Transcription Analysis of Chicken Embryo Fibroblast Cells Infected with a Recombinant Avian Leukosis Virus Isolate GX14FF03

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Abstract

Infection with recombinant avian leukosis virus (ALV) has previously been linked to malignancies and immunosuppression. However, the processes behind recombinant ALV's unique pathophysiology are poorly understood. The study aims to investigate the gene expression patterns of a recombinant ALV isolate GX14FF03 infected chicken fibroblast cells (CEFs) and used the RNA-Sequence technique to undertake a complete analysis of the mRNAs transcribed. As a consequence, a total of 907 significant differentially expressed genes (SDEGs) were identified. Among these SDEGs, the most significantly up-regulated gene was interleukin 8-like 1 (IL8L1), while the most significantly down-regulated gene was fibroblast growth factor 16 (FGF16). The 907 SDEGs were highly enriched ($p < 0.05$) for 252 Gene Ontology (GO) terms, including 197 BP, 3 CC, and 52 MF. According to the KEGG data analysis, SDEGs are implicated in eight significant pathways ($p < 0.05$). Furthermore, PPI network analyses revealed that IL8L1 interacts with 17 genes. These findings provide light on the molecular mechanisms of the recombinant ALV infection by explaining the mRNA expression profile in CEFs infected with GX14FF03 virus.

Background

Avian leukosis virus (ALV) is a retrovirus that can cause tumorigenesis in chickens when given exogenously [1]. ALV that infect chickens comprise 7 envelope subgroups designated A–E, J, and K [2]. Among these subgroups, subgroup J, first isolated in the United Kingdom in 1988, and which later proved to have a broader neoplastic spectrum [3]. Subgroups A and B are classical and common pathogenic exogenous viruses that induce lymphoid leukosis [4]. Despite current purification strategies, ALVs remains a serious problem in the poultry industry for there is no effective means of dealing with them [5].

Transcriptional profiling examination of the host response to an infectious agent, which provides new insights into the interaction between the host and the infectious organism [6]. Several *in vivo* and *in vitro* investigations have revealed the ALV infected birds and cells have differently expressed genes or proteins [7]. However, there are few studies that focused on the recombinant ALV [8]. In this study, RNA sequencing was performed to investigate the expression pattern of mRNAs in chicken embryo fibroblast cells infected by recombinant avian leukosis virus isolate GX14FF03.

Methods

Virus and cells

A recombinant avian leukosis virus isolate GX14FF03 (GenBank accession number KU923579) was previously isolated from a commercial native chicken in Guangxi Province [9]. The virus rGX14FF03 used in this study were rescued by using reverse genetics technique and identified [10]. Our previous research showed that this isolate caused body weight decrease and immunosuppression in experimental chickens. Chicken embryo fibroblasts (CEF) were cultured in Dulbecco's modified eagle medium (DMEM) containing 5% fetal bovine serum (FBS) at 37 °C and 5% CO₂ until they reached about 80% confluence.

Sample preparation and mRNA sequencing

The virus rGX14FF03 was diluted to 50 % tissue culture infective dose (TCID₅₀) of 10⁴ and inoculated into the CEFs for 2 h, then the cells were maintained in DMEM with 1% FBS, and harvested at 72 h of post-infection. The rGX14FF03 infected CEFs and control CEFs were processed for mRNA sequencing and analysis by Novoene company, Beijing, China. The Illumina Novaseq 6000 platform was used for mRNA sequencing.

Differential expression mRNA analysis

Differential expression mRNA analysis was performed using the DESeq2 R package (1.20.0). Genes with an adjusted P-adj<0.05 and log₂FoldChange>|2| found by DESeq2 were assigned as differentially expressed. Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the ClusterProfiler R package, in which gene length bias was corrected [11]. GO terms with corrected p-value less than 0.05 were considered as significantly enriched.

KEGG is a database resource for understanding high-level functions and utilities of the biological system [12]. ClusterProfiler R package were used to test the statistical enrichment of differential expression genes in KEGG pathways. KEGG pathways with corrected p-value less than 0.05 were considered as significantly enriched.

Protein-to-protein interaction (PPI) network analyses of SDEGs were performed using STRING database (<https://string-db.org/>) [13]. PPI network visualization was created with Cytoscape (version 3.8.2), and core proteins were identified by calculating the number of interactions between each network node [14].

Quantitative real-time PCR analysis for DEGs

The DEGs identified through transcriptome sequencing were validated using the reverse transcription quantitative real-time PCR (RT-qPCR). For RT-qPCR validation, IL6, IL10, FGF16, SMOC1, TLR15, IL8L1, MYLK, and ACOD1 were used. Primer Express® software for the Quantitative real-time PCR (qPCR) was used to design target-specific primers for the randomly selected genes, as indicated in Table 1. Total RNA samples were extracted and reverse transcribed to cDNA according to the manufacturer's guidelines of the used RNA extraction kit (TIANGN, China) and PrimeScript™ kit (Takara, China). The final volume is 20 µl containing 0.4 µl Dye, 0.4 µl of each primer, 2 µl of cDNA, 10 µl SYBR and 6.8 µl water. Assays were performed in triplicate. Analysis was performed by the 2^{-ΔΔCT} method.

Table 1
Primers for RT-qPCR in this study

Name	Primer sequence	Name	Primer sequence
IL6F	AAATTCGGTACATCCTCGACGG	TLR15F	AACCTGGTGCATTTGAGAACCTGC
IL6R	GGAAGGTTTCAGGTTGTTTTCTGC	TLR15R	TTTCAGGTGAGGTGCAAGACCAGA
IL10F	TTGCTGGAGGACTTTAAGGGT	IL8L1F	CCTCACTGCAAGAATGTGGA
IL10R	CTTGATGTCTGGGTCTTGGTT	IL8L1R	GGAGGAGGTAGGACGTTTTTG
FGF16F	GTGCATGGACCGGACTTTCTGTG	MYLKF	AAGATTGAAGGATACCCAGAC
FGF16R	TGTCAGCTTCTTCGACCCATAG	MYLKR	TCCATCGTTTCCACAATGAG
SMOC1F	GGCTATTAACCTCAGCAGCACCTACT	ACOD1F	TGCTGCTGCGTCCAAGTTT
SMOC1R	TGTTATTGTCCAACCTGGCTGAAGT	ACOD1R	GGGGCTTAGTCTGAGTGGC

Statistical analysis

Data were analyzed using GraphPad Prism, version 5.0, software and are expressed as means. The one-way ANOVA analysis was used to assess differences among clusters in the Statistical Package for Social Science Windows, version 19, software.

Data deposition

The raw sequence data were deposited in the NCBI database Sequence Read Archive with the accession number PRJNA757623.

Results

Evaluation of the transcriptome sequencing data

Total RNA was extracted from GX14FF03 or mock-infected CEFs to examine gene expression. At least 6.06 Gb of clean data from each sample (39.24GB) was obtained from the transcriptome sequencing. For all samples, the Q20 and Q30 percentages of clean data were higher than 97.50 % and 94.00 %, respectively. For all samples, the GC content of the clean data varied from 50.31 to 51.43 % (Table 2). Approximately 87.2-90.98% of clean reads were uniquely mapped to the reference *Gallus gallus* genome (Hisat2 v2.0.5.). Through normalisation and statistical analysis, a total of 907 significant differentially expressed genes (SDEGs) were identified, including 534 genes up-regulated and 373 genes down-regulated, 822 genes were known, and 85 genes were novel (Fig. 1A, Supplementary table S1). At the top 30 SEDGs, 26 were up-regulated and only 4 (FGF16, NKAN4, SMOC1, MYLK) were down-regulated (Fig. 1B). Interleukin 8-like 1 (IL8L1) was the most significantly up-regulated gene with a log2FoldChange of 7.618, while fibroblast growth factor 16 (FGF16) was the most significantly down-regulated gene with a log2FoldChange of -4.111 (Supplementary table S1).

Table 2
Summary statistics for sequence quality control and mapped data of samples

Sample	Raw reads	Clean reads	Q20 (%)	Q30 (%)	GC pct (%)	Total mapped	Unique mapped	Multi mapped
Mock 72h 1	47,504,588	45,787,868	97.50	94.00	51.08	40,732,727	39,928,848	803,879
Mock 72h 2	45,575,230	44,426,782	98.09	94.90	50.31	41,121,076	40,418,091	702,985
Mock 72h 3	41,586,088	40,408,138	98.01	94.57	50.49	37,390,145	36,755,609	634,536
ALV-B 72h 1	42,551,534	41,474,616	97.94	94.61	51.43	37,948,237	37,233,912	714,325
ALV-B 72h 2	46,824,782	45,451,720	98.11	94.99	50.79	41,871,050	41,088,886	782,164
ALV-B 72h 3	45,293,416	44,047,452	98.02	94.72	50.93	40,580,093	39,806,401	773,692

Gene Ontology (Go) Analysis

To generally described the functions of genes obtained from RNA-Seq, functional annotations were performed by comparing the sequences with the GO databases. Genes annotated by GO database were classified into the three GO categories, namely, biological progress (BP), cellular component (CC), and molecular function (MF) [15]. The significance of GO term enrichment in the DEGs was based on a p-value < 0.05. A total of 907 sDGEs were enriched for 252 GO terms were significantly enriched, including 197 BP, 3 CC (cell surface, external side of plasma membrane, receptor complex) and 52 MF (Supplementary table S2). We are concerned here with the top 30 GO terms, which contained 24 BP and 6 MF. (Fig. 2A, Table 3). In biological progress, the primary subcategories are the inflammatory response

Table 3
Functional analysis of DEGs using GO and showing the top 30 GO terms

Description	Category	P-value	Gene No.	Up Gene No.	Down Gene No.
inflammatory response	BP	1.77E-18	52	46	6
receptor regulator activity	MF	6.96E-16	49	36	13
receptor ligand activity	MF	2.32E-15	46	33	13
chemokine receptor binding	MF	1.42E-13	16	16	0
cytokine activity	MF	1.58E-13	25	24	1
chemokine activity	MF	2.22E-13	14	14	0
cell chemotaxis	BP	2.10E-12	33	26	7
positive regulation of immune system process	BP	5.19E-12	58	49	9
response to other organism	BP	1.08E-11	54	44	10
response to external biotic stimulus	BP	1.31E-11	54	44	10
regulation of immune response	BP	1.74E-11	50	44	6
response to biotic stimulus	BP	3.99E-11	54	44	10
regulation of signaling receptor activity	BP	9.03E-11	39	23	16
neutrophil migration	BP	1.04E-10	18	17	1
neutrophil chemotaxis	BP	1.37E-10	17	16	1
leukocyte chemotaxis	BP	1.38E-10	25	21	4
response to bacterium	BP	1.57E-10	40	33	7

Description	Category	<i>P</i> -value	Gene No.	Up Gene No.	Down Gene No.
granulocyte chemotaxis	BP	1.96E-10	18	17	1
granulocyte migration	BP	2.40E-10	19	18	1
response to molecule of bacterial origin	BP	3.81E-10	27	25	2
leukocyte migration	BP	4.68E-10	30	25	5
myeloid leukocyte migration	BP	1.15E-09	22	19	3
chemotaxis	BP	1.31E-09	44	36	8
taxis	BP	1.44E-09	44	36	8
activation of immune response	BP	2.04E-09	34	32	2
cell activation	BP	2.41E-09	53	40	13
immune response-regulating signaling pathway	BP	2.64E-09	31	29	2
cytokine-mediated signaling pathway	BP	2.65E-09	33	29	4
cytokine receptor binding	MF	3.12E-09	26	25	1
innate immune response	BP	3.65E-09	36	34	2

cell chemotaxis, the positive regulation of immune system process, the response to other organism, the response to external biotic stimulus, the regulation of immune response, the response to biotic stimulus and the regulation of signaling receptor activity. The inflammatory response was the most affected of the subcategories, including upregulated genes IL8L1, TLR15, PPBP, CD44, ADAM8, NFKBIZ, IL8, ELF3, F3, BDKRB, down-regulated genes GPRC5B and so on.

Kegg Pathway Enrichment Analysis

To understand the various biological processes of GX14FF03 infection, a KEGG pathway analysis based on the SDEGs was performed. The KEGG data analysis revealed that the SDEGs are involved in eight significant

pathways, included Cytokine-cytokine receptor interaction, Neuroactive ligand-receptor interaction, Toll-like receptor signaling pathway, Calcium signaling pathway, Phenylalanine metabolism, Intestinal immune network for IgA production, Cytosolic DNA-sensing pathway and Regulation of actin cytoskeleton (Fig. 2B, Table 4. Supplementary table S3). In the top three most significantly enriched pathways, IL8L1, CSF3, CCL19, IL8, IL13RA2, CCL20, IL1B, IL6, IL17C, RELT and some immune-related genes were enriched in cytokine-cytokine receptor interaction, and BDKRB1, PTGER2, GRIN2C, GRIA4, AGTR2, BDKRB2, ADORA2B, HTR2A, EDN, HTR7 and some other likes genes were enriched in Neuroactive ligand-receptor interaction, and IL8L1, IL8, IKBKE, chTLR1-type2, IL1B, IRF7, IL6, MAP3K8, CCL4, TLR1A and some other like genes were enriched in Toll-like receptor signaling pathway.

Table 4 KEGG pathway analyses of the differentially expressed genes. The first column indicates that the cell signalling pathways involved with differentially expressed genes. p-Value < 0.05 were used as the threshold to select significant pathways.

Tine	Name of pathway	<i>P</i> value	Gene No.	Up Gene No.	Down Gene No.
72h	Cytokine-cytokine receptor interaction	8.86E-15	37	35	2
	Neuroactive ligand-receptor interaction	1.54E-10	40	24	16
	Toll-like receptor signaling pathway	2.04E-06	15	15	0
	Calcium signaling pathway	0.000201252	20	14	6
	Phenylalanine metabolism	0.000592564	5	3	2
	Intestinal immune network for IgA production	0.001812759	6	6	0
	Cytosolic DNA-sensing pathway	0.001828423	7	7	0
	Regulation of actin cytoskeleton	0.003405639	18	11	7

Protein-protein Interaction Network (Ppi) Analysis Of Degs

PPI network analyses identified a total of 359 interaction relationships among 480 proteins (Fig. 3). The SDEGs SAA, BDKRB2, BDKRB1, C3AR1, POMC, AGTR2, IL8L1, IL8L2, SSTR2, GRM3, HTR1D, GPR183, P2PY4, CCL20, CCL1 and CCL19 gene shave more than 15 interactions, and there play important roles in maintaining the connection of the whole network (Fig. 3B, Supplementary table S4).

Validation of the RNA-seq results by Quantitative real-time PCR (RT-qPCR)

We randomly chose eight genes (IL6, IL10, FGF16, SMOC1, TLR15, IL8L1, MYLK, and ACOD1) for RT-qPCR investigation to confirm the consistency and repeatability of SDEGs discovered through the transcriptome

sequencing. The findings revealed that these genes were highly significantly elevated and were consistently up-regulated or down-regulated with gene expression variations based on RNA-Seq (Fig. 4) implying that the SDEGs derived from transcriptome sequencing were credible.

Discussion

Viruses may infect hosts in a variety of ways [16], changing the host's cellular metabolic network to create an appropriate intracellular milieu for the viral life cycle [17]. Molecules of the host are altered to regulate viral infection, and these alterations may be reflected in the transcriptional profile [18]. For many years, research on ALV had been continuing [5]. In CEFs with the ALV-J infection, previous research revealed that 36 differentially expressed lncRNAs and 91 genes were differentially expressed [19]. During ALV-J infection, 228 lncRNAs were differently expressed in HD11 cells and 361 lncRNAs in CEFs, according to another research [20]. The majority of these researches are focused on ALV-J, with only a few studies focusing on the recombinant ALV. Recombinant ALV, to our knowledge, has a particular proportion in clinical situations [10].

The transcriptional alterations produced by the recombinant ALV isolate GX14FF03 infection in CEFs were investigated using RNA sequencing in the current study. At 72 hours post-infection, SDEGs were identified in CEF infected with ALV. In addition, this study revealed a total of 19,895 genes, including 907 SDEGs ($P\text{-adj} < 0.05$ and $\log_2\text{FoldChange} > |2|$). The RT-qPCR analysis of the expression variations of randomly selected 8 genes revealed a high connection with the RNA-Seq results. This supported the findings from the RNA-Seq analysis being reliable..

IL8L1 was the most significantly upregulated gene among 907 SDEGs. In chickens, IL8L1 is the chicken homologue of CXCL8 (also known as interleukin-8, IL8), a member of the CXC family. CXCL8, a pro-inflammatory factor, is associated to the incidence and progression of a variety of inflammatory diseases, including proliferation, angiogenic responses, tumour metastasis, and encouraging infected tissue healing [21]. Previous studies showed that IL8L1 has a causal role in establishing acute inflammation; play important role in PPI network; probably associates with vIL8, which recruits T cells to B cells infected with MDV and leads to transduction of viral signal from B to T cells [22]. IL8L1 was enriched in 15.87% (40/252) GO terms, and the rate was high as 86.67% (26/30) in the top 30 GO terms. Besides, IL8L1 was enriched in cytokine-cytokine receptor interaction and toll-like receptor signaling pathway. PPI network analyses resulted showed that IL8L1 shown interactions with IL6, CCL19, GRM3, BDKRB2, POMC, P2RY4, IL8L2, BDKRB1, GPR183, SAA, CCL20, THR1D, IL10, CCL1, C3AR1, AGTR2, and SSTR2. It is imperative to study the specific mechanism of IL8L1 involved in the recombinant ALV infected CEFs.

The findings revealed eight major pathways, with the cytokine-cytokine receptor interaction signaling pathway being particularly active. Cytokines have an important role in immunological control, cell development and differentiation, and inflammation [23]. Furthermore, we were drawn to the Toll-like receptor signaling pathway. Toll-like receptor signaling pathways are critical components of innate immune responses and play a role in viral infection resistance [24]. Studies show that TLR signaling pathway might contribute to controlling HBV infection [25], substantial reductions in the mRNA expression of TLR1, TLR2,

TLR3 were detected in the surviving BM-DCs following ALV-J infection [26]. In the present study, IL8L1, IL8, IKBKE, chTLR1-type2, IL1B, IRF7, IL6, MAP3K8, CCL4, TLR1A, IL-12B, and LY96 were found to be involved in the Toll-like receptor signaling pathway. However, whether the Toll-like receptor signaling pathway affects ALV-B infected CEF cells need to be further studied.

Conclusions

In the present study, we characterized the transcriptome profile of CEFs infected with a recombinant ALV isolate GX14FF03. We observed a highly correlated expression pattern of mRNAs induced by the recombinant ALV with coding genes that are involved in antiviral innate immunity. The dynamic changes of differentially expressed genes of GX14FF03 infection contributed to understanding the molecular mechanisms of ALV-host interaction. In the future, more research is required to elucidate the mechanisms underlying the functions of the mRNAs involved in the recombinant ALV infection.

Abbreviations

ALV

avian leukosis virus; CEFs:chicken fibroblast cells; SDEGs:significant differentially expressed genes; IL8L1:interleukin 8-like 1; FGF16:fibroblast growth factor 16; GO:Gene Ontology; DMEM:Dulbecco's modified eagle medium; FBS:fetal bovine serum; TCID50:50 % tissue culture infective dose; PPI:Protein-to-protein interaction; RT-qPCR:reverse transcription quantitative real-time PCR; qPCR:Quantitative real-time PCR; BP:biological progress; CC:cellular component; MF:molecular function.

Declarations

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Authors' contributions

Conceptualization: WPK, WJ conceived and designed the experiments. LQH collected the samples and data; WPK, LQH,DQM, LM performed the experiments; WPK analyzed the data and wrote the manuscript. DQM, LM reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

No animal experiment was involved in this study.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest for this manuscript.

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Figures

Figure 1

Gene-expression profiling of CEFs upon recombinant ALV stimulation.

(A) Volcanic plot of DEGs. The Red dot represents significantly up-regulated genes (N=534), The green dot represents significantly down-regulated genes (N=373), and blue dots represent non-differentially expressed genes (N=18988). (B) Heat-map expression profiles of 30 significantly dysregulated mRNAs between recombinant ALV-infected and uninfected CEF cells, at 72 hours after infection.

Figure 2

Functional enrichment analysis of differentially expressed mRNAs in CEFs of different groups during the recombinant ALV infection. (A) Top 30 GO terms analysis of differentially expressed mRNAs in the ALV positive group vs Control group; (B) Scatter diagram of KEGG signal pathway. Horizontal axis: rich factor, vertical axis: signal pathway. rich factor: the ratio of the number of differentially expressed genes in a signal pathway to all annotated genes; only the top 20 gene regulation entries are displayed.

Figure 4

Validation of RNA-Seq data (for mRNAs) by real-time PCR. Average fold changes of gene expression in CEF cells at 72 h following recombinant ALV, as determined by qRT-PCR and RNA-seq analysis. The expression of selected mRNAs in recombinant ALV-infected CEF cells together with the expression of mock-infected controls was validated by qRT-PCR using a pair of specific primers and a probe for each mRNA. Data are expressed as the mean $\pm$ SD of triplicate reactions for each gene transcript.

Supplementary Files

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