Pakistan J. Zool., vol. 49(3), pp 1107-1110, 2017. **DOI**: http://dx.doi.org/10.17582/journal.pjz/2017.49.3.sc1

Short Communication

RNA-Seq Reveals Differentially Expressed Genes of Pig Vaccinated with Modified Live Attenuated Porcine Epidemic Diarrhea

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Article Information
Received 15 June 2016
Revised 20 August 2016
Accepted 28 August 2016
Available online 25 May 2017

Authors' Contributions
SKP and JYJ analysed the data, wrote and revised the manuscript. ESC and YDJ collected the data and performed the experiments. CSP designed the study and drafted the manuscript.

Key words
Porcine epidemic diarrhea virus, Peripheral blood mononucleated cell, RNA sequence, Differentially expressed gene, Gene ontology

ABSTRACT

Porcine epidemic diarrhea (PED) was triggered by pathogenic coronavirus causing watery diarrhea and high mortality in piglets. This post-viral syndrome results in severe economic losses in the pig industry. However, limited information on the porcine epidemic diarrhea virus-infected pigs is available. Thus, the objective of this study was to identify transcriptomes with RNA sequencing (RNA-seq) in peripheral blood mononucleated cell (PBMC) of piglets, Large White x Landrace crossbreds, infected with live strain PED virus (PEDV). Several gene ontology (GO) and differentially expressed gene (DEG) are also identified by RNA-seq. The analyzed transcriptome provides the 1805 DEGs and gene networks involving cellular processes. Between vaccinated and unvaccinated groups, there were 13 and 17 up- and down-regulated DEGs (2-fold; FDR < 0.05; $P < 0.001$), respectively, in PBMC of PEDV. Most of significant DEGs were involved in multiple cellular processes of cell growth and immune system. Thus, results from our current study indicate that DEG analysis of RNA-seq data help us better understand the precise mechanisms of PEDV in pig PBMC.

Introduction

Porcine epidemic diarrhea (PED) causes severe health problem of pigs and thus significantly impacting on productivity and welfare of swine farms. Porcine epidemic diarrhea virus (PEDV)-infected piglets show symptoms including reduced appetite, vomiting, watery diarrhea, and dehydration leading to high mortality in weaning period (Chae *et al*., 2000; Song *et al*., 2012; Stevenson *et al*., 2013).

As an enteric disease of pigs, PED is highly contagious and thus leads to huge economic losses in pig industry in most countries (Song *et al*., 2012; Lee, 2015). The impact of PEDV infection on the reproductive performance of gilts and sows depends on the period of pregnancy, during which females are exposed to the pathogen and the parity number (Lin *et al*., 2016). Therefore, understanding the genetic variation and diversity of PEDV is an important to prevent and control this disease. This study evaluated transcriptomes in peripheral blood mononuclear cell (PBMC) of piglets, Large White x Landrace crossbreds, infected with live strain PEDV to determine the complement nucleotide sequence of the PEDV. The PBMCs play a pivotal role in maintaining the homeostasis of immune response by monitoring the invasions and fighting against the infection (Yee *et al*., 2013). Therefore, the purpose of this study was to compare transcriptomes with RNA seq analysis in PBMCs between control and vaccinated piglets. Results from our study will help understand the mechanisms by which PEDV affects the host immune system and develop the rapid diagnosis method and effective viral gene vaccines.

Materials and methods

In this study, the animal experimental procedures

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0030-9923/2017/0003-1107 $ 9.00/0

were performed and approved by the Institutional Animal Care and Use Committee of the National Institute of Animal Science (NIAS), Republic of Korea.

Nine female pigs (14-week-old, 61.52 ± 2.66 kg), Large White x Landrace crossbreds, were housed in individual pens with natural ventilation system. Five pigs were vaccinated into subcutaneous at 1 week with $10^{4.5}\times$medium tissue culture infective dose ($TCID_{50}$) 1 mL of KPED-9 strain known as live strain of PED virus (Green Cross Veterinary Product Co., Ltd., Yongin, Korea) and remained were injected with 1 mL PBS as control. After 2 week, a vaccination was given again for boosting. Blood samples were collected from the internal jugular vein using the heparinized VACUTAINER® tubes (BD, Franklin Lakes, NJ, USA) at 4 week post-vaccination. PBMCs were adjusted to 1×10^6 PBMCs/ml in RPMI 1640 medium.

RNA seq analysis was performed on PBMCs. Total RNA was isolated from PBMCs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and purified using the RNeasy MinElute Cleanup Kit (Qiagen) according to the manufacturer's instructions. Processed RNAs were then sequenced by using NextSeq500 (Illumina Inc, San Diego, CA, USA). The reads were mapped to the pig reference genome (Sscrofa 10.2) in Tophat (v2.0.9, http://ccb.jhu.edu/software/tophat/) using Bowtie2. The aligned reads were added to Cufflinks (v2.2.0, http://cufflinks.cbcb.umd.edu/index.html). Assemblies were merged by Cuffmerge with Cuffdiff. Finally, Cuffdiff takes CXB files produced by Cuffquant to report genes and transcripts that are differentially expressed, and for normalization class-fpkm method was applied. For the DEGs analysis, using status code that cuffdiff provides, genes that only have "OK" status were obtained. After that, 2-fold change was calculated. The up- and down-regulated genes were 622 and 1183, respectively (Supplementary Table I). Gene contents in the identified mRNAs were retrieved from the Sscrofa 10.2 genome assembly using the BioMart (http://www.biomart.org/webcite).

The DAVID functional annotation tool (http://david.abcc.ncifcrf.gov/webcite) was used to perform gene ontology (GO) classification annotation of mRNAs. Also, we assessed the pathway analysis using Pathway Studio 9.0 software (Ariadne Genomics) and constructed a network based on the cellular processes of identified genes in PBMCs. The processes were evaluated by searching the database (ResNet 9.0) with identified genes from RNA-seq analysis.

Results and discussion

The GO analyses revealed 88 GO terms, of which 56 were statistically significant ($P < 0.05$). The significant GO terms were mainly involved in cell growth (GO:0001558), cell size (GO:0008361), cell activation (GO:0050865), cell death (GO:0010941), response to wounding (GO:0009611) and regulation of leukocyte activation (GO:0002694), defense response (GO:0006952), and other basic metabolic processes. There were also some enriched charts with T cell activation (GO:0050863), B cell activation (GO:0050864), cell signaling (GO:0007267) and immune system process (GO:0002684) (Supplementary Table II). We also analyzed the cellular processes using Pathway Studio software (version 9.0) from identified genes between control and experimental pig groups. Here, we showed that the number of gene on network was different compare to imported genes. The imported genes were involved in T-cell response and activation, immune response, cell death, inflammatory response (Supplementary Fig. 1).

On the basis of 1805 DEGs, 18 genes were significantly up-regulated (FDR < 0.05, $P < 0.001$) in PBMC of PEDV vaccinated group compared with unvaccinated group. The characterized 13 DEGs (2-fold up-regulated), such as *v-myb avian myeloblastosis viral oncogene homolog* (*MYB*), *recombination activating gene-1* (*RAG-1*), *epithelial cell adhesion molecule* (*TACSTD1*), *Secale cereale 75k gamma secalin gene* (*75k gamma secalin*), *snRNA* (*U6*), *microRNA mir-155* (*ssc-mir-155*), *microRNA let-7c* (*ssc-let-7c*), *NCK interacting protein with SH3 domain/cadherin, EGF LAG seven-pass G-type receptor 3* (*CELSR3,NCKIPSD*), *cAMP-regulated phosphoprotein, 21kDa* (*ARPP21*), *interleukin 20 receptor beta* (*IL20RB*), *DNA nucleotidylexotransferase* (*DNTT*), *collagen, type V, alpha 2* (*COL5A2*), and *microRNA mir-374a/microRNA mir-374b/microRNA mir-421/microRNA mir-545* (*ssc-mir-374a*) are shown in Supplementary Table I. Especially, the *RAG-1* (approximately 102-fold), as the lymphocyte-specific recombination enzyme, plays a crucial role in lymphoid cell development. *RAG-1* disruption causes a lack of immune deficiency in PBMC including T cell and B cell. Therefore, *RAG-1* was reconstituted by PEDV vaccination. *MYB* (2-fold) is associated with the promoter of *mir-155* gene, which has an important role in immune response. The *MYB* gene directly regulates the expression of *mir-155* (70-fold). The level of *mir-155* was significantly higher in *MYB* over-expressing B cell chronic lymphocytic leukemia patient cells (Vargova *et al*., 2011). These results indicate that *MYB* and *miR-155* increase by PEDV vaccination and associated with immune response. *DNTT* (7-fold) normally expressed in the number of the early lymphoid precursor. *DNTT* has roles in development of T cell in accordance with *RAG-1* and *-2* in human intestine (Taplin *et al*., 1996). Increase in the expression of *DNTT* gene might induce the immune response including lymphoid cell. Overall, our findings

suggest that the representative genes (*e.g. RAG-1, MYB, miR-155, IL20RB,* and *DNTT*) are associated with T cell and B cell activation of immune system. Twenty two genes were significantly down-regulated (FDR < 0.05, $P < 0.001$) in PBMC of PEDV vaccinated group compared to unvaccinated control. The characterized 17 DEGs (2-fold down-regulated), such as *neuron-derived orphan receptor-1 alfa* (*NOR-1*), *heparin-binding EGF-like growth factor* (*HBEGF*), *trafficking protein particle complex 9* (*TRAPPC9*), *lysozyme* (*LYZ*), *von Willebrand factor A domain containing 1* (*VWA1*), *cyclin-dependent kinase inhibitor 1A* (*p21, Cip1*) (*CDKN1A*), *putative ISG12(a) protein* (*ISG12(A)*), *HECT and RLD domain containing E3 ubiquitin protein ligase 5* (*HERC5*), *guanine nucleotide binding protein (G protein), gamma 11* (*GNG11*), *leucine rich repeat neuronal 2* (*LRRN2*), *myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)* (*MX1*), *plasminogen activator, urokinase* (*PLAU*), *DiGeorge syndrome critical region gene 8* (*DGCR8*), *nuclear receptor subfamily 4, group A, member 2* (*NR4A2*), *protein phosphatase 1, regulatory subunit 3B* (*PPP1R3B*), *tumor protein p53 inducible nuclear protein 2* (*TP53INP2*), and sialoadhesin (SIGLEC1) are shown in Supplementary Table II. *HBEGF* (4-fold) is a member of the epidermal growth factor family of growth factors that stimulate growth and differentiation. The expression of *HBEGF* inhibits inflammatory cytokines. The *CDKN1A* is involved in the regulation of transcription, apoptosis, DNA replication and repair, and cell motility (Abbas and Dutta, 2009; Cazzalini *et al*., 2010). *CDKN1A* is a well-known cell cycle arrestor by inhibiting the activity of cycle-dependent kinases (Dotto, 2000). The *PLAU* and its cellular receptors were identified to be associated with the development of inflammatory, immune, and coagulation responses (Del Rosso *et al*., 2011). *PLAU* gene expression was regulated by leukocytes with extracellular matrix for migration among tissues (Ng *et al*., 2015). *NR4A2* is an important nuclear factor linking gastrointestinal inflammation and cancers (Han and Cao, 2012). It serves as a candidate therapeutic target for the inflammation-related gastrointestinal cancers. Finally, 9 genes among 30 candidate genes identified by biological pathway analysis were directly regulated in PEDV vaccinated PBMC (Supplementary Fig. 1). Expression of these genes was validated by real-time PCR. Some genes, however, did not match with those identified by RNA seq analysis between unvaccinated- and vaccinated groups (data not shown). In summary, significantly up- and down-regulated genes were identified and functional analysis of RNA seq data revealed that those genes are associated with cell proliferation, inflammatory response, T cell response, cell death, and immune response. The comprehensive transcriptomes were constructed by using RNA-seq.

In conclusion, our results demonstrate that the bioinformatic analysis of PBMC from pigs vaccinated with PEDV provides us better understanding of precise mechanisms underlying cellular signal transduction pathways of the PED.

Acknowledgments

This work was supported by a grant (PJ00812704) from the Next-generation BioGreen 21 Program (No. PJ00812704), Rural Development Administration, Republic of Korea; and 2016 Postdoctoral Fellowship Program of National Institute of Animal Science, Rural Development Administration, Republic of Korea.

Conflict of interest statement

We declare that we have no conflict of interest.

Supplementary Materials

Supplementary Table I and II, and Figure 1 are available at http://dx.doi.org/10.17582/journal.pjz/2017.49.3.sc1

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