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Two Novel Splice Variants of the *Ovis aries* Toll-like Receptor 4

[1,2]Bao-Lu Zhang, [1]Jin-ling Du, [1]Hong-Bing Han, [1]Zheng-xing Lian and [3]Ning Li

[1]Laboratory of Animal Genetics and Breeding, Ministry of Agriculture, College of Animal Science and Technology, China Agricultural University, Beijing 100193, People's Republic of China

[2]College of Animal Science and Veterinary, Shenyang Agricultural University, Shenyang 110161, People's Republic of China

[3]State Key Laboratory of Agrobiotechnology, China Agricultural University, Beijing 100193, People's Republic of China

Corresponding Author: Dr. Zheng-xing Lian, Laboratory of Animal Genetics and Breeding, Ministry of Agriculture, College of Animal Science and Technology, China Agricultural University, Beijing 100193, People's Republic of China Tel: +86-010-62732681 Fax: +86-010-62732463

ABSTRACT

The objective of this project was to investigate the regulate function of transcript variants of *Ovis aries* toll-like receptor 4 (TLR4). Transcript variant may act as a regulator in many bio-processes. In mouse, alternative Toll-like receptor 4 mRNA splicing generates a soluble receptor that can negatively regulate TLR4 signaling; therefore this study was carried out to investigate whether similar mechanisms are also presented in sheep. Two transcript variants of *Ovis aries* TLR4 gene were found. Sequence bio-informative analysis was performed and results reflected one harbors an alternative exon between the second and third exons, whereas in the other the second exon is excluded in the mRNA. Effects of Lipopolysaccharides (LPS) on TLR4 transcript variants expression were studied on fibroblasts. Due to transcript variants of *Ovis aries* TLR4 may play different roles in immunity response, real-time PCR with variants specific primer pairs was used to study transcript variants expression patterns. The two novel variants showed opposite expression patterns compared with that of the reported variant during LPS stimulation. Furthermore, overexpression assay were performed, results indicated the one with an alternative exon had negative regulation function in LPS-induced tumor necrosis factor-alpha (TNF-a) transcription and taken together, the present study may provide an alternative therapy strategy for exdotoxin shock in Veterinary. In this study, reverse-transcription PCR combined with DNA sequencing were used to discover the novel transcript variants; real-time PCR was employed to measure the genes expressions; overexpression assay was performed to study effect on immunity response by transcript variant.

Key words: Toll-like receptor 4, transcript variant, gene cloning, fibroblasts

INTRODUCTION

The innate immune system is the first line of defense against infection. Immune sentinel cells, including macrophages, dendritic cells, endothelial cells and fibroblasts, recognize Pathogen-Associated Molecular Patterns (PAMP) on the surface of pathogens (Silzle *et al*., 2004). These cells identify invading microorganisms through a number of Pattern-Recognition Receptors (PRR) (Rock *et al*., 1998). The Toll-like receptors (TLRs) form a family of type I transmembrane PRRs. To

date 10 members of this family have been indentified in sheep (Chang *et al.,* 2009); each contains a highly similar cytoplasmic portion, toll/interleukin-1 receptor (TIR) domain, with homology to the interleukin (IL)-1 receptor. The extracellular regions of the TLRs contain leucine-rich repeats (LRRs) that bind to specific components of pathogens. TLR4 is considered to be the major PRR that binds lipopolysaccharide (LPS), a surface component of Gram-negative bacteria (Hoshino *et al.*, 1999). In concert with CD14 and MD2, TLR4 triggers a signaling cascade that leads to activation of Tumor Necrosis Factor (TNF) expression, a key regulator of several pro-inflammatory cascades. The immune response to LPS, particularly in the initial steps, is governed by complex regulatory mechanisms (Fujihara *et al.,* 2003). Because TLR signaling plays a critical role in both innate and adaptive immunity, strict regulation is required to avoid adverse effects and several negative regulators of TLR pathways have been identified (Wells *et al.*, 2006; Lang and Mansell, 2007).

Alternative splicing is frequently encountered in eukaryotes; more that 40% of human genes are subject to alternative splicing; the majority of these are involved in the immune and nervous systems (Johnson *et al.*, 2003). This mechanism increases the functional diversity of proteins encoded by a single gene. A soluble form of the related receptor TLR2 was previously identified as a negative regulator of IL-8 and Tumor-Necrosis Factor (TNF) during LPS challenge in human (LeBouder *et al.*, 2003). TLR4 appears to play a similar role and a soluble variant of the mouse TLR4 protein has been reported to inhibit LPS-induced NF-κB activation and TNF production (Iwami *et al.*, 2000). Because TLR4 receptors are widely distributed in the immune system we investigated the expression of TLR4 variants in fibroblasts, a type of non-professional antigen presenting cell; in response to LPS stimulation *in vitro*.

As no any alternative splice of *Ovis aries* TLR4 has been reported, consisted with human and mouse reports, with two novel transcript variants of *Ovis aries* TLR4 being found. Preliminary results indicated transcript variant of *Ovis aries* may play a negative roll in LPS-media signaling transduction.

MATERIALS AND METHODS

Primary skin tissue was isolated from an aborted 3-month-old Suffolk sheep embryo in Oct. 2009 at experimental farm of China Agricultural University (CAU). All the laboratory works were carried out at Laboratory of Animal Genetics and Breeding in CAU.

Fibroblast cell isolation and culture: Briefly, skin tissue was cut into small fragments, transferred to 100 mm Petri dishes and fibroblasts were cultured in DMEM (Gibco) supplemented with 15% fetal bovine serum (Gibco), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Sigma). Incubation at 37°C under humidified 5% CO_2. Culture media (10 mL) was added to each dish and fibroblasts were detached enzymatically 2 to 3 days later and passaged. Cells from passages 5 to 8 were used in this study. To investigate the expression of TLR4 variants, fibroblasts were stimulated with 100 ng mL^{-1} LPS (Sigma) in cell culture media and samples were withdrawn for RNA extraction at 0.5, 2, 4, 8, 12 and 24 h following LPS stimulation.

Sequencing of TLR4 genomic DNA and cDNA: DNA was isolated from cultured sheep fibroblast cells using a standard phenol-chloroform method (Sambrook and Russell, 2001). PCR amplification of TLR4 sequences employed (5'-3') 1F, ATG GCG CGT GCC CGC CG 1; 1R, AAG TGA ATG AAA AGG AGA CCT CA; 2F, CCA GGT TCC CAG AAC TGC AA; 2R, GGC TCC CCA GGC TAA ACT CT; 3F, GGA GAC CTA GAT GAC TGG GTT G and 3R, CTA TAG GGC TCG CGT

ACC AC. PCR reactions were performed with Phusion DNA polymerase (NEB). Reactions were initiated with 2 min denaturation at 94°C followed by 35 cycles of 94°C for 30 sec; 56, 62 or 60°C for 30 sec and 3 min extension at 72°C, followed by a final extension step of 10 min at 72°C.

Total cellular RNA was prepared using TRIZOL reagent (Invitrogen) and treated with DNase I (Fermentas) according to the manufacturer's instructions. cDNA was synthesized from 2 μg of total RNA by extension of oligo-dT(17) with 200 U of M-MLV (Promega). Primers used to amply TLR4 transcript variants were TLR4F, ATG GCG CGT GCC CGC CG and TLR4R, CTA TAG GGC TCG CGT ACC AC, corresponding to the first and third exons. PCR was carried out as described above with an annealing temperature of 60°C and products were separated by electrophoresis on 1.5% agarose gels and visualized by staining with ethidium bromide. PCR products were inserted into vector pGEM T-easy (Promega) and then triply sequenced using an ABI PRISM_3730 Genetic Analyzer (Applied Biosystems).

Expression plasmid: For an expression plasmid of transcript variant GU461887, the coding region was amplified from isolated alternative spliced cDNA and cloned into *Ecor*R I and *Sma* I sites of expression plasmid pIRES2. The new expression plasmid was named as pIRES2-soTLR4.

Transcript variant overexpression: Fibroblasts were plated into 6-well plate at 5×10^5/well the day before transfection. Either expression plasmids or empty vectors (4 μg) were transfected using Superfect (Qiagen) according to the manual. At 24 h after transfection, cells were stimulated with various concentrations of LPS (10, 100 and 1000 ng mL^{-1}) for 2 h. Cells transfected with empty vectors and untreated with LPS were used as control samples.

Real-time PCR: Genomic DNA contamination was tested by PCR amplification of β-actin using the following primers: β-actin F, AGA TGT GGA TCA GCA AGC AG and β-actin R, CCA ATC TCA TCT CGT TTT CTG, generating a 450 bp product from genomic DNA. Variant-specific primers were Var1F, CTG AAT CTC TAC AAA ATC CC; Var1R, CTT AAT TTC GCA TCT GGA TA; Var2F, CCG ACA ACA TCC CCA TAT CAA; Var2R, GGG AAC ACA CAA CTG GAA GCA; Var3F, GGA CCC TTG CGT ACA GAT G; Var3R, GAT GTC CAA TGG GGA AGT CA. All PCR products were confirmed by DNA sequencing. Total TLR4 expression levels were analyzed using primers TLR4TF, GGG TGC GGA ATG AAC TGG TA and TLR4TR, CAC CTT ACG GCT CTT GTG GAA. Primers specific for TNF-α were TNF-αF, AAC AGG CCT CTG GTT CAG ACA and TNF-αR, CCA TGA GGG CAT TGG CAT AC. Real-time PCR was performed using MX300P (Stratagene) with a RealMasterMix SYBR Green kit (Tiangen). Relative expression levels were normalized to the β-actin internal control (Schmittgen and Livak, 2008).

Bioinformatic analysis: Sequence analysis of nucleotides or proteins was carried out using the BLAST program (NCBI). Predictions of protein structure were performed on the Signal IP Server (http://www.cbs.dtu.dk/ services/SignalP/) and the TMHMM Server (http://www.cbs.dtu.dk/services/TMHMM-2.0/).

RESULTS AND DISCUSSION

Identification of ovine TLR4 splice variants: Genome sequences are highly conserved between bovine and sheep (De Gortari *et al.*, 1998). Sequence alignment between the bovine TLR4 DNA genomic sequence and the previously published ovine TLR4 cDNA revealed that ovine TLR4

primary transcript is likely to encompass three exons and two introns. The full length of the sheep TLR4 genomic DNA sequence was estimated to be approximately 10 Kb, Three pairs of overlapping PCR primers were designed for PCR amplification of the TLR4 gene. Products were confirmed by sequencing (the genomic sequence was submitted to Genbank; accession GU461886). Alignment was carried out between the previously published cDNA sequence (NM_001135930.1) and the TLR4 genomic DNA sequence, confirming that the ovine gene comprises three exons and two introns (Fig. 1a). The first two exons are 90 and 167 bp in length, respectively, however RT-PCR results revealed the presence of two additional bands on gel electrophoresis (Fig. 1b). These were also cloned and sequenced. This revealed that one contained an alternative exon of 222 bp between the second and third exons (GenBank accession GU461887) (Fig. 2a). The other variant harbored a deletion of 167 bp deletion corresponding to exon 2 (accession GU461888) (Fig. 2b). Notably, the alternative splice-variant GU461887 was generated by unconventional non-AG/GT mRNA splicing; both splice variants contained one or more in-frame stop codons.

Analysis of the predicted amino acid sequences indicated that the major transcript encodes a polypeptide of 840 amino acids (aa) with an extracellular domain of 633 aa, a transmembrane domain of 23 aa and an 84 aa intracellular domain. The two new splice variants encode truncated polypeptides of 86 aa and 33 aa corresponding to the N-terminus of TLR4 (Fig. 3).

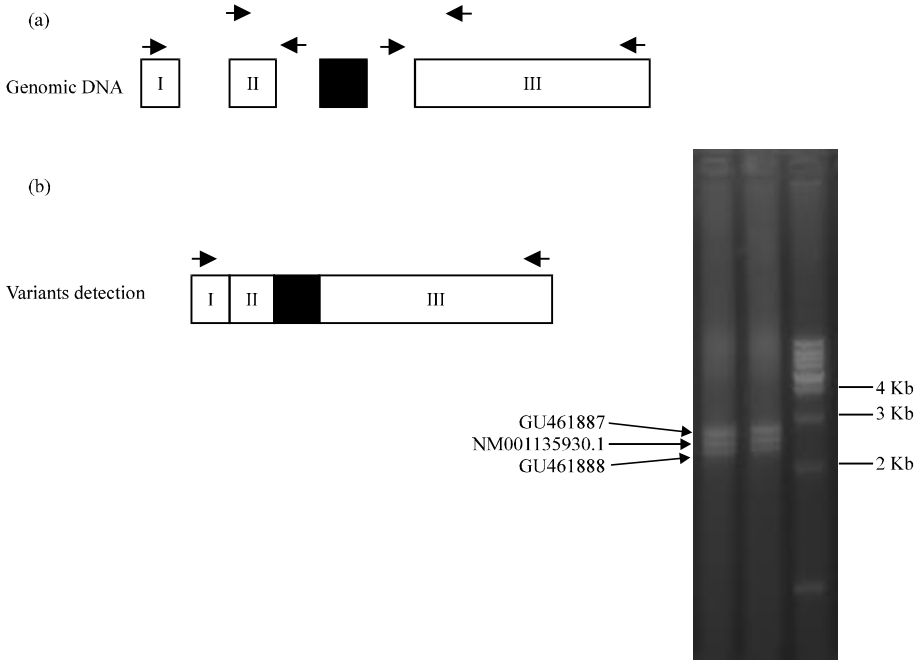

Fig. 1: Structure of sheep TLR4 gene and detection of variant transcripts. Boxes and lines denote exon and intron sequences respectively. (a) Structure of the ovine genomic TLR4 gene; primers used for PCR are indicated by arrows. (b) Detection of TLR4 transcript variants using RT-PCR primers based on sequences of the first and third exons (left, arrowed); the right panel shows agarose gel electrophoresis of the RT-PCR products obtained from ovine fibroblasts revealing two minor products, GU461887 and GU461888, in addition to the reported product (NM_001135930.1); right lane, size markers; left lanes, RT-PCR products

Fig. 2: Sequences alignment between all three splice variants. RT-PCR of splice variants of ovine TLR4 aligned with the major cDNA NM_001135930.1. (a) Splice variant GU461887 contains an additional exon of 222 bp; (b) GU461888 cDNA lacks exon 2

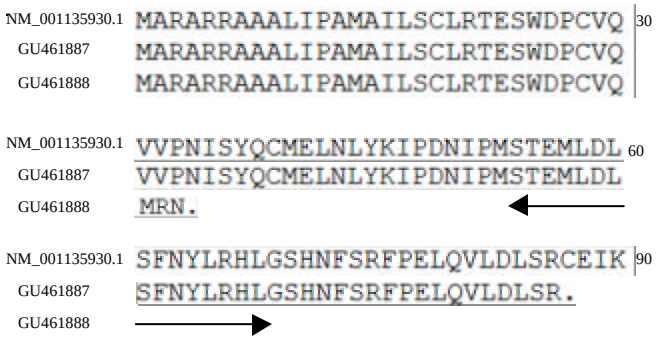

Fig. 3: Polypeptide sequences alignment between three splice variants. Polypeptide sequences encoded by the two novel splice variants of ovine TLR4. Both contain additional in-frame stop codons leading to predicted translation products of 86 and 33 aa, respectively. The C-terminus of GU461887 encode polypeptide exhibit a LRR feature (underline)

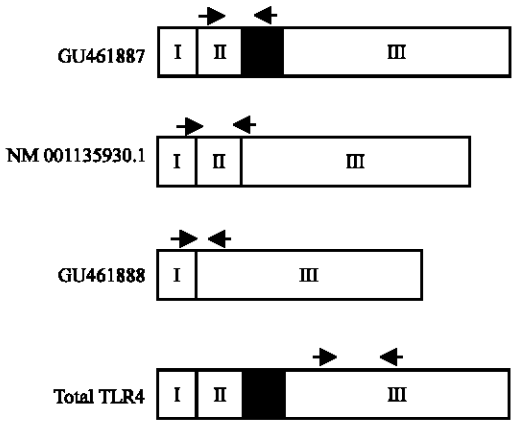

Fig. 4: Variants specific PCR primers design. Measurement of mRNA levels of different TLR4 splice variants by RT-PCR. Exons and introns are depicted as boxes and lines, respectively; the black box denotes the alternative exon. Variants specific primer pairs designed to detect the specific transcript variants, or total TLR4 transcript levels (exon 3 primers), are arrowed

To investigate LPS effect on the expression of the splice variants, primers were designed to allow detection variants individually (Fig. 4). Specificity of the primer combinations were confirmed by amplification of cloned variant cDNAs; RT-PCR and DNA sequencing was then used to confirm that these specific transcript variants were present in LPS-stimulated fibroblasts (data not presented). This permitted the design of primer pairs based on exon 3, a genomic region present in all cDNA products, in order to compare the expression levels of the different splice variants.

Levels of TLR4 variant transcripts following LPS stimulation: The production of mRNAs corresponding to the different splice variants in response to LPS stimulation was investigated. Fibroblasts were treated with LPS between 0.5 and 24 h; RNA was extracted at different time points and analyzed by RT-PCR. Total TLR4 and the previously reported alternative splice variant mRNA levels responded similarly to LPS stimulation (Fig. 5a-e). Immediately following stimulation (0.5 h) there was a decrease in transcript levels this was followed at 8 h by up-regulation and then return to near-baseline values by 24 h (Fig. 5a). Both of the new variants, GU461887 and GU461888 (Fig. 5c, d) responded with a similar pattern of expression, initially a slightly decrease in expression followed by elevation at 8 h and subsequent decline.

TNF-α expression during LPS stimulation: Following LPS stimulation TNF-α mRNA levels were determined. There was an immediate and dramatic upregulation at 0.5-2 h after LPS treatment. This upregulation increased levels to 9-fold above baseline (Fig. 5e). Thereafter there was a steep decrease in expression levels and by 12 h post-stimulation mRNA levels were not significantly different from baseline.

Effect of TLR4 transcription variant overexpression: To determine if the product generated by transcript variant GU461887 had any biological function. Fibroblasts cells were transfected with expression vector pIRES-soTLR4. Twenty-four hours post transcfection, cells were stimulated with different concentrations of LPS for 2 h. Expression of TNF-α in each group was determined by real-time PCR. As shown in Fig. 6, over expression of transcript variant GU461887 inhibited TNF-α expression in ovine fibroblasts.

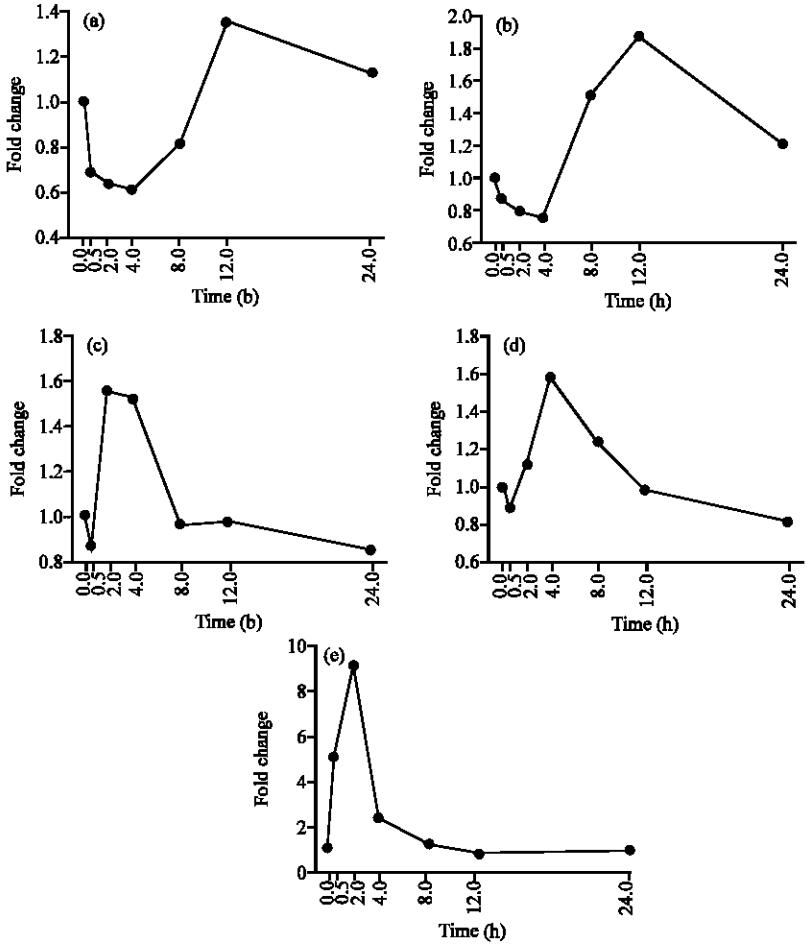

Fig. 5: Expression levels of variants TLR4 and TNF-α transcripts following LPS treatment of ovine fibroblasts. Transcript levels were measured by RT-PCR and normalized to levels of the β-actin internal control. (a) Total TLR4 transcript levels showing a transient decline followed by upregulation from 8 to 12 h after stimulation followed by return towards baseline values. (b) Levels of the major transcript encoding TLR4 (NM_001135930.1) showing a similar pattern with total TLR4 transcription following LPS stimulation (c, d). Expression levels of variants GU461887 and GU461888, showing an initial transient decline followed by upregulation at 0.5-8 h and subsequent decline lower than the baseline. (e) TNF-α mRNA levels showing dramatic upregulation at 0.5-2 h post LPS treatment and rapid return towards baseline values. Transcript levels in untreated cells were set as 1.0. The experiments were repeated three times; values presented are the results of one representative experiment

Bacteria infection cases world wide range diseases each year (Shayegh *et al.*, 2008). Subsequently, performances of livestock were dramatically influenced (Neelesh, 2007). TLR-mediated immunity response is the first line for bacteria infection. Several earlier studies have addressed the expression of the ovine TLR4 gene and polypeptide during pathogeny challenge (Menzies and Ingham, 2006; Hillman *et al.*, 2008; Nalubamba *et al.*, 2008). In most these studies, activation of TLR4 expression was monitored by measuring the levels of transcripts and variants

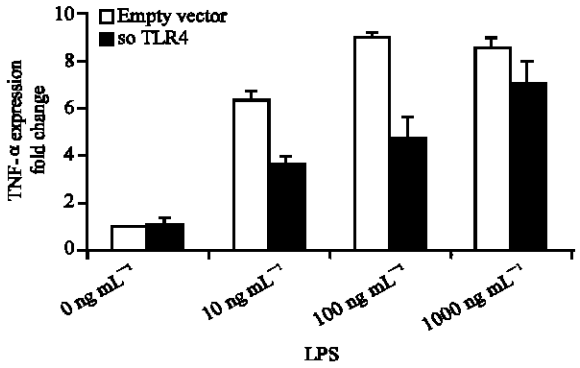

Fig. 6: Transcript variant overexpression assay. Overexpression of GU461887 inhibits transcription of TNF-α in ovine fibroblasts. Each group was transfected with either empty vectors or transcript variant (GU461887) expression vector (pIRES2-soTLR4). Twenty-four hours post transfection, cells were stimulated with various concentration LPS for 2 h. Results clearly showed TNF-α expression inhibited by overexpression of GU461887 in different LPS concentrations

harboring the third exon of the previously reported transcript variant. Recent study showed that more than 40% human genes transcripts are known to undergo alternative splicing (Johnson *et al*., 2003), notably many of those genes involved in immunological responses. Alternative spliced proteins are likely act as a regulator in immunity response (Modrek *et al*., 2001). This study focused on TLR4 splice variants and two new mRNA splice variants are reported. The first, GU461887, harbors an alternative exon of 222 bp between the second and third exons and that contains an in-frame stop codon near the beginning of the protein-coding sequence. The second variant, GU461888, lacks the second exon and also contains an in-frame stop codon. Similar to that of *Sx1* in *Drosophila*, both of them generate transcript variant by including or excluding stop codens (Bopp *et al*., 1991).

A reliable method was developed allowing for monitor TLR4 transcript variants individually after LPS stimulation. First of all, the total TLR4 expression of ovine fibroblasts, detecting by a pair of primers which located on the third exon of TLR4, was rapidly down-regulated within the first 0.5 h following LPS stimulation, subsequently increased (8-12 h) and declined thereafter. In contrast, an upregulation pattern was observed on monocyte (Armstrong *et al*., 2004). The temporarily lose of expression is similar to that previously reported in dendritic cells (Visintin *et al*., 2001; Jaregova *et al*., 2007). As it had been proved, TLR4 expression showed distinct patterns in different type of cells (Jaregova *et al*., 2007). In parallel, expression of TNF-α, a marker of LPS-derived activation, was dramatically elevated within 0.5 h, indicating that LPS-induced immunity responses were immediately triggered. Frequently, the degradation of TLR4 observed on monocyte associates with LPS tolerance, helping animal survives from bacteria infection (Lehner *et al*., 2001). It is notable that fibroblasts is a kind of antigen-presenting cells that can trigger the immunological responses through the release of large mount pro-inflammatory cytokines (Buckley *et al*., 2001; Kaufman *et al*., 2001; Smith *et al*., 1997). Due to the fibroblast is the most common cell type within the body, it is reasonable that TLR4 expression of fibroblasts is tightly regulated.

The expression of NM001135930.1 was coordinate with that of total TLR4. This indicated that NM001135930.1 was the functional transcript variant. While, similar upregulation transcript

patterns were observed in the two novel alternative splice variants. Consist to the previously report on human and mouse (Iwami *et al*., 2000; Jaregova *et al*., 2007), all variants were constantly transcribed. It was possible that these transcript variant may have biological function. Alternative splicing is a powerful and efficiency regulatory mechanism which able to regulate gene expression and protein production. It was frequently observed in immunity system to regulate immunity response during pathogen infection (Ip *et al*., 2007). An up-regulated transcript variant of IL-1 receptor accessory protein gene (IL-1R AcP) which encode a soluble form hIL-1R AcP protein had been proved inhibiting excessive cellular responses (Jensen *et al*., 2000).

The predicted protein which generated by GU461887 containing 86 aa, lacking the transmembrane domain and intracellular domains, would represent a new TLR4 secretory product. Sequence prediction reveled transcript variant GU461888 potentially encoding an alternative product of 33 aa in length. Doman prediction showed these were mainly belonging to signal peptide. For type transmembrane proteins, intracellular domain is essential for toll-like receptor proteins activating of downstream signaling pathways. Previous report (Sandor *et al*., 2003) demonstrated intracellular domains mutated TLR1 and TLR2 failed for launch immunity response. The extracellular segments of TLR proteins are distinctively composed of 22-28 aa-long LRRs (Hoshino *et al*., 1999). Biological function of LRRs motifs are bind to specific components of pathogens. Sequence analysis revealed the C- terminus of predicted protein encoded by GU461887 represent LRR domain feature (Fig. 3). Thus, it is possible that this truncated protein could able to binding affinities and would not active the TLR4 pathway due to lake of intracellular domain. As a result, such a small protein could regulate TLR signaling. A transcript variant of mouse TLR4 generates a soluble TLR4 (smTLR4) protein without the transmembrane and intracellular domains upregulated during LPS stimulation; its overexpression was shown to inhibit LPS-induced release of inflammatory factors (Iwami *et al*., 2000). Interestingly, resembling results were observed in our study. Transient transfection assay confirmed that overexpression GU461887 inhibited the transcription of TNF-α (Fig. 6). The possible reason for suppression of LPS-induced release of inflammatory factors by such small protein is interfering with the interaction between TLR4 and its co-receptors including MD2 and CD14 (Liew *et al*., 2005). Alternative splicing is an efficiency regulatory mechanism which is able to regulate gene expression and protein production.

In conclusion, two new sphce variants of ovine TLR4 have been sequenced. Both variants were upregulated following LPS stimulation of fibroblasts and TNF-α expressions were down regulated when overexpression of GU461887. This might provide an alternative therapy strategy for exdotoxin shock. Further experiments will be required to address how TLR4 variant impact LPS-induction of immune and inflammatory responses.

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