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Insights into leghorn male hepatocellular cells response to fowl adenovirus serotype 4 infection by transcriptome analysis

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ABSTRACT

Fowl adenovirus serotype 4 (FAdV-4), a member of the *Aviadenovirus* genus of the Adenoviridae family, causes hepatitis–hydropericardium syndrome (HHS) in chickens. It causes mortality of up to 80% in 3–6-week-old broilers, posing a substantial threat to the poultry industry. However, the specific host responses to the virus are not well understood. To better understand the interactions between the host and FAdV-4 and to explore the pathogenesis of this virus, a high-throughput RNA-seq technology was utilized with leghorn male hepatocellular (LMH) cells at 12, 24, and 48 h after FAdV-4 infection. We identified a total of 7000 differentially expressed genes (DEGs), which were enriched in a variety of biological processes and pathways using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. Several immune related pathways, including Toll-like receptor (TLR) signaling pathway and cytokine–cytokine receptor interaction pathway, were activated after the FAdV-4 infection. The transcriptional data were validated by quantitative real-time PCR. The expression profiles of 10 genes involved in FAdV-4-infected chicken livers, including TLR2A, TLR3, TLR5, MyD88, IL12B, IL15, IL18, CCL20, TNFRSF21, and CD30, were consistent with RNA-seq profiles. By transfecting small interfering RNA into LMH cells, our results confirmed that MyD88 mediated FAdV-4-induced inflammation. To our knowledge, this was the first study to use transcriptome analysis to investigate host responses to FAdV-4 infection. These findings provide insights into the mechanisms of FAdV-4 pathogenesis and host-FAdV-4 interaction.

1. Introduction

Fowl adenoviruses (FAdVs) belong to the genus *Aviadenovirus* of the family Adenoviridae. *Aviadenovirus* comprises five species (*Fowl aviadenovirus* A, B, C, D, and E) and 12 serotypes (FAdV-1 to 8a and −8b to 11) (Hess, 2000; Steer et al., 2009). Among the 12 FAdV serotypes, fowl adenovirus serotype 4 (FAdV-4) is the causative agent of hepatitis–hydropericardium syndrome (HHS) in poultry (Mazaheri et al., 1998). HHS is characterized by the accumulation of clear, straw-colored fluid in the pericardial sac, and by an enlarged, discolored liver with multifocal areas of necrosis and infiltration of mononuclear cell containing basophilic intra-nuclear inclusion bodies (Ganesh and Raghavan, 2000). It mainly occurs in 3–6-week-old broilers, leading to mortality of up to 80%; thereby, causing substantial economic loss to the poultry industry (Chandra et al., 2000).

FAdV-4 belongs to FAdV-C, and it can be transmitted both horizontally and vertically. Liver is one of the major target organs of FAdV-4. This virus can be isolated from liver homogenates of infected chickens (Li et al., 2016; Liu et al., 2016; Vera-Hernandez et al., 2016), and it can also propagated and cause cytopathic effects (CPEs) in a liver cell line, namely leghorn male hepatocellular (LMH) cell line (Li et al., 2016; Pan et al., 2017; Vera-Hernandez et al., 2016). Although FAdV-4 has been existing and prevalent for 30 years since first been identified in 1987, little is known about the virus pathogenesis and host responses to it. In a previous study, a reduction of $CD4^+$ and $CD8^+$ cells were detected in the spleen and thymus of a FAdV-4 virulent strain infected chickens. And a severe depletion of lymphocytes was also observed in the bursa of Fabricius (Schonewille et al., 2008). Moreover, it was reported that after a nonpathogenic FAdV-4 ON1 strain infection, the expression levels of IFN-γ and IL-10 increased in liver, whereas those of IFN-γ and IL-18 decreased in the spleen (Grgic et al., 2013). To date, few reports have been established to elucidate the molecular mechanism of FAdV-4 pathogenesis. Previous studies have determined the gene expression profiles of cancer cell lines or noncancerous cell lines infected with mammalian adenovirus by transcriptomic analyses. In particular, researchers have assessed the gene expression profiles of

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HeLa cells infected by human adenovirus type 2 (Granberg et al., 2005; Zhao et al., 2003), of human melanoma cells infected by human adenovirus serotype 5 (Dorer et al., 2011; Volk et al., 2005), and of the human hepatoma cells HuH7 infected by an adenovirus (Martina et al., 2007). Researchers have also revealed the transcriptional profiles of primary mouse embryo fibroblasts infected by human adenovirus serotype 5 vector (Hartman et al., 2007) and of primary human lung fibroblasts (IRM-90) infected by human adenovirus serotype 2 (Zhao et al., 2012, 2007). However, no transcriptome profile related to an avian adenovirus infection has been established so far as we know.

In this study, we used FAdV-4 to infect LMH cells and obtained the transcriptome profiles at 12, 24, and 48 h post infection (hpi) through RNA-seq. We further investigated the gene expression profiles of FAdV-4-infected chicken liver and the role of MyD88 in FAdV-4-induced inflammatory response. To our knowledge, this work is the first to report avian-adenovirus induced gene expression profiles and to provide a comprehensive understanding of the host and virus interactions.

2. Materials and methods

2.1. Virus and cells

The FAdV-4 virulent strain HB1502 (GenBank accession N.: KX421401.2) was isolated by our laboratory from the liver homogenate of infected birds. The freeze–thaw liver homogenate supernatant was inoculated to 9-day-old SPF-embryonated chicken eggs *via* the chorioallantoic membrane route for three passages. Then the virus was purified by plaque assay in LMH cells. The purified virus showed similar virulence to that of its parental virus after infecting chickens. The LMH cells were maintained in F12 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 0.1 mg/ml streptomycin. One-step growth curve was monitored as previously described (Alexander et al., 1998). LMH cells were seeded in 35 mm cell culture dishes (Corning, USA) and allowed to grow overnight to reach approximately 80% confluence. Then FAdV-4 was inoculated onto each dish at a multiplicity of infection (MOI) of 5. After 1 h adsorption in serum-free medium at 37 °C and 5% CO_2, the cells were washed three times with phosphate buffered saline (PBS). Then, 3 ml F12 medium containing 2% FBS was added to each dish. The cells, together with cell culture media, were collected at 6, 12, 24, 36, 48, 60, 72, 84, and 96 hpi by freezing and thawing three times. The virus titers of each time point were determined by plaque assay.

2.2. Virus inoculation

LMH cells were cultured for approximately 24 h to reach about 80% confluence. Then the cells were washed three times with PBS and infected with the FAdV-4 isolated strain at a MOI of 5. Non-infected LMH cells were used as negative controls. After 1 h adsorption in serum-free medium at 37 °C, the medium was replaced with 2% FBS F12 medium. The FAdV-4 infected and non-infected cells were collected at 12, 24, and 48 hpi. There were six groups: 12 h mock, 24 h mock, 48 h mock, 12 h infected, 24 h infected, and 48 h infected group. Each group was processed with three independent replicates.

2.3. Library construction and RNA sequencing

An RNeasy mini kit (Qiagen, Germany) was used to isolate total RNA from 18 samples (six groups) according to the manufacturer's instructions. Total RNA was purified by RNeasy micro kit (Qiagen, Germany) and RNase-Free DNase Set (Qiagen, Germany). RNA concentration was determined by NanoDrop ND-2000 (Thermo Fisher Scientific Inc., USA). RNA integrity was checked using an RNA Integrity Number (RIN) generated by an Agilent Bioanalyzer 2100 (Agilent technologies, USA). RNA samples with RIN ≥ 7 were used for library construction. The library was constructed using the TruSeq® RNA Sample Preparation Kit (Illumina, USA) following the manufacturer's protocol. mRNA was initially purified using poly-T oligo-attached magnetic beads. The purified mRNA was fragmented into 100 bp to 400 bp small pieces using divalent cations at 94 °C for 8 min. The double-strand cDNA was synthesized by priming with random hexamers. The cDNA fragments were then subjected to an end repair process to add a single nucleotide A (adenine). The repaired fragments were ligated to sequencing adapters. The products were purified by agarose gel electrophoresis as a template and amplified by PCR. The final cDNA libraries were quantified by Qubit® 2.0 Fluorometer (Life Technologies, USA) and validated by Agilent Bioanalyzer 2100 (Agilent Technologies, USA). Finally, 18 libraries were sequenced on the Illumina HiSeq 2500 (Illumina, USA). Library construction and sequencing were performed by Shanghai Biotechnology Corporation (Shanghai, China).

2.4. Identification of differentially expressed genes

Clean reads were obtained by Seqtk (version:1.0-r82-dirty) using default parameter to filter out all ribosome RNA reads, sequencing adapters, fragments shorter than 25 bp, and low-quality reads with error ratio ≤ 0.01. The clean reads were aligned and mapped to the chicken reference genome (Genome Assembly: Gallus_gallus-5.0 GCA_000002315.3) using Hisat2 (version 2.0.4) under spliced mapping algorithm with default parameter (Kim et al., 2015). The expression abundance of the mapped gene reads was normalized by fragments per kilobase of exon model per million mapped reads (FPKM) using StringTie (version: 1.3.0) with default parameter (Pertea et al., 2016, 2015). The mean FPKM value of three replicates in each group was calculated. For differentially expressed gene (DEG) analysis, three pairwise comparisons (12 h infected *vs*. 12 h mock, 24 h infected *vs*. 24 h mock, and 48 h infected *vs*. 48 h mock) were conducted. The DEGs were selected using edgeR (Robinson et al., 2010) with the threshold of false discovery rate (FDR) adjusted p-value ≤ 0.05 and $|\log_2$ fold-change$| \geq 1$. We set dispersion 0.01 in edgeR (version: 0.16). The DEGs identified at 12, 24, and 48 hpi were used to perform functional classification using GO and KEGG analysis, respectively. The GO and KEGG pathways were considered significantly enriched when p-value < 0.05. The RNA-seq raw data have been deposited in the NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/). The GEO accession number is GSE106839.

2.5. Animal experiment and ethics statement

A total of 20 35-day-old SPF leghorn chickens (Vital Merial, Beijing, China) were raised in separated negative-pressure isolators and randomly divided into four groups. Each group contained five chickens. One group was inoculated intramuscularly with 200 µl of PBS as a negative control. Chickens in the other three groups were inoculated intramuscularly with a lethal dose of 200 µl (10^7 TCID50 /ml) FAdV-4 HB1502 strain each. The negative control group was humanely euthanized at 0 hpi. The other three groups were humanely euthanized at 12, 24, and 48 hpi. Liver samples were collected and grinded with liquid nitrogen. The expression levels of TLR2A, TLR3, TLR5, MyD88, IL12B, IL15, IL18, CCL20, TNFRSF21 and CD30 were assessed by quantitative real-time polymerase chain reaction (qRT-PCR). The expression levels of these genes were normalized to those of the negative control group. The animal experimental protocol used in this study was approved by and performed in accordance with the Biological Studies Animal Care and Use Committee in Hubei Province, China (Permit Number: 11401000028015). All efforts were made to minimize animal suffering.

2.6. qRT-PCR

Total RNA of virus-infected and non-infected LMH cells, and liquid