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ORIGINAL ARTICLE

# Transcriptome based analysis of apoptosis genes in chickens co-infected with avian infectious bronchitis virus and pathogenic *Escherichia coli*

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## ABSTRACT

**Background and Objectives:** Infection with Infectious bronchitis virus (IBV) and avian pathogenic *Escherichia coli* (APEC) is an important respiratory infection worldwide. Apoptosis is a physiological process of cell death that occurs as part of normal development and responds to a variety of physiological and pathophysiological stimuli. The identification of molecular mechanisms of action or inaction of key apoptotic proteins is important. This study aimed to investigate apoptotic related genes in the trachea tissue of infected (IBV variant 2, and APEC serotype O78: K80) SPF chickens group compared to the control group.

**Materials and Methods:** Forty SPF chickens was divided into 2 groups. Differential transcriptional profile in the infected SPF chickens trachea tissue was compared to those of control group in the early stage of infection by Illumina RNA-seq technique paired-end and strand-specific sequencing. Differentially expressed genes (DEGs) of transcriptome profiling of the trachea from the infected group were identified. Gene ontology category, KEGG pathway, and STRING analysis were analyzed to identify relationships among differentially expressed genes.

**Results:** Twenty-eight apoptotic genes were identified. They consisted of six pathways related to cell death: the extrinsic pathway, intrinsic pathway, endoplasmic reticulum stress pathway, MAPK signaling pathway, and cell death by NFkB and activates mTOR pathway and some regulator and apoptosis inhibitors.

**Conclusion:** All of the apoptotic genes in our study were up-regulated. Among these genes, the more fold change value was for TRADD and BCL2A1 genes, and the less fold change value was for MAP3K14, NFKB1, PIK3CB, and ITPR2 genes.

**Keywords:** Chickens; Infectious bronchitis virus; *Escherichia coli*; Apoptosis; Ribonucleic acid sequencing

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## INTRODUCTION

The Infectious bronchitis virus (IBV) and secondary bacterial infections with avian pathogenic *Escherichia coli* (APEC) usually causes high economic losses among the poultry industry (1). Understanding the immune mechanisms and interactions between pathogens and hosts is important for prevent-

ing infection. Apoptosis is a form of programmed cell death by receptor activation and is a conserved process. It is an important part of the host's innate immune response, which helps in early control of the various infections (2). One of the strategies that supports intracellular survival and persistence of the viruses, bacteria, and parasites is inhibited host cell by apoptosis and the reorganization of cellular receptors, or by the activation of apoptotic proteins such as caspases. Many pathogenic bacteria and viruses can repress apoptosis during infection by encoding proteins to escape from immune attack, or viruses can promote apoptotic death of the host cells (3).

There are four apoptotic pathways consist of: the internal mitochondrial pathway (the intrinsic pathway), the death receptor-mediated external apoptotic pathway (the extrinsic pathway), the endoplasmic reticulum stress pathway, and the B-granzyme-mediated apoptosis pathway. The protein molecules that were involved in the apoptosis mechanisms are Cysteinyl aspartate specific proteins (Caspase), adapter proteins, B-cell lymphoma-2 (Bcl2), an inhibitor of apoptosis proteins (IAPs) (4).

Regulation of intrinsic apoptosis processes is by the BCL-2 and caspase families of proteins (5, 6). Caspases can be classified into three groups: apoptosis initiator (CASP2, CASP8, CASP9, and CASP10), inflammatory CASPs (CASP1, CASP4, CASP15, CASP11, and CASP12), and apoptosis executioner (CASP3, CASP6, and CASP7) (7). The inhibitors of apoptosis proteins (IAP) can regulate the activity of caspases. Cathepsin D can also regulate apoptosis (8). Other factors for regulating immune responses, apoptosis, and cell survival, are the mitogen-activated protein kinases (MAPKs) families and NFKB transcription factors (9).

Three MAPK families have been characterized: Jun kinase (JNK/SAPK), extracellular signal-regulated kinase (ERK), and p38 MAPK (10). Caspase-8 is a cell-extrinsic apoptotic signal that can cause transduction by death receptors (DRs), DR4, DR5, and Fas into a death-inducing signaling complex (DISC) (11, 12).

One of the molecules that are transducer metabolic and mitogenic signals to promote cell growth, migration, proliferation, and apoptosis are phosphoinositide 3-kinases (PI3Ks) family and PTPs (13). Also, receptor-interacting protein kinase (RIPK) 1 and RIPK3 that can be induced by tumor necrosis factor (TNF) signaling, are essential kinases for the regulation of necrosis (14). This study aimed to investigate the effects of mix-infection with IBV and APEC on activation of host apoptosis on the chicken trachea and identifying genes involved in apoptosis by RNA-seq.

## MATERIALS AND METHODS

**Virus and bacterial strains.** Avian infectious bronchitis virus variant 2 from the virus collection and avian pathogenic *E. coli* serotype O78: K80 from the University of Tehran culture collection was used in this study.

**Chicken challenge and tissue collection.** Forty SPF (specific pathogen-free) chickens were divided equally into two groups: one experimental group and one control group. All groups were kept in separate isolators. At 12 days of age, each chicken was inoculated via oculonasal inoculation of 100 µl of $10^4$ $EID_{50}$ infective doses of IBV, and 72 hours later were inoculated via the intratracheal route of 100 µl of $10^8$ CFU/ml infective dose of APEC, and control groups were inoculated with 100 µl PBS. Chickens were euthanized at 3 days post-injection (dpi), and the tracheal organs were harvested. A piece of trachea was added directly to the RNAlater solution (Thermo Fisher Scientific) and then stored at -80°C. A piece of trachea samples from the challenge group and control group were included in the qPCR validation of the RNA-seq results for IBV detection and culture for APEC's colony count.

**RNA isolation.** According to the manufacturer's instruction, total RNA from samples with the adequate virus and bacterial load was extracted from samples of control and challenged groups using TRIzol reagent (Thermo Fisher Scientific). On-column DNA digestion was performed using RNase-free DNase (SinaClone) to remove contaminating genomic DNA. RNA samples quantified using an Agilent Bioanalyzer 2100. These total RNAs were subjected to the Beijing Genome Institute (BGI) (Shenzhen, China) for RNA-seq and transcriptome analysis.

**Illumina library construction, RNA sequencing, and analysis.** Illumina HiSeq 2500 platform, paired-end, and strand-specific sequencing produced an average read length of ~150 bp. Acquired

data were processed to obtain raw reads, which were then extracted for quality control, trimming, read mapping, and identified differentially expressed genes (DEGs) using CLC Biomedical software (Trial version) that were aligned using reference genome (Gallus Gallus reference) extracted of NCBI. For DEGs analysis, after normalized data by CLC Biomedical software and statistical analysis by Beggarly test, DEGs were extracted by comparing gene expression levels between the infected and control groups. For visualization and discovery analysis of DEGs to perform functional annotation, GO (Gene Ontology) analysis was performed by Enrichr (http://amp.pharm.mssm.edu/Enrichr), then, terms with corrected FDR<0.05 were considered significantly enriched in differentially expressed genes. Obtained DEGs were analyzed for pathway enrichment analysis based on the KEGG (Kyoto Encyclopedia of Genes and Genomes) database (FDR<0.05) to explore the various biological processes. The apoptosis pathway was selected among the obtained pathways, and the genes group was collected, then genes were enriched in the STRING database for predicted protein interactions.

## RESULTS

**Functional analysis of apoptosis genes.** After the analysis of DEGs by comparing extracted genes between the infected group and the control group, this was identified that all of the apoptosis genes were up-regulated. The list of apoptotic genes is in Table 1 from most fold change value to least, respectively. The more fold change value was for TRADD and BCL2A1 genes that TRADD is one of the proteins associated with the extrinsic pathway, and BCL2A1 is a member in the intrinsic pathway, and the less fold change value was for MAP3K14, NFKB1, PIK3CB, and ITPR2 genes (Table 1).

The genes were enriched in the STRING database for predicted protein interactions. The apoptotic genes contained 28 nodes associated with 79 interactions (edges) (Fig. 1).

## DISCUSSION

This novel experiment characterized the type of apoptotic pathways in the chicken trachea mix-in-

**Table 1.** KEGG pathway analysis for apoptotic genes. Pathway analysis enrichment using DEGs between control and the group of inoculated with IBV and APEC. FDR < 0.05 was used as a threshold to select significant KEGG pathways. The KEGG ID was gga04210.

| Genes | Fold change value | Genes | Fold change value |
|---|---|---|---|
| TRADD | DIV | RIPK1 | 16 |
| BCL2A1 | DIV | DAB2IP | 14 |
| MAPK1 | 553 | PTPN13 | 13 |
| APAF1 | 228 | TNFSF10 | 13 |
| TRAF2 | 199 | PIK3R2 | 13 |
| PIK3CA | 133 | CTSC | 11 |
| CASP3 | 107 | BID | 10 |
| CASP7 | 65 | CFLAR | 10 |
| RAF1 | 52 | CASP2 | 9 |
| CTSD | 48 | FADD | 9 |
| ITPR3 | 40 | MAP3K14 | 8 |
| PIK3CD | 26 | NFKB1 | 8 |
| EIF2AK3 | 16 | PIK3CB | 8 |
| ATM | 16 | ITPR2 | 3 |

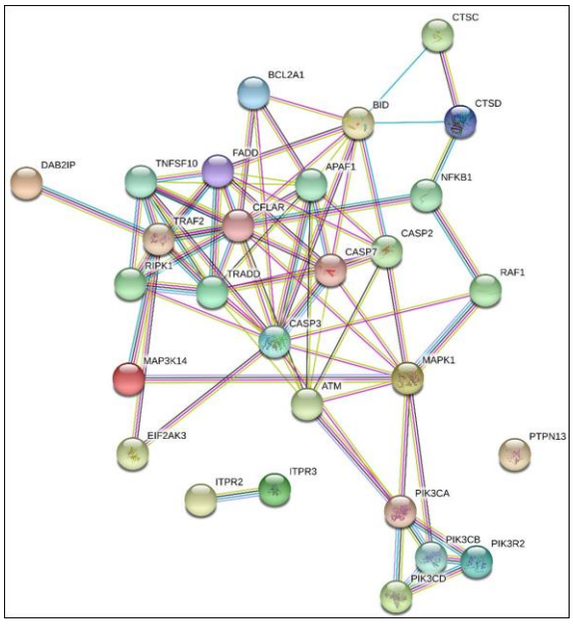


**Fig. 1.** STRING analysis of the relationship between differentially expressed genes (FDR < 0.05) related to the apoptotic pathways in up-regulated genes in the mixture infection group with IBV and APEC. The network nodes represent the proteins encoded by the DEGs, and the edges represent protein-protein interactions.

fected with IBV (variant 2) and APEC (O78: K80) in the early phase of infection by survey transcriptome changes in the chicken trachea with focuses on the use of the strand-specific NGS method.

Apoptosis is a conserved process for early control of the various infections that is consists of many pathways. The cells can sense the presence of pathogen proteins and eliminate them by inducing apoptosis (15). In this study, we found a total of investigated twenty-eight apoptotic genes (Table 1) were up-regulated, and they consisted of six pathways related to cell death, and some regulator and apoptosis inhibitors. In the extrinsic pathway by transduction of cell-extrinsic apoptotic signals by the death receptors (DRs), the caspase-8 recruiting into a death-inducing signaling complex (DISC) and induce a conformational change in the receptor that leads to recruitment of TNFR1-associated death domain protein (TRADD) or Fas-associated death domain protein (FADD) that they lead to apoptosis (16). In this study, the expression of TRADD was higher than FAAD. This study also demonstrated the PTPN13 gene, which is an enzyme member of the protein tyrosine phosphatase (PTP) family, that can interact and dephosphorylate the Fas receptor, which suggested this is the role of this protein in Fas-mediated programmed cell death (17). In previous studies, PLCB2, TRAF2, JAK1, CBLB, FOS, SOCS4, TNFRSF1A, CASP8, and TRADD in *E. coli* infection were shown (18, 19).

In the intrinsic pathway, the release of the pro-apoptotic proteins including cytochrome c, activates the BCL-2 family members Bax and Bak results in MOMP (mitochondrial outer membrane permeabilization), from the inter-membrane space into the cytosol (20, 21). Cytochrome c bind to APAF1 and forming the apoptosome and activating caspase-9, and this molecule cleave and activate caspase-3 and caspase-7 (22). In present research showed BCL2A1 and BID. BID is the proapoptotic Bcl2 family member that can migrate to mitochondria by protease-cleaved (Truncated BID (tBID)) (23), and APAF1 is related to this pathway. Current research found ATM that ATM is a substrate for caspase 3 *in vitro*. ATM can prevent DNA repair and DNA damage signaling by cleavage during apoptosis and generate kinase-inactive (24). The presence of caspase-1, caspase-9, caspase-18, Bcl-2L1, BCL2-antagonist/killer 1, Mcl-1, caspase-3, Bax, and Bak in IBV infection (25, 26) and the presence of caspase 3/7 in APEC (27) was shown previously.

Between these two pathways, most of our study genes were for the intrinsic pathway. However, we know that the molecules in one pathway can influence the other, and two pathways are linked (28). A group of negative regulators of both caspases and cell death is an inhibitor of apoptosis proteins (IAP) (29). In our study, anti-apoptotic protein CFLAR, RIPK1, and TRAF2 were found. CFLAR and TRAF2 suppressed CASP8 activation (30). RIPK1 kinase activity and RIPK3 have both caspase-dependent and independent mechanisms that contribute to TNF induced apoptosis. In addition to the above, two regulators, CTSD (Cathepsin D) and CTSC, were found in our study, too, that they both are an important regulator of apoptotic pathways in cells and autophagy (31, 32).

The endoplasmic reticulum stress pathway was another apoptotic pathway that was found in our study. Three ER-resident transmembrane proteins ERN1 (endoplasmic reticulum to nucleus signaling 1), ATF6 (activating transcription factor 6), and EIF2AK3 (eukaryotic translation initiation factor 2-α kinase 3) are the stress sensors of ER, among them EIF2AK3-EIF2S1 or the ERN1-MAPK8/9/10 pathway is a crucial mediator of ER stress-induced autophagy (33, 34), that EIF2AK3 was found in our study. The previous study showed that in mammal Vero cells, when infected with IBV, the endoplasmic reticulum stress pathway was reported. Also, IBV was found to induce ER stress in infected cells at a late stage of infection (35), while this study showed that infection with a mix of IBV and APEC induced this form of apoptosis in trachea cells in the early phase of infection.

Also, ITPR2 and ITPR3 were found in this study. Apoptosis is one Ca2+-mediated event influenced by ITPR isoforms such as ITPR3 and ITPR2 (36, 37).

Cell death by NFKB was another pathway in our study. NFKB consists of five subunits: RelB, p65 (RelA), p50 (NFKB1), c-Rel, and p52 (NFKB2) that they are homo-and hetero-dimmers. The most well-known NFKB dimer is p50: p65 (38), that they are generally thought to be transcriptional repressors by bounding to DNA (39). In our study, NFKB was shown to be one of the subunits of NFKB.

Raf-1 is one of the upstream component in the MAPK that can activate MEK (MAP kinase kinase) and then activates MAP kinase by phosphorylation (42). Subsequently other downstream kinases can be activated by MAP kinase (43).

Previously MAPK8 was shown to be the main pathway for TNFSF10-induced autophagy (44). Our study demonstrated that MAPK14, MAPK1, TNFSF10, and RAF-1, also exist in this pathway.

Other protein groups that contribute to cell death are PI3K complexes. In our study PI3KCA was found, PI3KCB, PI3KCD, and PIK3R2. Class I PI3Ks form a functional heterodimer composed of two subunits consist of catalytic (110 kDa (p110)) and regulatory (85 kDa (p85)). The two regulatory subunits are p85β (PIK3R2) and p85α (45). The catalytic p110 subunit has four isoforms consist of PI3KCA, PI3KCG, PI3KCB, and PI3KCD. PI3K activates protein kinase B (PKB), which activates the mTOR pathway (46). The previous study showed PIK3CD and PIK3R1 in APEC infection, too (19).

The pathways responsible for causing cell death, in this review were the extrinsic pathway, intrinsic pathway, endoplasmic reticulum stress pathway, MAPK signaling pathway, and cell death by NFkB and activates mTOR pathway. Although many apoptotic and anti-apoptotic proteins are involved in different apoptotic pathways, research is still required in this field from a deeper perspective and the therapeutic side.

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