INTRODUCTION

Innate immunity provides the first line of defense against pathogenic organisms through a set of germ-line encoded receptors called pattern recognition receptors (PRR). There are 4 types of PRR: Toll-like receptors (TLR), retinoic acid-inducible gene I (RIG-I)-like receptors (RLR), nucleotide oligomerization domain (NOD)-like receptors (NLR), and C-type lectin receptors (CLR; Akira et al., 2006; Geijtenbeek and Gringhuis, 2009).

Toll-like receptors, which are a group of evolutionarily conserved type-I transmembrane receptors, consist of an extracellular leucine-rich repeats (LRR) domain, a transmembrane domain, and an intracellular Toll/interleukin-1 receptor (TIR) domain (Akira and Takeda, 2004). The extracellular LRR domain functions in ligand recognition. The different structures of the LRR domain in variable TLR correspond to a variety of pathogen-associated molecular patterns (PAMP) expressed by microorganisms, such as peptidoglycan, lipoprotein, lipopolysaccharide, and flagellin in bacterial pathogen components and double-stranded RNA in the viral genome (Akira and Takeda, 2004). The TIR domains are the most conserved structures in TLR (Beutler and Rehli, 2002). Following TLR recognition of PAMP, the TIR domains bind to the TIR domain of adapter molecules including myeloid differentiation factor 88 (MyD88) and TIR-containing adapter molecule-1 (TICAM-1; also called TRIF). The TLR signaling via MyD88-dependent or -independent pathways results in activation of an appropriate immune response (Beutler and Rehli, 2002).

In mammals, TLR3 recognizes double-stranded RNA from viral sources through its ectodomain, which induces receptor dimerization required for adaptor-mediated signal transduction (Alexopoulou et al., 2001). The TLR3 mediates signaling via the adaptor protein, TICAM-1, which activates the transcription factors interferon regulatory factor 3, nuclear factor-κB and activator protein 1. This leads to the induction of type I interferon, cytokine/chemokine production, and dendritic cell maturation, which then enables the activation of natural killer cells and cytotoxic T-lymphocytes (Matsumoto et al., 2011).

The TLR3 has been identified in a variety of species, including mammals, fish, and birds (Rock et al., 1998; Yilmaz et al., 2005; Yang and Su, 2010). Here, the full-length of the Muscovy duck TLR3 (MdTLR3) cDNA was characterized as a novel member of the TLR family. The gene expression of MdTLR3 was investigated in
various tissues in uninfected ducks and the expression profile of MdTLR3 was further examined at different times postinfection with the H5N1 highly pathogenic avian influenza virus (HPAIV).

**MATERIALS AND METHODS**

**Virus**

The A/Duck/Guangdong/212/2004 (H5N1) virus (DK212) used in this study was isolated from ducks in the Guangdong Province of China in 2004 and identified as H5N1 avian influenza A virus by means of hemagglutination inhibition and neuraminidase inhibition tests. The virus was purified and propagated in the allantoic cavity of 10-d-old specific-pathogen-free embryos. Allantoic fluid pooled from multiple eggs was clarified by centrifugation and frozen in aliquots at −70°C. All experiments were carried out in Animal Biosafety Level 3 (ABSL-3) facilities.

**Bird Experiments**

One-day-old healthy Muscovy ducks were purchased from a duck farm in Guangzhou and housed in isolators. Muscovy ducks were confirmed serologically negative for avian influenza by agar gel precipitation tests and hemagglutinin inhibition assays as previously described (Sun et al., 2011).

At 4 wk old, 3 uninfected ducks were killed and tissues were collected for tissue distribution analyses, including brain, crop, trachea, heart, liver, spleen, lung, kidney, muscular stomach, glandular stomach, pancreatic gland, muscle, skin, duodenum, ileum, colon, cecum, rectum, and bursa.

Twenty-eight ducks (aged 4 wk) were divided into 2 groups. In the first group, 14 ducks were intranasally inoculated with DK212 [10^6 50% egg infective doses (EID50) in 0.2 mL]. In the second group, 14 ducks were inoculated with 0.2 mL of PBS as negative controls. At 24, 48, and 72 h postinoculation, 3 individuals from each group were killed, and brain, spleen, and lung tissues were harvested immediately for RNA extraction. The remaining ducks were observed for clinical symptoms for 14 d. All experiments were carried out in ABSL-3 facilities.

**Degenerate Primer Design and PCR Amplification**

To identify the TLR3 cDNA sequence from Muscovy duck, a degenerate PCR primer was designed based on the multiple alignment of previously reported TLR3 sequences: human (Homo sapiens; GenBank accession number NM_003265), mouse (Mus musculus; NM_126166), and chicken (Gallus gallus; NM_001011691). Amplification was performed with the degenerate primers idT3f and idT3r (Table 1), using the cDNA generated from Muscovy duck spleen. The PCR conditions consisted of an initial denaturation at 94°C for 5 min; 35 cycles of denaturation, annealing, and extension at 94°C for 30 s, 57°C for 30 s and 72°C for 1 min; and a final elongation step at 72°C for 7 min. The PCR product was ligated into the pMD19-T vector (Takara Biotechnology Co. Ltd., Dalian, China), transformed into the competent Escherichia coli JM109 cells, and plated on LB agar. Positive colonies containing an insert of the expected size were identified by colony PCR. Three of them were picked up and sequenced by Shanghai Invitrogen Biotechnology Co. Ltd. (Shanghai, China).

**Cloning the Full-Length cDNA of MdTLR3**

The 5′- and 3′-SMART rapid amplification of cDNA ends (RACE) PCR (Clontech, Mountain View, CA) was performed for cloning the full-length cDNA of MdTLR3. To obtain the 3′ unknown region, primer pairs 3rdT3f-1/ Universal Primer A Mix (UPM) and 3rdT3f-2/Nested Universal Primer A (NUP; Table 1), were used for the primary PCR and the nested PCR, respectively. The amplified PCR product was cloned and sequenced. Similarly, the 5′ end of MdTLR3 was obtained by the same method using primer pairs 5rdT3r-1/UPM and 5rdT3r-2/NUP (Table 1). The full-

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence of oligonucleotide (5′ → 3′)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>idT3f</td>
<td>AACATTGTTGRTACATTGCTGATMATTC</td>
<td>Gene cloning</td>
</tr>
<tr>
<td>idT3r</td>
<td>CYTCAARTGGATGAGMAGWACMA</td>
<td></td>
</tr>
<tr>
<td>5rdT3r-1</td>
<td>TTAACCTCATGACCTTGTGAGCTTGTGA</td>
<td>5′-RACE</td>
</tr>
<tr>
<td>5rdT3r-2</td>
<td>GTCCCAAGCAGAAATGGTCCCC</td>
<td>3′-RACE</td>
</tr>
<tr>
<td>3rdT3f-1</td>
<td>TGCTGATCTAAAGCAGACTGTTGC</td>
<td>Race method</td>
</tr>
<tr>
<td>3rdT3f-2</td>
<td>TGGCTCAGCTTTCAGAAGCCTTGGAG</td>
<td></td>
</tr>
<tr>
<td>Universal Primer A Mix</td>
<td>Long: CTAATACGACTCACTATAGGGCA AGCAGTGGTATCAACGCAGAGT</td>
<td></td>
</tr>
<tr>
<td>Short: CTAATACGACTCACTATAGGGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nested Universal Primer A</td>
<td>AAGCAGTGGTATCAACGCAGAGT</td>
<td></td>
</tr>
<tr>
<td>udT3f</td>
<td>AGTAGAAAGCAGACTGTTAAGA</td>
<td>Sequence verification</td>
</tr>
<tr>
<td>udT3r</td>
<td>TGATAGAAAGCAGACTGTTAAGA</td>
<td></td>
</tr>
<tr>
<td>qdT3f</td>
<td>GAGGTACCCCAAGTGTTAAGA</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>qdT3r</td>
<td>GTATAGGGTTAAGAAGTGTGTTAAGA</td>
<td></td>
</tr>
<tr>
<td>qdGAPDHf</td>
<td>ATGTTTCGATGTGCGTTGCGTCTGA</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>qdGAPDHr</td>
<td>CTTCTTCGGGCTGCGGTGCGTCTGA</td>
<td></td>
</tr>
</tbody>
</table>

1 R = A/G; M = A/C; Y = C/T; W = A/T. RACE = rapid amplification of cDNA ends. qRT = quantitative real-time.
length cDNA sequence was confirmed by sequencing the PCR product amplified by primer pairs udT3f and udT3r (Table 1) within the predicted 5′ and 3′ untranslated regions, respectively.

**Bioinformatic Analysis**

Sequence homology was obtained by BLAST program analysis using the nucleotide database of the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/blast; Altschul et al., 1990). Amino acid sequences were aligned using ClustalW (http://www.ebi.ac.uk/clustalw) and edited with BOXSHADE (http://bioweb.pasteur.fr/seqanal/interfaces/boxshade.html). Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de/) was used to predict the domain structure of MdTLR3 (Letunic et al., 2006).

**Gene Expression of MdTLR3 in HPAIV-Infected and Uninfected Ducks**

Total RNA was extracted from various Muscovy duck tissues using the RNeasy plus Mini Kit (Qiagen, Carlsbad, CA) according to the instructions provided by the manufacturer. Total RNA (1 µg) was reverse transcribed with the SuperScript III First Strand synthesis system (Life Technologies, Carlsbad, CA). Quantitative real-time (qRT) PCR was performed using the Quantifast SYBR Green PCR kit (Qiagen). Primers used for qRT-PCR were designed with the software Oligo 6 (Molecular Biology Insights Inc., Cascade, CO). Primer pairs (Table 1) were selected based on specificity determined by dissociation curves. The qRT-PCR was carried out using a 7500 Fast Real-Time PCR system (Applied Biosystems, Rotkreuz, Switzerland). The PCR conditions as follows: 1 cycle of 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 34 s. Dissociation curves of the products were generated by increasing the temperature of samples incrementally from 55 to 100°C as the final step of the PCR. For the purposes of assay validation, purified products were cloned into pMD19-T and sequenced to verify correct target amplification.

**Calculations and Statistics, and Nucleotide Sequence Deposition**

The relative expression ratios of target gene in tested group versus those in control group were calculated by the 2−ΔΔCt method using the duck housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH; AY436595) as the endogenous reference gene to normalize the level of target gene expression (Livak and Schmittgen, 2001). Standard deviations were calculated using the relative expression ratios of 3 replicates for each gene measured. Statistical analyses were performed using the GraphPad Prism 5 software (Graph-Pad Software Inc., San Diego, CA). The value of P < 0.05 was considered to be significant.

The cDNA sequence of the MdTLR3 gene was deposited in GenBank under accession number JQ 910167.

**RESULTS AND DISCUSSION**

**cDNA Cloning and Sequence Analysis of MdTLR3**

The full-length MdTLR3 cDNA sequence was obtained by homologous cloning and RACE techniques from Muscovy duck spleen cDNA. A 983-bp fragment was amplified using degenerate primers idT3f and idT3r. This fragment exhibited significant homology to the middle region of the Gallus gallus TLR3 gene using the BLAST server of the National Center for Biotechnology Information. Based on the sequence of the amplified 983-bp segment, primers 3rdT3f-1 and 3rdT3f-2 were designed and used with adaptor primers UPM and NUP to amplify the 3′ unknown region. Alignment of sequences from all homologous clones yielded a 1,601-bp consensus sequence. The clone included a poly (A) tail, suggesting that this expressed sequence tag represented the 3′ region of the MdTLR3 gene. The 5′ unknown region was obtained by the same method using primer pairs 5rdT3r-1/UPM and 5rdT3r-2/NUP. A product of 1,333 bp from nested PCR perfectly overlapped the initial 1,601-bp consensus sequence. These fragments were aligned to yield a 2,836-bp full-length cDNA sequence, which was confirmed to represent a single mRNA by amplification of a single 2,745-bp product from spleen cDNA using primers udT3f and udT3r.

Sequence analysis of the product revealed the full-length transcript of Muscovy duck MdTLR3 including the 5′UTR, coding sequence, 3′UTR and poly (A) tail. The longest open reading frame of the MdTLR3 began at nucleotide 70 and terminated at nucleotide 2757, encoding an 895 amino acid polypeptide. The amino acid sequence was predicted based on the open reading frames of MdTLR3. The deduced polypeptide has a calculated molecular mass of 102,467 Da and isoelectric point of 8.00. Like other TLR, the domain structure of MdTLR3 shows a typical type I transmembrane protein with an extracellular LRR domain at N terminus, a transmembrane domain, and an intracellular TIR domain at the C-terminus (Figure 1).

**Homology Analysis of MdTLR3**

Sequence alignments were performed to determine the percentage homology of MdTLR3 with other known TLR3 genes. MdTLR3 shared higher amino acid sequence similarity with the chicken TLR3 gene (87.3%) and did moderate similarity with the human and mouse TLR3 genes (62.0 and 60.2%, respectively). The TIR domains are the most conserved structures in TLR and, therefore, are more desirable for tracing the ancestry...
of TLR among diverse species such as mammals and aves (Beutler and Rehli, 2002). Interestingly, the differences between Muscovy duck and chicken TLR3 were largely located in the TIR domain, with 85.0% amino acid identity in the TIR domain compared with 88.3% in the LRR domain.

**Figure 1.** Amino acid sequence alignment of *MdTLR3* protein with human (accession numbers; NP_003256), mouse (NP_569054), and chicken (NP_001011691) Toll-like receptor 3 (TLR3) proteins. Alignment was performed using the CLUSTALW program and edited with BOXSHADE. Black shading indicates amino acid identity; gray shading indicates similarity (50% threshold). LRR, leucine rich repeat. TIR, Toll-interleukin 1 receptor signaling domain. The TLR3 sequences are shown for Muscovy duck (Md), human (Hu), mouse (Mo), and chicken (Ch).

**Tissue Distribution of MdTLR3 Expression**

Expression of *MdTLR3* mRNA in normal tissues was analyzed by qRT-PCR analysis. The tissues examined included those with primary immunological function (spleen and bursa), epithelial cells (kidney and liver),
neurological (brain), muscle (heart and muscle), as well as tissues that interface with the internal (various regions of the small and large intestine) and external (skin) milieu.

The MdTLR3 mRNA was constitutively expressed in all tissues analyzed except muscle, with higher levels detected in trachea, pancreatic gland, spleen, duodenum, and lung; moderate levels in crop, liver, colon, cecum, ileum, and rectum; and low levels in brain, heart, and skin (Table 2). Assessment of human, mouse, fish, and chicken tissue-specific expression of TLR has revealed distinct distribution patterns for each TLR (Sebastiani et al., 2000; Zarember and Godowski, 2002; Jault et al., 2004; Iqbal et al., 2005). The MdTLR3 was found to be expressed in a wide range of tissues, thus indicating a role in the immune surveillance system of various organs.

MdTLR3 mRNA Expression After HPAIV Infection

Two families of PRR, RLR, and TLR, play essential roles in recognition of viral nucleic acids to trigger antiviral innate immunity (Thompson and Locarnini, 2007; Takeuchi and Akira, 2010). The RIG-I are involved in clearing influenza infections in ducks, although this molecule may be absent in chickens, thus providing a plausible explanation for the increased susceptibility to influenza viruses found in chickens compared with ducks (Barber et al., 2010). Toll-like receptors such as TLR3, TLR7, and TLR8 are also important PRR for viral recognition (Boehme and Compton, 2004). In duck, TLR7 has been identified and involved in the antiviral immune response after H5N1 avian influenza virus infection (MacDonald et al., 2008; Liang et al., 2011).

To establish whether TLR3 may be associated with the immune response to viruses in Muscovy duck, we investigated the expression of this molecule during infection with HPAIV. In noninfected ducks, basal levels of TLR3 mRNA expression in the brain were far lower than in spleen and lung (Table 2). Ducks inoculated with DK/212 displayed increased levels of TLR3 mRNA relative to the controls in the brain at all time points (Figure 2). The MdTLR3 transcripts in the brain were upregulated significantly at 24 h (1.94-fold, P < 0.05), reached a peak at 48 h (4.64-fold, P < 0.05), and recovered to normal levels at 72 h. This observation is similar to that reported in chickens, where upregulation of TLR3 in brain was found in chicken at 24 h after HPAIV infection (Karpala et al., 2008).

In this study, MdTLR3 expression was downregulated during the test period in the spleen and lung. In contrast, a previous study in chickens showed upregulation of TLR3 in both tissues at 24 h after HPAIV infection (Karpala et al., 2008). Furthermore, after infection with grass carp reovirus, the susceptible hosts Gobio cypius rarus and Ctenopharyngodon idella exhibited upregulation of TLR3, whereas the resistant host, Cyprinus carpio, exhibited downregulation of TLR3 (Su et al., 2008, 2009; Yang and Su, 2010). Moreover, downregulation of TLR5 mRNA during chronic infection has been demonstrated in humans, mice, and Ictalurus sp. (Muthukuru et al., 2005; Ortega-Cava et al., 2006; Bilodeau-Bourgeois et al., 2008). The isolate virus, DK/212, used in this study is not fatal to Muscovy ducks and causes few symptoms (Su et al., 2011), indicating that the Muscovy duck is a resistant host for this virus. This may account for the observed downregulation of TLR3 mRNA in these tissues.

In conclusion, MdTLR3 is a novel member of the TLR family that exhibits some distinctive features and involved in the early stage of antiviral innate immune responses. However, the detailed mechanism of the antiviral functions of TLR3 in Muscovy duck requires further investigation.

ACKNOWLEDGMENTS

This work was supported by grants from the Natural Science Foundation of Guangdong Province (no. 1025106420100004, no. 1015106420100021, and no. 2ND = not detected.

Table 2. Quantitative analysis of tissue distribution of Toll-like receptor 3 (TLR3) transcripts in healthy Muscovy ducks

<table>
<thead>
<tr>
<th>Tissue</th>
<th>TLR3 gene relative expression level (mean ± SD)</th>
<th>Tissue</th>
<th>TLR3 gene relative expression level (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>0.24 ± 0.03a</td>
<td>Pancreatic gland</td>
<td>134.44 ± 14.24a</td>
</tr>
<tr>
<td>Crop</td>
<td>8.44 ± 0.99a</td>
<td>Muscle</td>
<td>ND2</td>
</tr>
<tr>
<td>Trachea</td>
<td>324.25 ± 28.20a</td>
<td>Skin</td>
<td>0.12 ± 0.01a</td>
</tr>
<tr>
<td>Heart</td>
<td>0.21 ± 0.04a</td>
<td>Duodenum</td>
<td>23.62 ± 1.81a</td>
</tr>
<tr>
<td>Liver</td>
<td>7.20 ± 0.95a</td>
<td>Ileum</td>
<td>2.34 ± 0.17a</td>
</tr>
<tr>
<td>Spleen</td>
<td>43.87 ± 1.78a</td>
<td>Colon</td>
<td>4.94 ± 0.14a</td>
</tr>
<tr>
<td>Lung</td>
<td>18.76 ± 1.34a</td>
<td>Cecum</td>
<td>2.64 ± 0.17a</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.23 ± 0.33</td>
<td>Rectum</td>
<td>1.88 ± 0.14</td>
</tr>
<tr>
<td>Muscular stomach</td>
<td>0.62 ± 0.15</td>
<td>Bursa</td>
<td>1.00 ± 0.08</td>
</tr>
<tr>
<td>Glandular stomach</td>
<td>1.61 ± 0.33</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*a*Indicates there was a difference (P < 0.05) between control and test tissues.

1Each result represents the level of target gene mRNA relative to those in bursa, expressed as the mean ± SD of triplicate from quantitative real-time PCR assays.

2ND = not detected.
REFERENCES


Figure 2. The expression profiles of Muscovy duck Toll-like receptor 3 (MdTLR3) mRNA in virus tissue by quantitative real-time PCR. (A) Relative Toll-like receptor 3 (TLR3) mRNA expression pattern in brain, (B) in spleen, and (C) in lung. The controls were inoculated with PBS; the experimental ducks were infected with DK/212. Each bar represents the level of target gene mRNA relative to those in control group. *The difference (P < 0.05) between experimental group and control group. Error bars indicate SD.

5200638), the National Natural Science Foundation of China (No. 31172343), the Science and Technology Projects of Guangdong Province (No. 2010B020307005), the Earmarked Fund for Modern Agro-Industry Technolo-

ogy Research System (nyctx-42-G3-03), and High-level Talents in University Project of Guangdong Province.
tor 3 in grass carp (*Ctenopharyngodon idella*). Fish Shellfish Immunol. 27:433–439.


