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Global transcriptional profiles in peripheral blood mononuclear cell during classical swine fever virus infection

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ABSTRACT

Classical swine fever virus (CSFV) is an etiologic agent that causes a highly contagious disease in pigs. Laying a foundation to solve problems in its pathogenic mechanism, microarray analysis was performed to detect the gene transcriptional profiles in peripheral blood mononuclear cells (PBMC) following infection with a Chinese highly virulent CSFV strain Shimen. Three susceptible pigs were inoculated intramuscularly with a lethal dose (1.0×10^6 $TCID_{50}$) of CSFV. Pigs showed classical CSF signs, depletion of lymphocytes and monocytes consistent with CSFV infection, and the CSFV genome was also confirmed in the PBMC. The PBMC were isolated at 1, 3, 6 and 9 days post-inoculation (dpi). Total RNA were extracted and subjected to microarray analysis. Data showed that expression of 847 genes wherein 467 genes were known function and the remaining 380 genes were unknown function, and 541 up- and 306 down-regulation, altered after infection. There were 54, 181, 438 and 354 up- and 61, 120, 218 and 145 down-regulated genes presented on 1, 3, 6 and 9 dpi, respectively. These genes were involved in immune response (14.5%), apoptosis (3.3%), signal transduction (7.6%), transcription (4.4%), metabolism (11%), transport (3.9%), development (6.8%) and cell cycle (3.7%). Results demonstrated its usefulness in exploring the pathogenic mechanisms of CSFV.

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1. Introduction

Classical swine fever virus (CSFV) belongs to the genus *pestivirus* within the family *Flaviviridae* and causes a highly contagious viral disease in pigs (Heinz et al., 2004). Classical swine fever (CSF) epidemic usually results in high fever, hemorrhagic syndrome, high mortality, and causes considerable economic loss of the pig industry worldwide, so it was classified as a List A disease by Office International des Epizooties (OIE). Abortions, stillbirths or weak piglets that die within 2 days of birth are also observed in pregnant sows infected. Although some countries have succeeded in eradicating CSF through vaccination and health policies, the disease prevalence still remains variable in other countries (Meuwissen et al., 1999).

Some studies on the pathogenesis of CSFV so far demonstrated that CSFV caused both B and T lymphocytes depletion in the circulatory system as well as in lymphoid tissues (Susa et al., 1992; Pauly et al., 1998), especially depletion of $CD4^{+}$ and $CD8^{high+}$ T lymphocyte subpopulations occurred before the onset of viraemia during CSF (Lee et al., 1999; Summerfield et al., 2001a). The low density blood granulatic cells induced during CSF are targets for virus infection (Summerfield et al., 1998a). Further studies assured that CSFV induces apoptosis in lymphocytes and in bone marrow neutrophil-lineage cells (Summerfield et al., 1998b, 2001b; Sato et al., 2000; Sánchez-Cordón et al., 2002; Choi et al., 2004; Sánchez-Cordón et al., 2005). Bensaude et al. (2004) reported that CSFV induced an initial and short-lived increase in the transcript levels of the proinflammatory cytokines interleukins 1, 6 and 8 at 3 h followed by a second more sustained increase 24 h post-infection. Transcriptional levels for the coagulation factor, tissue factor and vascular endothelial cell growth factor involved in endothelial cell permeability were increased (Bensaude et al., 2004).

As a crucial component of the host immune system, mononuclear cells play critical roles in innate immune defenses as well as in the initiation of the adaptive immune responses, owing to their production of immunomodulatory factors and specific antibodies. The course of mononuclear cells infected by CSFV will be inevitable to lead to all-round cellular response to invaders, including the activa-

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tion of transcriptional factor, the synthesis and secretion of various cytokines. To escape immunological defense, many viruses are capable of replicating in mononuclear cells for altering their function, or resulting in immunosuppression of the host (Chinsakchai and Molitor, 1992; Shimizu et al., 1996; Ramiro-Ibanez et al., 1997).

Recently, the interaction between CSFV and host cells had been reported by several researches (Carrasco et al., 2004; Zaffuto et al., 2007; Borca et al., 2008; Shi et al., 2009). The pathogenic mechanisms involved in the lymphopenia, thrombocytopenia, coagulation disorders and atrophy of the thymus and bone marrow observed in CSF remain unclear. Increasing information about the change of host gene expressions of pigs after CSFV infection is available. Since the mononuclear cells in peripheral blood play central roles in host immune system, it is necessary to investigate a dynamic, global transcriptional profiling of mononuclear cells during CSFV infection.

Various technologies are now available to identify host genes that might be essential for the life cycle of CSFV, important in antiviral defense, or might be activated nonspecifically in CSFV infection. But cDNA microarray analysis is performed easily for the detection of different classes of gene changes in mRNA levels and to assess different levels of gene expression (Zhu et al., 1998; Geiss et al., 2000; Wieland et al., 2004). Using microarray, it is now possible to define changes in gene expression that assess interaction between host cells and viruses, and to obtain specific insights into the molecular mechanism of viral pathogenesis (Brukman and Enquist, 2006).

The outcome of CSFV infection is determined by the convergence of several different virus–host interactions, including those that contribute to virus replication, spread, pathogenic effects on cells, and antiviral responses. To analyze how CSFV modulate the transcription of peripheral blood mononuclear cells (PBMC) genes and interfere with their biological function, Affymetrix GeneChip® Porcine Genome Array was used in this study to explore the gene transcriptional profiles in PBMC at very early (1 dpi), early (3 dpi), mid (6 dpi) and late (9 dpi) stages of CSFV infection. This study was conducted to elucidate the interaction between CSFV and host at cellular level and provide a better understanding of CSFV replication and pathogenesis.

2. Materials and methods

2.1. Animals and virus

Five pigs used in this study were 75 days old, Large White × Landrace, obtained from a BVDV-negative pig farm that BVDV antibody was detected periodically. All pigs were free of specific antibodies by ELISA and viral nucleic acid by RT-PCR or PCR for CSFV, porcine reproductive and respiratory syndrome virus, porcine circovirus type 2 and pseudorabies virus. Five pigs were randomly divided into two groups, the 1st group of three pigs was used for CSFV test, the second group of two pigs was used as normal control. To avoid contaminating each other by secretions and excretions, each pig was housed in an independent and clean room. A highly virulent CSFV strain Shimen was used to challenge.

2.2. Test of pigs infected with CSFV

Three pigs were inoculated intramuscularly with 1 ml each, containing 1.0×10^6 $TCID_{50}$ of the CSFV strain Shimen. The remaining two pigs were mock inoculated with phosphate buffered saline (PBS). Rectal temperature and clinical signs of pigs were recorded. Pre-inoculation blood samples 4 days before inoculation were obtained from pigs regarded as negative control and blood samples were collected aseptically from anterior vena cava at 1, 3, 6 and 9 days post-inoculation (dpi). Blood tubes containing 5% EDTA were used for separation of PBMC as well as lymphocyte and monocyte counts. Cell counts were performed using automated hematology analyzer (Bayer, Germany). The pre-inoculated and inoculated blood samples of three pigs were used for real time RT-PCR and microarray analysis. The two mock pigs were served as negative control for clinical signs observation.

2.3. Isolation of PBMC

EDTA stabilized blood samples were diluted twice in PBS. Diluted bloods were overlaid on Ficoll-Paque (density 1.077 g/l, TBD Sciences, Tianjin, China) and PBMC were isolated from interface after density gradient centrifugation at $800 \times g$ for 20 min. Contaminating erythrocytes were lysed by a single treatment with pH 7.4 NH_4Cl buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$) for 5 min. PBMC were washed three times (pelleted at $500 \times g$, 5 min) using PBS with purity of >99% determined by Wright-Giemsa staining.

2.4. Microarray analysis

Total RNA were extracted from PBMC using Trizol reagent (Invitrogen, Carlsbad, CA) as per manufacturer's instructions, and then digested with DNase I at 37 °C for 15 min to remove contaminating DNA. RNA quantities and qualities were determined by spectrophometer and electrophoresis. The samples with bright bands corresponding to ribosomal 28S and 18S RNA in a ratio of intensities of 1–2:1 were used for microarray analysis and real time RT-PCR.

Affymetrix GeneChip® Porcine Genome Array, which contains 23,937 oligonucleotide transcript probe sets to interrogate 23,256 transcripts in pig, represents 20,201 genes, was obtained from CapitalBio Corporation (Beijing, China), a service provider authorized by Affymetrix Inc. (Santa Clara, CA). Briefly, 1 μg of total RNA was used for cDNA synthesis, then produce biotin-tagged cRNA using GeneChip IVT Labeling kit (Affymetrix). cRNA was hybridized to each GeneChip array for 16 h at 45 °C (Affymetrix GeneChip Hybridization Oven 640). After hybridization, the GeneChip arrays were washed, and then stained with streptavidin-phycoerythrin on an Affymetrix Fluidics Station 450 followed by scanning with the Affymetrix GeneChip Scanner 3000.

The hybridization data were analyzed using GeneChip Operating Software, which uses statistical criteria to generate a 'present' or 'absent' call for genes represented by each probe set on the array. Afterwards, genes with 'absent' scores were filtered out and the remaining genes were analyzed. Significance Analysis of Microarrays (SAM) was used to identify genes that are differentially expressed in CSFV-infected samples compared with negative control samples. SAM identifies genes with statistically significant changes in expression by assimilating a set of gene-specific *t*-tests, and provides an estimate of the false discovery rate (FDR) from randomly generated data. Genes with scores higher than a threshold value or genes with FDR value lower than the threshold value were deemed potentially significant. Furthermore, fold-change analysis which calculates the ratios of geometric means of expression intensities of CSFV-infected samples relative to controls was performed. These ratios were reported as the up- or down-fold change. To select the differentially expressed genes, we used threshold values of ≥ 2 and ≤ -2-fold change between CSFV-infected samples and negative control samples and a FDR significance level of <5%.

2.5. Real time RT-PCR

CSFV genome in PBMC were quantified by SYBR Green I real time RT-PCR amplifying a 115 bp fragment of the 5′ untranslated

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