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HPAI-resistant Ri chickens exhibit elevated antiviral immune-related gene expression

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ABSTRACT

Background: Highly pathogenic avian influenza viruses (HPAIVs) is an extremely contagious and high mortality rates in chickens resulting in substantial economic impact on the poultry sector. Therefore, it is necessary to elucidate the pathogenic mechanism of HPAIV for infection control.
Objective: Gene set enrichment analysis (GSEA) can effectively avoid the limitations of subjective screening for differential gene expression. Therefore, we performed GSEA to compare HPAI-infected resistant and susceptible Ri chicken lines.
Methods: The Ri chickens *Mx*(A)/*BF2*(B21) were chosen as resistant, and the chickens *Mx*(G)/*BF2*(B13) were selected as susceptible by genotyping the Mx and BF2 genes. The tracheal tissues of HPAIV H5N1 infected chickens were collected for RNA sequencing followed by GSEA analysis to define gene subsets to elucidate the sequencing results.
Results: We identified four differentially expressed pathways, which were immune-related pathways with a total of 78 genes. The expression levels of cytokines (*IL-1β*, *IL-6*, *IL-12*), chemokines (*CCL4* and *CCL5*), type interferons and their receptors (*IFN-β*, *IFNAR1*, *IFNAR2*, and *IFNGR1*), Jak-STAT signaling pathway genes (*STAT1*, *STAT2*, and *JAK1*), MHC class I and II and their co-stimulatory molecules (*CD80*, *CD86*, *CD40*, *DMB2*, *BLB2*, and *B2M*), and interferon stimulated genes (*EIF2AK2* and *EIF2AK1*) in resistant chickens were higher than those in susceptible chickens.
Conclusions: Resistant Ri chickens exhibit a stronger antiviral response to HPAIV H5N1 compared with susceptible chickens. Our findings provide insights into the immune responses of genetically disparate chickens against HPAIV.

Keywords: Influenza A virus; RNA-seq; GSEA; Signaling pathway; Ri chicken

INTRODUCTION

H5N1, one of the highly pathogenic avian influenza viruses (HPAIVs), can cause diseases in humans and many other organisms, including chickens [1]. H5N1 has spread globally after first appearing in Asia, killing tens of millions of poultry, and in extreme cases they may cause pandemics [2]. Studies are needed to clarify the mechanisms underlying avian influenza virus (AIV) disease in chickens that control H5N1 infection.

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Conflict of Interest

The authors declare no conflicts of interest.

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There are two prevalent methods to identify differentially expressed genes (DEGs) involved in particular pathways or functions [3]: (i) a simple hypergeometric enrichment test and (ii) a gene set enrichment analysis (GSEA). The difference between the two methods is the null hypothesis. In a simple hypergeometric enrichment test, a gene set overlapping test between the analyzed DEGs with a predefined cut-off and functionally categorized genes is performed [4]. The null hypothesis is that the genes of a pathway are not enriched in the DEGs. In contrast, GSEA uses the expression values of all genes assessed. The null hypothesis is that genes from the set occur randomly in the ranked list. GSEA is a computational method that evaluates microarray data at the gene set level. GSEA can identify gene sets with significant genome-wide variation [5]. Furthermore, GSEA helps detect small changes in individual genes [3] with strong cross-correlation between genes in one gene set.

In present study, Ri chickens, well-defined, native breed of yellow feathered chickens in Vietnam, were used as the experimental animals [6]. The chickens were selected and bred as resistant or susceptible chickens depending on *Mx* and *BF2* genotyping results. Mx proteins are key components of resistance against influenza virus in mice [7] and other viral families [8]. Antiviral activity was determined as serine to asparagine substitution at position 631 [9]. Several MHC alleles have an effect on the antiviral response of the host. The *BF2*-B21 haplotype is related to resistance to H5N1 virus infection was demonstrated in a previous study [7]. In contrast, *BF2*-B13 haplotype-related chicken mortality to HPAIV H5N1 and Marek's disease herpesvirus infection is higher than those associated with other haplotypes [10]. Our previous research showed that resistant Ri chickens are more resistant to HPAIV/H5N1 [11]. To profile the transcriptomes in the case of AIV infection, we infected two genetically different Ri chicken lines with HPAIV/H5N1 and collected tracheal tissue for high-throughput RNA-seq and GSEA analysis.

MATERIALS AND METHODS

Experimental animals, genotyping and HPAIV infection

Forty Ri chickens free of commonly occurring pathogens and parasites, a local chicken breed in Vietnam, were selected as resistant and susceptible lines and raised for 4 wk prior to HPAIV/H5N1 infection. All experimental and animal management procedures were approved by the Ministry of Agriculture and Rural Development of Vietnam (TCVN 8402:2010/TCVN 8400-26:2014).

Genotype analyses of *Mx* and *BF2* were performed for resistant and susceptible trait selection. For the *Mx* protein, animals with the 631-allele polymorphism A were selected as resistant chickens, whereas those with the 631-allele polymorphism G were selected as susceptible chickens. In addition, among the *BF2* haplotypes, B21 is a resistant trait, and B13 is a susceptible trait. Therefore, chickens with the *Mx*(A)/*BF2*(B21) genotype were selected as HPAIV-resistant Ri chickens, and chickens with the *Mx*(G)/*BF2*(B13) genotype were selected as HPAIV-susceptible Ri chickens. Twenty chickens from each line were randomly assigned to control and infection groups [12]. The A/duck/Vietnam/QB1207/2012 (H5N1) virus strain was harvested from the allantoic fluid of 10^4 egg infectious dose (EID50). The virus (200 μL) was administered through inoculation intranasally, and daily observations for disease and mortality were made at the Department of Biochemistry and Immunology of the National Institute of Veterinary Research.

Tissue collection and RNA isolation

At day 3 post infection, tracheal samples were collected following the procedure described in the World Health Organization manual on animal influenza diagnosis and surveillance. The tracheal samples were stored in liquid nitrogen, homogenized, transferred to the laboratory, and stored at −80°C. Total tracheal RNA of both groups was isolated using TRIzol reagent (Thermo Fisher Scientific, USA) as indicated in the manufacturer's instructions.

RNA preparation and sequencing

All RNA samples were used for library preparation using the MGIEasy RNA Directional Library Prep Kit (MGI Tech., China). RNA quality control and RNA sequencing were performed by LAS Inc. (Korea) on the Illumina MGISEQ platform (Illumina Inc., USA). RNA quality was measured by the absorbance at 260, 280, and 230 nm (LAS Inc.).

RNA-seq data analysis

The analysis required initial checks on sequence quality using the read filtering tool FastQC (v0.11.5) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapters and raw quality reads were trimmed from next-generation sequencing raw data using the Skewer program (version 0.2.2.). After the trimming process, cleaned high-quality reads were mapped to the reference genome (chicken gg6) obtained from the University of California Santa Cruz database (https://genome.ucsc.edu) using STAR software version 2.6. (https://github.com/alexdobin/STAR). Consensus sequences were obtained from quality-trimmed reads mapped to the reference sequences.

GSEA

A gene set of the expressed genes was analyzed through GSEA using GSEA v4.1.0 software (http://software.broadinstitute.org/gsea/index.jsp) based on Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis data. GSEA ranked all expressed genes based on the significance of differential gene expression between the resistant and susceptible groups. The enrichment score (ES) of each gene set was measured using both groups' ranked gene lists and was used for the enrichment plot. The normalized ES (NES) was determined for each gene set. The core enriched genes of pathways were indicated as heatmaps. We quantify enrichment magnitude and statistical significance based on $|NES| > 1$, nominal p value ≤ 0.05, and false discovery rate (FDR) ≤ 0.25.

Protein-protein interaction (PPI) network construction

The immune-related pathway used in the PPI analysis was determined using the GSEA data. Core genes were determined based on the core enhancement of the genes shown in the selected pathway. To determine the relationship between core genes, the Search Tool for Retrieval of Interacting Genes (STRING) database (http://www.string-db.org/) was used to obtain the PPI network.

Validation of RNA-seq by reverse transcription followed by quantitative polymerase chain reaction (RT-qPCR)

The synthesis of complementary DNA (cDNA) from the total RNA of Ri chickens were conducted using a RevertAid First Strand cDNA Synthesis Kit (Invitrogen, USA). The primers design was performed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (**Supplementary Table 1**). qPCR was carried out using qPCR 2x PreMIX (SYBR Green with low ROX; Dyne Bio INC, Korea) based on the manufacturer's instructions, using a CFX connect real-time PCR (Bio-Rad, USA). For 20 μL of reaction mix, 10.0 μL 2× SYBR Green

Master Mix, 1.0 μL of 10 μM forward and reverse primer mix, 7.0 μL RNase-free ddH2O, and 1.0 μL (100 ng) of template cDNA was combined. The thermal incubation steps were 95°C for 5 min followed by 40 cycles of 95°C for 30 sec, annealing at 55°C to 60°C for 30 sec, and extension at 72°C for 30 sec. Each experiment was performed in triplicate. The housekeeping gene *GAPDH* was used to normalize cDNA quantity.

Statistical analysis

Statistical analysis was performed using IBM SPSS ver. 26 (IBM, USA). Data are presented as means ± standard error of the mean of values obtained from three independent experiments per group (n = 3). Statistical comparisons were performed using Student's *t*-test for two-group comparisons in triplicate. Differences with $p < 0.05$ were considered significant.

RESULTS

RNA sequencing analysis

The experimental chickens had tracheal hemorrhage and ruffled feathers after the HPAIV/H5N1 infection. All chickens in the experiment for gene expression evaluation were sacrificed. The tracheal H5N1-infected samples were subjected to quality control followed by high-throughput sequencing. We found that there was a significant difference in gene expression levels between the two chicken groups at day 3 post-infection. Three samples from each group were selected: R1D3I, R2D3I, and R3D3I from the resistant chicken infection group; S1D3I, S2D3I, and S3D3I from the susceptible chicken infection group (**Supplementary Table 2**).

The statistics of the raw and clean reads of individual transcriptomes are shown in **Supplementary Table 2**. There were cDNA sequencing data of six libraries between 5.7 GB and 9.5 GB per chicken. After sequence data filtering, 18.9–31.6 million clean reads per H5N1-infected chicken sample were obtained.

Supplementary Table 3 shows the number of mapped reads, percentages, and transcripts. More than 84.3% of filtered reads from each library were mapped to the chicken reference genome.

KEGG pathways analyzed by GSEA

The immune-related KEGG pathway enrichment analysis was performed by GSEA. Only the gene sets with $p < 0.05$ and FDR < 0.25 were considered significantly enriched [3]. The immune-related pathways that were found to be significantly enriched are shown in **Table 1**.

Table 1. The list of KEGG immune-related pathway enrichments analyzed by gene set enrichment analysis

KEGG pathway	Size	ES	NES value	NOM *p*-value	FDR *q*-value	Core enrichment genes
Toll-like receptor signaling pathway	70	−0.474	−1.775	0.000	0.119	FOS, CCL4, IRF5, IFNAR1, STAT1, CASP18, IFNW1, PIK3CA, PIK3CD, MAP2K3, NFKBIA, IL8L1, MAPK9, TLR5, SPP1, IL1B, MAP2K2, MAPK12, MAP3K8, IL6, MAP2K1, IL12A, IKBKE, TLR3, CD86, TLR1B, TLR4, TRAF3, TLR2B, RAC1, TLR1A, LY96, MAP2K4, IRF7, TIRAP, CCL5, MAPK13, MAPK11, JUN, TICAM1, CD40, TAB1, MAPK14, MAP3K7, TAB2, RIPK1, IFNAR2
Herpes simplex virus I infection	114	−0.406	−1.650	0.003	0.010	IKBKE, TLR3, B2M, CYCS, CASP9, SRC, TRAF3, TLR2B, EIF2AK2, IFNGR1, BID, TRADD, JAK1, EIF2AK1, FASLG, TMEM173, IRF7, STAT2, TAPBP, MAVS, CCL5, TICAM1, CD74, TAB1, C3, MAP3K7, SRSF7, PPP1CA, TAB2, BLB2, SRSF5, IFNAR2, DMB2
NOD-like receptor signaling pathway	101	−0.407	−1.642	0.001	0.088	JAK1, TMEM173, TANK, IRF7, STAT2, MAVS, CCL5, MAPK13, GBP, ATG5, MAPK11, TAB3, JUN, TICAM1, CYBA, TAB1, MAPK14, MAP3K7, RIPK2, TAB2, RIPK1, NFKBIB, IFNAR2
RIG-I-like receptor signaling pathway	46	−0.412	−1.423	0.050	0.221	CYLD, TRADD, TMEM173, TANK, IRF7, MAVS, MAPK13, ATG5, MAPK11, MAPK14, MAP3K7, RIPK1, NFKBIB

KEGG, Kyoto Encyclopedia of Genes and Genomes; ES, enrichment score; NES, normalized enrichment score; FDR, false discovery rate; NOM, nominal; NOD, nucleotide oligomerization domain; RIG-I, retinoic acid-induced gene I.

Four categories of immune-related pathways were identified after KEGG pathway enrichment analysis: Toll-like receptor (TLR) signaling pathway (NES, –1.77; FDR *q*-value, 0.119), Herpes simplex virus I infection (NES, –1.65; FDR *q*-value, 0.010), nucleotide oligomerization domain (NOD)-like receptor signaling pathway (NES, –1.64; FDR *q*-value, 0.088), and retinoic acid-induced gene I (RIG-I)-like receptor signaling pathway (NES, –1.42; FDR *q*-value, 0.221) (**Fig. 1**). All genes from the four immune-related pathways were analyzed and filtered by core

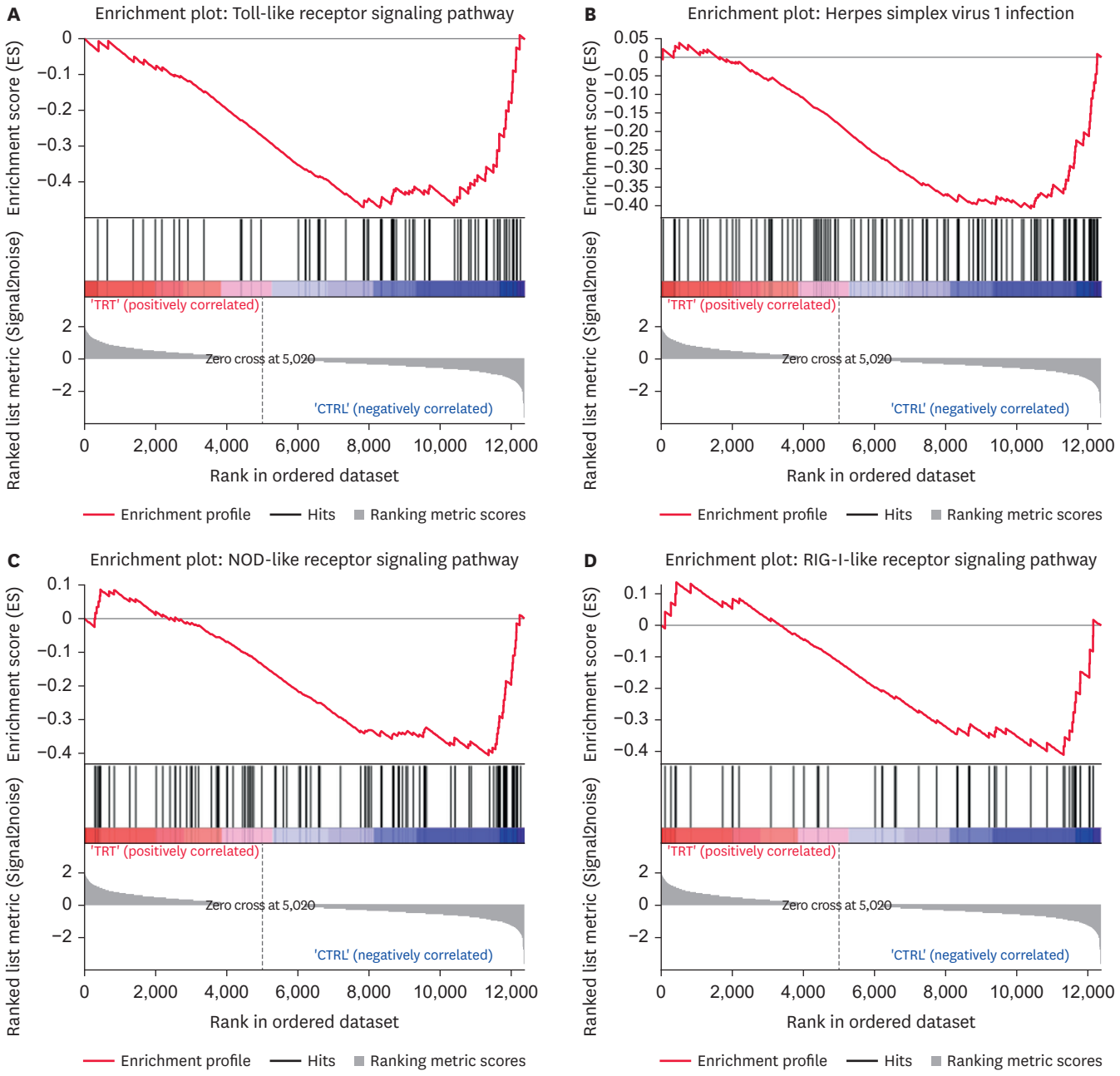

Fig. 1. Enrichment plot for four immune-related pathways in H5N1 virus-infected susceptible and resistant Ri chickens detected by GSEA. (A) Toll-like receptor signaling pathway. (B) Herpes simplex virus I infection. (C) NOD-like receptor signaling pathway. (D) RIG-I-like receptor signaling pathway. Bottom, the plot of the ranked list of all genes. Y-axis, the value of the ranking metric; X-axis, the rank for all genes. Middle, the location of genes from the immune-related pathway within the ranked list. Top, the running enrichment score for the gene set as the analysis walks along with the ranked list. The score at the peak of the plot is the ES for this gene set, and the genes that appear before or at the peak are defined as core enrichment genes for this gene set.
GSEA, gene set enrichment analysis; NOD, nucleotide oligomerization domain; RIG-I, retinoic acid-induced gene I; ES, enrichment score.

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enrichment, providing the leading-edge subset within the gene set (**Supplementary Table 4**). The number of overlapping DEGs in the four immune-related pathways is shown in **Fig. 2**. All 24 common genes that contributed to the core enrichment among more than two pathways are shown in the heat map with individual samples per group (**Fig. 3**). All these genes were important in H5N1 resistance. Notable among them were adaptor protein TIR-domain-containing adapter-inducing interferon (TRIF) *TICAM1*, stimulator of interferon genes (STING) *TMEM173*, Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway genes *JAK1* and *STAT2*; mitochondrial antiviral-signaling protein (MAVS); and interferon regulatory factor 7 (*IRF7*), jun proto-oncogene, AP-1 transcription factor subunit (*Jun*).

For PPI analysis, the STRING PPI database was used. The nodes indicate the different interacting partners, and the edges represent the nature of the interaction. The network contained 24 nodes with 57 edges (vs. 3 expected edges), a clustering coefficient of 0.621, and an enrichment *p* value of less than 1.0e–16. The confidence score threshold was set at 0.7 (high) for analysis (**Fig. 4**). The results showed that almost all genes were connected except NFKB inhibitor interacting Ras-like 2 gene.

Transcriptional mRNA expression comparison by RT-qPCR

To verify the expression profiles of genes identified by RNA-Seq, the expression levels of 10 genes overlapping in two or more immune-related pathways were confirmed by RT-qPCR (**Fig. 5**). The expression levels of *IRF7*, receptor-interacting serine/threonine-protein kinase 1 (*RIPK1*), an inhibitor of nuclear factor kappa B kinase subunit epsilon (*IKBKE*), *Jun*, TGF-beta activated kinase 1 (*MAP3K7*) binding protein (TAB1 and *TAB3*), *JAK1*, *STAT2*, and *TLR3* were significantly upregulated in HPAIV-resistant chickens compared with HPAIV-susceptible chickens (**Fig. 5**). The relative expression levels verified by RT-qPCR were consistent with

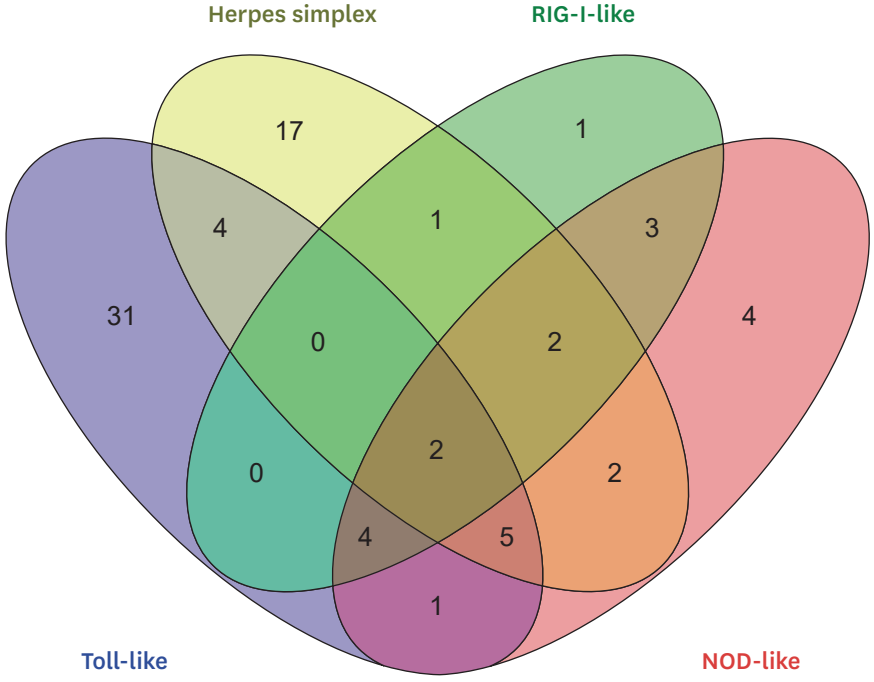

Fig. 2. The Venn diagram plots of gene distribution of immune-related Kyoto Encyclopedia of Genes and Genomes pathways enriched between two chicken groups.
NOD, nucleotide oligomerization domain; RIG-I, retinoic acid-induced gene I.

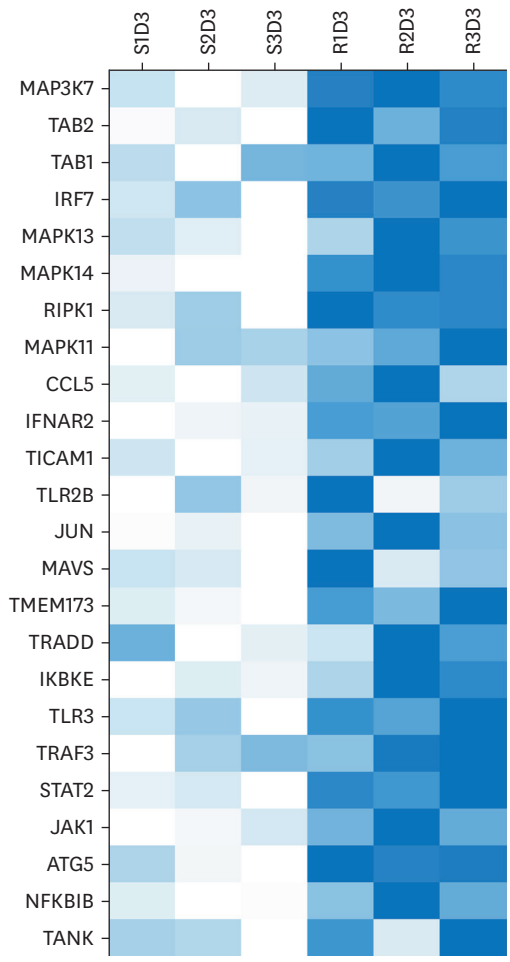

Fig. 3. Heat map of DEGs ranked by gene set enrichment analysis. White indicates DEGs with lower expression levels; blue indicates DEGs with higher expression levels, which were obtained from the expression values in reading count units after RNA-seq.
DEG, differentially expressed gene.

the RNA-seq data, and there were clear differences in the expression levels of these genes between the two HPAIV-infected lines (**Fig. 6**).

DISCUSSION

The present research studied the transcriptome profiles of HPAIV H5N1-infected tracheal tissues from Ri chickens using RNA sequencing. Ri chickens resistant or susceptible to HPAIV H5N1 were distinguished by genotyping *Mx*(A/G) and *BF2*(B21/B13) genes. Four immune-related pathways were found to be significantly expressed by GSEA.

In TLR signaling pathway, Herpes simplex virus I, NOD-like receptor signaling pathway, and RIG-I-like receptor signaling pathway, a total of 78 DEGs were identified (**Supplementary Table 4**). Avian influenza viral pathogen-associated molecular patterns are specifically recognized by host pattern recognition receptors (PRRs). Our results indicated that the expression levels of *TLR3*, TRIF (*TCAM1*), MAVS, STING (*TMEM173*), *IRF7*, AP-1 (*FOS* and *JUN*), and nuclear factor kappa B (NF-κB) pathway-related genes (*NFKBIA*, *NFKBIB*, and *IKBKE*)

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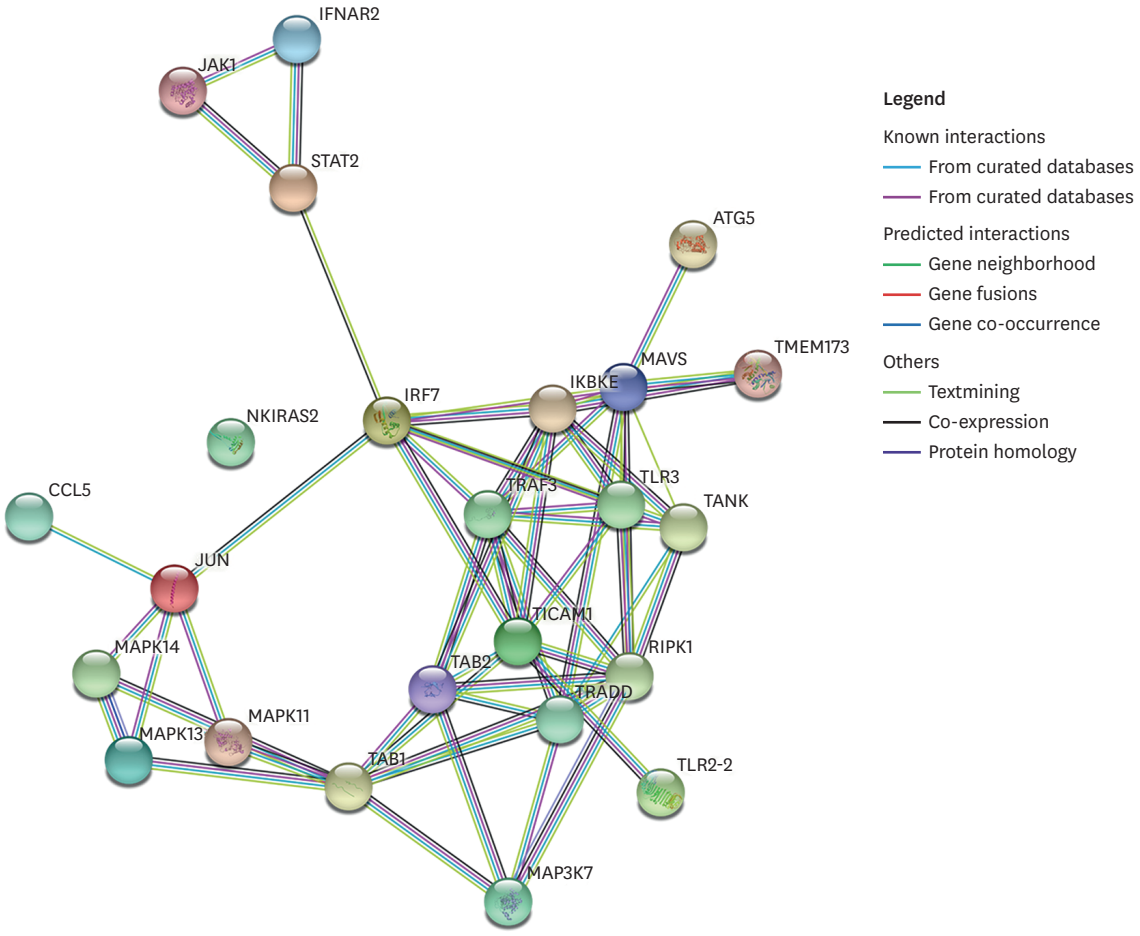

Fig. 4. The interact analysis of the 24 genes common between two or more Kyoto Encyclopedia of Genes and Genomes immune-related pathways, which were determined by gene set enrichment analysis in tracheal tissues of Ri chicken lines. This interaction analysis was obtained by the STRING version 11.0 (http://string-db.org/).

differed between the two chicken lines after H5N1 infection. Three distinct classes of PRRs are the TLRs, NOD-like receptors, and RIG-I-like receptors [13]. *TLR3* recognizes avian influenza double-stranded RNA through adaptor protein TRIF [14]. These adaptors activate the transcription factor *IRF7* and NF-κB pathways by MAVS [15]. MAVS, also in coordination with the STING, activates *IRF7*. Once activated, IRF7, NF-κB, and AP-1 are localized to the nucleus and induce interferon production in host cells.

PRR-mediated signaling activates antigen-presenting cells thereby regulating the expression of MHC class I and II molecules. So, co-stimulatory molecules (such as CD80 and CD86) and pro-inflammatory cytokines (type I interferons, *IL-1β*, *IL-6*, and *IL-12*) are observed [16]. This process leads to B cell activation and antibody production through co-stimulatory ligands (*CD40*) [17]. Our GSEA results indicated that the expression levels of *CD80*, *CD86*, *CD40*, *DMB2* (MHC class II), *BLB2* (MHC class II), and *β2m* (MHC class I) genes were highly upregulated in resistant chickens compared to susceptible chickens after H5N1 infection. These results suggested that the adaptive immune activity through the MHC class and their co-stimulatory molecules to B cells after H5N1 infection in resistant chickens was higher than that in susceptible chickens.

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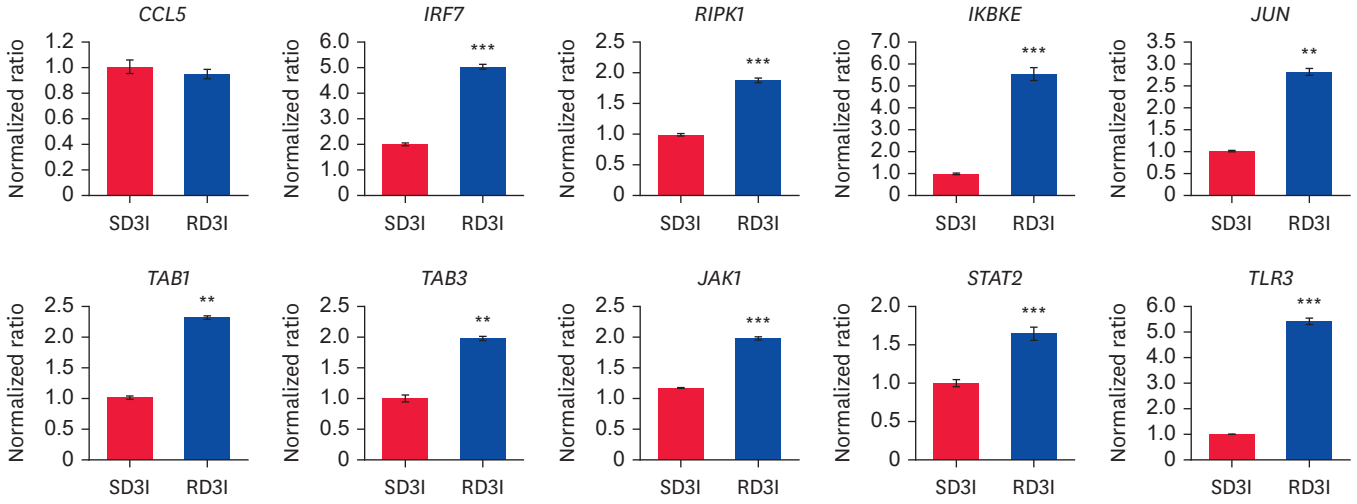

Fig. 5. RT-qPCR analysis of the expression levels of genes involved in four immune-related signaling pathways in two chicken groups after HPAIV/H5N1 infection. Relative quantitation data of RT-qPCR are performed as the mean ± SEM, normalized to GAPDH using the $2^{-\Delta\Delta Ct}$ method. Data are represented as the mean ± SEM of three independent experiments.
RT-qPCR, reverse transcription followed by quantitative polymerase chain reaction; HPAIV, highly pathogenic avian influenza virus; SEM, standard error of the mean.
$^{**}p < 0.01$; $^{***}p < 0.001$.

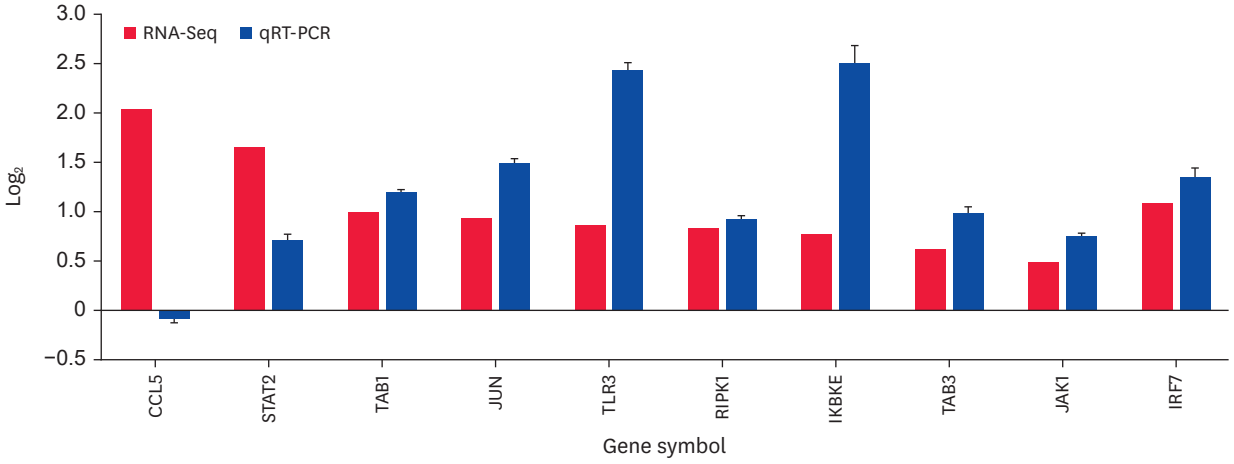

Fig. 6. Comparison of gene expression patterns revealed by RNA-seq and RT-qPCR data. Log_2 fold-changes are expressed as the ratio of gene expression levels after normalization to GAPDH.
FC, fold change.

Type I interferons, pro-inflammatory cytokines, and interferon stimulated genes are induced by *IRF7* and *NF-κB* [18]. Type I interferons lead to the expression of interferon stimulated genes by the Jak-STAT signaling cascade [19]. Interferons and interferon stimulated genes can inhibit viral replication by blocking viral entry into the host cells, binding to viral RNAs to prevent their translation, and regulating host antiviral responses [20]. Protein kinase R (EIF2AK2) represses the translation of viral mRNAs via the integrated stress response [21]. Our results indicated that the cytokines *IL-1β*, *IL-6*, *IL-8*, and *IL12A*; the chemokines *CCL4*, *CCL5*; interferons and their receptors *IFNAR1*, *IFNAR2*, and *IFNGR1*; Jak-STAT signaling pathway genes *STAT1*, *STAT2*, *JAK1*; interferon stimulated genes *EIF2AK2*, *EIF2AK1* were differentially expressed between the two chicken lines after H5N1 infection (**Supplementary Table 4**). These results suggest that, compared with susceptible chickens, resistant Ri chickens exhibit a higher antiviral response to HPAIV H5N1.

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In summary, we evaluated differentially expressed pathways in the tracheal samples of H5N1-infected resistant and susceptible Ri chickens by RNA sequencing and RT-qPCR. GSEA results indicated that the expression levels of PRRs, MHC class and their co-stimulatory molecules, cytokines, chemokines, interferons (type I and II), and interferon stimulated genes in H5N1-infected resistant chickens were higher than those in susceptible chickens. These findings indicate that HPAI-resistant Ri chickens exhibit elevated expression of antiviral immune-related genes as assessed by GSEA.

SUPPLEMENTARY MATERIALS

Supplementary Table 1

Primer sequences used for quantitative real-time polymerase chain reaction

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Supplementary Table 2

Summary of raw and clean reads of trachea tissue samples obtained from the resistant and susceptible chicken after 3 dpi highly pathogenic avian influenza virus H5N1 infection

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Supplementary Table 3

Sequence alignment of the tracheal tissue samples after 3 dpi highly pathogenic avian influenza virus H5N1

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Supplementary Table 4

The expression-fold changes and *p* values of 78 genes in the gene set enrichment analysis differentially expressed pathway were shown

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