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Characterization and Computational Investigation of Polymorphisms in the TIR Domain of the *TLR4* Protein in Holsteins Raised in Turkey

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Abstract

Increasing environmental temperatures due to climate change adversely affect livestock welfare and health. Moreover, temperatures increase the distribution and survival of parasites and infectious agents. Livestock diseases that cause significant economic losses are a worldwide concern. Toll-like receptor 4 (*TLR4*) is an ideal marker gene candidate, due to its critical role it plays in initiating the immune response against pathogens. In this study, the toll-interleukin-1 receptor (TIR) domain polymorphisms were investigated in the Holsteins raised in Turkey. by DNA sequencing. The effects of polymorphisms on the protein structure and function were evaluated by computational tools (I-Mutant Suite, Project Hope and PyMOL). The rs8193069 C>T polymorphism was detected in the TIR domain. The SNP causes Threonine to Isoleucine substitution at position 674 of the TLR4 protein which is the second amino acid of the TIR domain. The I-Mutant Suite predicted that Thr674Ile substitution could decrease protein stability (DDG= -0.40 Kcal/mol). Project Hope results showed that mutant and wild-type amino acids have different properties and that this can disturb the TIR domain. The Thr674Ile polymorphism observed in the TIR domain of *TLR4* protein in Holsteins raised in Turkey might affect the function of the *TLR4* by causing physicochemical changes.

Keywords: *TLR4*, TIR domain, polymorphism, in silico, cattle

Introduction

Global warming causes an increase in the distribution of parasites and infectious agents. Moreover, heat stress suppresses the immune system and enhances the susceptibility to pathogens [1-3]. The high cost of disease treatments [4] and the decrease in yield in livestock are fundamental problems for the livestock sector [5]. Therefore, improving different environmental conditions tolerance is an important goal for the livestock industry [6]. Determining the genetic background of the adaptation process in cattle breeds that are well adapted to harsh environmental conditions may provide essential data for marker-assisted breeding programs[7].

Toll like receptor (TLR) family are pattern recognition receptors (PRR) that are a vital component of the innate immune system. TLRs recognize pathogen associated molecular patterns (PAMP) and damage/danger associated molecular patterns (DAMP) [8,9]. PAMPs contain various conserved components of pathogens such as lipopolysaccharides, peptidoglycans, flagellin, bacterial DNA and viral double-stranded RNA [10]. DMAPs are cell-derived molecules that initiate immunity in response to various stress situations [11]. The ten TLR family members (TLR1–TLR10) are expressed in the bovine species [12]. The *TLR4* is one of the best defined TLRs that recognize the DAMPs and PAMPs (lipopolysaccharide from gram-negative bacteria) to evoke the host immune response during stress and infections [13]. The *TLR4* consists of the composed of 16 to 28 leucine-rich repeats extracellular ligand-binding domain and the intracellular toll-interleukin I receptor domain (TIR). The cattle *TLR4* gene located on chromosome 8 contains three exons and encodes 841 amino acids [14,15]. The TIR domain of *TLR4* protein is 143 amino acids (between 673 and 816) long. The highly conserved TIR domain is a protein-protein interaction motif module crucial for signal transduction [8]. It has been reported that *TLR4* expression is increased in cattle

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under heat stress. It provides heat tolerance by preventing the harmful effects of stress in cattle exposed to heat stress [16]. Heat shock proteins (HSP) are the primary stress tolerance proteins associated with the TLR pathway. HSP70 stimulates *TLR4* for the proliferation of dendritic cells [17]. The exonic [18], intronic [19] and promoter region [20] variations in the *TLR4* gene are associated with paratuberculosis in cattle. In addition, *TLR4* gene polymorphisms were associated with lower somatic cell counts, mastitis resistance and milk yield traits in cattle [18,21].

The *TLR4* is a potential candidate gene for marker research related to disease resistance due to its role in the initiation of the immune response against pathogens and its polymorphic nature [22]. The study aims to identify polymorphisms in the TIR domain in Holsteins raised in Turkey and to determine their effects on the structure and function of *TLR4* protein by computational tools.

Material and Methods

BSample collection and DNA extraction

The Holstein tissue samples were collected from carcasses after slaughter in the Thrace region of Turkey. The samples (n=56) were randomly selected from bulls identified as Holstein in the slaughterhouse documents. Because the samples were collected on different dates, cattle samples from different farms were included in the study. The muscle tissue samples weighing about 10 g were obtained from the neck of each carcass. The samples were collected in sterile containers and stored at -20 °C until molecular genetic studies. The genomic DNA extraction was performed using the phenol:chloroform:isoamyl alcohol method as described by Sambrook et al. [23].

PCR amplification and genotyping

The primer pair was designed to amplify the DNA fragment encoding the TIR domain of bovine *TLR4* protein using the NCBI-primer BLAST (Table 1). The positions of the primers on the bovine chromosome 8 were confirmed using the NCBI genome browser (Figure 1).

Table 1. Primer pair sequences, locations, annealing temperatures and PCR product size

Primer sequences (5'-3')	Chr8: Start-Stop	Annealing tempature (°C)	PCR product (bp)
F:CATCAGTGTGTCGGTGGTCA	107067492-107067511	62	639
R:GGGGATTCTCCTCCTCAGGT	107068130-107068111		

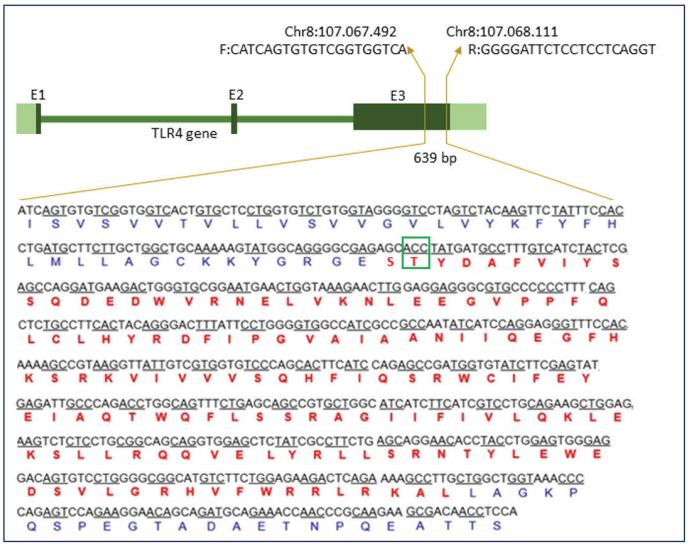

Figure 1. The binding position of the forward and reverse primer are shown on the TLR4 gene plot. The sequence of the amplified gene region and the amino acids it encodes are shown below. The amino acids that make up the TIR domain are written in red colour. The position of amino acid 674 in the TLR4 protein is marked with a green rectangle.

PCR amplification was carried out using a Proflex thermal cycler (Applied Biosystem) in a reaction volume of 25 µl, comprised of 1.0 µl (10 pmol) of forward primer, 1 µl (10 pmol) of reverse primer, 12.5 µl of PCR mastermix (K0171, Thermo Scientific), 5 µl of DNA template and 5.5 µl of nuclease free water. The PCR conditions involved initial denaturation at 95 °C for 3 min, followed by 35 cycles with denaturation at 95 °C for 30 s, annealing temperature ranging from 62 °C for 45 s, extension at 72 °C for 60 s followed by a final extension at 72 °C for 5 min. After running agarose gel electrophoresis, PCR products were visualized in a gel documentation system. Sequencing reactions were performed using a DTCS Quick Start sequencing kit (Beckman Coulter) and analyzed on a GenomeLab GeXP Genetic Analysis System (Beckman Coulter, USA). The DNA sequences were analyzed by BioEdit v7.2.5 [24] and Chromas v2.6.6 (Technelysium Pty Ltd, ASTL).

Statistical and computational analyses

The genotype, allele frequencies and Hardy-Weinberg Equilibrium of the bovine *TLR4* polymorphism were calculated using the Popgen v.1.32 software [25]. The predicted bovine *TLR4* protein structure (UniProt ID: Q9GL65) was retrieved from the AlphaFold [26] protein structure database. AlphaFold provides open access to protein structure predictions to accelerate scientific research. Computational analyses were performed using I-Mutant Suite [27], Project Hope [28] and PyMOL [29] (The PyMOL Molecular Graphics System, Version 2.5 Schrödinger, LLC) tools. I-Mutant Suite was used to evaluate the effect of an amino acid substitution on protein stability. This tool calculates the Gibbs free energy change (ΔΔG) between wild and mutant proteins.

According to the binary classification, ΔΔG<0 means decreased stability and ΔΔG>0 means increased stability. The Project Hope server was used to analyze the physicochemical and structural consequences of the amino acid mutation. The Project Hope server uses UniProt and DAS servers and predicts the 3-D structures of mutated proteins. The PyMOL (Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC) was used to visualize the protein structure. The mutant protein models were generated using the PyMOL mutagenesis wizard. PyMOL also was used to detect hydrogen bonds between residues.

Results

The DNA fragments (639 bp) encoding the TIR domain of the bovine *TLR4* protein were amplified successfully with the designed primer pair (Figure 2). Only one SNP (g. 107067611C>T) was identified in the exon three gene region coding for the TIR domain in the 56 Holstein cattle (Figure 3) (Table 2). The g.107067611C>T SNP (rs8193069) causes Threonine (Thr) to Isoleucine (Ile) substitution at position 674 of the *TLR4*. This mutation occurs at the second amino acid of the TIR domain (Figure 1).

The genotypic and allelic frequencies for rs8193069 are shown in Table 2. All three possible genotypes (TT, TC and CC) were identified and the most common genotype was CC (Frequncy= 0.839). The SNP fit in the Hardy–Weinberg equilibrium in Holstein cattle (P > 0.05) (Table 2). The C allele frequency was found 0.911; the T allele frequency was 0.089.

According to the Project Hope server report, there are differences in mutant and wild amino acid properties. The mutant and wild amino acids differ in size and hydrophobicity. The mutant residue is bigger and more hydrophobic than the wild-type residue. The mutation is located TIR domain according to the Uniprot database. The Thr674Ile mutation introduces an amino acid with different properties which can disturb this domain and abolish its function. The fasta sequence of the bovine *TLR4* protein was used as the input file for the I-Mutant Suite and the Thr674Ile polymorphism was analyzed by the binary classification prediction method. The mutation was predicted to decrease the stability of the *TLR4* structure (DDG= -0.40 Kcal/mol). The 3-D structure of the bovine *TLR4* protein was visualized with PyMOL. The ILE674 mutation was obtained using the mutagenesis wizard of the PyMOL. The PyMOL suggested four rotamers for the mutant amino acid. The one with the lowest energy was chosen as the best conformation. H bonds for mutant and wild residues were visualized in the PyMOL. It was determined that Thr674 formed three H-bonds with Glu672, Lys730 and Gln705 while Ile674 formed one H bond with Glu672 (Figure 4).

Figure 2. Agarose gel electrophoresis results of PCR products

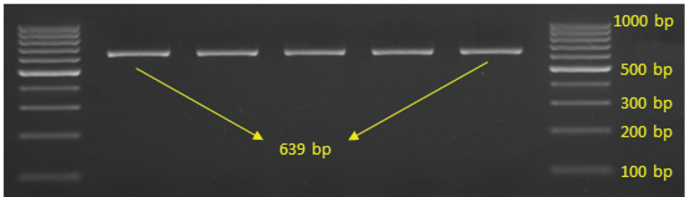

Figure 3. The sequencing chromatograms show that three genotypes for rs8193069 C>T polymorphism

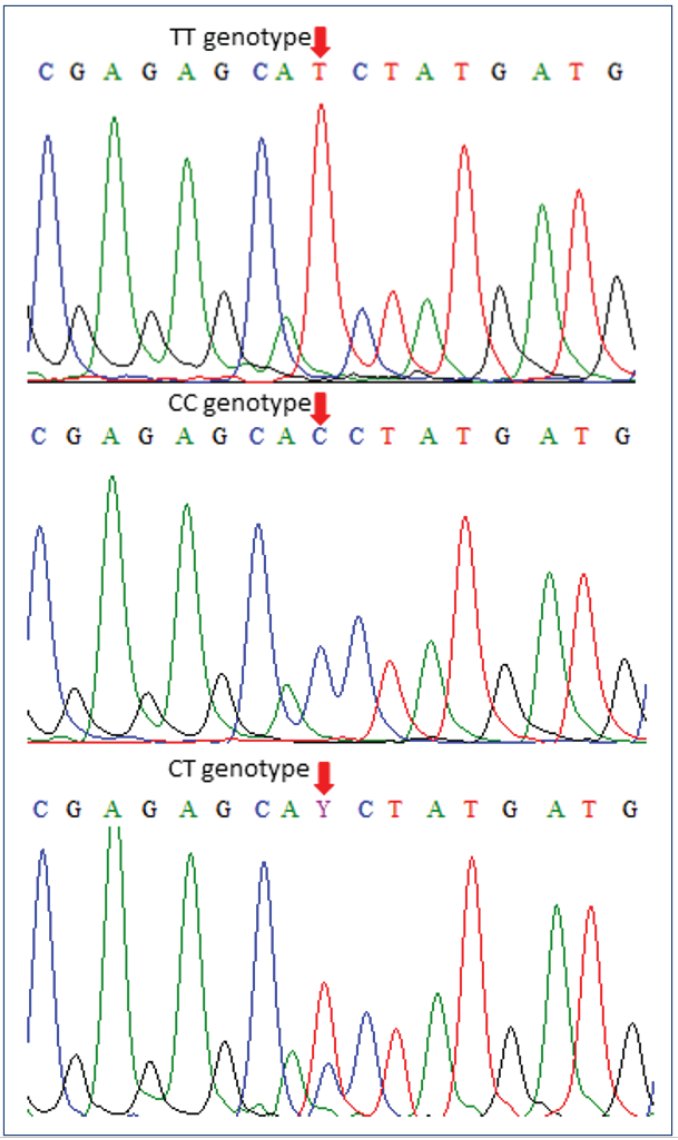

Table 2. The rs8193069 C>T polymorphism genotype and allele frequencies

g.107067611C>T (rs8193069)						
Cattle (n=56)	Genotypes			Alleles		HWE* P value
	CC	CT	TT	C	T	
Number	47	8	1	102	10	0.309
Frequency	0.839	0.143	0.018	0.911	0.089	

*HWE; Hardy-Weinberg equilibrium

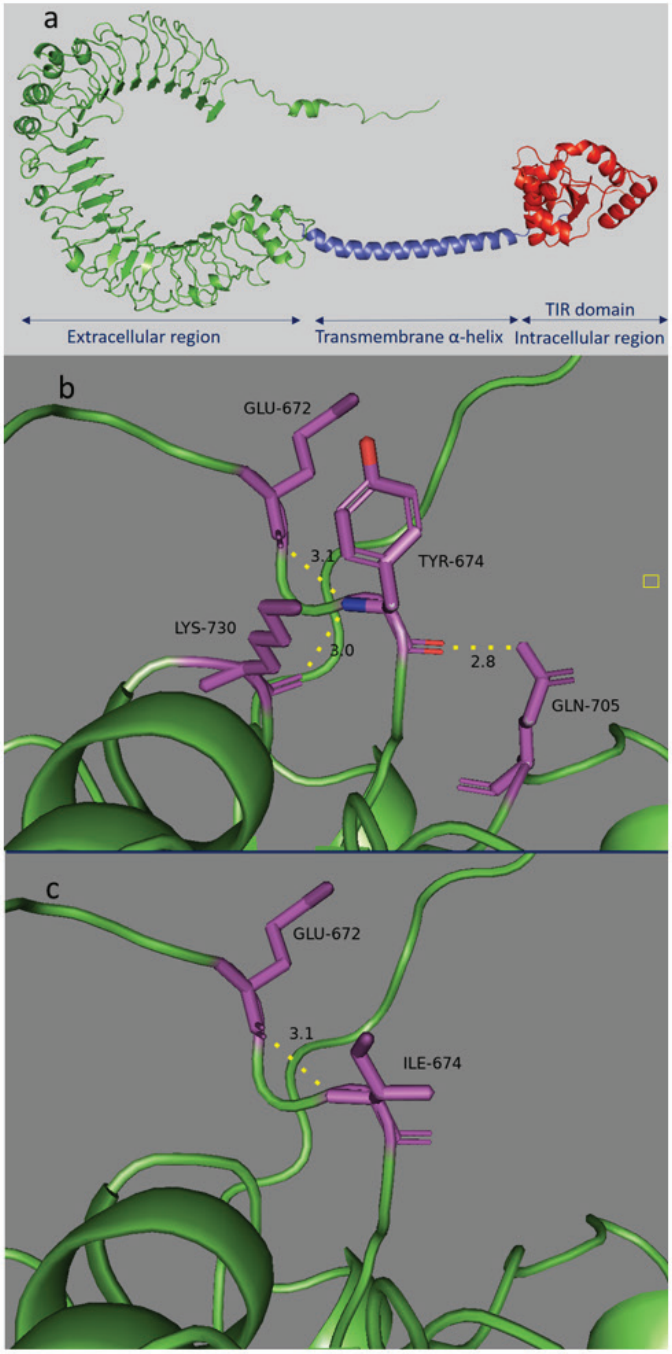

Figure 4. a- The 3-D structure of TLR4 protein; extracellular region (green), transmembrane α-helix (blue) and TIR domain (red). b- The wild Thr674 residue forms three H-bonds with Glu672 (3.1A), Gln70 (2.8 A) and Lys730 (3.0 A). c- The mutant Ile674 residue forms an H-bonds with Glu672 (3.1A).

Discussion

The *TLR4* is the critical receptor inducing the proinflammatory response that recognizes exogenous (PAMPs from gram-negative bacteria) and endogenous (DAMPs injured tissues and necrotic cells) stimuli [30]. When TLRs detect PAMPs and DAMPs, dimerization of the intracellular TIR region initiates innate immune responses. The TIR domain dimers interact with TIR-containing adapter proteins (MyD88, MAL, TRIF, TRAM and SARM), triggering the production of proinflammatory cytokines [31]. Residue substitutions in the TIR domain can reduce the recruitment of TIR-containing adapter proteins, resulting in a significantly reduced immune response [32].

Polymorphisms in the *TLR4* gene are associated with disease susceptibility [21], milk production traits [13] and heat stress response [16] in cattle. *TLR4* is an important molecular marker candidate as it is associated with issues that cause significant economic losses in the dairy industry worldwide. In this study, TIR domain polymorphisms and their structural effects on *TLR4* protein were investigated in Holsteins raised in Turkey. The rs8193069 C>T that caused the substitution (Thr674Ile) in the 2nd amino acid of the TIR domain was detected (Figure 1). Similar to the majority of previous studies [21,33-35], the C allele was found most frequent variant (0.911) for rs8193069 in Holsteins raised in Turkey. Chen et al. [21] found the C allele frequency of 0.922, and reported that the C allele was associated with both higher mastitis resistance and higher milk yield than in Holstein's T allele. Sharma et al. [35] found the C allele (frequency: 0.969) associated with a low somatic cell score in Canadian Holstein. Kulibaba et al. [36] reported that three cattle breeds (Ukrainian Black-and-White, Ukrainian Red-and-White and Ukrainian Grey) were monomorphic (CC) for the rs8193069 C>T polymorphism. Contrary to the results reported here, Mišeikienė et al. [37] observed a higher frequency of the T allele (0.510) than the C allele (0.490) in Lithuanian Holstein. They also reported no significant association between rs8193069 C>T polymorphism and mastitis resistance or milk composition [37]. Cattle breeds have been selected for milk yield traits and disease resistance for centuries. Therefore, the frequency of the T allele, which increases susceptibility to mastitis, may be shallow in Holstein cattle. Like most previous studies, it has been reported that the frequencies of alleles that are suggested to be associated with mastitis susceptibility are low in Holstein cattle [38-40]. Ağaoğlu et al. 41 reported that *TLR4* genotype frequencies associated with high milk yield and mastitis resistance are higher in Holsteins than in Turkish native cattle breeds.

The polymorphic nature of the *TLR4* gene in Holsteins raised in Turkey has been reported in previous studies [41-44]. Ağaoğlu et al. [41] identified two polymorphisms in the promoter (G-1539A) and 5' UTR (G+256C) regions of the *TLR4* gene using the restriction fragment length polymorphism (RFLP) method. Bilgen et al. [42] analyzed the *TLR4* gene with next generation sequencing and reported 75 SNPs. Arslan et al. [43] and Cinar et al. [44] determined the SNP (*TLR4* +10 C/T) in exon 1 of the TLR gene by the RFLP method. Similar to this study, Bilgen et al. [42] reported rs8193069 C>T polymorphism in Holstein. However,

a comparison between the two studies could not be made because genotype or allele frequencies were not reported in the manuscript of Bilgen et al. [42].

Nowadays, computational analyses are widely used to predict the effects of missense mutations on protein stability, structure, and function. In this study, three computational tools (I-Mutant Suite [27], Hope [28] and PyMOL[29]) were used to predict the effect of the SNP. The effect of Thr674Ile mutation on protein stability was investigated using the I-Mutant Suite. The Ile674 mutant was predicted to reduce *TLR4* protein stability (DDG= -0.40 Kcal/mol). Differences in the physical properties of polar threonine and non-polar isoleucine may lead to a decrease in *TLR4* stability. Decreased protein stability can result in increased protein degradation, aggregation, and misfolding of proteins [45]. Solanki et al. [46] reported that an amino acid substitution causing a decrease in the stability of bovine beta-defensin 129 protein affected bull fertility. Singh et al. [47] reported that missense mutations cause muscular dystrophy to reduce the stability of the dystrophin protein.

The Project Hope server was used to predict the effects of the mutation on hydrophobicity, amino acid size, charge and function. The Hope server determined that the mutant residue (Ile674) was both bigger and more hydrophobic than the wild residue (Thr674). The hydrophilic properties of amino acids in protein sequences regulate protein conformation and folding [48]. The more hydrophobic mutant residue may cause a loss of hydrogen bonds and disruption of correct folding [49,50]. It was confirmed by visualization in the PyMOL that the Ile674 mutation causes the loss of hydrogen bonds (Figure 4). In addition, hydrogen bonds contribute significantly to protein stability [51]. Previous studies have reported that hydrophobic changes in CSN3 [52], TLR2 [53] and APP [54] proteins can cause protein misfolding and decreased stability. Brennan et al. [55] reported that Thr>Ile substitution in the fibrinogen protein can cause hypofibrinogenaemia by disrupting protein function. Manal et al. [56] reported that a similar substitution affected the function of thrombin-activatable fibrinolysis inhibitor protein and caused breast cancer. These results suggest that the The674Ile mutation will cause changes in the physicochemical properties of the protein. This substitution in the TIR domain may affect the protein's function, increasing susceptibility to infections or stress.

In conclusion, rs8193069 locus was found to be polymorphic in Holsteins raised in Turkey. The frequency of the T allele, previously reported to be associated with mastitis susceptibility, was very low. These results should be confirmed in larger populations. The computational analysis showed that the T allele can negatively affect *TLR4* protein structure and function. Therefore, the rs8193069 variation may have the potential to be used as a molecular marker candidate in future studies.

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