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DETECTION OF POLYMORPHISMS AND PROTEIN DOMAIN ARCHITECTURES IN RABBIT TOLL-LIKE RECEPTOR 2

ZHANG X.Y., LEI M., XIE L., ZHANG C.X., ZHENG J., YANG C., DENG X.D., LI J.L., HUANG D.P., XIE X.H.

Research Institute of Rabbits, Sichuan Animal Science Academy, No. 7, Niusha Rd., 610066, Chengdu, China.

Abstract: Toll-like receptors (*TLRs*) recognise pathogen-associated molecular patterns (PAMPs) derived from pathogens and participate in activation of the immune responses. The *TLR2* gene can recognise PAMPs specific to bacterial diseases such as pneumonia. In the present study, we sequenced the coding regions of the *TLR2* gene in 18 rabbits from 5 breeds, including New Zealand White, Californian, Flemish Giant, Chinchilla and Fu Jian Yellow. In total, we discovered 11 single nucleotide polymorphisms (SNPs), including 4 non-synonymous SNPs located within the predicted *TLR* domains. Two non-synonymous SNPs (G205A and G265C) were located in the LRR (leucine-rich repeat) domains of the predicted protein, while another non-synonymous SNP (C943T) was situated in the regions involved in binding to ligands. In addition, one synonymous SNP (C1174T) was distributed in the nucleus regions of heterodimers formed. Then, we revealed five conservative regions in the LRR patterning by prediction and comparison of TLR2 protein domain architectures for multiple species. 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. The SNPs reported here will be useful to investigate the association between the *TLR2* gene and disease resistance in future studies.

Key Words: toll-like receptors, single nucleotide polymorphism (SNP), innate immunity, rabbits.

INTRODUCTION

A major challenge to the application of selection for resistance to disease is the exposure to pathogens. Challenging breeding animals is generally impracticable and cannot guarantee the animals' welfare. Therefore, identification of DNA markers for disease resistance is widely studied and many useful single nucleotide polymorphisms (SNPs) are reported (Nicholas, 2005). Testing candidate genes is an approach used to search for DNA markers. In terms of innate immune system, pattern recognition receptors specifically recognise the molecular patterns derived from pathogens. Toll-like receptors (*TLRs*) play important roles in recognising "pathogen-associated molecular patterns (PAMPs)" of pathogens (such as viruses, bacteria and fungi; Abreu *et al.*, 2010). They may also initiate the early immune response in both innate and acquired immunity (Vasselon and Detmers, 2002). Mammalian *TLR* proteins contain an extracellular domain that consists of leucine-rich repeat (LRR) domains and an intracellular region that consists of a Toll/IL-1 receptor (*TIR/IL-1R*). The LRR domains are involved in ligand recognition and the *TIR/IL-1R* mediates signal transduction. TLR2 recognises PAMPs which are specific to microbes (Akira and Takeda, 2004). TLR2 could identify mycobacterial lipoglycan and other bacterial cell wall macroamphiphiles (*Ray et al., 2013*). As a result, it is reasonable to consider that *TLR2* is a critical candidate gene for studies of resistance or susceptibility to bacterial infection in rabbits. It is well known that nucleotide change of the *TLR* genes may affect their ability to recognise PAMPs (Uenishi and Shinkai, 2009). Previous studies have demonstrated that the *TLR* genes could recognise slight differences among PAMPs (Janeway and Medzhitov, 2002). We can hypothesise that the sensitive recognition ability is due to polymorphisms in *TLRs*. Furthermore, many studies of *TLR* genes have revealed an association between polymorphisms in the *TLR* genes and disease (Bochud *et al.*, 2007; He *et al.*, 2007; Abu-Amero *et al.,* 2013).

Correspondence: Xie Xiao-hong, Zhangxiangyu757@163.com. Received *February 2013* - Accepted *December 2013.*
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Polymorphisms in the human *TLR2* gene protect against malaria (Greene *et al.,* 2012). The Arg753Gln mutation in the human *TLR2* gene is associated with vitiligo susceptibility and urinary tract infection (Tabel *et al.,* 2007; Karaca *et al.*, 2013). A16934T polymorphism in the human *TLR2* gene is associated with severity of atopic dermatitis (Potaczek *et al.*, 2011). In this study, we sequenced 18 rabbits from 5 breeds to discover genetic polymorphisms in the *TLR2* gene. SNPs, especially non-synonymous SNPs, discovered in this study will be useful to investigate the association of *TLR2* in disease resistance in future studies.

MATERIALS AND METHODS

DNA samples and extraction

The blood samples were collected from 18 buck rabbits of different breeds from different breeding farms. In addition to 6 Flemish Giant rabbits, there were 3 rabbits from other breeds. The DNA extracted from the muscle of a New Zealand White buck was used to conduct the confirmation of amplification products by basic local alignment search tool (BLAST) and optimise amplification conditions (Seabury *et al.,* 2007). DNA samples used for inspection were obtained from 5 breeds (New Zealand White, Californian, Flemish Giant, Chinchilla and Fu Jian Yellow) and stored at –20 °C after being extracted from whole-venous blood. These 5 breeds are the main types most widely used in production in China. Genomic DNA was extracted by 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 polymerase chain reaction (PCR) primers. Seven PCR primer pairs that produced overlapping fragments were designed according to the entire coding sequence (CDS) of the *TLR2* gene by using the Web interface for Primer 3 (http://frodo.wi.mit.edu/primer3) (Table 1). PCR reactions were performed in a volume of 25 µL consisting of the following: 50-100 ng g DNA, 0.4 µM each primer (forward and reverse), 2×Taq PCR MasterMix (Tiangen, Beijin, China). A biomet thermocycler was used to amplify the DNA fragments in the following thermal conditions: 3 min at 94 °C; 30 cycles×30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C; 5 min at 72 °C. The thermal cycling parameters of Primer 4 were the same as the above conditions except for annealing temperature, which was set to 58 °C.

All PCR amplicons were visualised via agarose electrophoresis and subsequently purified using the QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA, USA), following the manufacturer's recommendations. The PCR amplicons purified were sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3730XL sequencer (Applied Biosystems, Foster City, CA, USA).

Detection of polymorphisms

Data from automated sequencers may generally include miscalled bases at 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 detect polymorphisms within the sample. The SNPs

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

Fragment No.	Forward primer(5'-3')	Reverse primer(5'-3')	Expected fragment size (bp)
1	aggttggatccctggacaat	acagagatgtctccccaagg	451
2	gcctctctcgtccctgaaat	tcaggcacataagccagaag	471
3	ggaagcctttatgcctttgc	ttctcgcaggctgaattttt	683
4	aaagggtgaagagggtcaca	aaagacaggaagtcgcagga	625
5	agcagctcgaatcctttcag	gagttctccagctcctgcac	448
6	tcctgcgacttcctgtcttt	gcgtcgttgttctcatcaaa	581
7	catcgactgcatcgagaaga	accagttgcacagagacgtg	639

were automatically detected using Sequencher4.7 and each of them was verified by manual identification based on chromatograms. All heterozygous nucleotides were presented by the appropriate IUPAC-IUB code. Database searches were conducted using the NCBI (National Center for Biotechnology Information) Web site and the oryCun2 rabbit genome assembly (May 2010). Additionally, DNAMAN version 5.0 software package (Lynnon Corporation) was applied to perform the alignments of *TLR2* CDS and protein sequence of mammals.

TLR protein domain architectures were predicted and compared by the online utility SMART (http://smart.embl-heidelberg.de/) (Letunic *et al.*, 2012). For all protein domain searches and predictions, the normal mode of simple modular architecture research tool (SMART) was used with the following settings: include PFAM domains, signal peptides, and internal repeats. SMART was also used to reveal how predicted amino acid replacements influence the prediction of protein domain architectures for rabbit TLR2.

RESULTS

Polymorphisms detected

To identify nucleotide polymorphisms, we designed primer pairs that amplified reciprocally overlapping fragments. With these primers, 2723bp corresponding to rabbit *TLR2* were detected for 5 rabbit breeds and compared with the matched rabbit *TLR2* reference sequences (NM_001082781).

SNPs of the rabbit *TLR2* gene were identified by Sanger sequencing (Figure 1). We detected 11 SNPs, among which, 36% (n=4) were non-synonymous SNPs, 64% (n=7) were synonymous SNPs. Of the 11 SNPs, 91% (n=10) were transitions (A ↔ G; C ↔ T) and 9% (n=1) were transversions. A search of the published literatures on rabbit *TLR2* indicated that 8 of the polymorphisms identified are reported here for the first time. Through comparison of the rabbit genome sequence with the published cDNA sequence (NM_001082781), 3 SNPs (T488C, T567C and G1479A) can be found, which coincide with the SNPs reported in this research. In fact, the rate of occurrence for non-synonymous and synonymous SNPs in the CDS was one every 589 bp and one every 336 bp, respectively. The genomic positions of all SNPs, major and minor allele frequencies, SNP genotypes and amino acid positions are also provided in Table 2. Two non-synonymous SNPs distributed in the major protein domains predicted for rabbit TLR2, with predicted amino acid replacements observed within the LRR domains (Figure 2). The non-synonymous SNPs in the LRR domains may obviously change the ability to recognise extracellular pathogens. One non-synonymous SNP that changed the charges on the amino acids was identified at bases 265 in *TLR2*, and non-synonymous SNPs of this kind were also observed at other positions in the gene (Table 2).

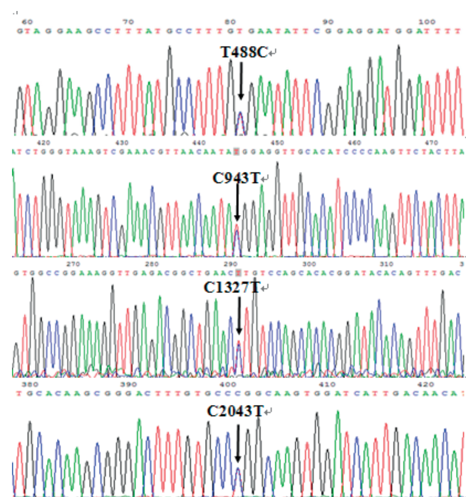

Figure 1: SNPs identified in the rabbit *TLR2* gene. Partial chromatograms show the heterozygosity of SNPs in samples. Arrows indicate position of nucleotide mutations.

Comparative prediction of protein domain architectures

Oryctolagus cuniculus TLR2 sequences are highly homologous to sequences from other mammalian species (Table 3). Through comparative prediction of TLR2 protein domain architectures via SMART for *O. cuniculus*, *B. Taurus*, *O. aries*, *C. familiaris*, *M. musculus*, *H. sapiens*, *R. norvegicus* and *S. scrofa*, we revealed five clusters of LRR patterning that were conserved among all species investigated (Figure 3). Moreover, comparative protein domain analysis for TLR2 showed that *C. familiaris* and *H. sapiens* were the species for which low-complexity regions were not confidently predicted by SMART. Furthermore, predicted signal peptides and Toll-interleukin-1 resistance (TIR)

Table 2: Distribution of single nucleotide polymorphisms (SNPs) among 18 individuals from 5 rabbit breeds[a].

Alleles[b]	Genomic position[c]	Observed frequencies	Amino acid position[d]	Amino acid[e]	Character	(SNP genotype) rabbit breed[f]	Domain
G/A	205	0.94/0.06	69	D/N	Anionic/ Neutral	(R)N, B	LRR[g]
G/C	265	0.91/0.09	89	D/H	Anionic/Cationic	(S)N, B	LRR
T/C	488	0.5/0.5	163	A/V	Non-polar/Non-polar	(Y)B, C, Fu, N, Ch	None
T/C	567	0.5/0.5	189	Y/Y	-	(Y)B, C, Fu, N, Ch	None
C/T	597	0.69/0.31	199	N/N	-	(Y)B, C, Fu, N, Ch	None
C/T	943	0.69/0.31	315	R/W	Cationic/Non-polar	(Y)B, C, Fu, N, Ch	None
T/C	1174	0.75/0.25	392	L/L	-	(Y)B, C, Fu, N	LRR
C/T	1327	0.69/0.31	443	L/L	-	(Y)B, C, Fu, N, Ch	None
G/A	1479	0.75/0.25	493	P/P	-	(R)B, C, Fu, N, Ch	LRR
T/C	1707	0.69/0.31	569	H/H	-	(Y)B, C, Fu, N, Ch	LRR
C/T	2043	0.64/0.36	681	P/P	-	(Y)B, C, Fu, Ch	TIR

[a] B: Flemish Giant; C: Californian; Ch: Chinchilla; N: New Zealand White; Fu: Fu Jian Yellow. [b] Alleles are depicted as major allele/ minor allele, including the NM_001082781 allele depicted in underlined text. [c] Genomic position based on the first base of the start codon. [d] Amino acid position based on the start codon. [e] Amino acid(s) match with the major and minor alleles, with predicted amino acid replacements. D: Aspartic Acid; N: Asparagine; H: Histidine; A: Alanine; V: Valine; Y: Tyrosine; R: Arginine; W: Tryptophan; L: Leucine; P: Proline. [f] All heterozygous SNPs were presented by the IUPAC codes. R: A or G; S: G or C; Y: C or T. [g] Leucine-rich repeat.

domains for TLR2 of all species were examined. Notably, one predicted amino acid replacement (R315W) encoded by a nsSNP could regulate the presence or absence of a LRR protein domain for *O. cuniculus* during sequential SMART analyses (Figure 3). The LRR protein domain did not occur for the rabbit TLR2 reference sequence, which encoded R at amino acid position 315. The predicted protein domain architectures for TLR2 were similar for all species investigated, implying that TLR2 in mammals are similar in function.

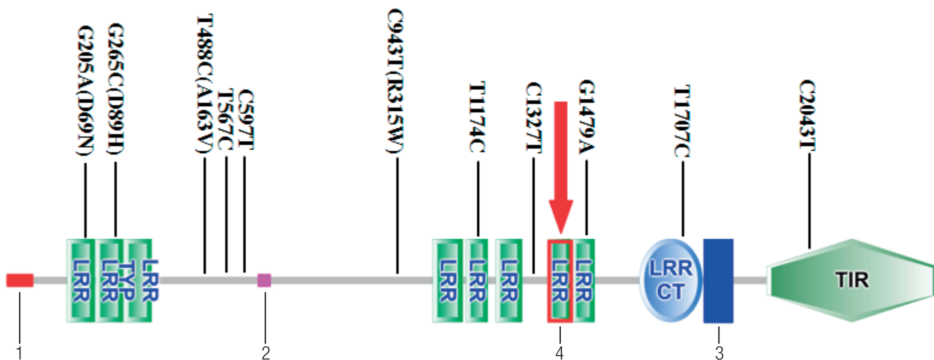

Figure 2: Distribution of SNPs in rabbit *TLR2* gene detected in the predicted protein structural domain. SMART-predicted domains and protein regions are indicated as follows: signal peptides are indicated by small red boxes (1); low-complexity regions are indicated in pink (2); leucine-rich repeats are depicted as LRR, LRR TYP, and LRRCT; vertical blue rectangles (3) indicate the transmembrane domain; TIR indicates Toll–interleukin 1-resistance homologous domain. Taking the start codon of this gene as the initial position, positions of nucleotides are used to label the locations of SNPs. The positions of amino acids which take the change are labelled in brackets along with the number and residues of amino acids (Shinkai *et al.*, 2006). A red arrow and red domain figure (4) label the novel LRR domain predicted by SMART.

Table 3: Homology rates between the coding regions and amino acid sequence of the *TLR2* of mammals.

Species	Common name	Accession number of GenBank	Homology(%) of coding regions	Homology(%) of amino acid sequence
Bos Taurus	Cattle	NM_174197	76.46	70.70
Ovis aries	Sheep	NM_001048231	76.21	70.70
Canis lupus familiaris	Dog	NM_001005264	76.25	70.83
Mus musculus	Mouse	NM_011905	71.84	69.43
Homo sapiens	Human	NM_003264	78.46	76.18
Rattus norvegicus	Rat	NM_198769	72.65	70.32
Sus scrofa	Pig	NM_213761	77.31	72.99

DISCUSSION

SNPs and conserved domains

The ectodomains of TLR molecules consist mainly of LRR domains, which are essential structures that distinguish PAMPs (Botos *et al.*, 2011). Analysis of polymorphisms in the rabbit *TLR2* gene indicates that SNPs within it are mainly located in the ectodomain encoding sequence. Comparisons of the SNP distribution in the *TLR* coding sequences of

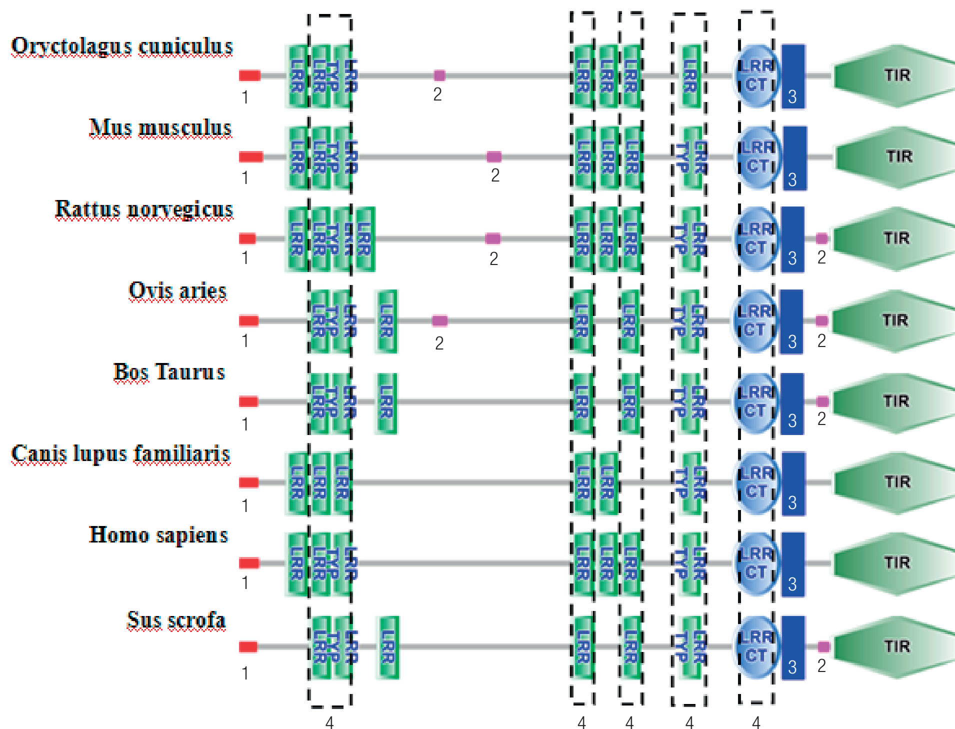

Figure 3: Comparison of TLR2 predicted protein domain architectures in different species of mammals. Domain architectures were predicted by SMART. Amino acid reference sequences for all species were retrieved from GenBank (*O. cuniculus* NP_001076250, *B. Taurus* NP_776622, *O. aries* NP_001041696, *C. familiaris* NP_001005264, *M. musculus* NP_036035, *H. sapiens* NP_003255, *R. norvegicus* NP_942064 and *S. S. scrofa* NP_998926). The LRR conserved domains all species have are labelled by a black dash box (4). SMART-predicted domains and protein regions are demonstrated as follows: signal peptides are indicated by small red boxes (1); low-complexity regions are indicated in pink (2); leucine-rich repeats are depicted as LRR, LRR TYP, and LRRCT; vertical blue rectangles (3) indicate the transmembrane domain; TIR indicates Toll–interleukin 1-resistance homologous domain (Seabury *et al.*, 2007).

pigs, cattle and humans reveal that while the non-synonymous SNPs are mainly located in sequences encoding the ectodomain, sequences encoding the LRR domains are particularly rich in non-synonymous SNPs (White *et al.*, 2003; Shinkai *et al.,* 2006; Tapping *et al.*, 2007). Two non-synonymous SNPs (G205A and G265C) and 2 synonymous SNPs (T1174C and G1479A) are located in the LRR domains of the predicted TLR2 protein sequence. The non-synonymous SNPs in the LRR domains may dramatically alter the ability of the molecule to identify extracellular pathogens (Fujita *et al.*, 2003). Furthermore, polymorphisms in the sequences encoding ectodomains that are involved in pattern recognition could improve recognition of the various kinds of PAMPs originating from rapidly evolving pathogens.

One study on the crystal structures of human and mouse TLR2-lipopeptide associations revealed that the N-terminal 266-355 amino acids participate in ligand binding (Jin *et al.*, 2007). The region of interest corresponds to positions 798-1065 in the *TLR2* coding sequence. Through comparison of the mRNA sequences of human, rat and rabbit homologues, we found that a non-synonymous SNP (C943T) is located in a region that may affect ligand binding. Non-synonymous SNPs within the region involved in ligand binding may change the specificity of ligands recognised by the TLR2 and TLR1/TLR6 heterodimers and increase the type of patterns recognised by the heterodimers, thereby affecting the survival of a population (Carrington *et al.*, 1999; Kruithof *et al.*, 2007). The nuclear regions of the heterodimer formed by TLR2 and TLR1 comprise amino acids 318-398 of the N terminus (corresponding nucleotides 954-1194) (Jin *et al.*, 2007), while the critical domain where TLR2 interacts with TLR6 comprises amino acids 318-404 (corresponding to nucleotides 954-1212) (Kang *et al.,* 2009). One synonymous SNP (C1174T) is located in this domain. However, as it does not change the amino acid it is unlikely to affect the heterodimers. In contrast, non-synonymous SNPs that modify the amino acid polarity in rabbit TLR2 may affect ligand binding. Furthermore, amino acid substitutions that alter the amino acid polarity may also have an impact on host immune responses and resistance to disease (Shinkai *et al.,* 2006). Three SNPs (G205A, G265C and C943T) that change the polarity of their corresponding amino acids are present in rabbit TLR2. G205A and G265C are located in the predicted LRR domains and may affect the function of rabbit TLR2; for example, its function in ligand recognition.

Nucleotide sequence comparisons indicate that the rabbit *TLR2* mRNA sequence shares a high degree of homology with sequences from other mammals. In addition, comparisons of the predicted mammalian protein domain architecture of TLR2 may reveal the conserved protein domains of the *TLR2* gene in mammals and identify similarities and differences in the LRR domains. Sequence conservation analysis of the predicted protein structures shows that there are 5 conserved LRR domains in mammalian TLR2. Notably, the non-synonymous G265C SNP is located in one of the conserved domains. Further research on the function of the conserved LRR domains and SNPs in these regions should uncover the important role that the conserved LRR domains and SNPs play in protecting mammals from pathogen invasion. There are some discrepancies between our predictions about the TLR2 mammalian protein domains and those of Seabury and Womack (2008). The main reason for this could be that we have not made predictions about regions of intrinsic disorder in the protein. Additionally, we used version 7 of the SMART database, whereas Seabury and Womack (2008), used version 5.0. Version 7.0 has an upgraded genomic mode protein database (Letunic *et al.*, 2006; Letunic *et al.*, 2012). In conclusion, the non-synonymous SNPs located in LRR domains (especially conserved LRR domains) and/or the central regions of heterodimers that alter amino acid polarity may have biological significance. These SNPs can be used as candidates for future research. Although synonymous SNPs do not change the amino acid sequence of a protein, they may influence gene expression, or the three-dimensional structure of the encoded protein (Sauna and Kimchi-Sarfaty, 2011; Brest *et al.*, 2011). Therefore, the synonymous SNPs reported herein merit further attention

Potential associations

Nowadays, few reports on associations between the rabbit *TLR2* gene and pathogens appear in the scientific literature. The abundance of rabbit *TLR2* mRNA was shown to increase significantly in lungs infected by pneumonia, which is mainly caused by bacterial infections (Kajikawa *et al.*, 2005). Additionally, *TLR2* mRNA expression was significantly increased in inflamed prostate tissue in humans (Vignozzi *et al.*, 2012). A recent study reported that polymorphisms in the rodent *TLR2* gene are associated with *Borrelia* infection in a wild bank vole population (Tschirren *et al.*, 2013). TLR2 recognises a variety of molecules, such as the peptidoglycans and lipoteichoic acid derived from Gram-positive bacteria, glycosylphosphatidylinositol from *Trypanosoma* spp. and zymosan from yeast (Takeda and Akira, 2005). Moreover, TLR2 forms a heterodimer with TLR1 or TLR6 to recognise mycobacterial-

derived triacylglyceride or diacylglyceride molecules (Krutzik *et al.*, 2003). The TLR2/TLR1 heterodimer can identify the lipoproteins from mycobacteria and meningococci (West *et al.*, 2006). Likewise, the TLR2/TLR6 heterodimer can recognise lipoprotein, peptidoglycan and *Bacillus anthracis* from mycoplasma, protozoa and fungi, respectively (Takeuchi *et al.*, 2001). Bacterial infections such as pneumonia present major challenges to production in the intensive meat rabbit industry. For the reasons given above, rabbit *TLR2* may be an important candidate gene for resistance to a variety of economically important infectious bacterial diseases. Studies on *TLR2* polymorphism in rabbits have, therefore, potential for improving disease control in rabbits via controlled breeding for disease resistance.

CONCLUSIONS

Our work was aimed at identifying polymorphisms in the rabbit *TLR2* gene and investigating how non-synonymous SNPs may influence the function of the predicted protein structure of this gene. We also examined structurally conserved domains in the predicted TLR2 proteins in different mammals and identified promising candidate polymorphisms for future association studies and signal transduction research. Finally, we characterised 11 SNPs and 5 LRR conserved domains. From these data, we identified the locations of the SNPs in the predicted protein, which allowed us to infer which polymorphisms had potential biological significance. *TLR2* could be an important candidate gene for resistance to infectious diseases in rabbits. Identification of the polymorphisms reported here increases the number of useful genetic markers for disease resistance mapping and association analyses. Future research will focus on using larger numbers of samples to verify the polymorphisms reported herein and conducting association studies on infectious disease resistance in rabbits.

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