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Transcriptome Analysis of Chicken Embryo Fibroblast Cell Infected with Marek's Disease Virus of GX0101ΔLTR

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■Keywords

Gene profiling; chicken; CEF cell; GX0101ΔLTR MDV.

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ABSTRACT

Marek's disease (MD), a lymphoproliferative disorder of chickens caused by the MD virus (MDV), is economically significant. The resistance/susceptibility to MD is controlled by host genetics. The host response to different virus strains varies. The pathogenicity of REV-LTR deleted GX0101ΔLTR MDV has been previously reported. However, the precise molecular mechanism of the response of chickens to GX0101ΔLTR remains unclear. The current study aimed at identifying the genes and pathways involved in the response to GX0101ΔLTR virus infection in specific pathogen-free chicken embryo fibroblast cells using global transcriptome analysis. A total of 1,633 genes associated with GX0101ΔLTR infection were identified. Functional analysis showed that the cytokine–cytokine receptor interaction plays an important role in the response to GX0101ΔLTR infection.

INTRODUCTION

Marek's disease (MD) is a chicken lymphoproliferative disease caused by MD virus (MDV) (Witter *et al.*, 2005). MD causes $1-2 billion annual losses to the industry (Morrow and Fehler, 2004). Field strains continue to evolve with increasing virulence (Hunt & Dunn, 2013). We have reported the GX0101 strain of MDV, which was the first field isolate with an LTR insert of REV origin (Zhang & Cui, 2005). The pathogenicity of the REV-LTR deleted GX0101ΔLTR virus was slightly higher than its parental GX0101 clone, based on growth retardation, immunosuppression, mortality and tumorogenicity (Sun *et al.*, 2010). However, the virus-host cell interaction related to GX0101ΔLTR pathogenicity is still unclear.

The high-throughput microarray is one of the widely used technologies in transcriptome studies (Haq *et al.*, 2010; Liu *et al.*, 2001; Sarson *et al.*, 2008). The microarray technology was applied to analyze the global gene expression profile of CEF following GX0101ΔLTR infection in the current study.

MATERIALS AND METHODS

Chicken embryo fibroblast (CEF) cell culture

The primary CEF cells were isolated from 10-day-old specific-pathogen free (SPF) White Leghorn chicken embryos. Whole embryos were dissociated into single cell populations using 0.25% trypsin/1mM EDTA. The cells dissociated from the embryos were suspended in Dulbecco's modified Eagle's medium (DMEM, 0.45% glucose) plus 10% fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine in 10 cm tissue culture dishes (Corning, Shanghai, China). Cultured cells were grown at 37°C in a 5% CO_2

incubator until the cells reached confluent monolayers. Frozen cell stocks were prepared and stored in liquid nitrogen for further utilization.

MDV infection and sample collection

Four chicken embryos were used in the current study. The primary CEF cells collected from each of the four embryos were seeded into two individual flasks at a density of 5 × 10^6/flask. The cells in one flask were infected with GX0101ΔLTR (Sun *et al.*, 2010), while those in the other flask were mock-infected with DMEM. The infected or non-infected CEF cells were collected at 56 h post infection and treated with RNeasy reagent (Qiagen, Valencia, CA) for total RNA extraction. A total of eight samples were collected; four of which were infected cells and four were non-infected controls.

Total RNA isolation, experimental design, sample labeling, and microarray hybridization

The total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) from the infected and non-infected CEF cells, according to the instructions of the manufacturer. RNA concentration and integrity were checked using NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA) and Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA), respectively.

A custom chicken 4 × 44 K Agilent microarray, including both chicken and MDV genes, was designed based on the chicken genome assembly galGal4 and MDV annotation using Agilent earray tool (https://earray.chem.agilent.com/earray/). A paired comparison was performed to compare the infected and the non-infected (I/N) groups. Four biological replicates were used in each group with dye balance.

A 400-ng total RNA from each sample was used for labeling. The sample labeled with Cy3 or Cy5 was hybridized with another labeled with Cy5 or Cy3 and incubated for 17h at 65°C. The slides were washed according to the manufacturer's recommendations. All procedures were performed according to Agilent's recommendation and described in detail previously (Li *et al.*, 2008).

Microarray data analysis

The signal intensity of each probe was filtered against negative controls in the microarray. Data normalization was performed using locally weighted scatter plot smoothing (Yang *et al.*, 2002). The normalized natural log intensities were analyzed using a mixed model from SAS (SAS, Cary, NC) with a fixed effect of treatment (I or N) and dye (Cy5 or Cy3) and a random effect of slide and array. A comparison between the infected (I) and non-infected (N) groups was made. Accordingly, $p<0.05$ and fold change >1.5 were considered as significant. The microarray information of this experiment was deposited in NCBI's Gene Expression Omnibus (GEO) database (Barrett *et al.*, 2013). The accession numbers were as follows: platform: GPL18321; series: GSE59052.

The functional annotations of Gene ontology (GO) and pathway enrichment for significantly differentially up-regulated and down-regulated genes were performed using DAVID 6.7 (Huang *et al.*, 2009, Huang *et al.*, 2009). The differentially expressed genes were uploaded to the DAVID database as a gene list. The *Gallus gallus* whole genome was used as the background. Default setting was used. $p<0.05$ was considered significant.

RESULTS

Gene expression was significantly different between MDV-infected and non-infected CEF cells

A total of 1,633 genes presented significantly different expression between I and N groups ($p<0.05$, fold change > 1.5). Out of those genes, 952 genes were up-regulated and 681 were down-regulated. Furthermore, 15 genes of the 952 up-regulated genes presented higher than 100-fold change, and 39 a fold change higher than 20. The highest fold change (1046) was found for one chicken EST (BU246007), whereas the lowest (1.5) was found for a chicken EST (CR385211). Only two down-regulated genes presented higher than 10-fold changes [Preproinsulin and a chicken EST (BU236185)]. Fifty-six immune-related genes were differentially expressed between I and N groups, with the highest fold change (122.9) for the chicken MX gene. The lowest fold change (1.5) was found for chicken CD44 (Additional file 1).

Gene ontology annotation analysis

The Gene ontology (GO) annotation was performed for the genes with significant expression using the DAVID database. The GO biological process (BP) annotation was reported in the current study.

There were 73 enriched BP terms associated with the up-regulated genes (Table 1). These enriched BP terms were roughly divided into six groups as follows: 1) immune-related group; 2) signal transduction-

Table 1 – GO BP annotation for up-regulated genes

Terms	Fold enrichment
GO:0051058~negative regulation of small GTPase-mediated signal transduction	43.92
GO:0046580~negative regulation of Ras protein signal transduction	43.92
GO:0045628~regulation of T-helper 2 cell differentiation	43.92
GO:0001937~negative regulation of endothelial cell proliferation	32.94
GO:0001936~regulation of endothelial cell proliferation	18.82
GO:0045766~positive regulation of angiogenesis	17.57
GO:0045428~regulation of nitric oxide biosynthetic process	14.64
GO:0046637~regulation of alpha-beta T cell differentiation	13.18
GO:0030193~regulation of blood coagulation	13.18
GO:0006584~catecholamine metabolic process	11.98
GO:0045765~regulation of angiogenesis	10.98
GO:0002237~response to molecule of bacterial origin	10.46
GO:0032496~response to lipopolysaccharide	10.34
GO:0050920~regulation of chemotaxis	10.14
GO:0050818~regulation of coagulation	10.14
GO:0045580~regulation of T cell differentiation	9.76
GO:0050795~regulation of behavior	9.41
GO:0010810~regulation of cell-substrate adhesion	8.78
GO:0002819~regulation of adaptive immune response	8.78
GO:0000187~activation of MAPK activity	8.78
GO:0045619~regulation of lymphocyte differentiation	8.37
GO:0046634~regulation of alpha-beta T cell activation	8.24
GO:0006954~inflammatory response	8.24
GO:0002250~adaptive immune response	8.24
GO:0032103~positive regulation of response to external stimulus	7.99
GO:0019216~regulation of lipid metabolic process	7.64
GO:0051272~positive regulation of cell motion	7.08
GO:0032101~regulation of response to external stimulus	6.76
GO:0006955~immune response	6.73
GO:0002696~positive regulation of leukocyte activation	6.59
GO:0030335~positive regulation of cell migration	6.51
GO:0002684~positive regulation of immune system process	6.48
GO:0050867~positive regulation of cell activation	6.27
GO:0043405~regulation of MAP kinase activity	6.06
GO:0009617~response to bacterium	5.99
GO:0006935~chemotaxis	5.86
GO:0009968~negative regulation of signal transduction	5.80
GO:0051251~positive regulation of lymphocyte activation	5.78
GO:0051051~negative regulation of transport	5.67
GO:0010648~negative regulation of cell communication	5.49
GO:0002694~regulation of leukocyte activation	5.49
GO:0007243~protein kinase cascade	5.42
GO:0048584~positive regulation of response to stimulus	5.32
GO:0050863~regulation of T cell activation	5.23
GO:0009611~response to wounding	5.23
GO:0050865~regulation of cell activation	5.07
GO:0051249~regulation of lymphocyte activation	4.88
GO:0050870~positive regulation of T cell activation	4.88
GO:0000165~MAPKKK cascade	4.88
GO:0001817~regulation of cytokine production	4.48
GO:0042060~wound healing	4.39
GO:0006952~defense response	4.39
GO:0040012~regulation of locomotion	4.32
GO:0051347~positive regulation of transferase activity	4.25
GO:0043549~regulation of kinase activity	4.23
GO:0051270~regulation of cell motion	4.18
GO:0030334~regulation of cell migration	4.14
GO:0051338~regulation of transferase activity	4.13
GO:0043062~extracellular structure organization	3.92
GO:0045859~regulation of protein kinase activity	3.80
GO:0045860~positive regulation of protein kinase activity	3.72
GO:0007626~locomotory behavior	3.51
GO:0043085~positive regulation of catalytic activity	3.28
GO:0042325~regulation of phosphorylation	3.24
GO:0008285~negative regulation of cell proliferation	3.18
GO:0019220~regulation of phosphate metabolic process	3.11
GO:0043068~positive regulation of programmed cell death	3.03
GO:0010942~positive regulation of cell death	2.99
GO:0010033~response to organic substance	2.95
GO:0044093~positive regulation of molecular function	2.88
GO:0042127~regulation of cell proliferation	2.66
GO:0043067~regulation of programmed cell death	2.47
GO:0042981~regulation of apoptosis	2.31

related; 3) metabolism- related; 4) circulatory system; 5) cell communication; and 6) others.

The four BP terms associated with the down-regulated genes were significantly enriched (Figure 1). Epithelial cell differentiation and epithelium development were related to epithelium function with the fold enrichments of 6.12 and 3.20, respectively. Cell adhesion and extracellular matrix organization had fold enrichments of 2.64 and 4.97, respectively.

The enriched BP terms associated with the up-regulated genes were further clustered using the Categorizer tool (Figure 2). The enriched terms were roughly clustered into 19 groups, including 16 immune-related groups and 3 non-immune related groups. Three non-immune related groups were protein metabolism, cell adhesion, and lipid metabolism, which accounted for 7.06%, 1.18%, and 1.18% of all groups, respectively. The top eight groups were lymphocyte activation, stress response, regulation of lymphocyte activation, T-cell activation, cell death, response to external stimulus, protein metabolism, and response to biotic stimulus, which were 12.94%, 11.67%, 10.59%, 8.24%, 7.06%, 7.06%, 7.06%, and 5.88% of all groups, respectively.

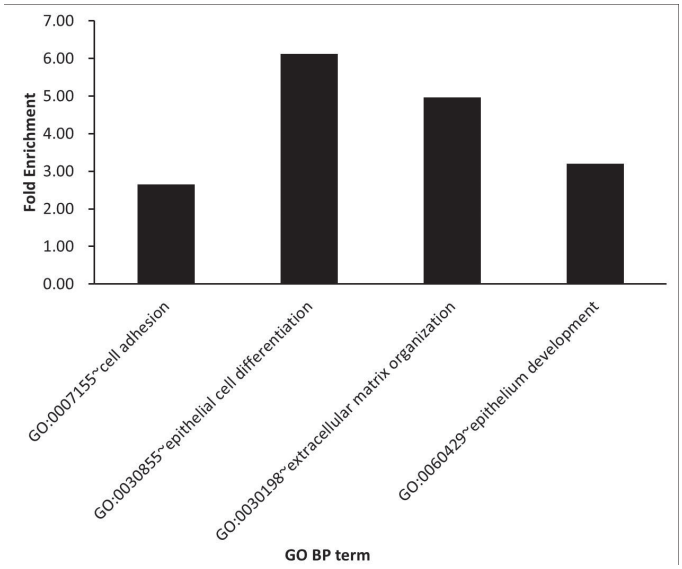

Figure 1 – GO BP annotation associated with down-regulated genes.

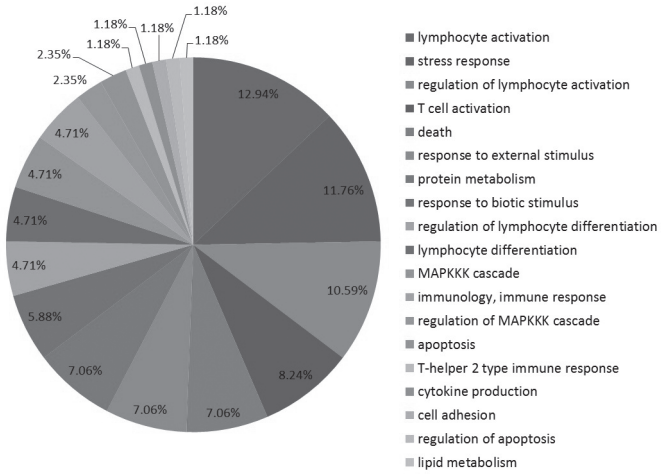

Figure 2 – GO term classification count using the Categorizer. The GO terms were classified based on the Immune_class classification method in Categorizer tool.

Pathway annotation for the significantly-expressed genes

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation results showed that four KEGG pathways associated with the up-regulated genes were significantly enriched (Table 2). These enriched pathways were the cytosolic DNA-sensing pathway, Toll-like receptor (TLR) signaling pathway, cytokine-cytokine receptor interaction, and focal adhesion with fold enrichments of 9.37, 3.89, 3.64, and 2.24, respectively. The enriched cytokine-cytokine receptor interaction pathway included IL6, IL1b, IL15, IL4R, IL17RA, CC5, CXCL14, TGFBR2, and the TNF family (i.e., TNFRSF21, TNFRSF15, and TNFSF10).

No enriched pathway was associated with the down-regulated genes.

Validation of microarray results using quantitative real-time PCR

The quantitative real-time PCR (qRT-PCR) was performed to validate the microarray data. The same RNA samples were used. Twelve differentially-expressed genes associated with immune response functional terms were selected for the qRT-PCR validation. The qRT-PCR results of the 11/12 validated genes were consistent with the microarray results in terms of significance and regulation direction (Table 3). The BCL6 showed up-regulation in both microarray and qRT-PCR results, but did not present significant expression by qRT-PCR. All the validated down-regulated genes presented higher fold changes in the qRT-PCR test compared with the microarray analyses. Five of the eight up-regulated genes presented higher fold changes in the microarray analysis than in the qRT-PCR test.

DISCUSSION AND CONCLUSIONS

The high-throughput microarray is one of most widely-used technologies to identify the transcriptome associated with a specific trait (Chiang *et al.*, 2008; Lee *et al.*, 2010; Li *et al.*, 2010; Li *et al.*, 2011; Sandford *et al.*, 2011, Allen *et al.*, 2012; Sandford *et al.*, 2012, Subramaniam *et al.*, 2013). Several studies have been performed to identify the gene expression profile in *in-vivo* and *in-vitro* MDV infections using microarray analysis (Yonash *et al.*, 1999; Morgan *et al.*, 2001; Sarson *et al.*, 2006; Kano *et al.*, 2009; Heidari *et al.*, 2010; Smith *et al.*, 2011; Lian *et al.*, 2012, Subramaniam *et al.*, 2013).. The molecular mechanism of the host response to GX0101ΔLTR MDV was elucidated in the current study.

Table 2 – KEGG pathway annotation for up-regulated genes

Terms	Genes	Fold enrichment
gga04623: cytosolic DNA-sensing pathway	IL6, IRF7, IL1B, NFKBIA, CCL5, ADAR	9.37
gga04620: Toll-like receptor signaling pathway	IL6, STAT4, MAP2K3, IRF7, IL1B, NFKBIA, CCL5, STAT1	3.89
gga04060: cytokine–cytokine receptor interaction	TNFRSF21, IL6, TGFBR2, TNFSF15, IL15, CCL5, IL17RA, TNFSF10, CNTF, CXCL14, IL4R, LOC777589, IL1B	3.64
gga04510: focal adhesion	ACTB, CAV2, CAV1, TNC, MYLK2, VAV2, FLNB, LOC777184, ACTG1, LOC776858, LOC768344, THBS1, LOC776816, FN1	2.24

Table 3 – Comparison of gene expression levels (fold change) between microarray and qRT-PCR results

Accession nos.	Gene symbol	Fold change (I/N)	
		Microarray result	qRT-PCR result
AB088533	MX	253.48**	9.77**
AF139097	IL15	3.07*	1.69*
BX929269	BCL10	1.53**	2.54**
AJ719545	BCL6	1.57*	1.52
AJ309540	IL6	3.39**	8.79**
Y15006	IL1B	1.92*	2.67*
AF222690	MMP9	1.63**	1.41**
L18784	TGFBR2	1.91*	1.63*
U66463	MMP16	-1.59**	-1.85*
D14459	CDH4	-1.67**	-1.82**
AF459439	CDH20	-3.12*	-3.57**
AF055342	CDH11	-2.17**	-2.63**

*represents a significant difference ($p<0.05$); ** represents a significant different ($p<0.01$)

The CEF is a reasonable model for studying the reaction to MDV infection (Subramaniam *et al.*, 2013). MDV infection includes the three following stages: early cytolytic infection starting at 2-7 dpi (day post infection), a latent phage initiated around 7-10 dpi, and a late cytolytic phase causing inflammation and transformation of latently-infected lymphocytes into tumor cells triggered between 14 and 21 dpi (Calnek, 1986, Calnek, 2001). Various expression profiles were discovered at different stages of MDV infection. The genes related to inflammation, cell growth, and differentiation and antigen presentation (e.g., MIP, IL-13R, MHC I, and MHC II) are induced both at 2 and 4 dpi when the CEFs are infected with MDV (Morgan *et al.*, 2001). More than one thousand genes in the present study were significantly expressed in the GX0101ΔLTR-infected CEF. MDV infection induced gene expression. It has been reported that more genes are up-regulated in CEF at 24, 48, and 96 h post MDV infection in both MD-resistant and -susceptible chicken lines (Subramaniam *et al.*, 2013). More up-regulated genes have been observed in the spleen at 2 and 5 days post MDV infection (Smith *et al.*, 2011). More genes are up-regulated on 5 and 10 days post infection in both MD-resistant and -susceptible chicken lines (Yu *et al.*, 2011). However, the opposite result has been found on 21 days post infection, where more genes were down-regulated in both MD-resistant and -susceptible lines (Yu *et al.*, 2011). The global gene expression associated with MDV infection shows temporal characteristics. The number of induced genes is greater at the early stage (2-10 dpi) and decreases at the late stage of MDV infection.

Cytokines are important mediators involved in cell-mediated immune responses to MDV infection and secreted as a result of antigen presentation to T cells (Haq *et al.*, 2013). The enriched cytokine-cytokine receptor interaction pathways are associated with the up-regulated genes in the chicken CEF, which indicated that the host proinflammatory response was stimulated at the early stage but weakened in the chicken spleen at the late tumor transformation phase following MDV infection (Lian *et al.*, 2012, Smith *et al.*, 2011). IL-6 and IL-18 are significantly up-regulated in the MDV-infected splenocytes of genetically susceptible chickens (Kaiser *et al.*, 2003), whereas IL-1b and IL-8 are up-regulated in resistant birds (Jarosinski *et al.*, 2005). IL-6 and IL-18 seem to be associated consistently with MD susceptibility, whereas both IL-1b and IL-8 were significantly expressed in the MDV-infected SPF CEF.

The TNF receptor superfamily and their ligands are mainly expressed on immune cells. Their immunomodulatory functions include the enhancement of dendritic cell survival and priming capacity for T cells, optimal generation of effector T cells, optimal antibody responses, and amplification of inflammatory reactions (Kwon *et al.*, 2003). The expression of the TNF (ligand) superfamily member 10 increases at 5 days post MDV infection, while the TNF (ligand) superfamily members 11 and 13b decrease at 15 dpi (Heidari *et al.*, 2010). TNFSF10, TNFSF13b, and TNFSF 18 are up-regulated at 5 dpi (Smith *et al.*, 2011). TNFRSF10, TNFRSF21, and TNFRSF15 are also up-regulated post MDV infection. In conclusion, we have identified the differentially expressed genes and pathways associated with GX0101ΔLTR infection in CEFs. The present findings will add to the current understanding of the mechanism behind MDV infection. The cytokine-cytokine receptor interaction plays important roles in the response to GX0101ΔLTR infection.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

XLI designed the microarray, analyzed data and drafted the manuscript. SS and NC carried out the cell infection and sample collection. HZ participated in experiment design and microarray design. XLIU validated the sequencing data through the qRT-PCR method. ZC provided the concepts of the study. All authors read, edited and approved the final manuscript.

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