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Comparative transcriptome analysis reveals induction of apoptosis in chicken kidney cells associated with the virulence of nephropathogenic infectious bronchitis virus

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ABSTRACT

Avian infectious bronchitis virus (IBV) that causes respiratory and nephritic diseases in chicken is a major poultry pathogen leading to serious economic loss worldwide. The nephropathogenic IBV strains cause nephritis and kidney lesions intrinsically and the pathogenic mechanism is still unclear. In the present study, SPF chicks were infected with three nephropathogenic IBVs of different virulence and their gene expression profiles in chicken kidney were compared at transcriptome level. As a result, 1279 differentially expressed (DE) genes were found in very virulent SCDY2 inoculated group, 145 in virulent SCK2 group and 74 in non-virulent LDT3-A group when compared to mock infected group. Gene Ontology (GO) and KEGG pathway enrichment analysis on SCDY2 group displayed that the up-regulated DE genes were mainly involved in cell apoptosis, and the down-regulated genes were involved in metabolic processes and DNA replication. Protein-Protein Interaction (PPI) analysis showed that DE genes in SCDY2 group formed a network, and the core of the network was composed by cell apoptosis and immune response proteins. The clustering of gene expression profile among the three virus inoculated groups indicated that the majority of up-regulated DE genes on apoptosis in very virulent SCDY2 group were up-regulated more or less in virulent SCK2 group and those down-regulated on innate immune response in SCDY2 group were also down-regulated differently in SCK2 group. In addition, the number of apoptotic cells detected experimentally in kidney tissue were very different among the three virus inoculated groups and were positively accordant with the viral titer, kidney lesions and viral virulence of each group. Taken all together, the present study revealed that virulent nephropathogenic IBV infection modified a number of gene expression and induction of apoptosis in kidney cells may be a major pathogenic determinant for virulent nephropathogenic IBV.

1. Introduction

Avian infectious bronchitis is one of the economically important virus diseases to poultry industry worldwide [1] and caused by infectious bronchitis virus (IBV), a positive-sense single-stranded RNA virus belonged to genus *Gammacoronavirus* [2–4]. The virus replicates at many epithelial surfaces of the chicken. IBV initially infects the upper respiratory tract and then spreads to kidney and oviduct, causing respiratory disease, nephritis and egg drop according the strain of the virus and the system involved [5–7]. The IBV genome is variable and variants of different genotypes have been reported worldwide [8]. Numerous serotypes and poor cross protection make it hard to control IBV spread completely in the commercial chicken farms [9–11].

The pathogenesis of IBV is complicated. In clinical case, infection of IBV is commonly followed by secondary bacterial/virus infections, which is the main cause of death and severe lesions [12]. Under the experimental conditions, the dynamic distributions of H120, M41 (respiratory type) and SAIBK (nephropathogenic) were described. They exhibited similar titers in lung, however titer of SAIBK in kidney was higher than H120, M41 at 4–10 days of post infection (dpi) [13]. Virus loads in kidney at early stage of infection may be responsible for interstitial nephritis and tubule lesions. In addition, using two dimensional gel electrophoresis, the differentially expressed proteins after infection with IBV strains were mainly relate to cytoskeleton, binding of calcium ions, the stress response, anti-oxidative and macromolecular metabolism [14].

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In order to understand the host cellular genes involved in the pathogenesis of IBV, high throughput techniques such as microarray [15–17] and RNA sequencing [18–20] were carried out. The immune responses to IBV were analyzed and a diversity of innate immunity and helper T-cell-type-1-biased adaptive immunity were activated in the host cell during early defense against IBV. Several immune factors were involved to active mucosal immunity, including toll-like receptors, type 1 interferon, interleukin 1 beta, complement, T-cell signaling molecules, surface markers and effector molecules [21]. The differential expression (DE) genes in chicken kidney after infection of ck/CH/LDL/091022 were also analyzed, 1777 DE genes were detected, including focal adhesion pathway, cytokine receptor interaction pathway, production of cell adhesion molecules and peroxisome function [15]. In addition, the DE genes in the lung tissues of IBV infected 18-days-old chicken and DE genes in the spleen of two chicken lines L10H and L10L were analyzed [16,22]. These studies exhibited a view of the molecular antiviral mechanisms of chicken response to the IBV infection. However, only one strain was used in these two reported experiments, it is difficult to compare the transcriptomics of these two strains under different animal experiments.

The infection of coronavirus has been reported with many functional and morphological changes in host cells that associated with significant changes in the patterns of expression of host cell genes [23]. Such as described in SARS-CoV [24] and mouse hepatitis virus (MHV) [25]. These results provided invaluable information on the cellular signaling pathways involved either in the cellular response to viral infections, or the viral manipulation of cellular machinery to ensure their own survival. Previous studies demonstrated that IBV could induce apoptosis in cultured cells, which may be related to the virus pathogenicity [26–28]. Only few recent studies have investigated the changes in the expression of cellular proteins during IBV infection in *ex vivo* or *in ovo* [29–31]. However, *in vivo* infection model could yield more biologically relevant insights into pathogenesis.

The present study aims to investigate gene expression profile in chicken kidney cells infected with nephropathogenic IBVs of different virulence. The comparative transcriptome analysis reveals that the interaction between virulent virus and chicken host cells might activate cell apoptosis and in turn resulted in kidney lesions.

2. Materials and methods

2.1. Viruses, animal experiment and sample collection

IBV viruses, SCDY2, SCK2 and LDT3-A, used in the study were nephropathogenic and maintained in SPF chicken embryo eggs in the laboratory of Animal Disease Prevention and Food Safety Key Laboratory of Sichuan Province. SCDY2 [32] and SCK2 [8] are very virulent and virulent nephropathogenic IBV strains respectively, isolated from Sichuan province, China. LDT3-A is an approved vaccine strain attenuated from highly nephropathogenic IBV tl/CH/LDT3/03 isolated in north China [33].

Forty white leghorn chicks hatched from SPF eggs (MERIAL-Beijing) were equally divided into four groups. At 15 days old, chicks in the three experimental groups were inoculated intranasally with 0.1 mL 10^5, 10^5 and $10^{3.5}$ median embryo infectious doses of SCDY2, SCK2 and LDT3-A, respectively. The same volume of phosphate buffer saline (PBS) were applied to each chick in the mock inoculated control group. The chicks were then housed in separate isolators.

Chicks inoculated SCDY2 showed clinical signs at 2 dpi and three chicks died at 5 dpi and one died at 6 dpi. The gross lesions were observed in kidney with swelling and uric acid salt deposits during autopsy of the died chicks. Chicks inoculated SCK2 showed typical symptoms and slightly kidney swelling was observed at 6 dpi. No clinical signs and kidney lesions were observed in the chicks inoculated LDT3-A or PBS.

Kidney tissues were taken from the four died chicks in SCDY2 inoculated group at 5 and 6 dpi and kidney samples were also collected in parallel from the killed chicks in SCK2 or LDT3-A inoculated and mock-inoculated groups. All samples were stored at −80 °C. The collection of tissue samples was performed in accordance with the Guidelines for Experimental Animals issued by the Ministry of Science and Technology of People's Republic of China.

2.2. RNA extraction, RNA sequencing and data analysis

Total RNA was isolated from collected kidney tissue with TRIzol reagent (Invitrogen, USA) following the manufacturer instructions. After quantification and qualification of the RNA samples, 3 μg RNA from each of three individuals per group at 5 dpi were mixed equally to form the input material for RNA sequencing. Libraries for RNA-seq were generated by using RNA Library Prep Kit for Illumina® (NEB, USA) and sequenced by paired-end reading on an Illumina Hiseq 4000 platform.

About 7.6 ± 1.31 G clean bases per sample were obtained and the clean reads were mapped to the genome *Gallus gallus* (http://ftp.ensembl.org/pub/release-76/fasta/gallus_gallus/dna/) with TopHat2. HTSeq v0.6.1 was used to count the number of reads mapped to each gene and FPKM (expected number of fragments per kilobase of transcript sequence per millions base pairs sequenced) of each gene was calculated based on the length of gene and the counts of paired-end reads mapped to the gene.

2.3. Analysis of DE genes

Gene differential expression analysis was carried out by using DESeq R package (1.18.0). Genes with an adjusted padj < 0.005 and Fold change ≥ 2 found by DESeq were assigned as DE genes. Gene Ontology (GO) enrichment of DE genes was analyzed by using of GOseq R package. The GO terms with P-value less than 0.05 were considered as significantly enriched. The enrichment of DE genes in KEGG pathways was analyzed with software KOBAS (2.0). Protein-protein interaction (PPI) of DE genes was constructed by inputting of DE genes involved in immune response, cytokine mediated signaling pathways, inflammatory response, apoptosis and cell cycling into STRING database on line (http://string-db.org/) and the PPI networks were visualized in cytoscape software. Clustering analysis of DE gene expression was carried out by hierarchical clustering based on FPKM of DE genes in three viruses inoculated and one mock inoculated groups. The clustered DE genes were further performed GO enrichment analysis to find out the genes related to immune response and apoptosis distributed in each cluster.

2.4. Validation of RNA-seq data and viral copies with RT-qPCR

To validate gene expression data created by RNA-seq, nine randomly selected genes with two apoptosis regulatory genes, Bax and Bcl2, were applied to revers transcription quantitative PCR (RT-qPCR). Total RNA extracted from the same tissues as used in RNA-seq was reverse transcribed by PrimerScript™ RT Reagent Kit (Takara). Primers for amplification of these genes were listed in Table S1 and the gene expression level was estimated by $2^{-\Delta\Delta CT}$ method based on relative expression to the reference gene, β-actin. The same strategy was used to validate viral RNA copies in kidney cells by amplification of IBV 5′-UTR (Table S1) as described previously [13].

2.5. Detection of apoptosis in kidney cells by TUNEL assay

Apoptotic cells were detected by the TUNEL assay using the FITC *in situ* cell death detection kit (Roche Diagnostics, USA) according to the manufacturer's instructions. Six sections of kidney tissue for each group were stained and the results were recorded by taking pictures under fluorescence microscope (40X). The ratio of apoptotic cells for each group were determined by counting of the apoptotic cells emitting