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Transcriptome analysis of the endangered Chinese giant salamander (*Andrias davidianus*): Immune modulation in response to *Aeromonas hydrophila* infection

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ABSTRACT

The endangered Chinese giant salamander (*Andrias davidianus*) is the largest extant amphibian species. Disease outbreaks represent one of the major factors threatening *A. davidianus* populations in the wild and the viability of artificial breeding programmes. Development of future immune therapies to eliminate infectious disease in *A. davidianus* is dependent on a thorough understanding of the immune mechanisms elicited by pathogen encounters. To this end we have undertaken, for the first time in amphibians, differential transcriptome analysis of the giant salamander response to *Aeromonas hydrophila*, one of the most devastating pathogens affecting amphibian populations. Out of 87,204 non-redundant consensus unigenes 19,216 were annotated, 6834 of which were upregulated and 906 down-regulated following bacterial infection. 2058 unigenes were involved with immune system processes, including 287 differentially expressed unigenes indicative of the impact of bacterial infection on several innate and adaptive immune pathways in the giant salamander. Other pathways not directly associated with immune-related activity were differentially expressed, including developmental, structural, molecular and growth processes. Overall, this work provides valuable insights into the underlying immune mechanisms elicited during bacterial infection in amphibians that may aid in the future development of disease control measures in protecting the Chinese giant salamander. With the unique position of amphibians in the transition of tetrapods from aquatic to terrestrial habitats, our study will also be invaluable towards the further understanding of the evolution of tetrapod immunity.

Abbreviations: AP, activator protein; B2M, beta-2-microglobulin; BCL, B-cell lymphoma; BCR, B-cell receptor; CCL, C-C motif chemokine ligand; CCR, C-C motif chemokine receptor; CD, cluster of differentiation; CFB, Complement factor B; CR1, complement component (3b/4b) receptor 1; CREB, cAMP-response element binding protein; CTL, c-type lectin; CLR, c-type lectin receptor; EGF, epidermal growth factor; GADS, glutamate decarboxylase; GILT, gamma-interferon-inducible lysosomal thiol reductase; HSP, heat shock protein; IGBP, Immunoglobulin binding protein; IGSF, immunoglobulin superfamily member; IKBKG, inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma; IKK, inhibitor of nuclear factor kappa-B kinase; IL, interleukin; IRF, interferon regulatory factor; JAG, protein jagged; JAK, Janus kinase; JAM, junctional adhesion molecule; JNK, c-Jun N-terminal kinase; KIF, kinesin family member; KLF, Kruppel-like factor; LAT, linker for activation of T Cells; LBP, lipopolysaccharide binding protein; LCK, lymphocyte-specific protein tyrosine kinase; MAF, macrophage activation factor; MAPK, mitogen-activated protein kinase; MBL, mannose-binding lectin; MIF, macrophage migration inhibitory factor; MRC, macrophage mannose receptor; MyD88, myeloid differentiation primary response 88; NFAT, nuclear factor of activated T-cells; NF-κB1, nuclear factor kappa-B p105 subunit; NOD, nucleotide-binding oligomerization domain-containing protein; PI3K, phosphoinositide 3 kinase; PAMPs, Pathogen-associated molecular patterns; PGRP, peptidoglycan recognition protein; PRR, pattern recognition receptor; RIP, receptor-interacting serine/threonine protein kinase; ROCK, Rho-associated coiled-coil containing protein kinase; SOCS, Suppressor of cytokine signalling; STAT, signal transducer and activator of transcription; TCR, T cell receptor; TLR, toll-like receptor; TNF, tumor necrosis factor; TOLLIP, Toll interacting protein; TRAF2, TNF receptor-associated factor 2; TRAM, translocating chain-associated membrane protein; TRIF, TIR-domain-containing adapter-inducing interferon-β; TYK2, tyrosine kinase 2; VEGF, vascular endothelial growth factor.

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1. Introduction

The Chinese giant salamander (*Andrias davidianus*) is the largest extant amphibian species ranked as a living fossil owing to its important evolutionary position representing the transition of animals from aquatic to terrestrial habitats (Zhu et al., 2014a,b). Its population has declined dramatically due to the reduction of suitable habits, over-harvesting, and increasing prevalence of infectious diseases (Che et al., 2014; Li et al., 2015). To facilitate future efforts to protect this valuable species, it has been included as a national class II protected species by the Chinese government and in the International Union for Conservation of Nature and Natural Resources (IUCN) Red list of Threatened Species. To relieve the pressure on salamander populations, artificial breeding practices have been carried out, to some extent, with many salamanders now farmed in mesocosms across China. However, the increasing outbreak of infectious diseases continues to threaten salamander populations and the viability of artificial breeding programmes (Gao et al., 2012; Chen et al., 2013).

Transcriptomic profiling of host responses to pathogens provides a powerful means of assessing the underlying immune mechanisms, which may enable the identification of novel targets for future immune therapies and disease control. In recent years, a growing number of transcriptomic studies have been undertaken in different vertebrate classes. These include the grey bamboo shark (*Chiloscyllium griseum*) (Krishnaswamy et al., 2014), zebrafish (*Danio rerio*) (Aanes et al., 2011), large yellow croaker (*Pseudosciaena crocea*) (Mu et al., 2010, 2014), frogs (*Xenopus tropicalis* and *Xenopus laevis*) (Ellison et al., 2014; Rosenblum et al., 2012), and the soft-shelled turtle (*Pelodiscus sinensis*) (Wang et al., 2013). The salamander had drawn considerable attention towards understanding the origin and evolution of immune systems owing to its evolutionary position in the transition of tetrapods from aquatic to terrestrial habitats. Transcriptomic analysis of spleen, skin and thymus of healthy salamander identified genes and pathways involved in immune response mechanisms (Che et al., 2014; Zhu et al., 2014a; Li et al., 2015). However, transcriptomic profiling of the Chinese giant salamander following pathogen infection remains to be elucidated.

Aeromonas hydrophila, a gram-negative bacterium, is a lethal pathogen towards many aquatic animals, including amphibians. *A. hydrophila* infection, in the early stage, can lead to acute damage of hepatic function with the liver, kidney, and intestines being the main target organs (Zhang et al., 2013). Immune responses evoked by *A. hydrophila* infection have been examined in fish, and are illustrated by broad changes in immune signalling mechanisms during infection (Lv et al., 2015). The current study is the first to conduct transcriptome (RNA-seq) analysis in *A. hydrophila*-challenged amphibians in order to elucidate the underlying immune mechanisms modulated by bacterial infection in the Chinese giant salamander (*A. davidianus*). This work provides valuable insights into amphibian-pathogen interactions and towards the development of future immunoprophylactic strategies to protect such endangered species.

2. Materials and methods

2.1. Ethics statement

This study was performed in accordance to the Chinese Academy of Sciences Guide for the Care and Use of Laboratory Animals. Ethical approval for this study was granted by the Ethics Committee of Animal Experiments (Institute of Hydrobiology; Permit Number: Y213531301). All surgery was performed under anaesthesia using 500 mg/L MS-222.

2.2. Salamanders and bacterial infection

Chinese giant salamanders (mean body weight 200 g) were obtained from a farm in Hubei province, China and acclimated in aerated water tanks for one week prior to experimentation. Tissue swabs were taken from spleen, heart and liver tissues, under sterile conditions, to check for the presence of any unforeseen bacterial infections. *A. hydrophila* (strain 4LNC209) was kindly provided by Professor Aihua Li (Institute of hydrobiology, Chinese Academy of Sciences). Salamanders in the experimental group were injected intraperitoneally, with *A. hydrophila* at a dose of 1.5×10^6 cfu/100 g body weight (Yu et al., 2014). Control animals were administrated with an equivalent volume of PBS vehicle. To analyze the transcriptomic changes during acute infection, livers from three *A. hydrophila* injected and three PBS injected animals were sampled at 12 h post injection.

2.3. RNA isolation, library preparation and sequencing

Two cDNA libraries, representing control and *A. hydrophila* infection groups, were generated from a pool of total RNA derived from three individual animals per library. Total RNA was extracted from liver tissues using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. RNA quality and quantity were assessed using an Agilent 2100 Bioanalyzer (RNA 6000 nano platform; Agilent Technologies) and a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, USA) respectively. Only high quality total RNA samples with RIN (RNA integrity number) values of >8.7 were used for cDNA library preparation. Equal quantities of total RNA from each group of 3 animals were mixed prior to Illumina cDNA library preparation. After DNase I treatment, mRNA was purified using oligo $(dT)_{25}$ magnetic beads (Dynabeads® oligo $(dT)_{25}$, Invitrogen) and broken into short fragments of 200–250 nucleotides using RNA fragmentation reagent (Ambion). cDNA, synthesized from fragmented mRNA, was end-repaired followed by adapter ligation using the TruSeq™ RNA library preparation kit (Illumina). Enrichment of cDNA Fragments 300–350 bp was undertaken using primers designed for TruSeq sequencing according to the manufacturer's instructions. Enriched PCR products were purified using Agencourt Ampure XP Beads (Beckman Coulter). The quality and quantity of each cDNA library was assessed using an Agilent 2100 Bioanalyzer and by real-time PCR respectively and sequenced on the HisSeq 2000 platform using a TruSeq SBS v3-HS kit (Illumina).

2.4. Assembly of transcripts and annotation

Sequence data were converted, by base calling, into raw reads and cleaned by removal of poly A/adaptor sequences and low quality reads. De novo transcriptome assembly was carried out using Trinity software (Grabherr et al., 2011), which was performed on an Intel PC (CPU: Xeon E5335, 2.0 GHz; RAM: 64 G) with RedHat Linux operating system. TIGR Gene Indices Clustering tools (TGICL) software (Pertea et al., 2003) was used to undertake further sequence splicing and acquisition of non-redundant unigenes. All unigenes were searched by BLAST analysis (*E*-value cut-off of 1.0E−5) against the non-redundant (NR) database in NCBI, Swiss-Prot and Clusters of Orthologous Groups (COGs) of proteins databases. Gene ontology (GO) annotation was acquired using the Blast2GO program (Conesa et al., 2005) and WEGO software (Ye et al., 2006). ESTScan software was used to determine the presence and direction of any coding regions found in unigenes with no BLAST hits (Iseli et al., 1999). Pathway analysis was performed using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg) (Kanehisa et al., 2012). Unigenes were subjected to BLASTX analysis against the KEGG database (*E*-value of <1.0E−5).