UNIVERSITI PUTRA MALAYSIA

APOPTOSIS AND TRANSCRIPTOME ANALYSES OF CRANDELL REESE FELINE KIDNEY CELLS FOLLOWING INFECTION WITH FELINE INFECTIOUS PERITONITIS VIRUS STRAIN WSU 79- 1146

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By

AHMAD NAQIB BIN SHUID

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the Degree of Master of Science

June 2013

Dedicated to:

My Father and Mother,

Shuid bin Din

Zainab bte Hashim

My beloved Brother,

Ahmad Fhyrun bin Shuid

Ahmad Nazrun bin Shuid

Ahmad Husaini bin Shuid

Whoever has provided me with care and compassion throughout my life.

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APOPTOSIS AND TRANSCRIPTOME ANALYSES OF CRANDELL REESE FELINE KIDNEY CELLS FOLLOWING INFECTION WITH FELINE INFECTIOUS PERITONITIS VIRUS STRAIN WSU 79- 1146

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Chairman: Prof Abdul Rahman Omar, PhD

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Feline infectious peritonitis (FIP) is a fatal, progressive and immune-augmented disease of cats caused by feline coronavirus (FCoV) infection. FIP pathology is characterized typically by severe systemic inflammatory damage of serosal membranes and widespread pyogranulomatous lesions. In addition, apoptosis of T-cells and changes in cytokine expression are observed in end-stage FIP. Despite of over 40 years of research, the mechanism of IPFV-induced disease and immunity remains unclear. Currently there is no diagnostic protocol that can discriminate the avirulent FCoV from FIPV and there is no effective treatment or vaccine available. In addition, the molecular pathogenesis of feline infectious peritonitis (FIPV) induced disease is poorly characterized. RNA sequencing using next-generation sequencing (NGS) technology allows better quantification of the expression levels of

the entire transcriptome with a high dynamic range. In the present study, analysis on mode of cell death of uninfected and FIPV infected CRFK was performed at different time points and followed by a comparative transcriptome analysis of FIPV strain 79 – 1146 infected Crandell Reese feline kidney (CRFK) cells at 9 hours post-infection was performed using Illumina Genome Analyzer. Sequence reads were assembled and analyzed using CLC bio Genomic Workbench software to generate RNA-seq library. Approximately 98 million sequenced reads were obtained from both uninfected and infected samples. A gene transfer format (GTF) annotated 2X whole genome shotgun sequencing of *Felis catus* from www.ensembl.org were used as reference in the RNA-seq analysis. Gene expression was estimated by calculating read density as 'reads per kilobase of exon model per million mapped reads' (RPKM) whilst, Gene Ontology analysis was performed to establish the function of differentially expressed genes among samples. From a total of 19046 annotated reference genes, 11124 genes were expressed in untreated and 11453 genes were expressed in infected CRFK cells. CLC bio Genomic Workbench further isolated a total of 1837 normalized differentially expressed genes (DEG) using Kal's test comparison feature set up at a false discovery rate (FDR) less than 0.05 (FDR <0.05) with increased and decreased in proportion fold change more or less than 2 ($-2 > X > 2$). Generated up-regulated and down-regulated DEGs were then subjected to bioinformatic analysis separately using Database for Annotation and Integrated Discovery (DAVID) which clustered 1403 DEGs from a total of 1837 normalized DEGs into 135 clusters of up-regulated and 170 clusters of down-regulated DEGs. Genes that belong to apoptosis, cell cycle and immune response clusters, together with the genes that were over- and under-expressed ($-\infty > X > \infty$) and other genes that were speculated of importance in FIPV immunopathology were selected for

further in silico analysis. From a total of 57 speculated DEGs in the cell cycle cluster, 20 genes (Gtse1, Anapc11, Uba7, Nek6, Mcm8, Ptp4a1, Eif4ebp1, Magoh, Ern2, Fbxw4, Cyld, Rad51, Nhej1, Neil, Nthl1, Apitd1, Htra2, Cdk2, Smad3 and Smarcb1) were hypothesized to be important in deregulation of cell cycle during FIPV infection. From the genes that involved in deregulation of apoptosis, at least 8 speculated pro-apoptotic genes (Arghdia, Htra2, Cidec, Nox5, Bnip3l, Ptprf, Smad3 and Traip) and 6 speculated anti-apoptotic genes (Bag4, Ywhaz, Cryab, Adam9, F3, and Api5l1) were speculated to be involved in deregulation of FIPV-induced apoptosis. Meanwhile, 9 deregulated genes (Poll, Sbno2, Polr3b, F3, Smad3, Shb, Nod2, Pdgfb and Fcn3) which might be involved in deregulating immune response. Apart from that, other genes that were not grouped under the immune response cluster such as Raf1, Nfatc2 and Chp2 were also hypothesized to play important role in immune responses against FIPV since previous studies showed that these genes deregulate T cells, B cells and natural killer (NK) cells activity. From a total of 5 over- and 8 under-expressed genes ($-\infty > X > \infty$), only 6 genes with known functions such as cytokine-chemokine genes (CCL-4 and G-CSF), heavy metals and glucocorticoids transcriptionally regulated gene (Mt2a), a nuclear transcription factor (Ankrd1), clathrin associated adaptor genes (Ap1s2) and an integral membrane protein encoding gene (Tgoln2) were identified. Furthermore, the most unique DEG in this study was Smad3 as it was found to be deregulated in all of the selected clusters. SMAD 3 is an intracellular signal transducer and transcriptional modulator as it was found to be deregulated in all the three chosen clusters. In conclusion, it can be suggested that apoptosis and not necrosis is the mode of cell death in FIPV infected CRFK cells while in transcriptomic study, from a total of 1837 significantly DEGs, a total of 57 DEGs were found in cell cycle cluster, 41 DEGs were found in

apoptosis clusters and 16 DEGs were found in immune system cluster. Further *in vitro* and *in vivo* studies are required to evaluate the involvement of these genes in FIPV immunopathology.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai keperluan untuk ijazah Master Sains

ANALISIS APOPTOSIS DAN TRANSKRIPTOMIK SEL GINJAL FELIN CRANDELL REESE YANG TELAH DIJANGKIT DENGAN VIRUS PERITONITIS BERJANGKIT FELIN STRAIN WSU 79-1146

Oleh

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Peritonitis berjangkit felin (FIP) adalah penyakit diperkuat imun progresif yang membawa maut pada kucing disebabkan oleh jangkitan virus korona felin (FCoV). Patologi FIP biasanya dicirikan dengan kerosakan teruk membran serosa akibat inflamasi secara sistematik dan penyebaran lesi pyogranulomatous. Selain itu, sel T apoptosis dan perubahan ekspresi sitokin dapat dilihat pada peringkat akhir FIP. Sehingga hari ini, tidak ada rawatan dan vaksin yang berkesan atau pun protokol diagnostik yang dapat membezakan FCoV yang tidak virulen daripada FIPV. Tambahan pula, patogenesis molekul penyakit teraruh FIPV adalah kurang jelas. Penjujukan RNA dengan menggunakan teknologi jujukan generasi baru (NGS) membolehkan pengkuantitian tepat tahap ekspresi transkriptom menyeluruh dilakukan dengan julat dinamik tinggi. Dalam kajian ini, analisi transkriptom

perbandingan sel ginjal kucing Crandall Reese (CRFK) yang dijangkiti dengan FIPV strain 79-1146 selepas 9 jam dijalankan menggunakan Illumina Genome Analyzer. Bacaan jujukan dibentuk dan dianalisi menggunakan perisian CLC bio Genomic Workbench untuk menghasilkan pustaka RNA-seq. Lebih kurang sebanyak 98 juta bacaan jujukan diperoleh daripada sampel normal dan sampel yang dijangkiti. Jujukan genom shortgun *Felis catus* 2X beranotasi dalam format gene transfer (GTF) daripada www.ensembl.org digunakan sebagai rujukan dalam analisis RNA-seq. Ekspresi gen yang dianggarkan melalui pengiraan bacaan padat dengan mengira bacaan per kilobase ekson per juta bacaan yang dipetakan (RPKM), sementara analisis ontologi gen dilakukan untuk menentukan fungsi gen ekspresi kebezaan sampel. Dari sejumlah 19,046 gen rujukan beranotasi, sebanyak 11,124 gen berjaya diungkap bagi sampel yang tidak dijangkit dan sebanyak 11,453 gen berjaya diungkap bagi sel CRFK yang dijangkiti. CLC bio Genomic Workbench seterusnya mengasingkan sejumlah 1837 gen terungkap kebezaan (DEG) ternormal menerusi perbandingan ujian Kal's dengan kadar penemuan palsu (FDR) kurang daripada 0.05 (FDR<0.05) dan perkadaran kali ganda melebihi atau kurang daripada 2 (-2 > X > 2). Daripada sejumlah 1837 DEGs, hanya 1403 DEGs berjaya dimuat naik ke pangkalan data DAVID secara berkelompok (Kelompok DEGs yang menaik kadar ekspresinya dan DEGs yang menurun kadar ekspresinya). Pangkalan data DAVID kemudiannya berjaya menghasilkan sejumlah 135 kelompok proses yang terdiri daripada DEGs yang meningkat kadar ekspresinya dan 170 kelompok DEGs yang menurun kadar ekspresinya. Gen yang terlibat dalam kelompok proses apoptosis, kitaran sel dan gerak balas imun bersama –sama dengan gen yang terlebih dan terkurang ekspresi (-2 > X > 2) dan gen lain yang dijangkakan penting dalam patologi imun FIPV dipilih berdasarkan info-info yang diperolehi melalui

pembacaan jurnal-jurnal yang berkaitan untuk analisi in silico seterusnya. Daripada sebanyak 57 DEGs yang tersenarai dalam kelompok proses kitaran sel, 20 gen (Gtse1, Anapc11, Uba7, Nek6, Mcm8, Ptp4a1, Eif4ebp1, Magoh, Ern, Fbxw4, Cyld, Rad51, Nhej1, Neil, Nthl1, Apitd1, Htra2, Cdk2, Smad3 dan Smarcb2) dijangkakan penting dalam mempengaruhi kitaran sel semasa jangkitan FIPV. Daripada gen-gen yang terlibat dalam penyahkawalseliaan apoptosis, sekurang-kurangnya sebanyak 8 gen pro-apoptosis (Arghdia, Htra2, Cidec, Nox5, Bnip3l, Ptprf, Smad3 dan Traip) dan 6 gen anti-apoptosis (Ywhaz, Cryab, Adam9, F3, Bag4 dan Api5l1) dijangkakan memainkan peranan penting dalam penyahkawalseliaan apoptosis teraruh FIPV. Manakala, 9 gen penyahkawalseliaan (Poll, Sbno2, Polr3b, F3, Smad3, SHB, Nod2, Pdgfb dan Fcn3) mungkin memainkan peranan dalam penyahkawalseliaan gerak balas imun. Selain daripada itu, gen lain yang tidak dikumpulkan di bawah kelompok proses gerak balas imun iaitu Raf1, Nfatc2 dan Chp2 mungkin memainkan peranan yang penting dalam gerak balas imun terhadap FIPV kerana kajian lepas menunjukkan gen ini terlibat dalam mengawal atur aktiviti sel T, sel B dan sel pembunuh semula jadi (NK). Daripada sejumlah 13 gen terlebih dan terkurang ekspresi ($-\infty > X > \infty$), hanya 6 gen dengan fungsi yang diketahui iaitu gen sitokin-kimokin (CCL4 dan G-CSF), gen yang transkripsinya dipengaruh oleh glukortikod dan logam berat (Mt2a), faktor transkripsi nuklear (Ankrd1), gen adaptor yang berkaitan dengan clathrin (Ap1s2) dan gene yang mengekod protein membran integral (Tgoln2) dikenal pasti. Disamping itu, DEG paling unik dalam kajian ini adalah Smad3, iaitu pemodulat transkripsi dan transduser isyarat intrasel kerana gen ini ditemui dalam ketiga-tiga kelompok proses yang dikaji. Kajian *in vitro* dan *in vivo* selanjutnya diperlukan bagi menilai penglibatan gen-gen ini dalam patologi imun FIPV.

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I certify that an Examination Committee has met on date of viva to conduct the final examination of name of student on his degree thesis entitled " Apoptosis and Transcriptome Analyses of Crandell Reese Feline Kidney Cells Following Infection with Feline Infectious Peritonitis Strain WSU 79-1146" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree.

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DECLARATION

I hereby declare the thesis is my original work except for quotations and citations which have been duly acknowledge. I also declare that it has not been previously or concurrently submitted for any other degree at University Putra Malaysia or other institutions

AHMAD NAQIB BIN SHUID

Date: 24 June 2013

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LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microlitre
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
CCoV	Canine Coronavirus
cDNA	Complementary Deoxyribonucleic Acid
CO_2	Carbon Dioxide
CPE	Cytophatic Effects
CRFK	Crandell Reese Feline Kidney Cell
°C	Degree Celsius
DAVID	Database for Annotation, Visualization and Integrated Discovery
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DEG	Differentially Expressed Genes
FBS	Fetal Bovine Serum
FCoV	Feline Coronavirus
FDR	False Discovery Rate
FECV	Feline Enteric Coronavirus
FIPV	Feline Infectious Peritonitis Virus
GTF	Gene Transfer Format
Gb	Gigabyte

g	Gram
H	Hour
IQR	Interquartile
i.e.	In example
Kb	Kilobase
KEGG	Kyoto Encyclopedia of Gene and Genomes
kDa	Kilodalton
L	Litre
Mg	Miligram
MEM	Minimal Essential Media
MHV	Mouse Hepatitis Virus
Min	Minute
Mins	Minutes
M	Median
ml	Mililitre
NCBI	National Centre of Biotechnology Information
NGS	Next Generation Sequencing
µg	Microgram
ng	Nanogram
OD	Optical Density
ORF	Open Reading Frame
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PE	Paired End
pH	Puissance hydrogen (Hydrogen-ion concentration)
PI	Propidium Iodide

PS	Phosphatidylserine
RIN	RNA Integrity Number
RNA	Ribonucleic Acid
RPKM	Reads per Kilobase of exon model per million napped reads
R	Range
rpm	Rotation per minute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RT	Reverse Transcriptase
SARS-CoV	Severe Acute Respiratory Syndrome Coronavirus
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl sulphate-polyacrylamide gel electrophoresis
SPSS	Statistical Package for Social Sciences
Secs	Seconds
T	Temperature
TAE	Tris-Acetate-EDTA
$TCID_{50}$	Tissue Culture Infectious $Dose_{50}$
TGEV	Transmissible Gastroenteritis Coronavirus
Taq	Thermus aquaticus
Tris	2-amino-2(hydroxymethy)-1,3 propandiol
UPM	Universiti Putra Malaysia
USA	United State of America
UV/Vis	Ultraviolet-visible Spectroscopy
UV	Ultraviolet
w/v	Weight/Volume
v/v	Volume/Volume

CHAPTER 1

INTRODUCTION

Feline coronavirus (FCoV) is a positive single stranded RNA virus that is ubiquitous in cat populations. Depending on the FCoV strains, the virus able to cause a different spectrum of diseases from a highly systemic immune-mediated fatal infection called feline infectious peritonitis (FIP) to asymptomatic mild enteritis. FIP is caused by a virulent form of feline coronavirus known as feline infectious peritonitis virus (FIPV) that arises from mutation of feline enteric coronavirus (FECV) (Vennema et al., 1998). Meanwhile, the mild enteritis in cats is caused by avirulent form of FECV. Several different strains of FIPV and FECV have been studied which include FIPV strain WSU 79-1146 and FECV strain WSU 79-1683, respectively (Pedersen et al., 2008).

FECV infect the cell of the intestinal mucosa and can cause from mild to moderate transient enteritis in kittens (Rottier et al., 2005). This is in contrast to FIPV which can cause fatal systemic disease. FIP was first recognized in the 1950's (Holzworth, 1963) and, FIP is considered the leading cause of death among pedigree cats and cats from shelters (Vennema et al, 1998). In addition, it has been suggested that the presence of cat antibodies might accelerated FIP development (Dewerchin et al., 2006). Despite over 40 years of research, the mechanisms of FIP-induced disease and immunity in cats are still not clear.

FIPV utilize several immune evasion mechanisms to avoid clearance of infected cells by the humoral immune response (Cornelissen et al., 2007). High humoral response without cell mediated immunity (CMI) resulted in most common form of FIP referred to as wet FIP. Wet FIP causes inflammation of the linings of the abdominal viscera, and less commonly of the thoracic organs. On the other hand, poor CMI response leads to dry forms of FIPV, characterized by type IV hypersensitivity (Paltrinieri et al., 1989). Dry FIP is the more chronic form of the disease that often ends up with jaundice, weight loss, diarrhea, ataxic and fever.

Numerous studies have indicated that viruses interact with host's cell cycle to disrupt host to cell function and helps viral replication which commonly lead to deregulation of cell growth and signaling networks (Krajcsi and Wold, 1998; O'Nions and Allday, 2004; Bai et al., 2005). Viral infection might cause G0 to G1 phase and G2 to M phase arrest, G1/S progression, DNA damage, increased in genomic maintenance, increased in DNA replication and increased proliferation activity. Apoptosis play an important role in tissue maintenance, deletion of aberrant cells and during embryonic development (Schutte et al., 1998). Apoptosis is controlled by a diverse range of extracellular and intracellular cell signals which may negatively or positively affect apoptosis. In FIP infected cats, the mechanisms of apoptosis induction in lymphoid tissue are unclear (Haagmans et al., 1996). Hence, knowledge on intracellular signaling activation and function in infected cells might help to unravel the key cellular regulation during viral infection.

Innate and adaptive immunity are two equally important component of the immune system. Innate immunity is rapid and nonspecific while adaptive immunity is

specific but requires time to response after infection. Helper T cells or CD4+ lymphocytes are mediators in adaptive immune response. There are two major subtypes of effector CD4+ T helper cell known as the Type 1 helper T cells (Th1) and Type 2 helper T cells (Th2) (Whitmire et al., 1998). Th1 dominates CD4+ T lymphocytes response in virus infection, which is influenced by the presence of interferon gamma (IFN-γ) and interleukin 12 (IL-12) (Schulz et al., 2009). Th2 with the help of interleukin 10 (IL-10) promote humoral immunity in response to viral infections (Bashyam, 2007). However, the role of humoral immunity in protection against FIPV is controversial and apart from that FIPV infection also impaires CMI responses. Antibody-dependent enhancement (ADE) might play a role in FIPV infection which is likely to be mediated by opsonisation of the virus facilitating viral uptake by macrophages (de Groot and Horzinek, 1995; Corapi et al., 1995). However, the role of ADE in natural infection is not clear as in the fields, cats were most likely to develop FIP on first exposure to FCoV (Addie et al., 1995, 2003).

With the advancement in next-generation sequencing (NGS) technology, RNA sequencing (transcriptome) is an important technique in gene expression study where the collection of various types of RNA including noncoding RNA from a specific cell, a group of cells or even an organism can be analysed. Currently, transcriptome study can be performed using a variety of platforms such as Illumina Genome Analyzer, Life Science's 454 Sequencing and ABI Solid Sequencing (Morozova and Marra, 2008). Hence, RNA sequencing facilitates genome-wide expression studies, which are not influenced by deductive assumptions and provide unbiased approach for investigating the pathogenesis of complex diseases.

Various bioinformatics tools are available to assemble annotate and analyse sequence data obtained from NGS technology. Transcriptomic data analysis can be performed by mapping reads with known reference genomes, and of related species, assembling estimated sequences tags (ESTs) from target species or de novo assembly of the reads that do not require supportive information derived from a related reference genome (Duan et al., 2012). De novo assembly of the transcriptome has some unique challenges such as identification and reconstruction of repetitive regions (Nijkamp et al., 2012) meanwhile mapping reads to related organism may result in loss of information and assembling ESTs requires the existence of comprehensive EST information that may lose specific tissue information (Duan et al., 2012). Analysis using RNA-seq would enable the mapping of short sequence fragments (reads) against reference genome. This new technology makes it possible to study differentially expressed genes, identify exons and introns, mapping their boundaries and the 5' and 3' ends of genes (Twine et al., 2011).

Transcriptome studies using both samples from in vitro and in vivo studies have been used to elucidate complex interaction during host-pathogen interactions (Van Baarlen et al., 2011; Bruno et al., 2010; Connolly et al., 2003). In addition, transcriptome study in cats infected with FIPV will provide information on the mechanisms of FIP infection in cats. So far, there is no study on the transcriptome of FIPV infected cats. This study was undertaken to investigate the in vitro differentially expressed genes in FIPV-infected and noninfected Crandell Rees Feline Kidney (CRFK) cells. To date, there are over 604,560 *Felis catus* contigs deposited in the National Center for Biotechnology whole genome shotgun sequencing project (WGS)

(http://www.ncbi.nlm.nih.gov.ilsprod.lib.neu.edu/Traces/wgs /?val =ACBE01) which of valuable resources of reference for bioinformatic analysis of feline transcriptome.

Hence, the RNA sequences obtained from this study were analysed using reference assembly approach where the data were processed via RNA-seq analysis and Kal's test using CLC bio Genomic Workbench. The differentially expressed genes were further analyzed using DAVID. Apart from that, Annexin V FITC was performed in order to determine apoptotic and necrotic activities in FIPV-infected CRFK cells. From this study, apoptosis is expected to be the major mode of cell death following FIPV infection to susceptible cells and transcriptomic profiling of FIPV infected cells would provide valuable information on gene functions and their interactions during FIPV infection.

The specific objectives of this study were:

1. To determine the mode of cell death in CRFK cells following infection with FIPV strain WSU 79-1146.
2. To extract high quality RNA from uninfected and FIPV-infected CRFK cells and to sequence and analyze the RNA sequences using NGS technology and bioinformatic tools, respectively.
3. To detect differentially expressed genes that of important in cell cycle, apoptosis and immune responses during FIPV infection using bioinformatic tools.

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