demonstrated that there were differences of normal anti-disease ability in Chinese indigenous chicken breeds and appeared no significant correlation with body size, product type and geographical location. The associated analysis of results showed that the SNPs of TLR4 gene in the study were not linked with potential major loci or genes affecting some resistant traits.

Key words: Chicken, TLR4 gene, polymorphism, resistant traits.

INTRODUCTION

In the modern livestock farming, diseases has severe effect not only livestock and poultry's health, especially communicable diseases, but also performances and the quality of animal products, huge economic losses were caused. Since 2000, resistant to diseases have become an important aim of improvement, breeding for disease resistance have been receiving more and more attention. Toll-like receptor stems from toll protein of *Drosophila melanogaster* and it not only participates in the process of polarity formation of *D. melanogaster* dorsoventral, but also can mediate *D. melanogaster* to produce innate immunity to infection of microbe directly (Medzhitov et al., 1997). In 1997, toll protein of man is homologous with *D. melanogaster*, which was found for the first time. Since TLRs have been reported, there are 13 members of TLR4 found in mammals (Beutler, 2005) (man and mice), and TLR11 was only found in mice, relating to anti toxoplasma infection and playing an important role in pathogen-associated molecular pattern (PAMP) of pathogenic bacteria in man and many species of animals (Hirschfeld et al., 2000; Ozinsky et al., 2000).

In the large family of TLRs, people's interests focused on TLR4, as a major receptor for the recognition of bacterial lipopolysaccharide, it can recognize many pathogenic microorganisms. The genetic difference in these receptor genes can cause difference in the resistance to various pathogens (Werling and Jungi, 2003; Machida et al., 2006; Akira and Takeda, 2004). Recognition ability of TLR4 to LPS among individuals is remarkably different. In 2000, Arbour's experiment results showed that the polymophism of coding region of TLR4 genes was the cause of these differences and individual gene functional variation was low-response to LPS (Werling and Jungi, 2003; Poltorak et al., 2000).

It is notable that the genetic variations at the TLR4 locus

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of humans and mice affect the susceptibility and resistance to diseases to a certain extent; SNPs of TLR4 also commonly exist in other animals, such as chicken, pig and cow. TLR4 genotype is usually related to the susceptibility and resistance to diseases in animals. For example, Leveque et al. (2003) proposed that the polymorphism of chicken TLR4 was related to the susceptibility to salmonella. Goldammer et al. (2004) reported that the copy number of TLR4 in the mammary gland of dairy cows increased with mastitis, Wang et al. (2007) analyzed genetic polymorphism of the TLR4 gene and its relationship to mastitis and Shinkai et al. (2006) and Zhou et al. (2008) discussed polymorphism in the coding region of swine TLR4 gene that consists of 3 exons and SNPs were found only in exon 3.

Now that TLR4 is known to play an extremely important role as a key linkage between innate immunity and specific immunity. But to date, no mutation has been detected in exon 2 in chicken. The correlation between polymorphism of TLR4 gene and its resistance to disease have rarely been reported in china. This study analyzed the polymorphism of chicken TLR4 exon 2 in the red jungle fowl and domestic and international chicken breeds using the PCR-SSCP method. The red jungle fowl is the ancestor of domestic chicken. General speaking, resistibility of Red Jungle fowl living in the natural state was strong. It looks forward to providing some experimental basis for further research on the functions of the TLR4 gene.

MATERIALS AND METHODS

Experimental material

15 chicken breeds: Wenchang chicken (WC)(70), Anka chicken (AK)(70), Chahua chicken (CH)(43), Gushi chicken (GS)(38), Henan Game (HG)(33), Silkies (SI)(41), Xianju chicken (XJ)(40), Luyuan chicken (LY)(44), Dagu chicken (DG)(35), Xiaoshan chicken (XS)(41), fatty chicken (FA)(42), and Tibetan chicken (TI)(24) were from local avian resources gene bank of Institute of Poultry Science, Chinese Academy of Agriculture; Huainan spotted-brown chicken (HS)(30) were obtained from Huainan Academy of Agricultural sciences of Anhui province; Wannan three-yellow chicken (WT)(30) were obtained from Chizhou city, Anhui province; Red Jungle fowl (RJ)(24) were collected from the Yunnan Wildlife Rescue and Rehabilitation Centre. Genome DNA was obtained by phenol and chloroform (1:1) extraction and stored at -20°C. The immunological traits were determined in accordance with the methods of Liying and Chun (An, 2000; Wu, 2007).

Primer design and PCR amplification

Based on the published red jungle fowl TLR4 gene sequences (No. AY064697) in GenBank, pair primers were designed using the Oligo 6.0 programs to amplify the exon 2 of TLR4 gene and detect the single nucleotide polymorphisms. Primer sequence: F:5′-ATC TGCCACAGGTCATCC-3′, R: 5′-AGCCACGAGACTCCAAA-3′. The predicted amplified fragment is 248 bp. The primers were synthesized by Shanghai BioAsia Biotechnology Co. Ltd, Taq DNA polymerase, dNTP were purchased from TaKaRa Biotechnology (Dalin) Co. Ltd.

Following optimization, PCR reactions were carried out in a total volume of 20 µl with 100 ng of genomic DNA, 10×PCR buffer 2.0 µl, 2.5 mmol/l dNTP 1.5 µl, 10 µmol/l primer(forward)1 µl, 10 µmol/L primer(reverse)1 µl, 1.0 U of Taq DNA polymerase (Fermentas) and ddH_2O 13.3 µl. The PCR amplification program: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 50 s, annealing at 60°C for 30 s, extension at 72°C for 50 s and a final extension at 72°C for 10 min. The products were examined by electrophoresis on 2% agarose gels stained with 0.25 µg/ml ethidiumbromide (Figure 1).

Single stranded sonformation polymorphism (SSCP) analysis

As the gel loading sample, 10 µl of loading buffer was mixed with 15 µl of PCR product, denatured at 98°C for 15 min and then incubated on ice for 10 min. All the denatured ice-cold mixtures were loaded onto 10% non-denaturing polyacrylamide gel (Acr: Bis= 49:1) for electrophoresis. The electrophoresis was carried out at 120 V for 12 to 14 h and the gel was subsequently silver stained.

Sequencing

According to the analysis results of PCR-SSCP, homozygotes of different genotypes were selected for sequencing. After electrophoresis detection, the targeted fragments in the PCR products were recovered by a gel extraction kit (BBI, Canada). After purification, these fragments were sent to Sangon Biotech (Shanghai) Co., Ltd. for a bi-directional sequencing.

Statistical analysis

The genotype and allele frequency distributions were compared by Chi-square test; POPGENE software was used to determine whether the individual variant was in Hardy-Weinberg equilibrium and estimate the genetic variation analysis in population. The statistical analyses were carried out using SPSS 13.0 software. The association between TLR4 and immunological traits was evaluated according to one-way analysis, using the following model:

$$Y = \mu + G + C + G \times C + e$$

Where, Y is the dependent variable (analyzed traits); µ is the overall mean; G is the genotype of TLR4 exon-2 (AA, AB and BB); C is the chicken population; G × C is the interactions between genotype and chicken population (all fixed effects) and e is the random error.

RESULTS

PCR-SSCP analysis results

PCR amplification was carried out using the designed primers. The length of the amplified fragment was in accordance with the predicted, without nonspecific bands. Therefore, SSCP analysis was performed. Three genotypes were detected and defined as AA, BB and AB, with two alleles of A and B (Figure 2).

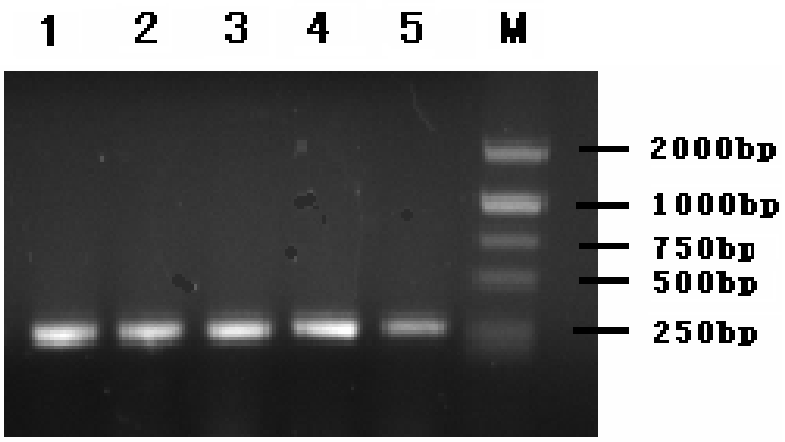

Figure 1. Agarose gel electrophoresis on PCR product of the exon 2 of TLR4 gene. M: marker; 1-5: PCR product.

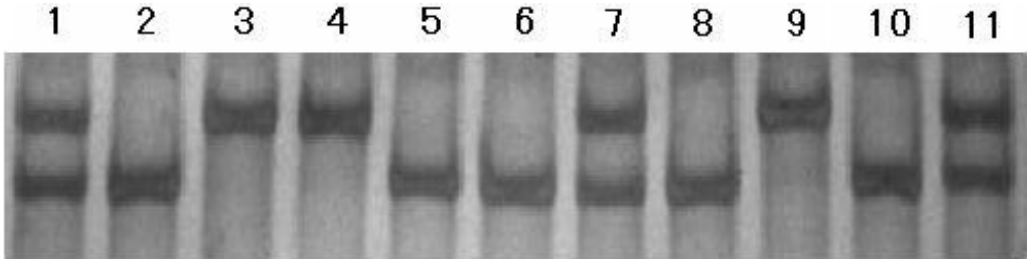

Figure 2. SSCP analysis on PCR product. 2, 5, 6, 8, 10, AA; 3, 4, 9, BB; 1, 7, 11, AB.

Sequencing analysis results

The sequencing results were compared with Red Jungle fowl TLR4 gene sequences (No. AY064697) in GenBank and with an identical sequence to the one from GenBank, genotype BB was defined as the wild type and AA had two G/A substitution mutations in 114 and 142 bp, respectively, namely G114A and G142A (Figure 3). Both mutations were located in exon 2.

After nucleotide sequence was translated into amino acid sequence and it showed that the amino acid of G114A did not change still Ile, so, the G114A was silent mutation. G142A changed the amino acid from Glu to Lys and the mutation was sense mutation.

The analysis of allele frequencies and genotype frequencies of TLR4 exon 2 in different chicken breeds

Samples from individual chickens of various breeds were assayed by PCR-SSCP. The frequencies were calculated for all genotypes and their alleles of each breed. Table 1 shows Tibetan chicken and red jungle fowl were detected only BB genotype and three genotypes of AA, BB and AB were appeared in the other chicken breeds. Through the test of Hardy-Weinberg with Chi-square test in 15 populations, almost all were in accordance with Hardy-Weinberg equilibrium, except Gushi chicken (Table 1).

The analysis of genetic variation in TLR4 gene

Result of Table 2 indicated that heterozygosity, PIC and effective numbers of alleles of the group detected average of 0.4921, 0.3710 and 1.9688. Heterozygosity and polymorphic information content (PIC) of Tibetan chicken and Red Jungle fowl were all 0, and in 13 other groups, heterozygosity, PIC and effective numbers of alleles of Henan Game were the highest, 0.5000, 0.3750 and 2.0000, respectively; while, the lowest in Wenchang chicken, which were 0.3368, 0.2801 and 1.5077, respectively (Table 2).

Interaction between the TLR4 gene exon-2 and genetic background of three chicken populations

The interactions between the genotypes of exon2 in TLR4

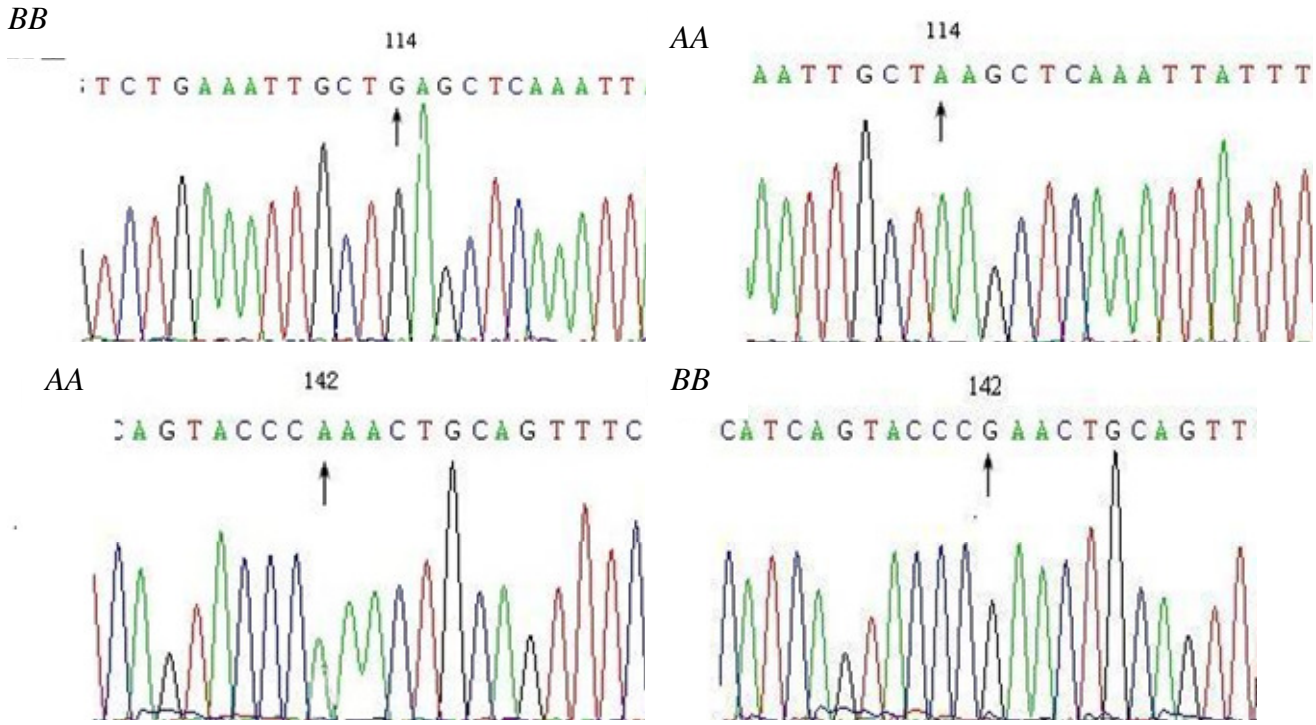

Figure 3. The mutation in 114 and 142 nucleotide acid between AA and BB genotype.

Table 1. Allele frequencies and genotype frequencies in TLR4 exon 2 of different chicken breeds.

Population	Number	Genotype frequency			Allele frequency		Chi-square test
		AA	BB	AB	A	B	x^2
WC	70	0.0429	0.6143	0.3429	0.2143	0.7857	0.00
AK	70	0.3143	0.2857	0.4000	0.5143	0.4857	1.23
CH	43	0.4419	0.1395	0.4186	0.6512	0.3488	0.22
GS	38	0.3158	0.5000	0.1842	0.4079	0.5921	7.95*
HG	33	0.2424	0.2424	0.5152	0.5000	0.5000	0.00
SI	41	0.6341	0.2927	0.2927	0.7805	0.2195	0.37
XJ	40	0.2250	0.2000	0.5750	0.5125	0.4875	0.62
LY	44	0.1136	0.4091	0.4773	0.3523	0.6477	0.01
DG	35	0.3429	0.1143	0.5428	0.6143	0.3857	0.26
XS	41	0.4634	0.1463	0.3903	0.6586	0.3414	0.24
FA	42	0.1905	0.3571	0.4524	0.4167	0.5833	0.11
WT	30	0.2333	0.3000	0.4667	0.4667	0.5333	0.02
HS	30	0.2000	0.2667	0.5333	0.4667	0.5333	0.15
TI	24	0.0000	1.0000	0.0000	0.0000	1.0000	0.00
RJ	24	0.0000	1.0000	0.0000	0.0000	1.0000	0.00
Average	/	0.2507	0.3912	0.3727	0.4371	0.5629	/

WC, Wenchang chicken; AK, Anka chicken; CH, Chahua chicken; GS, Gushi chicken; HG, Henan Game; SI, Silkies; XJ, Xianju chicken; LY, Luyuan chicken; DG, Dagu chicken; XS, Xiaoshan chicken; FA, fatty chicken; TI, Tibetan chicken; HS,

Table 2. The genetic variation analysis in TLR4 exon 2 of different chicken breeds.

Population	PIC	H	Ne
WC	0.2801	0.3368	1.5077
AK	0.3748	0.4996	1.9984
CH	0.3511	0.4543	1.8324
GS	0.3664	0.4830	1.9344
HG	0.3750	0.5000	2.0000
SI	0.2839	0.3426	1.5212
XJ	0.3748	0.4997	1.9988
LY	0.3522	0.4564	1.8395
DG	0.3616	0.4739	1.9007
XS	0.3486	0.4497	1.8172
FA	0.3680	0.4861	1.9460
WT	0.3739	0.4978	1.9912
HS	0.3739	0.4978	1.9912
TI	0.0000	0.0000	1.0000
RJ	0.0000	0.0000	1.0000
Average	0.3710	0.4921	1.9688

gene and genetic background of three chicken populations (WC, AK and RG) are shown in Table 3. Only the interleukin and SRBC had population effect ($P < 0.05$) and then, other traits were not significant in genotypes, populations and genotypes and populations effect ($P > 0.05$).

DISCUSSION

TLR4 gene is highly related to the susceptibility and resistance of animals to pathogens. For instance, Leveque et al. (2003) thought that TLR4 gene polymorphism was relevant to susceptibility to *Salmonella*. Generally speaking, resistibility of Red Jungle fowl living in the natural state and Tibetan chicken living in the semi-natural situation was strong. The exon 2 of the TLR4 gene is highly conserved in Red Jungle fowl and Tibetan chicken, with only the BB genotype detected. This suggested that BB genotype may be advantageous in chickens' immune response and B allele may be associated with general resistance. Genotypes AA, BB and AB can be measured in the other chicken breeds. It suggested that the appearance of A allele in the process of evolution may result from environment, selection intensity, artificial selection, evolutionary pressure and so on.

The results of Chi-square test showed that Gushi chicken noticeably deviate form Hardy-Weinberg equilibrium ($P < 0.05$) and the reason for this imbalance may be associated with artificial selection and might also be related to breed it. The other breeds and Red Jungle fowl were in accordance with Hardy-Weinberg equilibrium ($P>0.05$). Maybe the inherit advantage of different genotypes effect on the adaptability, because of the long-term evolution and breeding, the balance was achieved.

The exon 2 of TLR4 mutations was first detected in this study. G114A was detected and this was a silent mutation in exon 2 of TLR4 gene in this study, while, G142A was a sense mutation and changed the amino acid from Glu to Lys, that is to say, from acidic amino acid to basic amino acid. The change of acidity and alkali may cause the different isoelectric points, thereby causing change of protein structure and further lead to different functions. However, if the mutations are related to resistibility to disease, further research would reveal whether the mutation might serve as a marker of anti-disease characteristics or not.

Heterozygosity of population reflects the degree of genetic uniformity. The lower heterozygosity of population is the higher genetic uniformity. The results of this study indicated that the average heterozygosity and PIC among 15 populations were 0.4921 and 0.3710, respectively and showed that the degree of genetic variation was in the middle level and the level of genetic diversity was lower. Heterozygosity in Red Jungle fowl and Tibetan chicken were 0 and showed that the two populations basically were not genetic variation, heterozygosity of Henan game, 0.5000 was the highest and it showed that there were obvious genetic polymorphism and selection potentials in Henan Game. PIC is a good indicator of allele polymorphism. When $PIC > 0.5$, there was high polymorphism; when $0.25 < PIC < 0.5$, there was moderate polymorphism and when $PIC < 0.25$, there was low-grade polymorphisms. In this study, PIC in Red Jungle fowl and Tibetan chicken were 0 and showed that the two popu-

lations were not polymorphism; PIC in the others were between 0.25 and 0.50 and showed that there were

Table 3. Effects (P-value) of polymorphism of TLR4 gene exon 2 on chicken immune traits.

Trait	Genotype	Population	Genotype× population
Interleukin (pg/ml)	0.861	0.001	0.782
Neutrophilic granulocyte(GEAN)	0.612	0.426	0.375
Leukomonocyte	0.540	0.353	0.440
H/L	0.990	0.930	0.998
Sheep red blood cell (SRBC)	0.629	0.016	0.329

polymorphisms, which agreed with the results of heterozygosity. It showed that there were certain selection potentials in the populations and further breeding.

Effective numbers of alleles was also an indicator to reflect genetic variation, especially in conservation genetics, but the number of alleles easily influenced by sample size. In this study group, the average effective numbers of alleles was 1.9688. On the one hand, it explained that sample size in the study was relatively adequate, on the other hand, gene frequency distribution was relatively even on TLR4 gene in 15 populations. Therefore, analysis of genetic diversity showed higher validity and reliability.

At present, the function mechanism between the TLR4 gene and immunological traits are still unclear. But immunological traits are very important to keep up with growth and development. Therefore, different methods have been used to enhance resistance in poultry. The approach of candidate gene is a very powerful method to investigate associations of gene polymorphisms with economically important traits in farm animals. Many studies showed that TLR4 gene plays an important role in congenital immunity. In this study, the TLR4 gene was selected as a candidate gene to investigate associations of gene polymorphisms with some resistant traits in three chicken populations. The experiment results indicated that genotype and genotypes × populations had no significant effect in these metrical indexes. Only interleukin and SRBC had significant effect in populations, that is, the detected locus in the study had no significant effect in these resistant indexes from the measurement. According to experimental results, we conjecture that the SNPs may be invalid in molecular assistant selection (MAS) as a genetic marker for chicken resistance traits. Therefore, we will further test on other breeds and verify in order to enlarge the number of samples in the future. Simultaneously, we will carry on detection, other loci of TLR4 gene and hope on obtaining locus related to resistance traits.

Conclusion

Both G114A and G142A mutation on exon2 of TLR4 gene were detected exactly with PCR-SSCP method in group detected but not with the Red Jungle fowl and the Tibetan chicken. Almost all of the breeds, including the Red Jungle fowl, fit into Hardy-Weinberg equilibrium ($P > 0.05$), except Gushi chicken ($P < 0.05$). According to the analysis of population genetic variation, we detected that there were no polymorphism in Red Jungle fowl and Tibetan chicken and found that the remaining 13 breeds were at moderate polymorphism, therein, the genetic diversity in Henan Game was the highest.

The SNPs of the polymorphism of TLR4 gene in the study were not linked with potential major loci or genes affecting some resistant traits.

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