

DETECTING SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) IN TOLL-LIKE RECEPTOR 2 GENE IN THE RABBIT

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ABSTRACT

Toll-like receptors (*TLRs*) recognize pathogen-associated molecular patterns (PAMPs), which are derived from pathogen, and participate in activation of the immune responses as well. *TLR2* gene can recognize PAMPs specific to bacterial disease such as pneumonia. In the present study, we sequenced the coding regions of the *TLR2* gene in 15 rabbits from five breeds, including New Zealand White, Californian, Flemish Giant, Chinchilla and Fu Jian Yellow. In total, we discovered 11 single nucleotide polymorphism (SNPs), including four nonsynonymous SNPs located within the predicted *TLR* domains. Three replacements that change the amino acid characteristics were detected at bases 259, 319 and 997 in the *TLR2* gene. The SNPs in the *TLR2* gene may increase the probability of adaptation to variability of PAMPs due to the rapid evolution of pathogens and the possibility of survival in rabbit populations. These novel SNPs will be useful in future studies to investigate the association between the *TLR2* gene and disease resistance.

Key words: Rabbit, Toll-like receptors gene, single nucleotide polymorphism (SNP), Innate immunity.

INTRODUCTION

Toll-like receptors (*TLRs*) play important roles in recognizing "pathogen-associated molecular patterns (PAMPs)" of pathogens (such as viruses, bacteria and fungi). They may also initiate the early immune response in both innate and acquired immunity (Vasselon and Detmers, 2002). The mammalian *TLR* proteins contain an extracellular domain, which is consisted of leucine-rich repeats (LRRs), and an intracellular region consisted of a Toll/IL-1 receptor (TIR/IL-1R). The LRRs are involved in ligand recognition and the TIR/IL-1R mediates signal transduction (Kaisho and Akira, 2006). *TLR2* recognize PAMPs which are specific to microbes (Akira and Takeda, 2004). So, it is reasonably to hypothesize that the *TLR2* is a critical candidate gene for studies of resistance or susceptibility to bacterial infection in rabbits. The recent studies have demonstrated that the *TLR* genes could recognize slight differences among PAMPs (Janeway Jr and Medzhitov, 2002). It is well known that nucleotide change of the *TLR* genes may affect their capability to recognize PAMPs. Many studies of *TLR* genes have revealed an association between polymorphisms in the *TLR* genes and disease (Bochud *et al.*, 2007; Hawn *et al.*, 2007; He *et al.*, 2007). For example, the *Asp299Gly* mutation in the human *TLR4* gene increases risk of infection (Barber *et al.*, 2004). Mutation at *Arg392stop* in the human *TLR5* gene is associated with decreased risk of Crohn's disease (Gewirtz *et al.*, 2006). In the present study, we sequenced 15 rabbits

from 5 breeds to discover genetic polymorphisms in the *TLR2* gene. SNPs, especially nonsynonymous SNPs, discovered in this study will be useful for future studies to investigate the association of the *TLR2* in disease resistance.

MATERIALS AND METHODS

DNA samples and extraction

DNA which was extracted from muscle of a New Zealand White buck was used for primer optimization and identification of amplicon sequence could be identified via BLAST. Additional rabbit DNA samples of 15 unrelated rabbits from 5 breeds (New Zealand White, Californian, Flemish Giant, Chinchilla and Fu Jian Yellow) were extracted from whole-venous blood and readily stored at -20. Each breed included 3 individuals. These 5 breeds represent main breeds which are widely used in production. Genomic DNA was extracted using a Universal Genomic DNA Extraction Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions.

PCR and sequencing

The entire coding sequence (CDS) of the *TLR2* gene (NM_001082781) was used to design the PCR primers. Six PCR primer pairs that produced overlapping fragments within coding sequence of the *TLR2* gene were designed using the Web interface for Primer3 (<http://frodo.wi.mit.edu/primer3>). PCR primers for amplification are presented in Table 1. All PCR amplicons were visualized via agarose electrophoresis and subsequently purified using the QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's recommendations. The PCR amplicons purified were sequenced using an ABI 3730XL sequencer (Applied Biosystems, Foster City, CA, USA).

Table 1: PCR primers for amplification of rabbit *TLR2*

Fragment nos.	Forward primer (5' -3')	Reverse primer (5' -3')	Fragment size (bp)
1	aggttgatccctggacaat	gcctcagtgaggagaaccag	643
2	ggaagcctttatgcctttgc	ttctcgcaggctgaatttt	683
3	aaagggtgaagagggtcaca	aaagacaggaagtcgcagga	625
4	gacgtactgcatcccacaga	gagttctccagctcctgcac	645
5	tctcgcacttctgtcttt	gcgctggtgtctcatcaaa	581
6	catcgactgcatcgagaaga	accagttgcacagagacgtg	639

Detection of polymorphisms

Data from automated sequencers generally may include miscalled bases at each side of the sequence. Sequence quality was evaluated by Sequencer 4.7 (Gene Codes, Ann Arbor, MI, USA). Then, the low quality data at the ends of the sequence were trimmed by Sequencer 4.7. Each sequence from a single DNA sample was assembled to generate a consensus sequence for that DNA sample and to detect polymorphisms within the sample. Sequencer 4.7 provided an overview of the differences with the same coding relative to a selected Reference Sequence. These differences could be candidate SNPs. The SNPs were automatically detected

using Sequencher4.7 and each of them was verified by manual identification of the chromatograms. All heterozygous nucleotides were annotated with the appropriate IUPAC-IUB code.

RESULTS AND DISCUSSION

We obtained 6 fragments by PCR, and the length of these amplicons was 581-683 bp. The PCR amplicons produced mutually overlapping fragments within *TLR2* gene. Comparative sequence analysis of 2355 bp for five rabbit breeds revealed 11 SNPs in rabbit *TLR2*, of these, four were nonsynonymous SNPs. We anticipated that these SNPs attribute resistance to infection with particular pathogens. There was an average density of 1 SNP for 214 bp sequenced. Rabbit *TLR2* SNPs and predicted amino acid replacements are presented in Table 2. Nonsynonymous SNPs were identified within all major protein domains predicted for rabbit *TLR2*, with predicted amino acid replacements detected within the LRR and TIR domains. Polymorphisms within the LRR domains in *TLR2* may increase the scope of recognition of extracellular molecules in pathogen. Results from the previous studies have demonstrated the function of the LRR domains of *TLRs* in the recognition of PAMPs (Bell *et al.*, 2003).

Table 2: Distribution of single nucleotide polymorphisms (SNPs) among 15 individuals from five rabbit breeds^a.

Alleles ^b	Genomic position ^c	Observed frequencies	Amino acid position ^d	Amino acid ^e	Amino acid Character	(SNP genotype) rabbit breed	Domain
<u>G/A</u>	259	0.8/0.2	87	D/N	Anionic/Polar	(R)N, F	LRR
<u>G/C</u>	319	0.9/0.1	107	D/H	Anionic/Cationic	(S)N, F	LRR
<u>T/C</u>	542	0.53/0.47	181	A/V	Nonpolar/Nonpolar	(Y)F, C, Fu, N, Ch	None
<u>T/C</u>	621	0.53/0.47	207	E/E	-	(Y)F, C, Fu, N, Ch	None
<u>C/T</u>	651	0.73/0.27	217	V/V	-	(Y)F, C, Fu, N, Ch	None
<u>C/T</u>	997	0.73/0.27	333	R/W	Cationic/Nonpolar	(Y)F, C, Fu, N, Ch	None
<u>T/C</u>	1228	0.77/0.23	410	L/L	-	(Y)F, C, Fu, N	LRR
<u>C/T</u>	1381	0.73/0.27	461	L/L	-	(Y)F, C, Fu, N, Ch	None
<u>G/A</u>	1533	0.8/0.25	511	G/G	-	(R)F, C, Fu, N, Ch	LRR
<u>T/C</u>	1761	0.73/0.27	587	V/V	-	(Y)F, C, Fu, N, Ch	LRR
<u>C/T</u>	2097	0.7/0.3	699	G/G	-	(Y)C, Fu, Ch	TIR

^a Breed abbreviations as follows: F, Flemish Giant; C, Californian; Ch, Chinchilla; N, New Zealand White; Fu, Fu Jian Yellow

^b Alleles are depicted as major allele/minor allele, with the NM_001082781 allele depicted in bold, underlined text.

^c Genomic position based on GenBank accessions (NM_001082781).

^d Amino acid position based on GenBank accessions (NM_001082781).

^e Amino acid(s) encoded by the major and minor alleles, respectively, with predicted amino acid replacements.

^f Heterozygous SNP genotypes are depicted using the IUPAC codes for heterozygosity. Meaning of letters as follows: R, A or G; S, C or G; Y, C or T.

Recently, several studies have indicated that *TLRs* play critical roles in identification of invading PAMPs in chickens (Leveque *et al.*, 2003; Ye *et al.*, 2006). Zhou and colleagues reported that many of polymorphisms in the *TLR4* and *TLR9* genes have also been detected in sheep (Zhou *et al.*, 2007). A

total of 136 SNPs have been detected in the *TLR1*, *TLR2*, *TLR4*, *TLR5* and *TLR6* genes in 96 pigs from 11 different breeds. Among them, 63 were nonsynonymous SNPs. These nonsynonymous SNPs were clustered in the ectodomains of these *TLRs* (Shinkai *et al.*, 2006). In another study, 98 polymorphisms were detected within bovine *TLRs* 1, 5, and 10, including 23 nonsynonymous (Seabury *et al.*, 2007). The present study showed that there are some nonsynonymous SNPs and synonymous SNPs within the coding sequences of the rabbit *TLR2* gene. The SNPs may be useful for marker-assisted selection for disease-resistance breeding. By summing up the distribution of SNPs in the coding sequence of *TLRs* in pigs, cattle, rabbit, and humans, we found that nonsynonymous SNPs were mainly located in the sequences encoding ectodomains. A study of crystal structures of mouse *TLR2* associated with lipopeptide demonstrated that LRR of *TLR2* are direct binding regions for ligand recognition (Jin *et al.*, 2007).

Two substitutes that change the charges on the amino acids were detected at bases 259 and 319 in the *TLR2* gene. Moreover, polar changes of substitutions in amino acids were detected at bases 997 in *TLR2*. It is possible that these replacements in amino acids in *TLR2*, which change the amino acid characteristics, will change their extracellular pathogen recognition capabilities. Furthermore, amino acid replacements that induce a subtle change of the amino acid characteristics might influence on the resistance to diseases. For example, A nonsynonymous SNP at *A1775G* (amino acid *N592S*) in human *TLR5* is significantly associated with resistance to Legionnaires' disease (Hawn *et al.*, 2003).

CONCLUSIONS

The identification of polymorphisms reported here increases the resource of the genetic markers useful for mapping and association analyses with disease resistance. Further researches are needed to survey any potential associations, but nonsynonymous SNPs may be the candidates to conduct these studies.

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