

Single Nucleotide Polymorphisms in the Rabbit Toll-Like Receptor 4 Gene

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ABSTRACT

TLR4 could detect Gram-negative bacteria by identifying lipopolysaccharide (LPS) or lipid A; therefore, we think that TLR4 is a significant candidate gene for rabbit diseases resistance research. In this study, we have detected the genetic polymorphisms of TLR4 of 13 meat rabbits of 4 different breeds. DNA samples of 13 unrelated rabbits from 4 breeds (New Zealand White, Californian, Flemish Giant and Fu Jian Yellow) were extracted from whole-venous blood and readily stored at -20°C. 4 were Californian, 4 were Fu Jian Yellow, 3 were New Zealand White, 2 were Flemish Giant. Comparative sequence analysis indicated that 6 SNPs were identified, 5 of which were non-synonymous SNPs. Four nonsynonymous SNPs (G563A, G628T, A653C and A654C) were located in the LRR domains of the predicted protein. The nonsynonymous SNPs in the LRR domains, especially related to leucine residues, may dramatically alter the ability to identified extracellular pathogens. Besides, it is possible that these replacements in amino acids in TLR4, which change the amino acid characteristics, will change their extracellular pathogen recognition capabilities. The identification of these polymorphisms reported here increases the resource of genetic markers useful for mapping and association analyses with disease resistance. And the future researches are needed to survey any potential associations.

Key Words: Toll-like Receptors, Single Nucleotide Polymorphism (SNP), Innate Immunity, Rabbit

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 & Detmers 2002). TLR4 can identify various pathogenic microorganism components, induce the natural immunity and acquired immunity reaction of organism. TLR4 gene variance might influence the function of combining receptor with ligand and signal transduction capacity after combining with ligand, having an important influence on the pathogen recognition and disease resistance of the organism (Uenishi & Shinkai 2009). Researches indicate that there is a association between TLR4 gene polymorphism and inflammatory response to the host and susceptibility to the infectious diseases (Sharma et al. 2006). TLR4 is one of the important members of TLRs, belonging to cell-

surface TLR with structure divided into extracellular region, transmembrane domain and intracellular region. There are 18-31 leucine-rich repeats (LRRs), while extracellular region is composed of Tolhomology domain (TH domain), and oll/L-1-receptor homologous region (TIR).

There have been more reports on research of correlation between cattle TLR4 gene structure and its polymorphism and mastitis, TLR4 might play an important role in the response reaction of host to the intramammary infection (Werling et al. 2004; White et al. 2003). Researches on human diseases show that there is a significant correlation between TLR4 polymorphism and disease of digestive system and respiratory system (Lien et al. 2002). One case-control study demonstrates that there are associations between polymorphisms of TLR4 of the rabbit and susceptibility of digestive disorders. In this study, we have detected the genetic polymorphisms of TLR4 of 13 meat rabbits of 4 different breeds.

MATERIAL 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 13 unrelated rabbits from 4 breeds (New Zealand White, Californian, Flemish Giant and Fu Jian Yellow) were extracted from whole-venous blood and readily stored at -20. 4 were Californian, 4 were Fu Jian Yellow, 3 were New Zealand White, 2 were Flemish Giant. Genomic DNA was extracted using a Universal Genomic DNA Extraction Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions.

PCR and sequencing

The coding sequence (CDs) of the TLR4 gene (NM_001082732) was used to design the PCR primers. Four PCR primer pairs that produced overlapping fragments within CDS of the TLR4 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).

Data from automated sequencers generally have low quality data which may include miscalled bases at the each side of the sequence. Sequence quality was evaluated by

Sequencher4.7 (Gene Codes, Ann Arbor, MI, USA). Then, the low quality data at the ends of the sequence were trimmed by Sequencher4.7. 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. Sequencher 4.7 provided an overview of the differences with the same contig relative to a selected Reference Sequence. These differences could be candidate SNPs. The SNPs were automatically detected using Sequencher 4.7 and each of them was verified by manual identification of the chromatograms. All heterozygous nucleotides were annotated with the appropriate IUPAC-IUB code.

RESULT AND DISCUSSION

To identify nucleotide polymorphisms we designed primer pairs that amplified reciprocally overlapping fragments (Table 1). We obtained 4 fragments by PCR, and the length of these amplicons was 612-722 bp. Using these primers, 2080 bp corresponding to rabbit TLR4 were detected for 4 rabbit breeds and compared to the matched rabbit TLR4 reference sequences (NM_001082781). We detected 6 SNPs, of these, 83% (n = 5) were nonsynonymous SNPs, 17% (n = 1) were synonymous SNPs. Of the 6 SNPs, 50% (n = 3) were transitions (A ↔ G; C ↔ T) and 50% (n = 3) were transversions. In fact, the rate of occurrence (bp/polymorphism) of nonsynonymous SNPs and synonymous SNPs in the coding sequence (CDS) were 416 and 2080, respectively. The genomic positions of all SNPs, SNP genotypes and amino acid positions are also provided in Table 2. Nonsynonymous SNPs were identified within most major protein domains predicted for rabbit TLR4, with predicted amino acid replacements observed within the LRR domains.

Table 1. PCR primers for amplification of rabbit TLR4

Fragment nos.	Forward primer(5'-3')	Reverse primer (5'-3')	Fragment size (bp)
1	tgtggcccataatcttatcca	caccttgttgagcagtga	626
2	caaccaagaaattttcctaaga	gactccagtcctggggagtga	612
3	atgctttccaggacaacagg	ttctcaccagtcctcatcc	621
4	tgtgcatcacacctccaaat	ggaagccctcaggagtatt	722

Detection of polymorphisms

Table 2. Distribution of single nucleotide polymorphisms (SNPs) among 13 individuals from 4 rabbit breeds

Alleles ^a	Genomic Position ^b	Amino acid Position ^c	Amino Acid ^d	Character	(SNP genotype) rabbit breed ^e	Domain
<u>G</u> /A	563	188	S/N	-	R	LRR
<u>G</u> /T	628	210	A/S	Nonpolar/Polar	K	LRR
<u>C</u> /A	653	218	P/Q	Nonpolar/Polar	M	LRR
<u>C</u> /A	654	218	P/Q	Nonpolar/Polar	M	LRR
<u>G</u> /A	757	253	E/K	Anionic/Cationic	R	None
<u>G</u> /A	1095	365	R/R	-	R	None

^aThe NM_001082732 allele depicted in bold, underlined text^bGenomic position based on the first base of the start codon^cAmino acid position based on the start codon^dAmino acid(s) encoded by the alleles, with predicted amino acid replacements^eHeterozygous SNP genotypes are depicted using the IUPAC codes for heterozygosity

One nonsynonymous SNP that changed the charges on the amino acids was identified at bases 757 in TLR4. Moreover, polar changes of substitutions in amino acids was detected at bases 628, 653 and 654 in TLR4. It is possible that these replacements in amino acids in TLR4, 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).

TLRs play critical roles in identification of invading PAMPs in host (Janeway Jr & Medzhitov 2002), with recent study providing further evidence for this conclusion in domestic animals (Bochud et al. 2007;). We detected 6 SNPs in rabbit TLR4, we anticipated that these SNPs attribute resistance to infection with particular pathogens. Polymorphisms in the sequences encoding the regions might have allowed TLR4 to adapt to various kind of PAMPs derived from the rapid mutations of microbes. The polymorphisms of the rabbit TLR4 gene indicated that SNPs were mainly located in the sequences encoding the ectodomains of TLR4. The comparison of the distribution of SNPs in the coding sequences of TLR genes in pigs, cattle, and humans showed that the nonsynonymous SNPs were mainly located in the sequences encoding ectodomains- especially the sequences encoding LRRs

(Sherry et al. 2001; White et al. 2003; Shinkai et al. 2006).

The sequences encoding ectodomains of TLR4 played an important role in the recognition of pathogen associated molecular patterns. Polymorphisms in the sequences encoding ectodomains which involved in pattern recognition may improve the ability for recognition to various kinds of PAMPs originated by the rapid evolution of pathogeny. Nonsynonymous substitutions in the region directly binding to ligands may improve host immunity, thus, may increase the survival of the population (Carrington et al. 1999). It is generally known that the mutation of TLR4 coding sequences is likely to change the structure of protein encoded. And then, the structural alteration affects the function of identification. The nonsynonymous SNPs in the LRR domains, especially related to leucine residues, may dramatically alter the ability to identified extracellular pathogens (Fujita et al. 2003). The nonsynonymous SNPs which modified the amino acid polarity in rabbit TLR4 may affect binding to ligands. Furthermore, amino acid substitutions that alter the amino acid polarity might also impact the host immune responses and resistance to diseases. Three SNPs which changed the polarity of the amino acid residue were detected in rabbit TLR4. There were characteristic polymorphisms, such as G628T, C653A, C654A and G757A in TLR4. G628T, C653A and C654A, which located within the LRR domains predicted, might have a functional effect on rabbit TLR4, such as ligand

recognition. However, some SNPs have been found in human's TLR4, most of which are unfavorable to the individual diseases-resistance (Smirnova et al. 2001). TLR4 identifies the target molecules by the combined action of such adaptor proteins as CD14 and MD-2. The structural changes of extracellular region caused by mutation might have disadvantageous effects to the interaction between TLR4 and the adaptor proteins.

TLR4 recognizes molecules generated by Gram-negative bacteria. TLR4 plays a role in the recognition of various molecules, such as lipopolysaccharide (LPS) and lipid A. C3H/HeJ strain mice have a high tolerance to lipopolysaccharide (LPS), while C57BL/10ScCr strain wild mice are highly susceptible to Gram-negative bacterial infection, the former is a missense mutation resulting from the transformation of an amino acid from proline to histidine caused by a point mutation in TLR4 gene cytoplasm area, while the latter is defected in TLR4, indicating that TLR4 gene is the key point of LPS signal transmission (Poltorak et al. 1998). There is a correlation between TLR4 polymorphic site and infection of Pasteur pneumococcal. A study indicated that mutation of TLR4 protein site 259 associated with respiratory syncytial virus (RSV), the two haplotypes of TLR4 are also correlated with the infection of RSV (Puthothu et al. 2006). In addition, TLR4 gene is related with the diseases generated by sepsis, systemic lupus erythematosus, rheumatic arthritis, asthma etc. and microbial infection such as enteritis (Beuder 2002). Studies on association between TLR4 gene polymorphism (Asp299Gly and Thr399Ile) and gastritis showed that TLR4 gene Thr 399 Ile polymorphic site might be a pathogenic factor for gastritis, TLR4 gene might be related with chronic inflammation (Achyut et al. 2007).

At present, in TLR4 gene coding sequence, the polymorphisms of two mononucleotides Asp299Gly and Thr399Ile were researched most and it has been demonstrated that they were closely related with infectious diseases. The mutation of TLR4 allele Asp299Gly may increase the susceptibility of group to Gram-negative bacteria. It is generally believed that Asp299Gly and Thr399Ile of TLR4 associate with the inflammatory reaction. TLR4 allele polymorphism Asp299Gly is related with the

susceptibility of Gram-negative bacteria and sepsis, the carriers of this site are easier to infect Gram-negative bacteria and suffer from sepsis and the death rate is extremely high (Lorenz et al. 2002). The gene knockout mice experiment demonstrated that both TLR4 and MyD88 participated in the pathological process of atherosclerosis and there exists a correlation between Asp299Gly and atherosclerosis (Bjorkbacka et al. 2004; Michelsen et al. 2004). Further research indicated that compared with Thr399Ile, Asp299Gly might be easier to influence the function of TLR4 (Arbour et al. 2000).

CONCLUSION

Our work is aimed to identify the polymorphisms of rabbit TLR4 gene so as to provide ideal candidate polymorphisms for the next association analysis and signal transduction research. Finally, 6 SNPs were detected. The identification of these 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 these nonsynonymous SNPs are the best candidates to conduct such studies.

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