UNIVERSITI PUTRA MALAYSIA

GENE EXPRESSION PATTERNS INDUCED BY HUMAN RHINOVIRUS SPECIES B INFECTION USING AN In Vitro MODEL OF HUMAN TYPE II ALVEOLAR PULMONARY EPITHELIAL CELL LINE

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

October 2016

DEDICATION

Specially dedicated to

My beloved Mother and Father

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Doctor of Philosophy

GENE EXPRESSION PATTERNS INDUCED BY HUMAN RHINOVIRUS SPECIES B INFECTION USING AN *In Vitro* MODEL OF HUMAN TYPE II ALVEOLAR PULMONARY EPITHELIAL CELL LINE

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October 2016

Chairman : Professor Zamberi Sekawi, MD, MPath
Faculty : Medicine and Health Sciences

Human rhinoviruses (HRVs) belong to the family *Picornaviridae* are the most commonly isolated viruses in acute respiratory infections. It has been associated with exacerbations of lower respiratory tract infections especially among individuals with chronic respiratory disease such as chronic obstructive pulmonary disease (COPD) and asthma by induction of inflammation through perturbation in airway epithelial cells. Achieving a suitable treatment which can target both virus replication as well as inflammatory response is feasible through better understanding of the virus-cell interactions and host cell response to the infection. Although the differences between HRV species A/B/C have been presented in terms of clinical manifestations and disease prevalence, the experimental data is rather controversial regarding to HRV species B which may be clinically relevant and beneficial as drug targets. Genome-wide transcriptional analysis using microarray technology was used to understand whether human lung epithelial cells infected with HRV species B is associated with specific gene expression pattern at different time points after infection using an *in vitro* system.

HRV-B72 (ATCC® VR-1182™) was propagated in H1HeLa cells and subsequently concentrated by ultracentrifugation and purified through sucrose gradient. Then the virus titer was determined using plaque assay. Human lung alveolar epithelial cell line (A549) was used for subsequent infection and gene expression analysis. Cell viability was measured using both thiazolyl blue tetrazolium bromide (MTT) assay. Total RNA was extracted from HRV-infected and mock-infected cell monolayers at 6, 12, 24 and 48 hpi in three independent replicates. The transcriptional profiles of epithelial cells infected with HRV-B72 was analyzed using high-density oligonucleotide Affymetrix GeneChip ®PrimeView Functional categories enriched in differentially expressed genes at each time points were analyzed by DAVID bioinformatics resources. At the end of the current study, representative genes from different functional groups were selected for validation study using qRT-PCR assay.

The data acquired from experiments analyzing both intracellular and extracellular viral RNA level demonstrated significant replication of the HRV-B72 which further supports the *in vitro* proof for implicating of rhinovirus in lower airway epithelium. In total 991 genes were found differentially expressed during the course of infection. Of these, 459 genes were up-regulated whereas 532 genes were down-regulated. Differential gene expression at 6 hpi (184 genes up-regulated vs. 156 down-regulated) were significantly represented by gene ontologies related to the chemokines and inflammatory molecules indicating characteristic of viral infection. The 75 up-regulated genes surpassed the down-regulated genes (35) at 12 hpi and their enriched ontologies fell into discrete functional entities such as regulation of apoptosis, anti-apoptosis, and wound healing. At later time points of 24 and 48 hpi, predominated down-regulated genes were enriched for extracellular matrix proteins and airway remodeling events. Selected differentially expressed genes (DEGs) form diverse gene ontologies (CXCL8, CCl20, CXCL3, BCL2A1, FOSL1, JUN, EGR1 and DUSP6) showed consistent trend in RT-qPCR assay.

Our data provides a comprehensive image of host response to HRV-B72 infection using *in vitro* system. Profound gene expression modifications was observed in the context of pronounced virus propagation suggesting that HRV-B72 is potentially able to infect alveolar epithelial cells and induce cell transcriptional perturbations. HRV-B72 induced expression of CXC and CC chemokines. Uncontrolled expression of the chemokines can eventually lead to pathological inflammatory reactions. The study suggests the underlying molecular regulatory networks genes which might be involved in pathogenicity of the HRV-B72 and potential targets for further validations and development of effective treatment.

Abstrak tesis yang telah dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

CORAK EKSPRESI GEN TERARUH OLEH JANGKITAN RHINOVIRUS MANUSIA SPESIS B MENGGUNAKAN MODEL *In Vitro* TURUNAN SEL EPITELIAL PULMONARI ALVEOLAR MANUSIA JENIS II

Oleh

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Rhinovirus (HRVs) manusia tergolong dalam keluarga Picornaviridae adalah virus terpencil yang paling biasa pada pesakit dengan jangkitan pernafasan akut. Ia telah sering dikaitkan dengan eksaserbasi jangkitan saluran pernafasan bawah terutama dalam kalangan individu yang mempunyai penyakit pernafasan kronik seperti penyakit penghalang pulmonari kronis (COPD) dan asma. Secara induksi keradangan melalui usikan dalam sel-sel epitelium saluran udara dan bukannya mendorong kesan sitopati. Oleh itu untuk mencapai rawatan sesuai yang boleh menyasarkan kedua-dua replikasi virus dan juga tindak balas keradangan boleh dilaksanakan melalui pemahaman interaksi virus-sel dan tindak balas sel tuan rumah kepada jangkitan yang lebih baik. Walaupun perbezaan antara spesies HRV A, B dan C telah dibentangkan dari segi manifestasi klinikal dan prevalens penyakit, data eksperimen agak kontroversi mengenai perbezaan ekspresi gen tertentu spesies, terutama bagi kumpulan B yang mungkin relevan secara klinikal dan bermanfaat sebagai ubat sasaran bagi kajian akan datang. Kajian ini akan menyediakan kajian ekspresi gen komprehensif sel epitelium paru-paru manusia dijangkiti HRV kumpulan B menggunakan sistem in vitro. Analisis transkripsi genom-lebar (genome-wide transcriptional) menggunakan teknologi mikrotatasusunan digunakan untuk memahami sama ada sel-sel epitelium paru-paru manusia dijangkiti HRV spesies B dikaitkan dengan corak ekspresi gen tertentu pada titik masa yang berbeza selepas jangkitan menggunakan sistem in vitro.

HRV-B72 (ATCC® VR-1182™) telah disebarkan dalam sel H1HeLa dan kemudiannya dipekatkan oleh pengultraemparan dan ditulenkan melalui kecerunan sukrosa. Titer virus tulen telah ditentukan dengan menggunakan asai plak. Turunan sel epitelium paru-paru manusia (A549) telah digunakan untuk analisis jangkitan dan ekspresi gen seterusnya. Kebolehhidupan sel diukur menggunakan kedua-dua asai thiazolyl biru tetrazolium bromida (MTT) dan ujian pewarna pengecualian. Sampel telah disediakan dalam tiga replika berasingan dengan jarak selang minggu. Jumlah RNA telah dikeluarkan pada 6, 12, 24 dan 48 hpi dari ekalapisan sel jangkitan HRV

dan jangkitan pengolokan dalam tiga replikasi bebas. Profil transkripsi sel-sel epitelium dijangkiti HRV-B72 dianalisis menggunakan tatasusunan GeneChip ®PrimeView Affymetrix oligonukleotida berkepadatan tinggi. Kategori kefungsian diperkaya dalam gen terzahir pada setiap titik masa dianalisis oleh sumber bioinformatik DAVID. Pada akhir kajian ini, wakil gen daripada kumpulan kefungsian yang berbeza telah dipilih untuk kajian kesahan menggunakan asai QRT-PCR.

Data yang diperoleh daripada eksperimen menganalisis pada kedua-dua tahap virus RNA intraselular dan extraselular menunjukkan replikasi signifikan HRV-B72 yang seterusnya menyokong buktu in vitro untuk mengaitkan rhinovirus dalam epitelium saluran udara bawah. Keseluruhannya, 991 gen didapati pembezaan ekspresi semasa jangkitan. Daripada jumlah ini, 459 gen adalah pengawalaturan meningkat manakala 532 adalah pengawalaturan menurun. Penyata gen berbeza pada 6 hpi (184 gen pengawalaturan meningkat, vs 156 pengawalaturan menurun) diwakili dengan signifikan dengan gen ontologi berkaitan dengan chemokines dan molekul radang menunjukkan ciri-ciri jangkitan virus. 75 gen pengawalaturan meningkat melepasi gen pengawalaturan menurun (35) pada 12 hpi dan ontologi diperkaya mereka jatuh ke dalam entiti berfungsi diskret seperti peraturan apoptosis, anti-apoptosis dan penyembuhan luka. Pada titik masa 24 dan 48 hpi, gen pengawalaturan menurun yang didominasi telah diperkaya untuk aktiviti protein matriks extraselular dan pembentukan saluran udara. Ekspresi gen berbeza terpilih (DEGs) membentuk pelbagai gen ontology (CXCL8, CCl20, CXCL3, BCL2A1, FOSL1, JUN, EGR1 dan DUSP6) menunjukkan tren yang konsisten dalam asai RT-dPCR.

Data yang diperolehi dalam kajian ini memberikan imej menyeluruh pertama tindak balas sel epitelium manusia kepada jangkitan HRV kumpulan B menggunakan sistem in vitro. Modifikasi ekspresi gen mendalam telah dikenalpasti dalam konteks penyebaran virus ketara mencadangkan HRV-B72 berpotensi untuk menjangkiti sel-sel epitelium alveolar dan mendorong usikan transkripsi sel. HRV-B72 mendorong ekspresi chemokines CXC dan CC. Ekspresi tidak terkawal oleh chemokines akhirnya boleh membawa kepada tindak balas keradangan patologi. Kajian ini mencadangkan gen rangkaian pengawalan molekul tersirat yang mungkin terlibat dalam kepatogenan HRV-B72 dan target berpotensi bagi pengesahan selanjutnya dan pembangunan rawatan yang berkesan.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Superivisory Committee were as follows:

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This is to confirm that:

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LIST OF ABBREVIATIONS

ALRIs	Acute lower respiratory infections
AOM	Acute otitis media
ARIs	Acute respiratory infections
AURIs	Acute upper respiratory infections
BLAST	Basic Local Alignment Search Tool
CPE	Cytopathic effect
COPD	Chronic obstructive pulmonary disease
DAVID	Database for Annotation, Visualization and Integrated Discovery
dsRNA	double-stranded RNA
DE	Differentially expressed gene
FBS	Fetal bovine serum
g	Gram
GO	Gene ontology
hpi	hour post infection
HRV	Human rhinovirus
ICD	International Classification of Disease
IFN	Interferon
IRES	Internal ribosome entry site
ISGs	Interferon stimulated genes
MAPK	Mitogen-activated protein kinases
MTT	Thiazolyl blue tetrazolium bromide
Mx	Myxovirus-resistance
NTC	Negative template control
OAS	2′,5′-oligoadenylate synthetize

OD	Optical density
ORF	Open reading frame
PCA	Principle Component analysis
PKR	Protein kinase R
RMA	Robust Multichip Analysis
RNA	Ribonucleic acid
RTIs	Respiratory tract infections
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TLR	Toll-like receptor
TNS	Trypsin neutralizing solution
UTR	Untranslated region
µg	Microgram
µl	Microlitre

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background of the study

Respiratory tract infections (RTIs) are diseases which affect nasal and bronchial passages and the lungs. They are divided into acute infections such as bronchitis, bronchiolitis and pneumonia, and chronic respiratory disease including chronic obstructive pulmonary disease (COPD) and asthma (WHO, 2015). Acute respiratory infections (ARIs) are classified as acute upper respiratory infections (AURIs) or acute lower respiratory infections (ALRIs). According to the International Classification of Disease (ICD), AURIs are including acute nasopharyngitis (common cold), acute sinusitis, acute pharyngitis, acute tonsillitis, acute laryngitis and tracheitis. ALRIs are defined as those infections that affect airways below the epiglottis with manifestations of laryngitis, tracheitis, bronchitis, bronchiolitis, lung infections or any combination of them, or with upper respiratory infections (Lanata *et al.*, 2004). ALRIs have remained the third top major killer after ischaemic heart disease and stroke worldwide among all age groups during past decade and the first leading cause of death in low income countries (WHO, 2014).

Viral and bacterial pathogens are major cause of RTIs and viruses constitute much higher proportion of the infections, although respiratory viral infections result in only small proportion of severe or fatal outcome of the illnesses (Berman, 1991). Respiratory syncytial virus (RSV), human rhinovirus (HRV), influenza virus (IFV) parainfluenzavirus (PIV), adenovirus (ADV) are mostly implicated in ARIs in children. Other virus types including human metapneumovirus (HMPV), human coronaviruses (HCoV), bocavirus (HBoV) have also been implicated as aetiological agents of ARIs (Arden *et al.*, 2006; Kesson, 2007).

HRV was initially isolated by Price (1956) and Pelone (1957) using rhesus monkey kidney cultures infected with nasopharyngeal samples of patients with colds. HRVs in addition to other important pathogens including poliovirus, enterovirus and hepatitis A virus belong to the family *Picornaviridae*. HRVs are the most commonly isolated viruses in patients with ARIs and they cause approximately half of all common cold illnesses (Kusel *et al.*, 2006; Piotrowska *et al.*, 2009). HRV infection has been detected in similar frequency with RSV among hospitalized children with ARI (Miller *et al.*, 2009; Piotrowska *et al.*, 2009). HRV infections could be associated with asymptomatic to wheezing and pneumonia disorders among previously healthy individuals (Chung *et al.*, 2007; Peltola *et al.*, 2008). It has been frequently associated with lower respiratory tract infections especially among children with chronic disease (Cheuk *et al.*, 2007; Peltola *et al.*, 2009). Predominant clinical features associated with different age groups infected with HRV are including: bronchiolitis and pneumonia among young children, exacerbations of asthma in older children, exacerbations of COPD and pneumonia in older age adults (Papadopoulos, 2004; Peltola *et al.*, 2009). Studies also proposed that symptomatic

infancy HRV infections such as expiratory wheezing may be a risk factor for development of childhood wheezing (Kotaniemi-Syrjänen *et al.*, 2003; Lemanske *et al.*, 2005).

Similar to the other respiratory viruses, human airway epithelial cell is the primary target for HRV infection (Mosser *et al.*, 2002; Biacchesi *et al.*, 2006). Rhinovirus can infect epithelial cells of the alveoli and cause interstitial pneumonia and hyperplasia desquamation of the alveolar lining cells (Imakita *et al.*, 2000). Although the epithelial layer of the respiratory system serves as a barrier against pathogens in normal condition, it also plays critical role in infectious and inflammatory process, as well. Upon lung exposure to infectious agents, leukocytes are activated and migrated to the site of the infection through secretion of variety of immunoregulatory factors including pro-inflammatory molecules by airway epithelial cells. Although several studies have been conducted on HRV using primary human airway epithelial cells (Chen *et al.*, 2006; Proud *et al.*, 2008), it has been shown that human alveolar adenocarcinoma cell line (A549) with the properties of type II like alveolar epithelial cells is also a susceptible cell line and can induce chemokine expression in response to HRV infection (Johnston *et al.*, 1998; Kotla *et al.*, 2008). Therefore, the A549 cell line can be used as a model to study the response of lower airway epithelial cells to HRV infection.

HRV induced exacerbation of several diseases such as asthma and COPD is likely initiated by increased airway inflammation rather than through cytopathic effects on epithelial cells. On the other hand, cell cytotoxicity which is obvious in other viruses such as influenza is not a common phenomenon in HRV (Mosser *et al.*, 2002; Proud *et al.*, 2008). Therefore, better understanding of the virus-cell interactions and host cell response to the HRV infection is fundamental in order to search for suitable treatment with potential to target both virus replication and inflammatory responses. Investigation of virus induced host transcriptional changes can show virus pathogenesis as well as host antiviral response which provides the evidences to the disease manifestations caused by the virus (Leong *et al.*, 2005; Tang *et al.*, 2005).

1.2 Problem Statement

Understanding of the HRV pathogenesis is mandatory in order to develop an effective antiviral treatment and intervention strategies. Global host cell transcriptional response to HRV has been investigated in several *in vitro* and *in vivo* studies (Chen *et al.*, 2006; Proud *et al.*, 2008; Bochkov *et al.*, 2009). The focus of the studies was mainly on the HRV-A virus species. Therefore, it remains unclear whether there are any differences in terms of host cell response against different HRV species and strains and if these differences could be resulted in disease outcomes. The studies have found almost indistinguishable gene expression pattern between HRV-A major and minor groups using *in vitro* primary cell culture system. Although clinico-epidemiological studies have revealed specific features associated with HRV species, the transcriptomic data is rather limited and have not been extensively understood with respect to different species (Lau *et al.*, 2009; Etemadi *et al.*, 2013). The current gene expression analysis of HRV-B could help to understand

gene expression pattern and virus pathogenicity which might be clinically relevant and beneficial as drug targets for further studies.

1.3 Significance of study

The results which will be obtained in current study will help to understand the infection process of the HRV species B as an important human pathogen in the epithelial lung cells and will furnish a foundation to understand the pathogenesis by investigating the role of the induced genes and the pathways in future studies. The data obtained in this study will identify the novel genes related to different functional categories such as pro-inflammatory or antiviral response which may have impact on pathogenicity or antiviral state against HRV. The differences identified here may highlight different strategies of treatment depending on HRV species.

1.4 Hypothesis

In the current study we hypothesized that HRV-B infection could deregulate expression of genes in a timely manner with potential to introduce novel targets attributed to species B for further investigations. A discrete gene annotation and functional categories could be enriched along with development of infection. To address this hypothesis, we performed gene expression analysis of the RNA samples obtained from alveolar epithelial cells infected with of HRV-B using Affymetrix Genechip technology.

1.5 Objectives

➢ General objective

Genome-wide transcriptional analysis was employed to explore whether *in vitro* infection of human lung epithelial cells with HRV-B is associated with specific gene expression pattern in order to improve understanding of HRV-B pathogenesis and to introduce novel cellular targets for developing HRV treatment.

➢ Specific objectives

- To determine growth properties and pathogenicity of HRV-B72 in A549 cell line.
- To determine cytotoxicity of HRV-B72 infection on A549 cells
- To determine differentially expressed (up and down-regulated) genes in A549 epithelial cell line infected with HRV-B72 virus compared at 6, 12, 24 and 48 hour post infection (hpi).
- To investigate gene annotation and functional ontologies enriched in differentially expressed genes at 6, 12, 24 and 48 hpi.
- To validate differentially expressed genes from diverse functional groups using RT-qPCR.

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LIST OF PUBLICATIONS

Etemadi, M. R., Ling, KH, Chee, H., Abidin, SZ. Sekawi, Z., (2017), Rhinovirus infection induces a differential gene expression pattern in pulmonary epithelial cells, accepted: PLOS ONE.

Mohammad Reza Etemadi, Michael KH Ling , Chee Hui Yee1 and, Zamberi Sekawi1 'Early Stage Gene Expression Profile of Human Lung Adenocarcinoma Epithelial Cell Line Infected with Human Rhinovirus group B' Infectious Diseases & Microbial Genomics, 7th & 8 th April, 20152015, IOI Resort City, Malaysia

Etemadi, M. R, Othman, N., Savolainen-Kopra, C., Sekawi, Z., Wahab, N. Sann, L., (2103), Biodiversity and clinico-demographic characteristics of human rhinoviruses from hospitalized children with acute lower respiratory tract infections in Malaysia, Journal of Clinical Virology, 58(4), 671-677.

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