Original Article
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Cloning, expression and functional analysis of the duck Toll-like receptor 5 (TLR5) gene

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Toll-like receptor 5 (TLR5) is responsible for the recognition of bacterial flagellin in vertebrates. In the present study, the first TLR5 gene in duck was cloned. The open reading frame (ORF) of duck TLR5 (dTLR5) cDNA is 2580 bp and encodes a polypeptide of 859 amino acids. We also cloned partial sequences of myeloid differentiation factor 88, 2'-5'-oligoadenylate synthetase (OAS), and myxovirus resistance (Mx) genes from duck. dTLR5 mRNA was highly expressed in the bursa of Fabricius, spleen, trachea, lung, jejunum, rectum, and skin; moderately expressed in the muscular and glandular tissues, duodenum, ileum, caecum, and pancreas; and minimally expressed in the heart, liver, kidney, and muscle. DF-1 or HeLa cells transfected with DNA constructs encoding dTLR5 can activate NF-κB leading to the activation of interleukin-6 (IL-6) promoter. When we challenged ducks with a Herts33 Newcastle disease virus (NDV), mRNA transcription of the antiviral molecules Mx, Double stranded RNA activated protein kinase (PKR), and OAS was up-regulated in the liver, lung, and spleen 1 and 2 days post-inoculation.

Keywords: duck, innate immune response, myeloid differentiation factor 88, Newcastle disease virus, Toll-like receptor 5

Introduction

Innate immunity is the first line of host defense against invading pathogens. These innate immune responses are initiated by host pattern recognition receptors (PRRs), which recognize molecular structures of conserved pathogen-associated molecular patterns (PAMPs) expressed by microorganisms [10,13,25,28]. Toll-like receptors (TLRs) are important PRRs that recognize pathogenic motifs presented on the cell surface or within intracellular vesicles. These receptors consist of an extracellular leucine repeat (LRR) domain that mediates the detection of PAMPs, transmembrane domains, and intracellular Toll/interleukin-1 receptor (TIR) domains that are important for signal transduction [4,14,23]. Recognition of PAMPs by TLRs activates transcription factors and the expression of innate antiviral genes as well as pro-inflammatory cytokines [4,14].

Based on data from genomic analyses, it has become evident that the TLR system is part of ancient machinery that is evolutionary conserved with homologs present in nematodes, insects, fish, plants, mammals, and birds [16,27]. However, functional differences between related TLRs from different species exist, and have been instrumental in deciphering TLR function and evolution along with an organism's susceptibility to infection [16]. Currently, 13 murine TLRs, 10 human TLRs, and 10chicken TLRs have been characterized. However, many waterfowl TLRs have yet to be identified, including duck TLR5 (dTLR5).

Newcastle disease virus (NDV) can cause an economically significant and serious disease in almost all birds. Ducks are often resistant to NDV and avian influenza virus (AIV) that are capable of killing chickens [32]. The high level of resistance to NDV and AIV in ducks and geese has been linked to the molecular characteristics of retinoic acid-inducible gene I (RIG-I) [1]. Chickens lack RIG-I while ducks and geese not, which may increase susceptibility of chickens to AIV and NDV compared to ducks and geese [1]. Therefore, different avian species have different types of PRRs, and they may thus produce different antiviral innate immune responses. Consequently, it is

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meaningful to study the innate immune system of ducks, which may further reveal the molecular basis of ducks' high resistance to NDV and AIV.

Avian TLRs are different from those of other animals in many ways, including the presence of chicken TLR1La,chicken TLR1Lb, chickenTLR15, chickenTLR21, and pseudogene TLR8 as well as the absence of TLR9 [3]. The avian TLR repertoire has both common and unique features compared to that of mammals. Knowledge about the natural variation in TLR function and specificity will be conducive to our understanding of the evolution of the innate immune system and disease resistance mechanisms [15].

In mammals, TLR5 plays an important role in host defense against bacterial infections.TLR5 mainly recognizes flagellin, which contributes to the motility of bacterial pathogens [9]. This activates the myeloid differentiation factor 88 (MyD88) signaling pathway and nuclear factor kappa B (NF-κB)-dependent genes [6,8]. The MyD88 signal pathway is utilized by all TLRs except TLR3. Stimulation with TLR ligands recruits the interleukin-1 receptor-associated kinase (IRAK) family of protein kinases and MyD88 protein to activate tumor-necrosis-factor-receptor-associated factor 6 (TRAF6). The activation of TRAF6 causes activation of transforming-growth-factor-β-activated kinase 1 (TAK1) that leads to activation of activator protein1 (AP-1) and NF-κB through the mitogen-activated protein kinases (MAPK) and NF-κB kinase (IKK) complex, respectively [23]. Data from a previous study indicated that TLR5 plays a role in the recognition of flagellated pathogens in mammals [9], chickens [16], and geese [7]. However, TLR5 has not yet been identified in ducks and its function is not clear.

In the present investigation, we cloned the full-length dTLR5, partial sequences of duck MyD88, and two antiviral molecules (Mx and OAS), which laid a foundation for further functional studies. Gene expression of dTLR5 was measured in various tissues from uninfected ducks by quantitative real-time PCR (qRT-PCR). We also demonstrated that overexpression of dTLR5 in DF-1 and HeLa cells can activate NF-κB and subsequently induce IL-6 expression. These data promote our understanding of the relationship between TLR5 and innate immunity in avians including chickens and ducks.

Materials and Methods

Experimental birds

Fifteen one-day-old healthy Pekin ducks were purchased from the Chinese Gene Center for Waterfowl (Taizhou, China) and housed in isolators. The birds were confirmed to be serologically negative for NDV and AIV by agar gel precipitation tests and hemagglutinin inhibition assays.

Identification studies of duck TLR.5, MyD88, OAS and Mx genes

Total RNA was extracted from the spleen of 3-week-old Pekin ducks using an HP Total RNA kit (Omega, China) according to the manufacturer's instructions. The isolated RNA was incubated 15min at room temperature with RNase-free DNase I (Omega) to remove contaminating genomic DNA before being reverse transcribed into cDNA. The RNA concentration was measured using a NanoDrop 2000 (Thermo Scientific, USA). Next, 2 μg of total RNA was reverse transcribed with 1 μL M-MLV reverse transcriptase (Promega, USA) at 42°C for 2 h. To identify the TLR5, MyD88, OAS and Mx sequences of Pekin duck, PCR primers were designed based on multiple alignments of humans, mice, chickens and geese with Oligo Primer Analysis Software (ver. 6.71; Molecular Biology Insights, USA). The sequences of TLR5, MyD88, OAS and Mx of humans, mice, chickens and geese were obtained from the GenBank (National Center for Biotechnology Information, USA). Degenerate primers (Table 1) and cDNA were used to amplify these genes. The PCR mixture consisted of 10 μL of 5× PrimeSTAR buffer (Takara Bio, China), 30 μL nuclease-free water, 4 μL dNTP Mixture (Takara Bio), 1 μL of cDNA, 2 μL of each gene specific primer (20 mM, Table 1) and 1 μL of PrimeSTAR HS DNA polymerase. The PCR cycling conditions were: 30 cycles of 98°C for 10 sec, 58°C for 15 sec, 72°C for 30 sec; One cycle of 72°C for 5 min. The PCR product was cloned into a pMD19-T (Takara Bio) easy vector then used to transform competent DH5α cell (TransGen Biotech, China) that were plated on LB-agar dishes. Positive colonies were screened by colony PCR. The colony PCR mixture consisted of 10 μL of 2× Premix taq (Takara Bio), 7.5 μL nuclease-free water, 2 μL of bacterium fluid and 0.25 μL of each gene specific primer (20 mM, Table 1). The PCR cycling conditions were: 30 cycles of 98°C for 10 sec, 58°C for 30 sec, 72°C for 30 sec; One cycle of 72°C for 5 min. Five of the positive colonies were sent to a commercial company (Sangon Biotech, China) for sequencing. Rapid amplification of cDNA ends (RACE) PCR primers were designed based on the partial nucleotide sequences of dTLR5 with Oligo Primer Analysis Software (ver. 6.71; Molecular Biology Insights). 5' and 3' SMARTer RACE PCR (Clontech Laboratories, USA) was performed with the designed RACE PCR primers and SMART universal primer supplied in the kit (Table 1). The nucleotide sequences of dTLR5 were confirmed by sequencing the RACE PCR product as described above. Based on the sequences obtained by RACE RCR, gene-specific primers dTLR5 F and dTLR5 R (Table 1) were designed to amplify the full coding region.

Amino acid sequences were aligned using Clustal X software [20] and edited with Boxshade 3.3.1 in Mobyle system (the Institut Pasteur Biology IT Center and the Ressource Parisienne en Bioinformatique Structurale, France). Sequence homology and phylogenetic analyses based on the amino acid sequences

Table 1. Sequences and designated application of primers used in this study

Primer name	Primer sequence (5′-3′)	Usage
idTLR5 F	ATGATGTTACATCAACAGCTAGT	Degenerate PCR
idTLR5 R	TCAATGTGAGAGTGTCGCTACAGTC	
5′-RACE GSP1	TGCTTTCACACAGGTTGGATATGGC	RACE PCR
3′-RACE GSP2	CAACTATGTCTGCGATTGTACTTTAC	
UPM Long	CTAATACGACTCACTATAGGGCAAG	
UPM Short	CAGTGGTAT-CAACGCAGAGT	
NUP	AAGCAGTGGTATCAACGCAGAGT	
TLR5 F	TCGAGCTCATGATGTTACATCAACAGCTAGT	Full-length cloning
TLR5 R	TACTCGAGTCAATGTGAGAGTGTCGCTACAGTC	
idMyD88 F	TGAGGAGCTGGGCTGCGAATATT	Gene cloning
idMyD88 R	AGCGACACTGTGGTCCCGAAGGC	
idOAS F	GCCTGGTCAAGCACTGGTAC	Gene cloning
idOAS R	GGGTCGGCGGGATCCAGGAT	
qdTLR5 F	GCACTCCGGCTGTTTCAGAACA	qRT-PCR
qdTLR5 R	TGCTTTCACACAGGTTGGATATGGC	
qdMyD88 F	TGTCTTTGACCGGGACGTCTTG	qRT-PCR
qdMyD88 R	TGCACTTCACGGGAATCAGC	
qdPKR F	AATTCCTTGCCTTTTCATTCAA	qRT-PCR
qdPKR R	TTTGTTTTGTGCCATATCTTGG	
qdOAS F	TCTTCCTCAGCTGCTTCTCC	qRT-PCR
qdOAS R	ACTTCGATGGACTCGCTGTT	
qdMX-1 F	TGCTGTCCTTCATGACTTCG	qRT-PCR
qdMX-1 R	GCTTTGCTGAGCCGATTAAC	
qGAPDH F	ATGTTCGTGATGGGTGTGAA	qRT-PCR
qGAPDH R	CTGTCTTCGTGTGTGGCTGT	

TLR: Toll-like receptor 5, RACE: rapid amplification of cDNA ends, GSP: gene specific primer, NUP: nested universal primer A, UPM: universal primer mix, MYD88: myeloid differentiation factor 88, OAS: oligoadenylate synthetase, PKR: protein kinase, MX: myxovirus resistance, GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

were conducted using MegAlign software (DNASTAR, USA). A phylogenetic tree was constructed that contained the TLR5 of 27 different species: duck (*Anas platyrhynchos*, KF717594) goose (*Anser anser*, AEP71332.1), chicken (*Gallus gallus domesticus*, ACR26276.1), turkey (*Meleagris gallopavo*, ADX33343.1), zebra finch (*Taeniopygia guttata*, XP_002188762.1), human (*Homo sapiens*, AAI09119.1), chimpanzee (*Pan troglodytes*, NP_001123934.1), macaque (*Macaca mulatta*, NP_001123901.1), mouse (*Mus musculus*, NP_058624.2), rat (*Rattus norvegicus*, NP_001139300.1), rabbit (*Oryctolagus cuniculus*, AEA11027.1), pig (*Sus scrofa*, AGT79978.1), dog (*Canis lupus familiaris*, NP_001184105.1), gray wolf (*Canis lupus*, NP_001184105.1), cat (*Felis catus*, XP_004001379.1), cattle (*Bos Taurus*, AFR42399.1), water buffalo (*Bubalus bubalis*, AEY63776.1), American bison (*Bison bison*, AEY63777.1), sheep (*Ovis aries*, NP_001129398.1), chamois (*Rupicapra rupicapra*, AFR42404.1), deer (*Odocoileus virginianus*, AFR42402.1), Japanese flounder (*Paralichthys olivaceus*, AB562152), fugu rubripes (*Takifugu rubripes*, AAW69374.1), rainbow trout (*Oncorhynchus mykiss*, NM_001124744), carp (*Cyprinus carpio*, AGH15501.1), zebrafish (*Danio rerio*, NP_001124067.1), and rattlesnake (*Crotalus adamanteus*, AFJ51724.1).

Quantitative gene expression studies

For tissue distribution analyses, tissues including trachea, crop, heart, spleen, liver, lung, kidney, glandular stomach, stomach, pancreatic gland, bursa of Fabricius, ileum, jejunum, colon, caecum, rectum, leg muscle, and skin were collected from three uninfected Pekin ducks. To quantify gene expression of TLR5 in the various tissues, total RNA was isolated with an HP Total RNA Kit (Omega). The RNA concentration was measured using a NanoDrop 2000 (Thermo Scientific). Next, 2 μg of total RNA was reverse transcribed into cDNA as described above. The cDNA (1 μL) was amplified in a 20 μL reaction using a PTC-200 real-time PCR system (Bio-Rad Laboratories, USA). The primers for dTLR5 (q-dTLR5 F and

q-dTLR5 R) were designed with Oligo6 (ver. 6.71; Molecular Biology Insights) (Table 1). The qRT-PCR reaction consisted of 1 μL of cDNA sample, 8 μL nuclease-free water, 10 μL of 2× SYBR Green PCR master mix (Takara Bio), and 0.5 μL of each primer (10 mM). The PCR cycling conditions were: one cycle of 95°C for 30 sec followed by 40 cycles of 95°C for 5 sec, 57°C for 30 sec, and 72°C for 30 sec. A dissociation curve analysis was performed to verify the amplification of a single and specific product. The threshold cycle (CT) value was normalized relative to the housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase [GAPDH]). The relative expression levels for dTLR5 mRNA were measured using GAPDH as an internal reference using the comparative Ct ($2^{-\Delta\Delta Ct}$) method [20].

The animal experiment was conducted according to the Guidelines for Animal Experimentation of Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences (China). The NDV strain Herts33 used in the experiment was obtained from the China Institute of Veterinary Drug Control (Beijing, China). Twelve 2-week-old ducks were divided randomly into two groups of six. The ducks in group 1 were intramuscularly injected with Herts33 ($10^6$of 50% egg infectious dose [EID_{50}]). The ducks of group 2 were injected with PBS only as a control. On 1 and 2 days post-infection (dpi), three ducks in each group were sacrificed and the livers, spleens, and lungs were collected. The total RNA was isolated with an HP Total RNA Kit (Omega), reverse transcribed into cDNA and the mRNA expression levels of PKR, OAS, and Mx were measured by qRT-PCR as described above. The relative expression ratios of the target gene in the tested group versus those in the control group were calculated according to the $2^{-\Delta\Delta Ct}$ method relative to GAPDH as the reference gene [21]. Standard deviation (SD) values were calculated using the relative expression ratios of three individual replicate trials for each target gene measured. *P* values less than 0.05 were considered significant.

Functional studies

The construct pCAGGS-dTLR5 was created by inserting the full-length dTLR5 sequence into the *Sac I* and *Xho I* sites of the pCAGGS expression vector (Addgene, USA). A DNA fragment containing the dTLR5 ORF was amplified using primers presented in Table 1. The amplified PCR product was digested with *Sac I* and *Xho I* (Fermentas, USA), and ligated into the pCAGGS vector (designated as pCAGGS-dTLR5). Constructs expressing the human IL-6 promoter luciferase reporter (pGL3-IL6-Luc) and pGL-NF-κB-Luc containing four copies of the NF-κB binding positive regulatory domain (PRD) motif were purchased from Beyotime (China).

DF-1 chicken embryonic fibroblast cells produced from East Lansing strain eggs [31], and human cervical carcinoma HeLa cells were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco, USA) plus 10% fetal bovine serum (FBS; Gibco) and grown under 5% CO_2 at 37°C. Cells (1×10^5) were seeded in 24-well plates (Corning Life Sciences, USA) and cultured under 5% CO_2 at 37°C overnight. The cells were then cotransfected with 500 ng of pCAGGS-dTLR5 or empty vector, 100 ng of reporter plasmid (pGL-NF-κB-luc or pGL-IL6-luc), and 10 ng of the constitutive renillaluciferase reporter pRL-TK (Promega) with Fugen HD (Promega). Twenty-four hour after transfection, 50 ng/mL of purified flagellin (Sangon Biotech) was added to the cell culture medium. After stimulation for 6 h, the cells were washed with phosphate buffer saline (PBS) and lysed with the 1× Passive Lysis Buffer (PLB; Promega) for 15 min at room temperature. The cell lysates were harvested and luciferase activity was then measured using the Dual-Luciferase Reporter Assay System (Promega). Data are expressed as fold induction relative to cells transfected with the empty vector and represent the mean ± SD of three independent experiments.

Results

Identification of the TLR5, MyD88, OAS, and Mx genes in duck

Sequences of dTLR5, MyD88, OAS, and Mx were obtained by RT-PCR using RNA from Pekin ducks with primers specific for a conserved sequence of regions in orthologs from other species. Nucleotide sequences of dTLR5 (accession no. KF717594), MyD88 (accession no. KJ126990), and OAS (accession no. KJ126991) were deposited in GenBank (National Center for Biotechnology Information). TLR5 and OAS genes from duck shared an identity of more than 90% with

Table 2. Summaryof immune-related genes inducksexaminedin this study

Gene product	Accession number	Size (bp)	Homology (%)
TLR5	KF717594	2580	Goose (93.8%)
			Chicken (87.4%)
			Human (50.6%)
			Mouse (49.9%)
MyD88	KJ126990	730	Goose (94.7%)
			Chicken (88.5%)
			Human (73.5%)
			Mouse (70.2%)
OAS	KJ126991	285	Goose (93.8%)
			Chicken (87.4%)
			Human (50.6%)
			Mouse (49.9%)
Mx		152	Chicken (73.0%)
			Human (52.0%)
			Mouse (50.0%)

the goose genes, more than 73% with the chicken genes, and more than 49.9% with the human and mice genes (Table 2). Nucleotide homology of MyD88 ranged from 70.2% to 94.7%compared to the genes from goose, chicken, human, and mouse.

Full-length cloning and sequence analysis of dTLR5

The full-length cDNA of dTLR5 (accession no. KF717594) contains 2580 bp and encodes 859 amino acid residues. Multiple sequence alignment showed that the amino acid sequence of dTLR5 is 87.4%, 81.3%, and 50.6% identical to the TRL5 gene in goose (*Anser anser*, AEP71332.1), chicken (*Gallus gallus domesticus*, ACR26276.1), and human (*Homo sapiens*, AAI09119.1), respectively. With the SMART program, we predict the protein domains of dTLR5 (Fig. 1). Results of the analysis showed that dTLR5 consisted of a signal peptide sequence in the N-terminal region(the first 21 amino acid residues), 11 leucine-rich repeat (LRR) domains, a transmembrane domain, and a TIR domain in the carboxy-terminus (at positions 693-840).

Homology and phylogenetic analysis of dTLR5

Using the neighbor-joining (NJ) method, phylogenetic analyses were performed for the amino acid sequences of 27 different TLR5 proteins. The resulting phylogenetic tree was composed of three major branches (Fig. 2).TLR5 protein sequences from the avians, including goose, chicken, turkey, and zebra finch, were in the same subgroup with sequences from mammals, including human, chimpanzee, macaque, mouse, rat, rabbit, pig, dog, gray wolf, cat, cattle, water buffalo, American bison, sheep, chamois and deer. The piscine TLR5 protein sequences, including ones from Japanese flounder, fugu

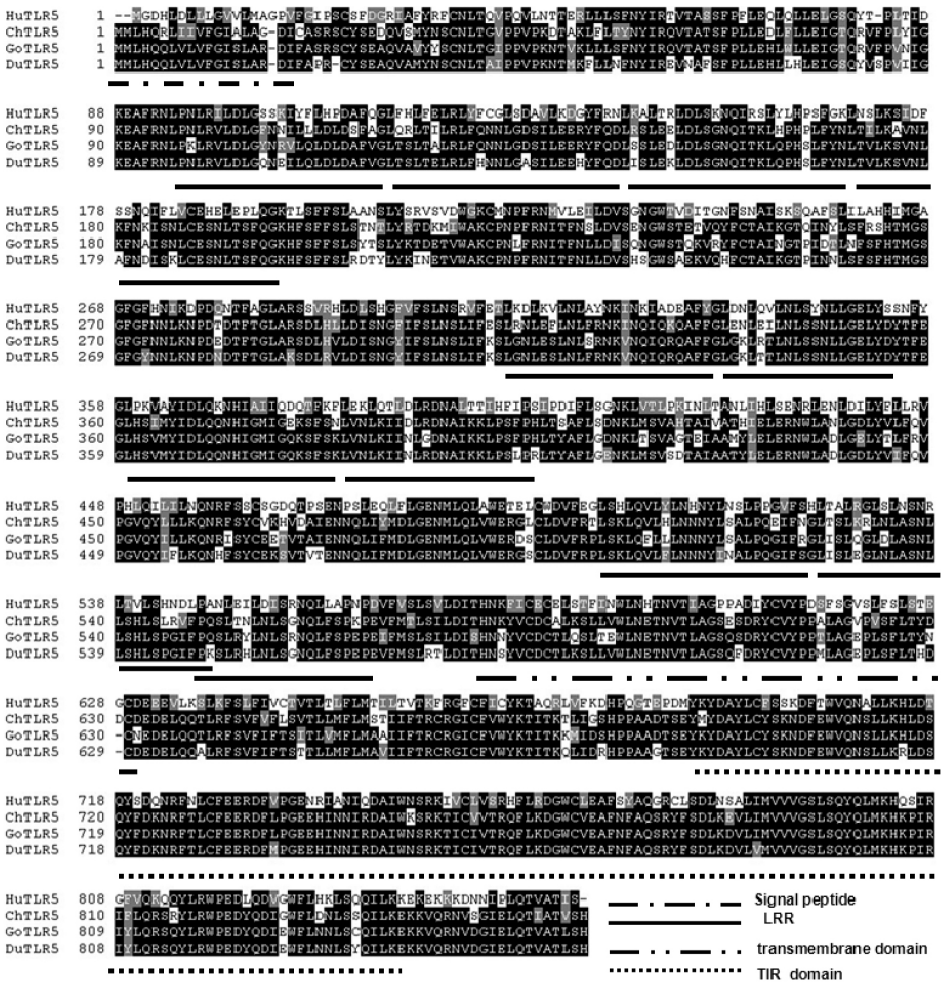

Fig. 1. Amino acid alignment of Pekin duck (dTLR5, accession no. KF717594), goose (GoTLR5, accession no. AEP71332.1), chicken (ChTLR5, accession no. ACR26276.1), and human (HuTLR5, accession no. AAI09119.1) TLR5. Alignment was performed using Clustal X software and edited with Boxshade. Black shading indicates amino acid identity and gray shading indicates similarity (50% threshold).

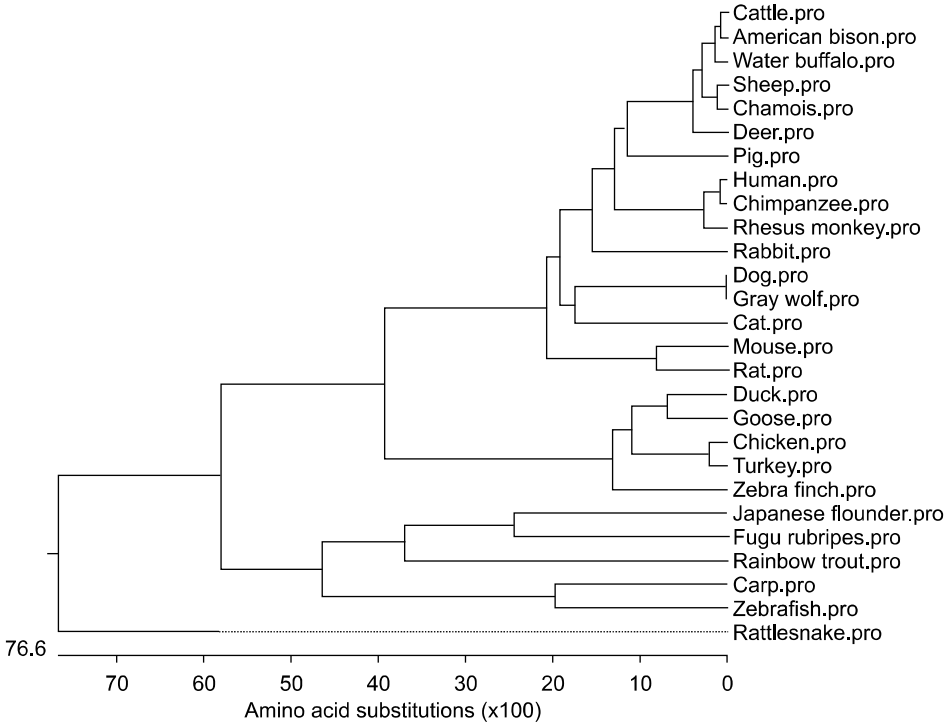

Fig. 2. Phylogeneticanalysis of TLR5. Aneighbor-joining (NJ) tree was constructed using MegAlign. The sequences were derived from the predicted amino acidsequences of thePekin duck TLR5 (KF717594) and GenBank entries for the domestic goose (*Anser anser*, accession no. AEP71332.1), chicken (*Gallus gallus domesticus*, accession number. ACR26276.1), cattle (*Bos Taurus*, accession no. AFR42399.1), chimpanzee (*Pan troglodytes*, accession no. NP_001123934.1), cat (*Felis catus*, accession no. XP_004001379.1), gray wolf (*Canis lupus*, accession no. NP_001184105.1), human (*Homo sapiens*, accession no. AAI09119.1), macaque (*Maca camulatta*, accession no. NP_001123901.1), mouse (*Mus musculus*, accession no. NP_058624.2), pig (*Sus scrofa*, accession no. AGT79978.1), rabbit (*Oryctolagus cuniculus*, accession no. AEA11027.1), rat (*Rattus norvegicus*, accession no. NP_001139300.1), sheep (*Ovis aries*, accession no. NP_001129398.1), turkey (*Meleagris gallopavo*, accession no. ADX33343.1), water buffalo (*Bubalus bubalis*, accession no. AEY63776.1), zebra finch (*Taeniopygia guttata*, accession no. XP_002188762.1), carp (*Cyprinus carpio*, accession no. AGH15501.1), fugu rubripes (*Takifugu rubripes*, accession no. AAW69374.1), zebrafish (*Danio rerio*, accession no. NP_001124067.1), rattlesnake (*Crotalus adamanteus*, accession no.AFJ51724.1), American bison (*Bison bison*, accession no. AEY63777.1), chamois (*Rupicapra rupicapra*, accession no. AFR42404.1), and deer (*Odocoileus virginianus*, accession no. AFR42402.1). The phylogenetic tree was generated with Clustal X software using the NJ method.

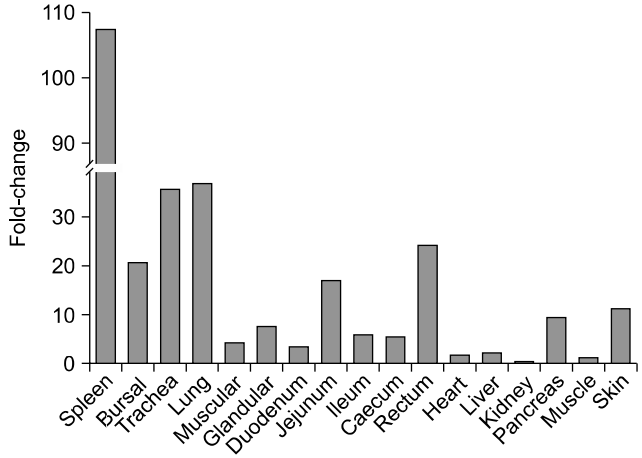

Fig. 3. Quantitative analysis of the tissue distribution of dTLR5 transcripts in healthy Pekin ducks. dTLR5 mRNA levels are expressed as the relative mRNAs relative to those in kidney.

rubripes, rainbow trout, carp, and zebrafish, were in another subgroup. Reptilian TLR5 (rattlesnake) was in the third subgroup. Duck and goose TLR5 were tightly clustered into one class, then further clustered with turkey and chicken, and more distantly clustered with zebra finch. This result is reflective of the genetic relationships among those species. Thus, the TLR5 gene has been conserved during the evolutionary process.

Tissue distribution of dTLR5 mRNA

In this study, the expression levels of dTLR5 mRNA in healthy duck tissues were analyzed by qRT-PCR. The tissues assessed included those with primary immunological (bursa of Fabricius and spleen), respiratory (trachea and lung), internal organ (liver and kidney), muscular (leg muscle and heart), and neurological (brain) functions as well as tissues that form an interface with the internal (various regions of the intestine) and external milieu (skin). dTLR5 mRNA was constitutively expressed in all

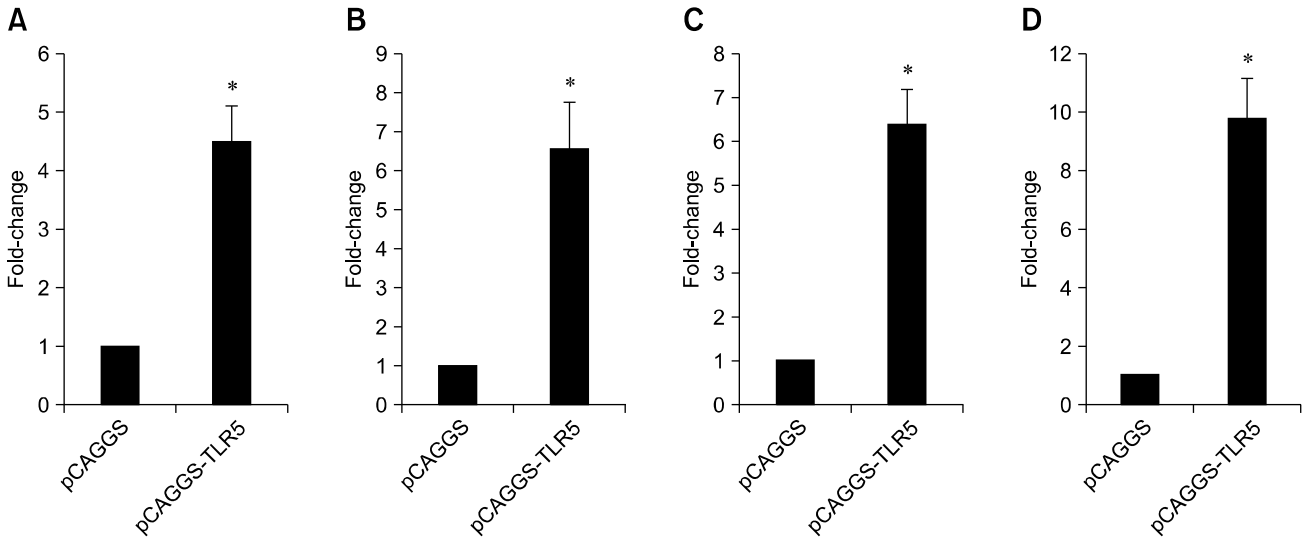

Fig. 4. Functionality of dTLR5. (A and B) HeLa (A) and DF-1 (B) cells were transfected with 0.1 μg/well of a reporter plasmid (pGL-NF-κB) along with 0.025 μg/well of pRL-TK plasmid and the expression constructs (pCAGGS-dTLR5 or empty vector) using Fugen HD. (C and D) HeLa (C) and DF-1 (D) cells were transfected with 0.1 μg/well of pGL-IL-6-Luc along with 0.025 μg/well of pRL-TK plasmid and the expression constructs (pCAGGS-dTLR5 or empty vector). Twenty-four hour post-transfection, 50 ng/mL of flagellin was added to the transfected cells and luciferase assays were performed after stimulation for 6 h using a Dual-Luciferase Assay Kit. Data are presented as the mean values from three independent experiments. Significance was analyzed with a two-tailed Student's *t*-test (*$p < 0.05$).

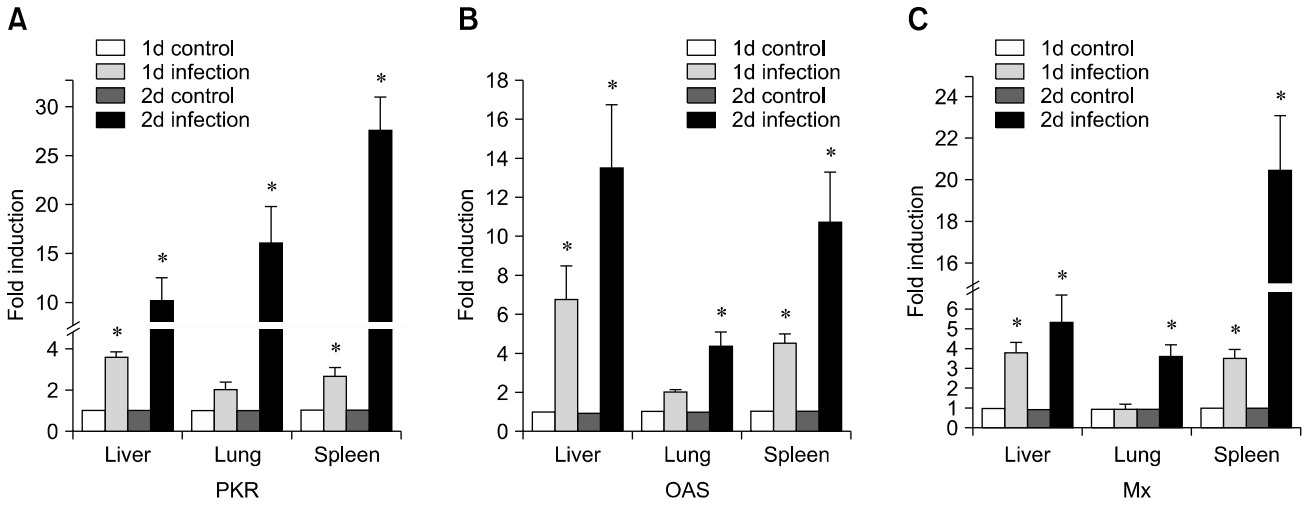

Fig. 5. Fold-changes in gene expression of PKR (A), OAS (B), and Mx (C) in different tissues of virus-infected ducks compared to control animals. The controls were inoculated with PBS while the experimental ducks were infected with Herts33 NDV. Each bar represents the level of target gene mRNA relative to that in the control group. *Significant differences ($p < 0.05$) between the experimental and control groups. Error bars indicate the SD.

tissues examined with highest levels in spleen, relatively high levels in trachea, lung, rectum, jejunum, and bursa; moderate levels in glandular stomach, muscular stomach, duodenum, ileum, caecum, liver, pancreas, and skin; and low levels in heart, leg muscle, and kidney (Fig. 3).

Functional analysis of dTLR5

To study the functionality of dTLR5, this gene was cloned into the pCAGGS expression vector and transfected into DF-1 (panel A in Fig. 4) and HeLa (panel B in Fig. 4) cells along with a NF-κB-luciferase reporter plasmid. After flagellin stimulation, luciferase activity was measured. The luciferase activities of dTLR5-transfected HeLa and DF-1 cells were

4.48- ($p < 0.05$) and 6.56-fold ($p < 0.05$), respectively, of that observed in cells transfected with the empty vector. This result is in agreement with data from a previous study [7]. To determine whether dTLR5 can drive IL-6 activation in DF-1 or HeLa cells, the luciferase reporter plasmid pGL-IL6-luc was co-expressed with the dTLR5 construct. It was found that overexpression of dTLR5 in both HeLa (panel C in Fig. 4) or DF-1 (panel D in Fig. 4) cells could effectively increase the activity of the IL-6 promoter after flagellin stimulation (HeLa, 6.41-fold, $p < 0.05$; DF-1, 9.72-fold, $p < 0.05$).

Expression of antiviral molecules in different tissues of ducks infected with Herts33

We analyzed the expression of PKR (panel A in Fig. 5), OAS (panel B in Fig. 5), and Mx (panel C in Fig. 5) during infection with Herts33 NDV. At 1 dpi, the expression of PKR was significantly up-regulated in both liver (3.6-fold, $p < 0.05$) and spleen (2.7-fold, $p < 0.05$). Expression in lung PKR was slightly elevated (1.99-fold, $p > 0.5$), but this change was not statistically significant. At 2 dpi, PKR expression was significantly up-regulated in all the tissues tested, especially in spleen (27.6-fold, $p < 0.05$). Levels of Mx gene expression were significantly increased all the tested tissues except for lung at 1 dpi. Expression of this gene was most significantly up-regulated in spleen (20.44-fold, $p < 0.05$). mRNA expression of the OAS gene was also increased in liver, lung, and spleen after Herts33 infection. Unlike Mx and PKR, OAS expression was especially strong in liver after infection with Herts33 while PKR and Mx expression was stronger in spleen.

Discussion

In the current study, we cloned the 2580 bp dTLR5 gene, which shared a high amino acid sequence similarity with goose and chicken TLR5 genes as well as a moderate similarity with mouse and human TLR5 genes. The dTLR5 protein secondary structure is similar to that of mammals, consisting of a signal peptide sequence, several leucine-rich repeats (LRR) domains, a transmembrane domain, and an intracellular Toll/interleukin-1 receptor (TIR) domain. We also identified partial sequences of the TLR5 adaptor protein MyD88 and antiviral factors Mx and OAS. All of these genes in duck are highly homologous to the chicken genes. These results show that ducks have a homologue of mammalian TLR5.

Most TLRs contain a conserved proline residue in the BB loop [33] (a loop which connects the βB strand and the αB helix) of the TIR domain. In TLR2 and TLR4, this proline residue was found to be essential for binding to the downstream adapter protein MyD88 [26,30]. A similar proline residue was discovered in the corresponding position of dTLR5. At this time, it is unclear whether this residue has a similar function in dTLR5 signaling.

Tissue and cell distribution are important characteristics of TLR5 function since these factors influence the capacity to detect the entry and growth of different microorganisms in various tissues. Understanding the distribution patterns of dTLR5 will enable a more concrete interpretation of immune induction and host-pathogen relationships that define infectious disease biology in ducks. We determined that dTLR5 mRNA was broadly expressed in most tissues of healthy duck. We also found that dTLR5 was highly expressed at sites of microbial contact with immune tissues such as spleen and bursa. This was evidence of a functional TLR5-mediated innate immune response to pathogenic challenges in duck. In addition, we observed that dTLR5 mRNA levels were higher in tissues with mucosa that form the first barrier which protects the body from invading foreign pathogens (*i.e.*, in direct contact with air or food). It is possible that this special distribution enables dTLR5 to respond to invading pathogens as soon as possible.

A previous study found that NF-κB is activated in HeLa cells overexpressing chicken TLR5 that are infected with Salmonella enteric serovar Enteritidis [17]. Fang et al. also indicated that goose TLR5 can respond to flagellin from Salmonella typhimurium and induce NF-κB-driven luciferase activity [7]. In our study, we found that dTLR5 can significantly increase the activity of NF-κB and the IL-6 promoter in DF-1 and HeLa cells.

Chicken PKR is a protein with antiviral properties that confer protection against vesicular stomatitis virus (VSV) infection [19]. Another recent report indicated that PKR plays an important role in the formation of antiviral stress granules involved in the orchestration of RIG-I and signaling components, and this process can be inhibited by non-structural protein 1 (NS1) of AIV [18]. The function of duck PKR is unknown. Chicken OAS encodes a domain typical of 2'-5'oligoadenylate synthetase proteins along with two ubiquitin-like (UbL) domains [29]. While human OAS protein lacks oligoadenylate synthetase activity, the UbL domains are necessary for antiviral activity [22]. Human OAS inhibits the replication of RNA viruses [12], and its expression level was up-regulated by influenza through interferon regulatory factor-3 (IRF-3) [24]. Mx, PKR, and OAS are antiviral genes in mammalian cells [5,11]. In avian cells, the functions of these genes are still unclear. Some studies suggested that chicken PKR and Mx genes fail to confer protection against H5N1 AIV infection [2,5]. In the present study, we found that Mx, PKR, and OAS were up-regulated in tissues from NDV-infected ducks. The functions of these three factors in duck, especially following exposure to NDV, require further study.

In conclusion, we identified the dTLR5 gene and characterized its predicted protein domains. We also identified partial sequences of the TLR5 adaptor protein MyD88 and antiviral molecules Mx and OAS. We determined that dTLR5mRNA is broadly expressed in most tissues of healthy

Pekin ducks. Overexpression of dTLR5 in DF-1 or HeLa cells appeared to activate NF-κB and subsequently up-regulated the activity of the IL-6 promoter. Future studies will be necessary to investigate the detailed role of dTLR5 in the innate immune response.

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Conflict of Interest

There is no conflict of interest.

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