Transcriptome Analysis of Thymus Tissues from Chinese Partridge Shank Chickens Before/After NDV LaSota Vaccine Injection Via High-Throughput RNA Sequencing

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Abstract

Background: Newcastle disease (ND), caused by virulent Newcastle disease virus (NDV), is a huge threat for poultry and birds. LaSota strain of NDV is a common live attenuated vaccine to control ND. In this study, high-throughput RNA sequencing was performed to explore thymus tissue transcriptome change and better manage “vaccine failure” in Chinese Partridge Shank Chickens at 0 h and 48 h post LaSota vaccine injection.

Results: 140 long non-coding RNAs (lncRNAs), 8 microRNAs (miRNAs) and 1514 mRNAs were differentially expressed post LaSota NDV vaccine inoculation. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis for vaccine-affected mRNAs and miRNA target genes was conducted. Moreover, 70 innate immune genes (31 up-regulated, 39 down-regulated) in GO, InnateDB and Reactome Pathway databases were found to be differentially expressed. Furthermore, the interaction network of proteins encoded by these dysregulated innate immune genes with known names was established by STRING website. Additionally, expression patterns of some dysregulated lncRNAs, miRNAs and mRNAs were further tested through RT-qPCR assay. Pearson correlation analysis for dysregulated transcripts validated by RT-qPCR assay was also performed. Based on the correlation data, 2 potential competing endogenous RNA (ceRNA) networks (ENSGALT00000060887/gga-miR-6575-5p/paraoxonase 1 (PON1) or MSTRG.188121.10/gga-miR-6631-5p/MMP9) were established.

Conclusions: Our data presented many differentially expressed lncRNAs, miRNAs, mRNAs and innate immune genes, and established 2 potential ceRNA networks (lncRNAs/miRNAs/mRNAs) in Chinese Partridge Shank Chickens in response to LaSota vaccine inoculation, which could deepen our understanding on host responses to NDV LaSota vaccine in Chinese Partridge Shank Chickens.

Background

Newcastle disease (ND), one of the leading devastating diseases in poultry and wild birds worldwide, has underwent several outbreaks and brought about enormous economic losses for poultry industry since its first official report ninety years ago (<https://www.oie.int/en/animal-health-in-the-world/animal-diseases/newcastle-disease/>) [1, 2]. Moreover, ND is a zoonosis (disease of animals that can also infect humans) that can give rise to mild conjunctivitis and influenza-like symptoms in human [2]. Newcastle Disease virus (NDV), a negative-sense single-stranded RNA virus, belongs to genus Avulavirus in the family of Paramyxoviridae. NDV strains can be categorized as lentogenic (mild), mesogenic (moderate), and velogenic (very virulent) pathotypes according to the difference of mean death time in chicken embryos [3]. Lentogenic NDV strains produce mild subclinical signs with negligible mortality, while velogenic strains can cause lethal haemorrhagic lesions, serious nervous and respiratory disorders, rapid transmission and high mortality in birds and poultry [3]. Once found, mesogenic and virulent NDV viruses need to immediately notify the Office of International Epizootics. Virulent strains of NDV are defined as the causative agent of ND by the World Health Organization for Animals (OIE). To date, more than 250 bird species especially gallinaceous birds (e.g. quail, chickens, and turkeys) have been reported with NDV infection [4–6].

Prophylactic vaccination is the main strategy to protect poultry from virulent NDV infection [7]. Currently, NDV vaccines can be mainly divided into 3 categories: live attenuated, inactivated and recombinant vaccines [8, 9]. Worldwide, live attenuated and inactivated NDV strains are extensively used as vaccines for the prevention of ND in poultry [6, 7]. Among these vaccination schemes, LaSota strain is the most frequently used live lentogenic vaccine formulation in many countries due to its outstanding immunogenicity [7, 8]. However, “vaccine failure” such as vaccines incomplete or improper immunization is a common issue [7]. Hence, it is imperative to have a deep insight into the molecular responses to NDV vaccines to better manage this problem.

Coding RNAs and non-coding RNAs including long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) have been emerged as crucial players in various biological process and host responses to disease and pathogens in animals including poultry [9–11]. Recently, high-throughput transcriptome sequencing (RNA-Seq) has attracted much attention from researchers due to its outstanding advantages such as little genomic sequence limitation and low background signal [12]. RNA-seq is a powerful technique that can simultaneously capture almost all coding and noncoding transcripts and decipher the information of transcriptome including lncRNAs, miRNAs, and mRNAs [13, 14]. RNA-seq not only can measure gene expression and discover novel RNAs especially non-coding RNAs, but also can identify differentially expressed genes under different conditions and investigate the host-pathogen interactions, which contributes to deepen our understanding on molecular pathology and help us to assess the changes associated with diseases [15, 16].

In this text, we took advantage of RNA-seq technology to have an in-depth exploration on transcriptome changes in response to NDV LaSota vaccine injection in Chinese Partridge Shank Chickens. Our data revealed that 140 long non-coding RNAs (lncRNAs), 8 microRNAs (miRNAs) and 1514 mRNAs were differentially expressed ($|\log_2\text{FoldChange}| > 1$, P value < 0.05) in thymus tissue samples of Chinese Partridge Shank chickens post LaSota NDV vaccine inoculation. Moreover, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis for vaccine-affected mRNAs and miRNA target genes was conducted. Additionally, expression patterns of 712 innate immune genes in InnateDB database and 319 innate immune genes in Reactome Pathway Database were measured in our experiments. Moreover, 70 innate immune genes (31 up-regulated, 39 down-regulated) were demonstrated to be differentially expressed. Also, expression patterns of some lncRNAs, miRNAs and mRNAs were further measured through RT-qPCR assay. Furthermore, 2 potential competing endogenous RNA (ceRNA) regulatory networks (lncRNAs/miRNAs/mRNAs) were established according to the co-expression data.

Methods

Sample collection

Specific pathogen-free (SPF) Chinese Partridge Shank Chickens were obtained from Beijing Merial Laboratory Animal Technology Co. Ltd (Beijing, China) and raised in the rooms at the biosafety level II facility. Chickens ($n=20$) were randomly divided into 2 groups (pre-inoculation and post-inoculation groups) with 10 chickens in each group. At the age of 30 days, the chickens in the post-inoculation group were inoculated with 0.2 ml of 10^5 50% egg-infectious dose (EID_{50}) Lasota suspension through eyes, respectively. Chickens were euthanized by the intravenous injection of pentobarbital sodium solution (90 mg/kg body weight) in each group. The thymus tissue samples were collected in RNase-free microtubes, immediately frozen in liquid nitrogen, and then stored at -80°C until RNA extraction.

Our study was approved by Animal Care and Use Committee of the Henan University of Animal Husbandry and Economy and was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Henan University of Animal Husbandry and Economy. All efforts were made to minimize suffering of birds.

Total RNA extraction, cDNA Library Construction and Sequencing

RNA was isolated from thymus tissue samples using Trizol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and then treated with a TURBO DNA-free™ Kit (Thermo Fisher Scientific) to remove DNA from RNA samples (Thermo Fisher Scientific). Next, the concentration and purity of RNA was measured using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and RNA quality was further tested using a RNA Nano 6000 Assay Kit (Agilent Technologies, Santa Clara, CA, USA) through Agilent Bioanalyzer 2100 (Agilent Technologies). After the removal of ribosomal RNA using the Ribo-Zero rRNA Removal Kit (Illumina), high-quality samples (RNA concentration ≥ 400 ng/ μl , OD_{260/280}: 1.8-2.2, RNA Integrity number ≥ 8) were used for the construction of following cDNA libraries of mRNAs and lncRNAs according to TruSeq RNA Sample Preparation Guide (Illumina, San Diego, CA, USA).

Briefly, mRNAs and lncRNAs were enriched using magnetic beads and then fragmented using divalent cations. Next, fragmented mRNAs were reversely transcribed into cDNAs, followed by the conversion of residual overhangs into blunt ends. Thereafter, Illumina PE adapter oligonucleotides were ligated with adenylated DNAs (3'ends). Subsequently, cDNA fragments were enriched by PCR amplification and purified through Agencourt AMPure XP System (Beckman Coulter, Beverly, CA, USA). Next, cDNA libraries were quantified by the Agilent high sensitivity DNA assay on Agilent Bioanalyzer 2100 (Agilent Technologies). Finally, signal strand cDNA libraries were sequenced on Illumina HiSeq 2500 instrument (Illumina) by Shanghai Personal Biotechnology Co. Ltd (Shanghai, China). Small RNA libraries were conducted through NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs, Ipswich, MA, USA) following the protocols of manufacturer. Briefly, RNA samples were ligated to RNA 3' and 5' adapters and then reverse-transcribed and amplified into cDNA libraries. After quantified and qualified by Agilent Bioanalyzer 2100 (Agilent Technologies), libraries were sequenced.

Raw data processing

The information of reference genome of *Gallus_gallus*.*Gallus_gallus*-5.0 (assembly GCA_000002315.3) established by Ensembl database (version 94.5) was shown in Table 1. Also, we provided the annotation information of *Gallus_gallus*.*Gallus_gallus*-5.0 (assembly GCA_000002315.3) genes in different databases including GO, KEGG and UniProtID databases (Table 2). The information of raw data was presented in Table 3. The information of clean data without reads containing 3' adaptors or average base quality < Q20 was provided in Table 4. After filtering, high-quality clean data were aligned to reference genome of *Gallus_gallus*.*Gallus_gallus*-5.0 (assembly GCA_000002315.3) using Tophat2. The alignment results were presented in Table 5.

Differential Expression analysis

Expression levels of lncRNAs and mRNAs were measured using the fragments per kilobase of transcript per million mapped reads (FPKM) method. Differentially expressed genes were selected using the condition of $|\log_2\text{FoldChange}| > 1$ and $P\text{value} < 0.05$.

Gene annotation

Gene ontology (GO) (<http://geneontology.org/>), Kyoto Encyclopedia of Genes and Genomes(KEGG) (<http://www.kegg.jp/>), UniProt Knowledgebase (<http://www.uniprot.org/help/uniprotkb/>), Enzyme Commission (EC) (<http://enzyme.expasy.org/>), Evolutionary Genealogy of Genes: Non-supervised Orthologous Groups (eggNOG) (http://eggnoG.embl.de/version_3.0/) databases were used to clarify gene function and discover vital regulatory pathways or networks. GO terms and KEGG pathways were regarded as significantly enriched when P value was less than 0.05.

Target prediction

The potential interaction between miRNAs and lncRNAs or mRNAs was predicted by miRanda software.

Reverse transcription-quantitative PCR (RT-qPCR)

RNA was extracted using Trizol Reagent (Thermo Fisher Scientific) and quantified using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) as described above. Then, RNA was reversely transcribed into cDNA first strands using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) and random primer (for mRNAs and lncRNAs) or stem-loop miRNA RT primers in Table 6. Next, cDNA was amplified using ITaq Universal SYBR Green Supermix (Bio-Rad) and specific primers in Table 7. Expression levels of lncRNAs, miRNAs and mRNAs were calculated using the formula of $2^{-\Delta\Delta CT}$ with GAPDH or 5S RNA as the internal control.

Statistics Analysis

Data was analyzed using GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA, USA). Student's *T* test was used to compare the difference between groups. Difference was regarded as statistically significant when *P* value was less than 0.05.

Results

Identification of differentially expressed lncRNAs

The effect of NDV vaccine on expression of 6491 lncRNAs was examined by RNA-seq in chicken thymus tissue samples. Results showed that expression levels of 140 lncRNAs (58 up-regulated and 82 down-regulated) ($|\log_2\text{FoldChange}| > 1$, *P* value<0.05) were significantly altered in thymus tissue samples of chickens after NDV vaccine treatment (Supplementary Table 1, Figure 1).

Identification of differentially expressed miRNAs and bioinformatics analysis of miRNA targets

Also, expression patterns of 837 miRNAs were measured in our project. Eight miRNAs (3 up-regulated, 5 down-regulated) ($|\log_2\text{FoldChange}| > 1$, *P* value<0.05) were found to be differentially expressed in thymus tissue samples isolated from chickens post vaccine treatment compared to control group (Supplementary Table 2, Figure 2A). In detail, expression of gga-miR-6631-5p, gga-miR-6575-5p and gga-miR-1727 was remarkably up-regulated and expression levels of gga-miR-1712-3p, gga-miR-12273-3p, gga-miR-6655-5p, gga-miR-1744-3p and gga-miR-6548-3p were notably down-regulated in thymus tissue samples of chickens post vaccine injection (Supplementary Table 2). It is well known that miRNAs can exert their function through regulating specific mRNAs. Hence, potential targets of differentially expressed miRNAs were examined by bioinformatics analysis using the miRanda software and the results were presented in Supplementary Table 3. To have a deep insight into the function of these miRNAs, GO and KEGG enrichment analysis for these miRNA target genes was carried out. The top 10 KEGG pathways enriched by these differentially expressed miRNA targets were Endocytosis (ko04144), Collecting duct acid secretion (ko04966), Epstein-Barr virus infection (ko05169), Viral carcinogenesis (ko05203), Peroxisome (ko04146), Plant-pathogen interaction (ko04626), Bacterial invasion of epithelial cells (ko05100), Longevity regulating pathway-multiple species (ko04213), Other types of O-glycan biosynthesis (ko00514), and Antigen processing and presentation (ko04612) (Supplementary Table 4 and Figure 2B). GO analysis for putative targets of differentially expressed miRNAs suggested that miRNA target genes were involved in the regulation of multiple biological processes, especially for cellular metabolic process (GO:0044237), metabolic process (GO:0008152), organic substance metabolic process (GO:0071704), primary metabolic process (GO:0044238), nitrogen compound metabolic process (GO:0006807), biosynthetic process (GO:0009058), organic substance biosynthetic process (GO:1901576), cellular biosynthetic process (GO:0044249), cellular nitrogen compound metabolic process (GO:0034641) and small molecule metabolic process (GO:0044281) (Top 10 GO terms in biological processes) (Supplementary Table 5, Sheet 1). Molecular function analysis in GO annotation showed that these miRNA target genes mainly exerted catalytic activity and binding activity with multiple molecules such as protein, enzyme, and nucleoside (Supplementary Table 5, Sheet 2).

Identification of differentially expressed mRNAs and related GO and KEGG enrichment analysis

Moreover, 1016 up-regulated mRNAs (Supplementary Table 3A, sheet 1) and 498 down-regulated mRNAs (Supplementary Table 3A, sheet 2) ($|\log_2\text{FoldChange}| > 1$, *P* value<0.05) were identified among 17131 mRNAs in thymus tissue samples of chickens at 48 h after NDV vaccine infection (Supplementary Table 6, Figure 3A). The top 10 up-regulated mRNAs with known symbols were cyclin dependent kinase 3(CDK3), thyrotropin releasing hormone receptor(TRHR), mesogenin 1 (MSGN1), chromosome 1 open reading frame 158 (C1ORF158), actin like 6B(ACTL6B), G protein subunit gamma transducin 2 (GNGT2), epidermal differentiation protein starting with MTF motif 4 (EDMTF4), myogenic factor 5(MYF5), avian beta-defensin 2 (AvBD2) and meiotic double-stranded break formation protein 4(ME14)(Supplementary Table 6,

sheet 2). The top 10 down-regulated mRNAs with known names were family with sequence similarity 181 member B(FAM181B), phosphatidylinositol glycan anchor biosynthesis class C (PIGC), ubiquitin like 4A (UBL4A), inka box actin regulator 1(INKA1), spermatogenesis associated 2 like (SPATA2L), NADH: ubiquinone oxidoreductase subunit B7 (NDUFB7), cyclin dependent kinase 2 associated protein 2 (CDK2AP2), adaptor related protein complex 5 subunit sigma 1(AP5S1), cytochrome c oxidase assembly factor COX14(COX14) and apolipoprotein L domain containing 1 (APOLD1) (Supplementary Table 3A, sheet 3).

Based on the outcomes of GO enrichment analysis, vaccine-influenced genes participated in the regulation of many biological processes, especially for transmembrane transport (GO:0055085), ion transport (GO:0006811), ion transmembrane transport (GO:0034220), inorganic ion transmembrane transport (GO:0098660), cation transport (GO:0006812), cation transmembrane transport (GO:0098655), calcium ion transmembrane transport (GO:0070588), calcium ion transport (GO:0006816), inorganic cation transmembrane transport (GO:0098662), metal ion transport (GO:0030001) (Top 10 terms in GO biological_process category) (Supplementary Table 3B, Sheet 2). Moreover, vaccine injection led to the abundant enrichment of genes belonging to cellular components such as nucleosome (GO:0000786), extracellular region (GO:0005576), DNA packaging complex (GO:0044815), integral component of plasma membrane (GO:0005887), intrinsic component of plasma membrane (GO:0031226), transporter complex (GO:1990351), transmembrane transporter complex (GO:1902495), protein-DNA complex (GO:0032993), plasma membrane part (GO:0044459) and ion channel complex (GO:0034702) (Top 10 terms in GO cellular_components category) (Supplementary Table 7, Sheet 3). Meanwhile, vaccine-affected genes were found to be involved in the regulation of molecular functions such as transmembrane transporter activity (GO:0022857), transporter activity (GO:0005215), inorganic molecular entity transmembrane transporter activity (GO:0015318), ion transmembrane transporter activity (GO:0015075), substrate-specific channel activity (GO:0022838), ion channel activity (GO:0005216), channel activity (GO:0015267), passive transmembrane transporter activity (GO:0022803), inorganic cation transmembrane transporter activity (GO:0022890), cation transmembrane transporter activity (GO:0008324) (Top 10 terms in GO molecular_function category) (Supplementary Table 3B, Sheet 4). KEGG analysis revealed that the injection of NDV vaccines led to differential expression of many genes, especially for those in Systemic lupus erythematosus (ko05322), Neuroactive ligand-receptor interaction (ko04080), Retrograde endocannabinoid signaling (ko04723), Staphylococcus aureus infection (ko05150), Complement and coagulation cascades (ko04610), Nicotine addiction (ko05033), Alcoholism (ko05034), Glutamatergic synapse (ko04724), GABAergic synapse (ko04727), and Asthma (ko05310) pathways (Top 10 KEGG pathway) (Supplementary Table 8, Figure 3B).

Screening and analysis of vaccine-influenced innate immune genes

Furthermore, differentially expressed innate immune genes were presented in Sheet 1 of Supplementary Table 4A. The information of these genes was integrated and shown in Sheet 3 of Supplementary Table 9. The innate immune genes with known names were transmembrane protein 173 (TMEM173), RNA binding motif protein 14 (RBM14), complement factor D (CFD), solute carrier family 11 member 1(SLC11A1), mannan binding lectin serine peptidase 2(MASP2), cytochrome b-245 alpha chain (CYBA), apolipoprotein A4 (APOA4), macrophage migration inhibitory factor (glycosylation-inhibiting factor) (MIF), retinoic acid receptor responder (tazarotene induced) 2 (RARRES2), collectin subfamily member 11 (COLEC11), tripartite motif containing 62 (TRIM62), otopetrin 1 (OTOP1), triokinase and FMN cyclase (TKFC) and serpin family G member 1 (SERPING1) (Supplementary Table 9, Sheet 2).

In view of conservation of innate immune responses among different organisms, we screened out more innate-immunity-related genes in InnateDB and Reactome Pathway databases. In this text, expression patterns of 712 innate immune genes in the InnateDB database (<http://innatedb.sahmri.com/annotatedGenes.do?type=innatedb>, Supplementary Table 10) were measured(Supplementary Table 4C sheet 1). Moreover, 50 innate immune genes(19 up-regulated, 31 down-regulated) were found to be differentially expressed in experimental group vs control group (Supplementary Table 11 sheet 2). Moreover, expression changes of 319 innate immune genes in the Reactome Pathway Database

(<http://reactome.ncpsb.org/PathwayBrowser/#/R-GGA-168249&PATH=R-GGA-168256&DTAB=MT>, Supplementary Table 12) were examined in our project (Supplementary Table 4E sheet 1). Among these genes, 15 innate immune genes (11 up-regulated, 4 down-regulated) were differentially expressed in experimental group vs control group (Supplementary Table 13 sheet 2). Finally, the information of 70 innate immune genes annotated by GO, Reactome pathway, and InnateDB databases (31 up-regulated, 39 down-regulated) was integrated into Supplementary Table 14.

Establishment of interaction networks among proteins encoded by these filtered innate immune genes

Next, the interactions among proteins encoded by filtered innate immune genes were analyzed through STRING: functional protein association networks (<https://string-db.org/cgi/network.pl?taskId=s7sL6l9NyZIM>). The image of interaction network was shown in Figure 4. The interaction edges with the combined score ≥ 0.4 were presented in sheet 1 of Supplementary Table 5A and related annotation information was displayed in sheet 2 of Supplementary Table 15. Moreover, KEGG function annotation analysis revealed that cytokine-cytokine receptor interaction pathway (matching proteins: Interleukin-10 (IL10), Interleukin-22 (IL22), Interleukin-5 (IL5), Interleukin-9 (IL9), prolactin (PRL), phagosome pathway (matching proteins: ATPase H⁺ transporting V0 subunit e2 (ATP6V0E2), collectin subfamily member 11 (COLEC11), cytochrome b-245 alpha chain (CYBA), nitric oxide synthase 1 (NOS1)) and Retinoic acid-inducible gene I (RIG-I)-like receptor signaling pathway (matching proteins: triokinase and FMN cyclase (TKFC/DAK), mitogen-activated protein kinase 10 (MAPK10), TMEM173 were significantly enriched by these screened innate immune genes (Supplementary Table 16 sheet 1). In addition, these innate immune genes led to the significant enrichment of 3 Cellular Component terms (Supplementary Table 16 sheet 2) and 41 Biological Process terms (Supplementary Table 16 sheet 3) in GO analysis. In addition, GO analysis for these dysregulated innate immune genes showed that CCAAT enhancer binding protein beta (CEBPB) and IL10 were involved in the negative regulation of neuron death.

RT-qPCR validation of some differentially expressed lncRNAs, miRNAs and mRNAs

Next, RT-qPCR validated that expression levels of MSTRG.22689.1, ENSGALT00000065826, ENSGALT00000059336 and ENSGALT00000060887 were notably up-regulated and MSTRG.188121.10 expression was remarkably down-regulated in thymus tissue samples of 10 random chickens at 48 h post NDV challenge compared to pre-challenge group (Figure 5A). Also, we demonstrated that gga-miR-6575-5p, gga-miR-6631-5p and gga-miR-1727 were highly expressed, while gga-miR-6655-5p and gga-miR-6548-3p were low expressed in thymus tissue samples of 10 random chickens at 48 h after NDV vaccine inoculation relative to pre-inoculation group (Figure 5B). In addition, RT-qPCR results substantiated that paraoxonase 1 (PON1), mitogen-activated protein kinase 10 (MAPK10) and cystic fibrosis transmembrane conductance regulator (CFTR) were highly expressed and matrix metalloproteinase 9 (MMP9) were low expressed in thymus tissue samples of 10 random chickens post NDV vaccine injection relative to pre-injection group (Figure 5C). However, there was no conspicuous difference in the expression of gga-miR-1744-3p (Figure 5B), gga-miR-1712-3p (Figure 5B), E2F transcription factor 1 (E2F1) (Figure 5C) and nuclear factor kappa B subunit 2 (NF- κ B2) (Figure 5C) between pre-inoculation and post-inoculation groups.

Co-expression analysis of dysregulated lncRNAs, miRNAs and mRNAs

Next, Pearson correlation analysis revealed that gga-miR-6631-5p expression was negatively associated with the expression of ENSGALT00000065826 or MSTRG.188121.10 in thymus tissue samples of chickens after NDV vaccine injection (Figure 6A). Moreover, there was a negative correlation between gga-miR-6575-5p expression and ENSGALT00000060887 or MSTRG.188121.10 expression in thymus tissue samples of 10 random chickens post NDV vaccine challenge (Figure 6A). In addition, MSTRG.188121.10 expression was positively related with MMP9 expression, but was inversely correlated with CFTR expression in thymus tissue samples of chickens following NDV vaccine treatment (Figure 6B). ENSGALT00000060887 expression was found to be positively associated with PON1 or MMP9 expression in thymus tissue samples of chickens after NDV vaccine administration (Figure 6B). Next, the correlation of mRNAs and

miRNAs with shared related lncRNAs was further analyzed. Results showed that there was a negative correlation between gga-miR-6631-5p and MMP9 expression in thymus tissue samples of chickens post NDV vaccine administration (Figure 6C). Also, gga-miR-6575-5p expression was found to be inversely related with PON1 expression in thymus tissue samples of chickens post NDV vaccine challenge (Figure 6C). Based on the co-expression data of ENSGALT00000060887, gga-miR-6575-5p and PON1, we supposed that ENSGALT00000060887 might function as a molecular sponge of gga-miR-6575-5p to sequester gga-miR-6575-5p from PON1 in chickens post NDV vaccine injection. MSTRG.188121.10 might regulate MMP9 expression through acting as a competing endogenous RNA (ceRNA) of gga-miR-6631-5p in chickens post NDV vaccine injection.

Discussion

Vaccination has been well documented as an effective preventive strategy to protect animals from infectious diseases by stimulating the immune system [17, 18]. To better utilize vaccines, it is requisite to gain a comprehensive knowledge and understanding on all possible vaccination effects (e.g. mode of action, risks, advantages, and disadvantages). In this study, transcriptome analysis by RNA-seq technology was performed to more deeply investigate the cellular and molecular responses to LaSota NDV vaccine in Chinese Partridge Shank Chickens.

In this text, expression patterns of 6491 lncRNAs, 17131 mRNAs and 837 miRNAs were examined in thymus tissue samples of Chinese Partridge Shank Chickens before/after LaSota vaccine injection by RNA-seq and miRNA-seq. Among these transcripts, expression of 58 lncRNAs, 3 miRNAs and 1016 mRNAs were notably up-regulated and expression of 82 lncRNAs, 5 miRNAs and 498 mRNAs was markedly down-regulated in thymus tissue samples of Chinese Partridge Shank Chickens post LaSota NDV vaccine inoculation compared with pre-inoculation group. Moreover, GO and KEGG analysis for vaccine-affected mRNAs and putative target genes of dysregulated miRNAs was conducted in our project, which was presented in Supplementary Table 2B-2D, Supplementary Table 3B and Supplementary Table 3C.

Live vaccines are known for their stronger protective effects by virtue of their capacity to efficiently induce a series of robust immunity responses [7, 8, 19]. In view of the conversation of innate immune responses among different organisms [20], more innate immune genes were picked out through GO, InnateDB and Reactome Pathway analysis. Combined with the expression data of mRNAs, we discovered that expression patterns of 712 innate immune genes in InnateDB database and 319 innate immune genes in Reactome Pathway Database were measured by RNA-seq. Moreover, 70 innate immune genes (31 up-regulated, 39 down-regulated) annotated by GO, InnateDB and Reactome Pathway databases were demonstrated to be differentially expressed in post-vaccine group vs pre-vaccine group. Furthermore, the interaction network of these dysregulated innate immune genes with known names was established using STRING website. KEGG enrichment analysis revealed that cytokine-cytokine receptor interaction pathway (matching proteins: IL10, IL22, IL5, IL9 and PRL), phagosome pathway (matching proteins: ATP6V0E2, COLEC11, CYBA and NOS1) and RIG-I-like receptor signaling pathway (matching proteins: DAK, MAPK10 and TMEM173) were significantly enriched by these filtered innate immune proteins. RIG-I receptor has been found to be involved in the early response against NDV [21]. In addition, GO analysis for these dysregulated innate immune genes showed that CEBPB and IL10 were involved in the negative regulation of neuron death. Moreover, previous studies demonstrated that TMEM173 can induce pro-inflammatory and innate immune responses against infection and cancer progression [22, 23]. TMEM173 potentiated spinal cord injury by eliciting pro-inflammatory responses via activating NF- κ B and mitogen activated protein kinase pathway in lipopolysaccharide-incubated mouse microglia [24]. These data suggested that CEBPB, IL10, and TMEM173 were closely associated with innate immune, inflammation and neurological damage in chickens post LaSota vaccine infection. Complement system is a vital part in host innate immune response [20, 25, 26]. In this text, complement cascade-related genes such as C1QB, C6, C8A, CFI, and MASP2 were also identified by Reactome pathway analysis (Supplementary Table 5B, sheet 4). Previous studies showed that PON1 functioned as a crucial player in the innate immune response to pathogens [27, 28]. Moreover, Aharoni *et al.* demonstrated that PON1 inhibited macrophage pro-inflammatory responses in

J774.A1 macrophage-like cell line and mouse bone marrow-derived macrophages [29]. E2F1 [30, 31], CFTR[32, 33], MAPK10[34], MMP9[35, 36] and NF- κ B2 [37, 38]also have been reported to be involved in the regulation of innate immune responses. In addition, GO, InnateDB and Reactome Pathway annotation analysis revealed that E2F1, CFTR, MAPK10, MMP9 and NF- κ B2 were innate immune genes. Hence, expression patterns of these differentially expressed genes, miRNAs (gga-miR-6631-5p, gga-miR-6655-5p, gga-miR-6575-5p, gga-miR-6548-3p, gga-miR-1744-3p, gga-miR-1727 and gga-miR-1712-3p) and lncRNAs (MSTRG.188121.10, ENSGALT00000065826, ENSGALT00000059336, ENSGALT00000060887 and MSTRG.22689.1) were further examined through RT-qPCR assay. In line with RNA-seq data, RT-qPCR assay further validated that lncRNAs (MSTRG.22689.1, ENSGALT00000065826, ENSGALT00000059336 and ENSGALT00000060887), miRNAs (gga-miR-6575-5p, gga-miR-6631-5p and gga-miR-1727) and genes (PON1, MAPK10, and CFTR) were highly expressed and MSTRG.188121.10, gga-miR-6655-5p, gga-miR-6548-3p andMMP9 were low expressed in thymus tissue samples of 10 random chickens post NDV challenge relative to pre-challenge group. However, no notable difference in expression of gga-miR-1744-3p, gga-miR-1712-3p, E2F1 and NF- κ B2 was observed in thymus tissue samples of chickens before or after NDV vaccine injection.

Recently, ceRNA hypothesis has attracted much attention from researchers in elucidating the molecular basis of non-coding RNAs including lncRNAs [39]. ceRNA hypothesis proposes that lncRNAs can perform as the molecular sponge of miRNAs to attenuate the inhibitory effect of miRNAs on target genes [40, 41]. Hence, co-expression patterns of these dysregulated lncRNAs, miRNAs and mRNAs validated by RT-qPCR assay were explored in our project. Pearson correlation analysis presented that gga-miR-6631-5p expression was negatively associated with the expression of ENSGALT00000065826 (lncRNA), MSTRG.188121.10 (lncRNA), or MMP9 in thymus tissue samples of chickens after NDV vaccine injection. Moreover, MSTRG.188121.10 expression was positively related with MMP9 expression in post-challenge group. These data suggested that MSTRG.188121.10 might act as a ceRNA of gga-miR-6631-5p to regulate MMP9 expression in chickens post NDV vaccine inoculation and there existed a possible link between ENSGALT00000065826 and gga-miR-6631-5p. Also, an inverse correlation between MSTRG.188121.10 and CFTR expression was found in post-inoculation group, suggesting the close link of MSTRG.188121.10 and CFTR. In addition, gga-miR-6575-5p expression was inversely correlated with ENSGALT00000060887 (lncRNA), MSTRG.188121.10 (lncRNA) or PON1 expression in thymus tissue samples of chickens post NDV vaccine challenge. ENSGALT00000060887 expression was positively associated with PON1 expression in post-inoculation group. These outcomes suggested that ENSGALT00000060887 could regulate PON1 expression by functioning as a molecular sponge of gga-miR-6575-5p and there was a possible negative regulatory relationship between MSTRG.188121.10 and gga-miR-6575-5p. Furthermore, ENSGALT00000060887 expression was positively associated with MMP9 expression in post-challenge group, suggesting the relevance of ENSGALT00000060887 and MMP9 in chickens inoculated with NDV vaccine.

Although previous studies have performed the transcriptome analysis to explore host immune responses against NDV LaSota vaccine infection in embryos[42], spleen [43], trachea [44]from Leghorn and Fayoumi chickens and chick embryo fibroblasts [45], these documents focused on the investigation of differentiated expressed genes including some innate immune genes. To our knowledge, the relationships among lncRNAs, miRNA and mRNAs have not been examined by transcriptome analysis in thymus tissue samples of Chinese Partridge Shank Chickens after LaSota vaccine injection. In this study, 140 lncRNAs, 8 miRNAs and 1514 mRNAs were found to be differentially expressed in thymus tissue samples of Chinese Partridge Shank chickens post LaSota NDV vaccine inoculation. Moreover, GO and KEGG analysis for differentiated expressed mRNAs and target genes of dysregulated miRNAs was conducted in our project. Furthermore, 70 innate immune genes in GO, InnateDB and Reactome Pathway databases were found to be differentially expressed in post-challenge group versus pre-challenge group. Moreover, 2 ceRNA networks (ENSGALT00000060887/gga-miR-6575-5p/PON1 and MSTRG.188121.10/gga-miR-6631-5p/MMP9) were established based on the co-expression data. In addition, some possible regulatory networks of lncRNAs, miRNA and mRNAs were estimated based on the co-expression data. Due to the diversity of host responses against NDV vaccines in different chickens, our data have vital values in coping with the “vaccine failure” problem and exploring host-pathogen interactions in Chinese native chickens.

Conclusion

In conclusion, differential transcriptome analysis of thymus tissues revealed that 70 innate immune genes were differentially expressed in Chinese Partridge Shank chickens post LaSota NDV vaccine inoculation versus pre-challenge group. Moreover, lncRNAs (MSTRG.22689.1, ENSGALT00000065826, ENSGALT00000059336, ENSGALT00000060887, and MSTRG.188121.10), miRNAs (gga-miR-6575-5p, gga-miR-6631-5p, gga-miR-1727, gga-miR-6655-5p and gga-miR-6548-3p) and innate immune-related mRNAs (PON1, MAPK10, CFTR and MMP9) were differentially expressed in post-challenge group versus pre-challenge group. In addition, co-expression analysis revealed 2 potential ceRNA networks (ENSGALT00000060887/gga-miR-6575-5p/PON1 and MSTRG.188121.10/gga-miR-6631-5p/MMP9) in Chinese Partridge Shank chickens post LaSota NDV vaccine inoculation. These data can deepen our understanding on innate immune responses and host-pathogen interactions induced by LaSota NDV vaccine in Chinese native chickens, which contributes to the better management of “vaccine failure” problem.

Abbreviations

RNA-seq
RNA sequencing
ND
Newcastle disease
NDV
Newcastle disease virus
lncRNAs
long non-coding RNAs
miRNAs
microRNAs
GO
Gene Ontology
KEGG
Kyoto Encyclopedia of Genes and Genomes
ceRNA
competing endogenous RNA; PON1 = paraoxonase 1
CDK3
cyclin dependent kinase 3
TRHR
thyrotropin releasing hormone receptor; MSGN1 = mesogenin 1
C10RF158
chromosome 1 open reading frame 158
ACTL6B
actin like 6B
GNGT2
G protein subunit gamma transducin 2
EDMTF4
epidermal differentiation protein starting with MTF motif 4
MYF5
myogenic factor 5
AvBD2
avian beta-defensin 2

MEI4
meiotic double-stranded break formation protein 4
FAM181B
family with sequence similarity 181 member B
PIGC
phosphatidylinositol glycan anchor biosynthesis class C
UBL4A
ubiquitin like 4A
INKA1
inka box actin regulator 1
SPATA2L
spermatogenesis associated 2 like
NDUFB7
NADH: ubiquinone oxidoreductase subunit B7
CDK2AP2
cyclin dependent kinase 2 associated protein 2
AP5S1
adaptor related protein complex 5 subunit sigma 1
COX14
cytochrome c oxidase assembly factor COX14
APOLD1
apolipoprotein L domain containing 1 (APOLD1)
MAPK10
mitogen-activated protein kinase 10
CFTR
cystic fibrosis transmembrane conductance regulator
MMP9
matrix metalloproteinase 9
E2F1
E2F transcription factor 1
NF- κ B2
nuclear factor kappa B subunit 2

Declarations

Ethics approval and consent to participate: All animal experiments were performed with the approval of Animal Care and Use Committee of the Henan University of Animal Husbandry and Economy and performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Henan University of Animal Husbandry and Economy.

Consent for publication: Not applicable.

Availability of data and material: The data displayed in this manuscript is available from the corresponding author upon reasonable request.

Competing interest: The authors have no conflict of interest to declare.

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Authors' contributions: FN designed and performed the experiments, wrote the manuscript. JZ, ML, XC, and YJ contributed to experimental work. HD and JZ participated in the data analysis. HL and LG involved in the design of this research. Moreover, LG revised the manuscript. All authors have read and approved the final manuscript.

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Tables

Table 1 Reference genome information	
Genome	Gallus_gallus.Gallus_gallus-5.0.dna.toplevel.fa
genebuild by	Ensembl
Database version	94.5
Base Pairs	1285637921

Table 2 Chicken gene annotation information in different databases		
Database	Number	Percentage
NCBI_GI	12312	67.1
eggNOG	1158	6.31
KEGG	9014	49.13
GO	15747	85.83
EC	2675	14.58
UniProtID	8761	47.75
Ensembl	18346	100

Table 3 Raw data statistics						
Sample	Reads No.	Bases (bp)	Q30 (bp)	N (%)	Q20 (%)	Q30 (%)
ctl1	112006994	16913056094	15556184034	0.016238	96.64	91.97
ctl2	107861238	16287046938	14921050839	0.010633	96.46	91.61
ctl3	101546874	15333577974	14003424760	0.005531	96.26	91.32
48h1	108620036	16401625436	15386850552	0.002824	97.37	93.81
48h2	106833502	16131858802	15119130773	0.002845	97.34	93.72
48h3	101846738	15378857438	14396829696	0.002809	97.26	93.61
Note:						
Bases (bp): Total number of bases.						
Q30 (bp): Base number of base recognition accuracy rate > 99.9%.						
N (%): Percentage of ambiguous bases.						
Q20 (%): Percentage of bases with recognition accuracy rate > 99%.						
Q30 (%): Percentage of bases with recognition accuracy rate > 99.9%.						

Table 4 Clean data statistics				
Sample	Clean Reads No.	Clean Data (bp)	Clean Reads %	Clean Data %
ctl1	111188706	16735182418	99.26	98.94
ctl2	106983056	16101011058	99.18	98.85
ctl3	100626522	15110019778	99.09	98.54
48h1	108187642	16231587710	99.6	98.96
48h2	106428002	15989418284	99.62	99.11
48h3	101429340	15240892986	99.59	99.1
Note:				
Clean data: removing reads with 3' adaptor and mean quality score < Q20				
Clean Reads No.: read number of high-quality sequences				
Clean Data (bp): base number of high-quality sequences				
Clean Reads %: percentage of clean reads on total sequencing reads				
Clean Data %: percentage of high-quality sequence base number on total sequencing base number				

Table 5 RNAseq Map statistics				
Sample	Clean_Reads	Total_Mapped	Multiple_Mapped	Uniquely_Mapped
ctl1	111188706	90835667 (81.70%)	3410734 (3.75%)	87424933 (96.25%)
ctl2	106983056	87490661 (81.78%)	3258412 (3.72%)	84232249 (96.28%)
ctl3	100626522	80628531 (80.13%)	3009815 (3.73%)	77618716 (96.27%)
48h1	108187642	88886144 (82.16%)	3497429 (3.93%)	85388715 (96.07%)
48h2	106428002	87593246 (82.30%)	3605116 (4.12%)	83988130 (95.88%)
48h3	101429340	82519425 (81.36%)	3459058 (4.19%)	79060367 (95.81%)
Note:				
Clean Reads No.: total number of aligned sequences				
Total Mapped: sequence number aligned to reference genome, percentage: Total Mapped number/Clean Read number				
Multiple Mapped: sequence number aligned to multiple positions, percentage: Multiple Mapped number/Total Mapped number				
Uniquely Mapped: sequence number aligned to one position, percentage: Uniquely Mapped number/Total Mapped number				

Table 6 Reverse-transcription primers for miRNAs (stem-loop primers)	
gga-miR-6631-5p	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGgcagaacc-3'
gga-miR-6655-5p	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGaggataca-3'
gga-miR-6575-5p	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGaagagctt-3'
gga-miR-6548-3p	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGacagcaac-3'
gga-miR-1744-3p	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGtcagtcctt-3'
gga-miR-1727	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGccttcagt-3'
gga-miR-1712-3p	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGccaaactc-3'

Table 7 Quantitative PCR primers		
miRNAs	forward primers	reverse primers
gga-miR-6631-5p	5'- ACACTCCAGCTGGGGAAGAGAATGCTGTGG- 3'	5'-TGGTGTCTGGAGTCG-3'
gga-miR-6655-5p	5'- ACACTCCAGCTGGGTCTGCTAGGAGGCTGTG- 3'	5'-TGGTGTCTGGAGTCG-3'
gga-miR-6575-5p	5'- ACACTCCAGCTGGGTTGTCAGCTTGGGGAA- 3'	5'-TGGTGTCTGGAGTCG-3'
gga-miR-6548-3p	5'- ACACTCCAGCTGGGAGAGGTGCCCCGCTGT-3'	5'-TGGTGTCTGGAGTCG-3'
gga-miR-1744-3p	5'- ACACTCCAGCTGGGACTTCAACAGGAGCAA-3'	5'-TGGTGTCTGGAGTCG-3'
gga-miR-1727	5'- ACACTCCAGCTGGGAAGCTGCTCTAATGAAC- 3'	5'-TGGTGTCTGGAGTCG-3'
gga-miR-1712-3p	5'- ACACTCCAGCTGGGTTCAGTTATCAGTGGA-3'	5'-TGGTGTCTGGAGTCG-3'
mRNAs		
ENSGALG00000009689(PON1)	5'-CCTCATGGGATCAGCACTTAC-3'	5'- CACTTGTCTCAGAAGGTCGTGTC- 3'
ENSGALG00000003045 (E2F1)	5'-CACCACAGCCACAGGATTAC-3'	5'- GAAGTCCCCAAAGTCACAGTC- 3'
ENSGALG000000032986(CFTR)	5'-GGGGAGGTCACCAAATCTGT-3'	5'- AGGGTGTATGAGCAGCGTTC- 3'
ENSGALG000000011109(MAPK10)	5'-TACAGCGTGGAAGTGGGTGA-3'	5'- GCACAACTATTCCCTGAGCC- 3'
ENSGALG00000006992(MMP9)	5'-CCACTCGTCCTTCTGGAAATC-3'	5'- CCCTCTTGGTGAGCACATCTT- 3'
ENSGALG00000005653(NF-κB2)	5'-AGCAGTGGGAACCTCACTCTG-3'	5'- TCGTCGTCCTCATAGAACCG-3'
lncRNAs		
MSTRG.188121.10	5'-AGACGTTACAGCAACCTGGG-3'	5'- AGTCCTTGGACTGAGCAACG- 3'
ENSGALT00000065826	5'-GACAGGACGTTTGCTCCTCA-3'	5'-CTTCTCCCCACATAACGCA- 3'
ENSGALT00000059336	5'-GGAGAAGACGGGCATTTCCCT-3'	5'- TTAGACCGTTTTGCCCCAG-3'
ENSGALT00000060887	5'-CGGAAAGTGTCGTTGCAGTC-3'	5'- AGAGCAGACGCATCTGAAGG-

		3'
MSTRG.22689.1	5'-GTTCCAGGCATCTCGGGTTT-3'	5'- AATCAGGCTTGCCACCATACT- 3'

Figures

Figure 1

Volcano plot of up-regulated, down-regulated, and non-differentiallncRNAs in vaccine-treated group vscontrol group.

Figure 2

(A) Volcano plot of up-regulated, down-regulated, and non-differential miRNAs in vaccine-treated group compared with control group. (B) Top 20 KEGG pathways enriched by putative target genes of differentiated expressed miRNAs.

Figure 3

(A) Volcano plot of up-regulated, down-regulated, and non-differential mRNAs in vaccine-treated group relative to control group. (B) Top 20 KEGG pathways enriched by differentiated expressed mRNAs.

Figure 4

Interaction network of proteins encoded by filtered dysregulated innate immune genes ($|\log_2\text{FoldChange}| > 1$, $P\text{ value} < 0.05$). Confidence score of protein and protein interaction was equal to or higher than 0.4.

Figure 5

RT-qPCR validation of some differentially expressed lncRNAs, miRNAs and mRNAs. (A-C) Expression levels of several lncRNAs (MSTRG.22689.1, ENSGALT00000065826, ENSGALT00000059336, ENSGALT00000060887 and MSTRG.188121.10) (A), miRNAs (gga-miR-1744-3p, gga-miR-1712-3p, gga-miR-6575-5p, gga-miR-6631-5p, gga-miR-1727, gga-miR-6655-5p and gga-miR-6548-3p) (B) and mRNAs (PON1, MAPK10, CFTR, MMP9, E2F1, and NF- κ B2) (C) were further examined through RT-qPCR assay in thymus tissue samples of 10 random chickens at 0 h and 48 h post NDV injection.

Figure 6

Pearson correlation analysis of differentially expressed lncRNAs, miRNAs and mRNAs in thymus tissue samples of 10 random chickens at 48 h after NDV inoculation.

Supplementary Files

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