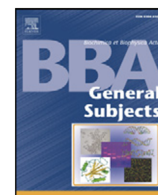
Biochimica et Biophysica Acta 1790 (2009) 267–274

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen



Polymorphism distribution and structural conservation in RNA-sensing Toll-like receptors 3, 7, and 8 in pigs

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A R T I C L E   I N F O

Article history:
Received 6 October 2008
Received in revised form 7 January 2009
Accepted 12 January 2009
Available online 20 January 2009

Keywords:
Swine
Toll-like receptor
Genomic structure
Single nucleotide polymorphism

A B S T R A C T

Background: Viral genomic RNA—both single-stranded (ss) and double-stranded (ds)—is recognized by RNA-sensing Toll-like receptors (TLRs), notably TLR3 (dsRNA), TLR7 (ssRNA), and TLR8 (ssRNA). However, our knowledge of the roles of porcine TLR3, 7, and 8 in antiviral immunity is inadequate.
Methods: From information on exon–intron boundaries obtained through comparisons of the genomic and cDNA sequences, polymorphisms in the coding sequences of each gene were detected in 84 male pigs of 11 breeds.
Results: Genomic structures are conserved between pigs and humans. The RNA-sensing *TLR* genes had fewer polymorphisms causing amino acid alterations than did the cell-surface *TLR* genes, but the alterations were distributed with a similar bias toward ectodomains.
Conclusions: The low level of diversity of substitutive polymorphisms in RNA-sensing *TLRs* than cell-surface *ones* implies that polymorphisms severely affecting function have been eliminated by selection pressure during longstanding pig breeding.
General significance: Recognition of virus-derived RNA is critical in host defense against infection. These results should provide a useful clue to analysis of the association between polymorphisms in RNA-sensing TLRs and disease resistance.

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1. Introduction

In terms of host defenses, pathogen recognition and the following activation of the innate and adaptive immune system are mediated by pattern recognition receptors such as Toll-like receptors (TLRs). TLRs recognize the characteristic molecular patterns (pathogen-associated molecular patterns, PAMPs) of microorganisms such as viruses, bacteria, fungi, and protozoa. The human *TLR4* (*hTLR4*) was isolated by a pattern search of the Toll/Interleukin-1 receptor (IL-1R) domain (TIR domain) of *Drosophila* Toll [1] in an expressed sequence tag (EST) database [2]. With the exception of *hTLR6*, which was derived from a placental cDNA library [3], *hTLR1* to *5* [4] and *10* [5] were also found in an EST database, and *hTLR7*, *8*, and *9* were harvested from a genomic DNA database by a homology search against the extracellular domain of *hTLR4* [6,7]. So far, 10 human *TLRs* have been reported, and 10 or more orthologous genes have been identified in many animals. Correlations among TLRs from various species have been revealed by analysis of a phylogenetic tree comprising six major families [8]. From a different perspective, the TLRs have been split into two groups by their cellular localization. Briefly, the cell-surface group (TLR1, 2, 4, 5, 6, and 10) principally recognizes unique bacterial cell-body, -wall or -surface components and the cytoplasmic vesicle group (TLR3, 7, 8, and 9) senses ectopic pathogen-derived nucleic acids [9].

Ten porcine *TLRs* (*poTLR1–10*) equivalent to the 10 human *TLRs* have been completely cloned and characterized [10–13]. Three RNA-sensing poTLRs (poTLR3, 7, and 8), belonging to the cytoplasmic vesicle group, have been reported recently [14–16]. TLR3 generally recognizes the synthetic dsRNA-analog polyinosine–polycytidylic acid (polyI:C), which mimics viral dsRNA formation during virus multiplication [17]. PolyI:C stimulation of porcine alveolar macrophages induces greater production of type-I interferons (IFNs) than occurs with stimulation of peritoneal macrophages [18]. PolyI:C activates interferon regulatory factor (IRF)-3 and IRF-7 promoters in the presence of poTLR3; on the other hand, imiquimod (R837), which is a synthetic antiviral compound and an imidazoquinoline derivative, activates only the IRF-7 promoter in the presence of poTLR7 [14]. R837 triggers nuclear factor kappa B (NF-κB) activation through TLR8 as well as TLR7 in pigs, although hTLR8 does not show recognition of R837 [16]. Such a case is observed in hTLR7 and hTLR8, which commonly recognize another imidazoquinoline derivative, resiquimod (R848), although murine TLR8 does not activate NF-κB after

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doi:10.1016/j.bbagen.2009.01.002

Table 1
Primer sequences used in this study

Gene	Objective		Forward (5′ to 3′)	Reverse (5′ to 3′)	Size (bp)
TLR3	Isolation	Full-length cDNA	TACCTGAAGAAGATAAACAAGGC	AGGAAAATTACTCCTTTAATCTC	3046
	SNP search	Fragment 1	AAGTTGTGAGTAGCGAGGTC	ATTCTTCACCTTGATAATTAG	689
		Fragment 2	AACGTTCTGTACTAGCCATG	CCTACGCCACAGCTTTCAGC	891
		Fragment 3	CAGACTCCATTGTATTTTAG	AGGTATCTGTTGTAGGAAAG	824
		Fragment 4	AACTCCTTCTCAAGTTTGCG	ACTGGGAGACCATGATATTG	902
		Fragment 5	TTCCTACAACAGATACCTAG	CTGCATATTCAAACTGCTCTG	884
		Fragment 6	GAAAGCATTGCCTGGTTTGT	TCTGGAATCTCCTCAAGAAA	844
		fragment 7	TTACTAACATTATTGCTCAC	TACTCTTACCTATAGATATG	512
TLR7	Isolation	Full-length cDNA	GCCCATCTCTTCACCTCCGAAGATTC	CAACCCTGTTTCTATACATACCAG	3707
	SNP search	Fragment 1	TTTGGTAGTCTCAAGTGTCG	GAAGTTAGACAGACCAAGTG	531
		Fragment 2	ATGCAGCAAACTGCACTTGC	AGAGAGTTACTGTGTAGACGT	975
		Fragment 3	TCAGTCAACTGCAAGTTCTG	ACTGCCATTAAGAGCTTGGC	869
		Fragment 4	ATGAGTATGCAAGGAGTTGC	TTTCTAGATTCTGCAGATAC	705
		Fragment 5	GAGACGGTGATAACAGATAC	CTAGGCTGTCTCTTTGAACA	1244
		Fragment 6	TGCATCATAATCGGTTTCTG	CAACCCTGTTTCTATACATACCAG	1206
TLR8	Isolation	Full-length cDNA	GCGAGGTTCTGCTGATGGTAG	CGAACCACGACCAAACATCACCGAGG	3266
	SNP search	Fragment 1	TGTTGAGGGAACTTCAAAGC	GTCAGGTAGCGAAAAGCCAG	928
		Fragment 2	ACGAATTTGAAGGTGCTGTC	TGCCAGTACCTGTCATCTTG	1295
		Fragment 3	GACTTTGATGATGACGCTGC	GTGACCTTCAGATTCTCATC	752
		Fragment 4	CAGATAGCCTATCTAAATTC	CGAACCACGACCAAACATCACCGAGG	1037

stimulation with R848 [19,20]. The ssRNA virus genome is commonly known to be a physiological agonist of mouse TLR7 and hTLR8; hence it is considered that there are species-specific differences in the recognition of viruses [21]. RNA oligonucleotide (ORN) containing a guanosine (G)- and uridine (U)-rich sequence derived from foot-and-mouth disease virus (FMDV) activates NF-κB and induces the expression of IFN-α via poTLR7 [15]; moreover, ORN derived from the U5-region of human immunodeficiency virus-1 (HIV-1) stimulates dendritic cells and macrophages in humans and mice [21].

The pathogens causing notifiable swine infectious diseases consist largely of RNA viruses, including FMDV, but knowledge of the roles played by poTLR3, 7, and 8 in immunity against such viruses is inadequate. We therefore completely sequenced the cDNA and genomic fragment inserted in bacterial artificial chromosome (BAC) clones including these TLRs, and we attempted to clarify their genomic structures by comparing the sequences of the cDNAs and the genomic sequences. From an analysis of exon–intron genomic structures we elucidated nucleotide polymorphisms, including insertion/deletions (indels) and single nucleotide polymorphisms (SNPs), within open reading frames (ORFs). The rates of occurrence of polymorphisms in the RNA-sensing *poTLRs* were significantly lower than in cell-surface *TLRs* (*poTLR1*, *2*, *4*, *5* and *6*) [13].

2. Materials and methods

2.1. Cell preparation

Peripheral blood lymphocytes (PBLs) were separated from a Landrace pig and maintained in RPMI1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA).

2.2. RNA extraction, cDNA synthesis, reverse transcription-polymerase chain reaction (RT-PCR), and cDNA cloning

Total RNA was extracted from Landrace PBLs with ISOGEN (Nippon Gene Co., Ltd, Tokyo, Japan), and then the total RNA was treated with DNaseI (Nippon Gene) to prevent contamination by genomic DNA. To perform rapid amplification of cDNA ends (RACE), cDNA was synthesized from 1 μg of DNaseI-treated total RNA by using a Marathon cDNA Amplification Kit (BD Biosciences, Palo Alto, CA, USA) in accordance with the manufacturer's instructions. The RACE primers (data not shown) were designed from partial nucleotide sequences of *poTLR3* (accession number CJ014653.1) and *poTLR7* (accession numbers CJ024769.1, CJ015502.1, and BW956357.1) in Pig Expression Data Explorer (PEDE) [22], and *poTLR8* cDNA (accession number NM_214187.1), obtained from the DDBJ/EMBL/GenBank

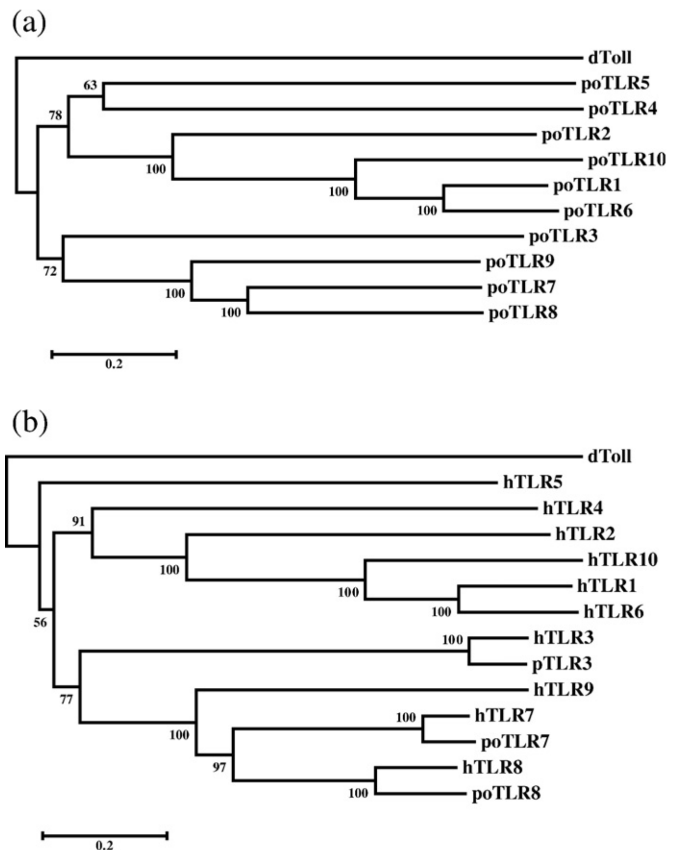

Fig. 1. Phylogenetic trees of poTLR3, 7, and 8 with other poTLRs (a) and with hTLRs (b). The accession numbers of the entire amino acid sequences obtained for the TLRs were as follows: hTLR1, NP_003254.2; hTLR2, NP_003255.2; hTLR3, NP_003256.1; hTLR4, NP_612564.1; hTLR5, NP_003259.2; hTLR6, NP_006059.2; hTLR7, NP_057646.1; hTLR8, NP_619542.1; hTLR9, NP_059138.1; hTLR10, NP_112218.2; poTLR1, BAD91798.1; poTLR2, BAC99316.1; poTLR4, BAD36843.1; poTLR5, BAD91800.1; poTLR6, NP_998925.1; poTLR9, NP_999123.1; poTLR10, BAD91802.1. *Drosophila* Toll (dToll, NP_524518.1) was used as the outgroup. Bootstrap values for 1000 replicates are shown as percentages beside the branches.

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