Systems biology of human cells responded to influenza B virus infection

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ชีววิทยาเชิงระบบของเซลล์มนุษย์ที่ตอบสนองต่อการติดเชื้อไวรัสไข้หวัดใหญ่ชนิด บี

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กฤษฎา สิริวาสนาเมธา : ชีววิทยาเชิงระบบของเซลล์มนุษย์ที่ตอบสนองต่อการติดเชื้อไวรัสไข้หวัดใหญ่ชนิด บี. (Systems biology of human cells responded to influenza B virus infection) อ.ที่ปรึกษาหลัก : รศ. ดร.สัญชัย พยุงภร, อ.ที่ปรึกษาร่วม : อ. นพ.ไตรรักษ์ พิสิษฐ์กุล

เชื้อไวรัสไข้หวัดใหญ่ชนิด บี เป็นสมาชิกในวงศ์ *Orthomyxoviridae* ทำให้เกิดโรคไข้หวัดใหญ่ในมนุษย์ส่งผลให้เกิดปัญหาทางสาธารณสุขที่สำคัญทั่วโลก การศึกษาทางชีววิทยาเชิงระบบเป็นการศึกษาโดยใช้การวิเคราะห์หาความเชื่อมโยงของระบบโมเลกุลต่างๆ เป็นประโยชน์ต่อการทำนายการเปลี่ยนแปลงทางชีววิทยาอย่างเป็นระบบ ภายใต้การเปลี่ยนแปลงของเวลาและสภาพแวดล้อม ในการศึกษานี้การวิเคราะห์แบบมัลติโอมิกส์ถูกนำมาใช้ในการศึกษาการตอบสนองของเซลล์มนุษย์ต่อการติดเชื้อไวรัสไข้หวัดใหญ่ชนิด บี โดยศึกษาการแสดงออกของยีนต่างๆ ในทรานสคริปโตมซึ่งประกอบด้วย mRNA, lncRNAs และ miRNAs รวมถึงโปรติโอม ด้วยเทคโนโลยี high-throughput เช่น เทคโนโลยีเอ็นจีเอส และแมสสเปกโตรเมตรี ผลการศึกษาพบว่าในเซลล์ของมนุษย์ที่มีการติดเชื้อไวรัสไข้หวัดใหญ่ชนิด บี มีการเปลี่ยนแปลงระดับการแสดงออกของทั้ง mRNAs, lncRNAs, miRNAs และโปรตีน พบว่ายีนบางกลุ่มมีระดับการแสดงออกที่สูงขึ้น โดยพบทั้งในเซลล์ที่ติดเชื้อไวรัสไข้หวัดใหญ่ชนิด บี เชื้อสายวิคตอเรีย และยามากาตะ บางยีนตอบสนองแบบจำเพาะต่อเชื้อสายของไวรัส ในขณะที่ไม่พบกลุ่มยีนที่ถูกลดระดับการแสดงออกร่วมระหว่างไวรัสไข้หวัดใหญ่ชนิด บี ทั้งสองเชื้อสาย ทั้งนี้พบว่ายีน *MX1* มีการเพิ่มระดับการแสดงออกทั้งในระดับ mRNAs และโปรตีน โดยพบการเพิ่มการแสดงออกทั้งสองเชื้อสายของไวรัสไข้หวัดใหญ่ชนิด บี การแสดงออกของ lncRNAs พบว่ามีการเปลี่ยนแปลงระดับการแสดงออกอย่างจำเพาะต่อเชื้อสายของไวรัส สำหรับ miRNAs พบว่ามีการเปลี่ยนแปลงระดับการแสดงออกทั้งเพิ่มขึ้นและลดลง โดยจากการทำนายพบว่า miRNAs ดังกล่าวสามารถจับกับยีนเป้าหมายของเซลล์มนุษย์อย่างหลากหลาย นอกจากนี้ miR-30e-3p ที่พบว่ามีการเพิ่มการแสดงออกในกลุ่มเซลล์ที่ติดเชื้อไวรัส พบว่าสามารถจับกับยีน NA และ NP ของไวรัสได้โดยตรง ส่งผลให้เกิดการยับยั้งกระบวนการเพิ่มจำนวนของไวรัส

สาขาวิชา ชีวเคมีทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2561 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORD: Systems biology, Influenza B virus, microRNAs (miRNAs), Long non-coding RNAs (lncRNAs), Proteomics

Kritsada Sirivassanametha : Systems biology of human cells responded to influenza B virus infection. Advisor: Assoc. Prof. SUNCHAI PAYUNGPORN, Ph.D. Co-advisor: TRAIRAK PISITKUN, M.D.

Influenza B virus is a member of *Orthomyxoviridae* family which can cause influenza in human and affect to worldwide health problem. Systems biology is the computational modeling of molecular systems which is useful to predict how these biological systems change over time and under different conditions. In this study the multiomics was used to study human cellular response to influenza B virus infection. The transcriptome including mRNAs, lncRNAs and miRNAs and proteome were investigated by high-throughput technologies such as next-generation sequencing and mass spectrometry. The results showed that in human cells infected with influenza B virus, transcriptome including mRNAs, lncRNAs and miRNAs and proteome were changed. Some genes were up-regulated responding to the influenza B virus infection for both Victoria and Yamagata lineages. Some were lineage specific. Whereas there was no similar down-regulated genes for both lineages. The *MX1* genes was found to be up-regulated in both mRNAs and protein level in both Victoria and Yamagata lineages. For lncRNAs, the response to influenza B virus infection seems to be lineage specifically. The miRNAs profiling also showed the up-regulated and down-regulated miRNAs which were predicted to target several of human genes. In addition, the up-regulated miR-30e-3p was selected for functional study and found that it can directly target viral NA and NP inhibiting influenza B virus production.

Field of Study: Medical Biochemistry Student's Signature .....

Academic Year: 2018 Advisor's Signature .....

Co-advisor's Signature .....

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Kritsada Sirivassanametha

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Chapter 1 Introduction

Background and Rationale

Influenza B virus is a member of enveloped RNA virus in the family *Orthomyxoviridae* (1). Its genome contains 8 negative single strands RNA:polymerase basic-1 (PB1), PB2, polymerase acidic (PA), haemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M), and nonstructural protein (NS) (2). Influenza B virus also causes influenza, which is a worldwide infectious problem. The clinical symptoms generally similar to the infection of influenza A viruses including fever, muscle aching, headache, dry cough, fatigue and nasal congestion (3). There are several differences between influenza B virus and influenza A virus such as no subtypes in influenza B while the influenza A has varieties of HA and NA genes which can be used to in subtype classification (4). Genetic reassortment is a major process of evolution for influenza B virus producing new recombinant genome supporting viral adaptation, which can cause epidemic and pandemic occasionally around the world (5). Currently, two influenza A subtypes and 1 influenza B lineage are included in current trivalent seasonal influenza vaccines.

Even the influenza vaccine and antiviral drugs are available but the effectiveness of the vaccine and drugs are considerably low. The average percent effective of seasonal flu vaccination during 2004-2018 are around 30-50% (6-13). The anti-neuraminidase which used for influenza B infection treatment was also suggested to be more effective than influenza A infection (14) but some reports showed that the antiviral drug resistance was also found in influenza B (14, 15). Leading to the interest in searching for new alternative treatment for influenza B virus infection. The strategy

might be start by understanding the human cellular response to influenza B virus infection.

Systems biology is the computational modeling of molecular systems and the integrative interpretation of ever larger postgenomic datasets are accepted as useful tool for biological research (16). It has been responsible for some of the most important developments in the science of biomedical research. Systems biology is useful to predict how these biological systems change over time and under varying conditions, and to develop solutions to the health and environmental issues (17). Multi-omics refers to a systems biology analysis approach in which the data sets are multiple "omes", such as the genome, proteome, transcriptome, epigenome, and microbiome. This type of study uses the data from several omics technologies to understand the specific biological condition (18).

The insight of biological process of human cellular response in influenza B virus infection is still rare and unclear. It might be influenced by early studies that concluded that influenza B posed less of a public health problem than influenza A. Furthermore, because of the ability of influenza A to cause severe pandemics, it is more frequently a topic of press coverage than influenza B. In contrast to the popular opinion that influenza B has minimal impact, there are indications that the impact of influenza B is substantial. Therefore, this study aims to understand the human cells response to influenza B virus infection by using multi-omics study.

Research questions

1. Which human miRNAs change their expression level when infected with influenza B virus?

2. Which genes of human cells that change their expression levels when cells infected with influenza B virus?

3. Which LncRNAs changes the expression level when cells infected with influenza B virus?

Objectives

1. To find the candidate human miRNAs, lncRNAs, mRNAs and proteins that change their expression levels when infected with influenza B virus.

2. To identify the interaction between multiomes of responses to influenza B virus infection.

Hypotheses

1. Influenza B virus infection alters the cellular miRNAs, lncRNAs, mRNAs and proteins expression which lead to the host responses to viral infection.

2. The responded miRNAs, lncRNAs, mRNAs and proteins interact to each other playing roles in cellular responses to influenza B virus infection.

Key words

Systems biology

Influenza B virus

microRNAs (miRNAs)

Long non-coding RNAs (lncRNAs)

Proteomics

Operational definition

MiRNA mimics are small, chemically modified double-stranded RNAs that mimic endogenous miRNAs and enable miRNA functional analysis by up-regulation of miRNA activity.

MiRNA inhibitors are small, chemically modified single-stranded RNA molecules designed to specifically bind to and inhibit endogenous miRNA molecules and enable miRNA functional analysis by down-regulation of miRNA activity.

Mimics negative control are the double-stranded RNAs which designed to be negative control for miRNA mimics its sequences is not complement to human genes.

3'UTR reporter assay is the assay validating the direct binding of miRNA and its predicted target gene by ligating the target region into the 3'UTR of reporter gene (in this study is firefly luciferase) and measuring the reporter gene expression.

Relative luciferase activity refers to the expression ratio between the Firefly luciferase as the reporter gene and the *Renilla* luciferase as the control.

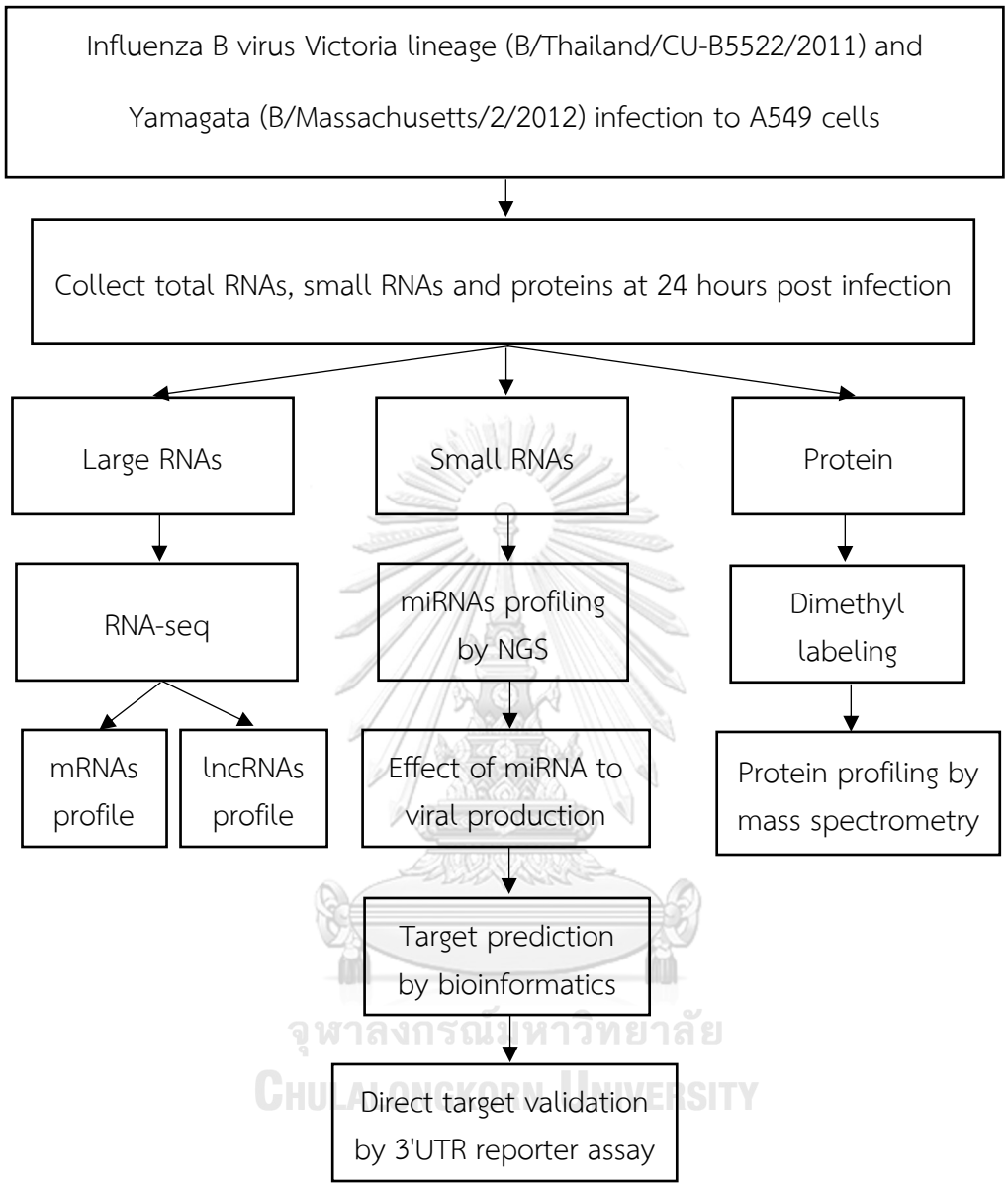
Expected Benefits and applications

1. Responded human miRNAs to influenza B virus infection at 24 and 48 hours post infection

2. Function of the responded miRNAs which play important role in viral gene silencing or viral replication inhibition

3. Two international publications

Procedure framework



Chapter 2 Literature review

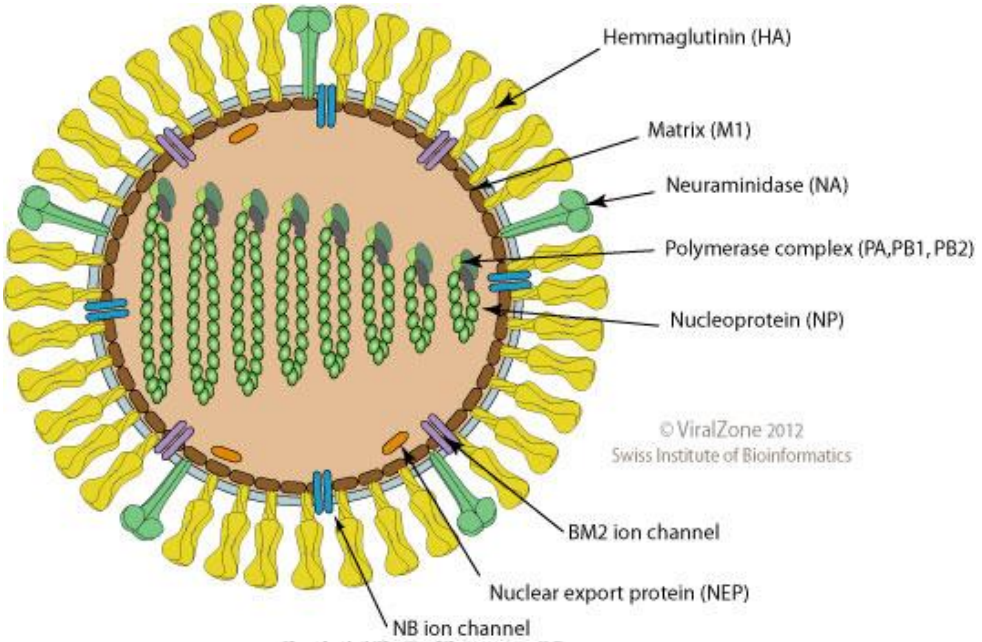
Influenza B virus

Influenza viruses are the global health problem in which each year thousands of people contract influenza viruses and develop an acute respiratory infection. The clinical symptoms which usually be classified as ILI (Influenza-like Illness), including fever, muscle aching, headache, dry cough, fatigue and nasal congestion. The causative agents of these infections are influenza A and B viruses. Although influenza B has been long overshadowed by influenza A, recent surveillance and epidemiological data suggest that it contributes to a higher global health problem of circulation and disease than traditionally thought (19).

Influenza B virus is a member of *Orthomyxoviridae* family. It is only known to infect humans, seals (1) and ferrets (20). The first identified influenza B virus was B/Lee/40 in 1940 (21). And in 1983, the emergence of a second lineage of influenza B was reported (22). Influenza B viruses can be classified into two major lineages base on the difference of HA glycoprotein: B/Victoria/2/87-like and B/Yamagata/16/88-like. Since 2001, both influenza B lineages have been co-circulating in each influenza season different to the pattern of previously dominance by a single lineage that occurred between 1985 and 2000 (23).

The virus particle is 80–120 nanometers in diameter and usually roughly spherical. The viral particles of all influenza viruses are similar in composition (24). These are made of a viral envelope containing two main types of glycoproteins, wrapped around a central core (Figure 1A). The central core contains the viral RNA genome and other viral proteins that package and protect this RNA (25).

(A)



(B)

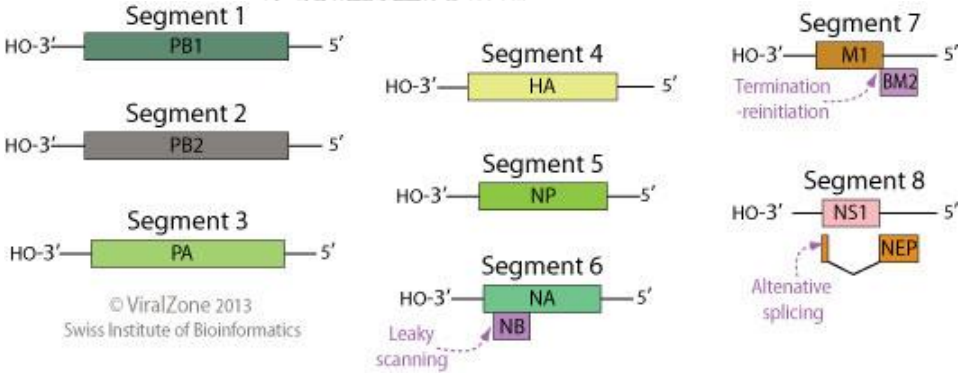


Figure 1 Influenza B virus

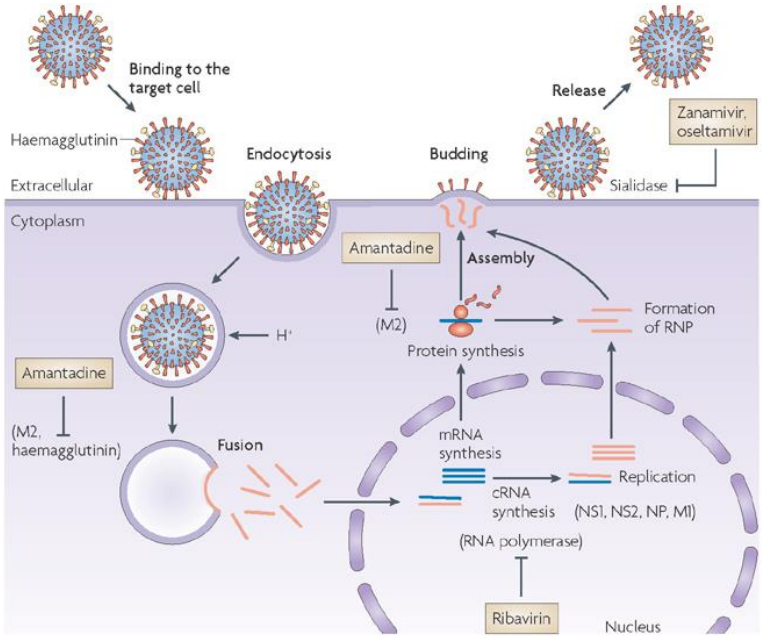
(A) The enveloped virion of influenza B virus

(B) RNA segments and coding proteins of influenza B virus

Even the influenza B virus is classified as the member of *Orthomyxoviridae* family like the influenza A virus by sharing similar structure such as the negative-sense, single stranded, segmented RNA genome. However, influenza B viruses have features distinctive from influenza A viruses that classified into a different genus. The genome of influenza B consists of 8 segments of single strand RNAs encoding 11 proteins: PB1, PB2, PA, HA, NP, NA, NB, M1, BM2, NS1 and NEP with some differences from influenza A viruses (Figure 1B). The HA and NA surface proteins of influenza B virus are antigenically diverse from those of influenza A viruses. Due to a lack of alternative protein production, influenza B virus encodes fewer viral proteins than the influenza A viruses including PB1-F2, N40, PA-X, and PA-M. But influenza B virus encodes NB protein from the NA gene. The NB protein is an ion-channel transmembrane protein that is incorporated into virions and required for efficient replication *in vivo* but is dispensable for *in vitro* growth (26). The matrix BM2 protein of influenza B viruses performs an ion channel function similar to the M2 of influenza A viruses which is resistant to the adamantane class of antiviral drugs (27).

Influenza B virus has no subtypes, in contrast of influenza A which has various subtypes according to variety of HA and NA genes (28). Genetic reassortment is a major process of evolution for influenza B virus in producing new recombinant genome supporting viral adaptation, which can cause epidemic and pandemic occasionally around the world. Previous studies suggested that the rates of evolutional rate of influenza type B viruses are 2-3 times slower than in influenza A (29). Influenza A viruses are able to undergo antigenic shift by genetic reassortment between different subtypes, while influenza B virus reassorts to make various mechanism of deletion, insertion, and substitution within different co-circulating strains.

Influenza B virus can replicate only in living cells (30). Its infection and replication is a multi-step process (Figure 2) : first, the virus has to bind to and enter the cell, next deliver its genome to produce new copies of viral proteins and RNA, then assemble these components into new viral particles, and lastly exit the host cell (24). Influenza B virus binds to the sialic acid receptors on the surfaces of host epithelial cells through hemagglutinin (31). After the hemagglutinin is cleaved by a protease, the viral particle is imported into the cell by endocytosis (32). The intracellular details of viral replication and cellular response are still being explicated. It is known that in the cytoplasm, the acidic conditions in the endosome because part of the hemagglutinin protein fuses the viral envelope with the vacuole's membrane. Then the M2 ion channel allows protons to move into the viral particle. The acidic condition causes the core of the virus to disassemble and release the viral RNA and core proteins (24). The M2 ion channel can be blocked by M2 inhibitor drugs such as amantadine (33). The core proteins and viral RNA form a complex that is transported into the nucleus, where the RNA-dependent RNA polymerase begins transcription of positive-sense viral RNA (34). The viral RNA is also exported into the cytoplasm for translation. Negative-sense viral RNAs that form the genomes of future viruses are assembled with RNA-dependent RNA polymerase and other viral proteins forming a new virion. The mature virus buds off from the cell in a sphere of host phospholipid membrane, acquiring hemagglutinin and neuraminidase with this membrane coat. As before, the viruses adhere to the cell through hemagglutinin; the mature viruses detach once their neuraminidase has cleaved sialic acid residues from the host cell (31). After the release of new influenza viruses, the host cell dies.



(35)

Figure 2 Replication of influenza virus

Moreover, the viral infection or the synthesized viral protein can alter the cellular activities or induce the cellular response such as degrading cellular mRNA and inhibiting translation of host-cell mRNAs (36). This alteration may cause by either the direct interaction between viral product and cellular mRNA or the alteration of cellular miRNAs expression.

Systems Biology

Systems biology is an approach in biomedical research to understanding the larger picture be it at the level of the organism, tissue, or cell by putting its pieces together. It is different from the reductionist biology, which involves taking the pieces apart (16). Systems biology is the computational modeling of molecular systems and the integrative interpretation of ever larger postgenomic datasets are accepted as useful tool for biological research. It has been responsible for some of the most important developments in the science of biomedical research. It is useful to predict how these systems change over time and under varying conditions, and to develop solutions to the health and environmental issues. This advantage can be applied to discover new biomarkers for disease, stratify patients based on unique genetic profiles, and target drugs and other treatments (17).

Multi-omics study is an approach for systems biology that integrate the data sets of different omic groups during analysis. By multi-omics integrates diverse omics data to find a coherently matching geno-pheno-envirotype relationship or association (18). The different omic strategies employed during multi-omics are genome, proteome, transcriptome, epigenome and microbiome. Transcriptomics is an approach used to identify the qualitative and quantitative RNA levels of their expression. Although only 2% of the DNA is translated to protein, almost 80% of the genome is transcribed (37). This includes the coding RNA, short RNA, including microRNA, piwi RNA, small nuclear RNA. It is crucial to understand which transcripts are expressed at a time in a specific condition. Apart from next generation sequencing, probe-based assays, and RNA-seq are also used in this approach. Proteomics is involved in identifying protein levels, modifications, and interactions at the level of genome (38).

The majority of proteins are regulated through post-translational modifications, such as phosphorylation, acetylation, ubiquitination, nitrosylation, and glycosylation. These modifications are involved in maintaining cellular structure and function. Mass spectroscopy based techniques are being used to analyse the global proteomic changes and quantifying the post translational modifications.

microRNAs

MicroRNAs (miRNAs) are small non-coding RNAs with approximately 22 nucleotides in length which play an important role in regulation of gene expression (39). The miRNAs biosynthesis transpires originally in nucleus where hundreds and thousands of nucleotides with hairpin structures, called primary miRNAs (pri-miRNAs) were transcribed (Figure 3). Then the primary miRNAs are cropped and trimmed to 60 to 100 nucleotides long with a stem loop structure called precursor miRNAs (pre-miRNAs). The pre-miRNAs are then exported to the cytoplasm by Exportin-5 and then processed by Dicer containing RNaseIII endonuclease activity (40). The Dicer removes the loop region of the hairpin, and releases the mature miRNA duplexes which approximately 22 nucleotides in length with 2 nucleotides overhanging on both 5’ and 3’ ends. As soon as the miRNA duplexes assembled with RNA induced silencing complex (RISC) and then one strand of miRNA is removed by a helicase activity of the RISC, the remaining miRNA strand guides the RISC to a distinctive target mRNA via base pairing (41). A complementary bindinging between miRNA and target mRNA leads to silencing activity through either mRNA degradation or translational repression. Therefore, miRNAs play an imperative and foremost undertaking in the regulation of gene expression in terms of gene silencing (42).

MiRNAs are involved in various biological processes, including development, differentiation, apoptosis and proliferation. Previous study suggested that miRNAs also evolve in viral infection response; hsa-miR-32 reduces the accumulation of the primate foamy virus type 1 (PFV-1)), miR-323 miR-491 and miR-654 targets the PB1 gene of H1N1 influenza A virus (A/WSN/33) and inhibit viral replication. The miRNAs response to influenza A virus infection were discovered in previous studies. But the insight of biological process of miRNAs involve in influenza B virus infection is still rare and unclear.

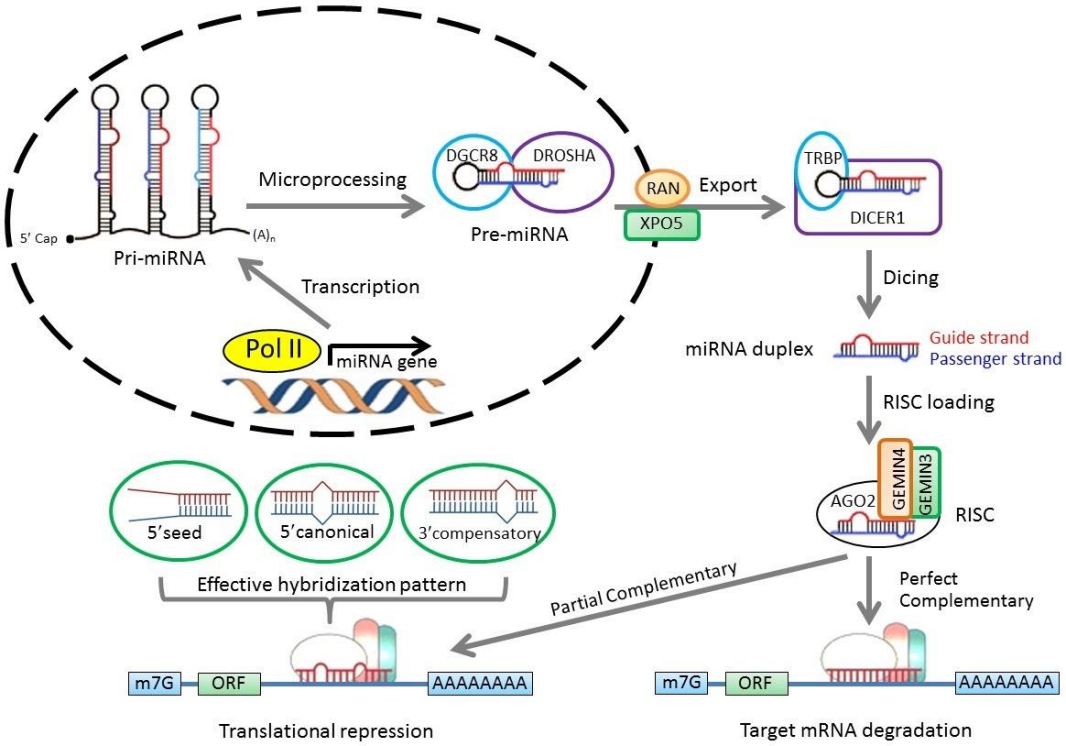
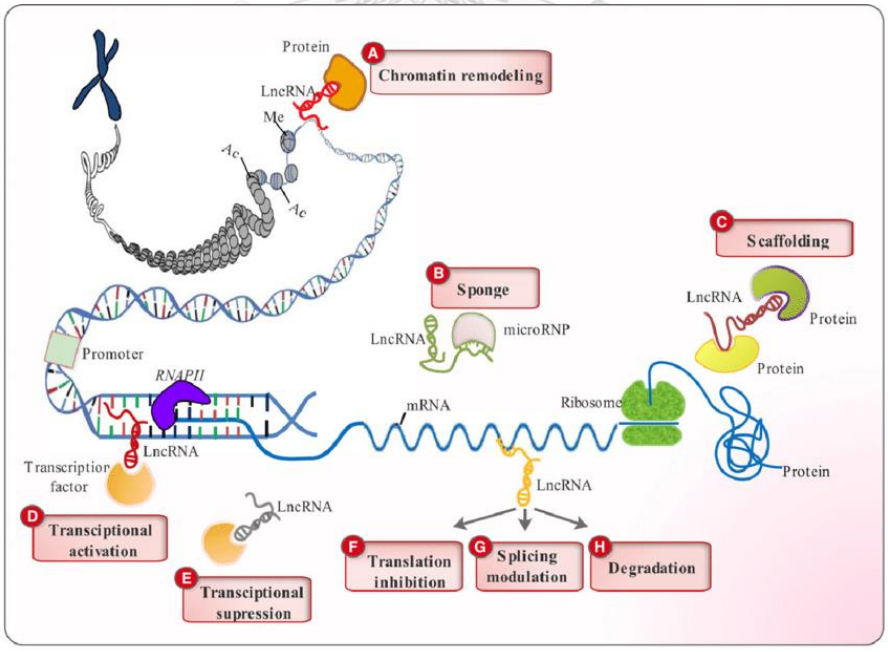


Figure 3 microRNA biogenesis

In general, the most important region for target recognition comprises the 2^{th}-8^{th} nucleotides from the 5' portion of the miRNA which known as the “seed” region; and binding sites are most commonly found in the 3'UTR of the target mRNAs. In addition, many examples have shown that miRNA binding sites can be located outside the 3'UTR and even lack of perfect seed pairing can be compensated by 3' complementarity. This seed-binding principle has been used as the major criteria for computational miRNA target prediction and generally combined with the secondary structure of the 3'UTR of the target gene. For example the well-known computational prediction tool such as TargetScan which is the computational tool predicting biological targets of miRNAs by searching for the presence of 8mer, 7mer, and 6mer sites that match the seed region of each miRNA (43). The biggest limitations of current algorithms for computational tools is that the predictions are in most cases restricted to the 3' UTRs of the mRNA in contrast to the recent experimental data indicating that a large proportion of the miRNA/mRNA interactions may occur through the coding sequences (CDS) or even the 5' UTR (44). In addition, none of current predicting methods considers the possibility of tissue-specific interactions. Therefore, experimental approaches may be used to confirm the biological targets of the miRNA. Base on the function of miRNAs that regulate gene expression, the most straight-forward experimental approach to identify the target of each miRNAs is transfection of specific miRNA mimics or inhibitors into the cells followed by high-throughput analysis of mRNA expression such as microarray or high-throughput sequencing, or proteomics (45).

Long non-coding RNAs

Long non-coding RNAs (lncRNAs) are a large and diverse class of transcribed RNA molecules with a length of more than 200 nucleotides that do not translate into proteins (46). lncRNAs are thought to include nearly 30,000 different transcripts in humans, therefore lncRNA transcripts account for the major part of the non-coding transcriptome (47). To date, very few lncRNAs have been characterized in detail. However, it is clear that lncRNAs are important regulators of gene expression. lncRNAs are thought to have a wide range of functions in cellular and developmental processes. lncRNAs may carry out both gene inhibition and gene activation through a range of diverse mechanisms (Figure 4), adding yet another layer of complexity to our understanding of genomic regulation (48). It is estimated that 25 – 40% of coding genes have overlapping antisense transcription, so the impact of lncRNAs on gene regulation is not to be underestimated.



(49)

Figure 4 Classification of lncRNAs functions

Protein mass spectrometry

Mass spectrometry (MS) is an analytical chemistry technique used for identification the type and amount of chemicals present in a sample by ionizing chemical compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge ratios and abundance of gas-phase ions. The applications of MS include protein identification which can be applied in biological research. There are two main ways MS is used to identify proteins. Peptide mass fingerprinting uses the masses of proteolytic peptides as input to a search of a database of predicted masses that would arise from digestion of a list of known proteins. If a protein sequence in the reference list gives rise to a significant number of predicted masses that match the experimental values, there is some evidence that this protein was present in the original sample. Peptide mass fingerprinting can be achieved with MS-MS. Tandem MS is a method for identifying proteins. Collision-induced dissociation is used in mainstream applications to generate a set of fragments from a specific peptide ion. The fragmentation process primarily gives rise to cleavage products that break along peptide bonds. Because of this simplicity in fragmentation, it is possible to use the observed fragment masses to match with a database of predicted masses for one of many given peptide sequences. Tandem MS of whole protein ions has been investigated recently using electron capture dissociation and has demonstrated extensive sequence information in principle but is not in common practice. This is sometimes referred to as the "top-down" approach in that it involves starting with the whole mass and then pulling it apart rather than starting with pieces (proteolytic fragments) and piecing the protein back together using de novo repeat detection (bottom-up).

Chapter 3 Research Methodology

Research design

This study used the experimental research which did not perform in neither animal model nor clinical sample. Thus, it was considered to be exempted for the ethic consideration under the review of Institutional Review Board (IRB) Faculty of Medicine, Chulalongkorn University.

Materials and Reagents

1. Annealing buffer preparation
    - Tris (AMRESCO)
    - NaCl (Merck)
    - EDTA (Sigma)
2. Restriction digestion
    - *Nhe*I (Thermo Scientific)
    - *Xho*I (Thermo Scientific)
    - 10X Tango™ Buffer (Thermo Scientific)
3. Cloning and plasmid extraction
    - T4 DNA Ligase (Thermo Scientific)
    - Difco™ Agar Technical (Becton Dickinson)
    - Bacto™ Tryptone (Becton Dickinson)
    - Bacto™ Yeast Extract (Becton Dickinson)
    - NaCl (Merck)
    - *E. coli* DH-5α (RBC Bioscience)
    - Amplicillin (General Drug House)

- Kanamycin (General Drug House)

- RBE Real Genomic HiYield™ Plasmid Mini Kit (RBC Bioscience)

4. Polymerase chain reaction (PCR)

- Themal Cycler instrument

- Distilled water (DW)

- Master mix (5 PRIME)

- primers (1st Base)

5. Agarose Gel Electrophoresis

- Agarose (Invitrogen)

- 1X TBE

- Ethidium Bromide (AMRESCO)

- DNA marker (1kbp and 100 bp (RBE Bioscience))

- Transilluminator

- Gel Electrophoresis

6. DNA extraction from agarose gel

- HiYield Gel/PCR DNA Fragments Extraction Kit (RBC Bioscience)

7. Cell culture

- DMEM (Thermo Scientific)

- Fetal Bovine Serum (FBS)

- Antibiotic-Antimycotic (Gibco)

- Trypsin 0.25% (Gibco)

- Tissue culture flask (TPP)

- 96-well plate (TPP)

- Serological pipette (TPP)

- 15 and 50 mL tube (Axygen Scientific)

- Incubator (BioSan)

- Centrifuge (Hettrich)

8. Transfection

- Lipofectamine® 2000 reagent (Invitrogen)

- Opti-MEM®I (1X) Reduced Serum Medium (Gibco)

9. Dual-Luciferase Assay

- Dual-Luciferase® Reporter Assay System (Promega)

- 96-well plate White Opaque (Coastar)

- Microplate Reader

10. General tools and equipment for research

- Heat box (Bioer Technology)

- Mini Centrifuge (Eppendorf)

- Incubator (Biosan)

- 0.2 and 1.5 mL microcentrifuge tube (Axygen)

- 10, 200 and 1,000 µl tips (Nepture)

- 3, 10, 20, 100, 200 and 1,000 µl Autopipette (Biohit)

11. Computational programs for data analysis

- miRBASE (http://www.mirbase.org)

- RNAhybrid

  (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html)

- BioEdit Sequence Alignment Editor Version 7.0.4.1

- Oligos version 9.1

Experimental procedure

Cell culture

Adenocarcinomic human alveolar basal epithelial cells (A549 cells) were used in *in vitro* study. The A549 cells were cultured in high glucose containing Dulbecco's modified Eagle medium (DMEM) with 1% (v/v) antibiotic/antimycotic and 10% fetal bovine serum (FBS). Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. Culture media was replaced every 3 days by using the following protocol;

1. Rinse cells with phosphate buffer saline (PBS).
2. To a T75 flask, add 2 mL of trypsin-EDTA to the flask and observe for cell layer detachment under the microscope. If cells are not detaching, place in incubator for 5 minutes.
3. Neutralize the trypsin-EDTA by adding 8 mL of complete media (DMEM with FBS); centrifuge the cells and remove the supernatant.
4. Add 6-8 mL of growth medium to pellet and gently mix the cells by pipetting.
5. Add aliquots of the cell suspension to culture flask with appropriate ratio.
6. Incubate at 37°C in 5% CO_2.

Viral infection

In this study, two lineages of influenza B viruses were used; Victoria (B/Thailand/CU-B5522/2011) and Yamagata (B/Massachusetts/2/2012. The A549 cells were divided into 3 groups; uninfected cells, infected with Victoria lineage and infected with Yamagata lineage. The viral infection protocol is described below;

1. Seed the A549 cells (3×10^5 cells/well) in DMEM medium without antibiotic/antimycotic in 6-well plates.
2. Prepare the influenza B viruses by diluting the virus stocks with DMEM medium to yield the desired concentration of 3×10^5 pfu/mL (MOI = 0.5).
3. When cells reached approximately 80% confluence, remove the cell culture media from each well and wash with 3 mL of PBS.
4. Add 500 µl of each virus suspension into each well and then incubate in a humidified atmosphere with 5% CO_2 at 37°C for 1 h with occasional shaking to allow the virus to adsorb into the cells.
5. Remove the virus suspension from each well and wash by 3 ml of PBS.
6. Add 3 ml of complete DMEM medium into each well and incubate at 37°C, 5% CO_2 for 24 h.

Total RNAs, small RNAs and proteins were extracted for mRNAs profiling, miRNAs profiling, lncRNAs profiling and protein profiling, respectively.

Small RNA extraction

The small RNAs were extracted from the cells by using microRNA purification kit (Norgen) as the follow protocol;

1. Aspirate media and wash cell monolayer with an appropriate amount of PBS. Then Aspirate PBS.
2. Add 350 µl of Buffer RL directly to culture plate.
3. Lyse cells by gently tapping culture dish and swirling buffer around plate surface for 5 minutes.
4. Transfer lysate to a microcentrifuge tube.

5. Assemble a gDNA Removal Column with a provided collection tube.
6. Apply up to 600 µl of the lysate prepared from Section 1 onto the column and centrifuge at 14,000 x g (~14,000 RPM) for 1 minute.
7. Retain the flowthrough for RNA Purification.
8. To every 100 µl of flowthrough from Step 2c, add 60 µl of 96 – 100 % Ethanol. Mix by vortexing.
9. Assemble an RNA Purification Micro Column with one of the provided collection tubes. Apply up to 600 µl of the lysate with the ethanol onto the column and centrifuge at ≥ 3,500 x g (~6,000 RPM).
10. Apply 400 µl of Wash Solution A to the column and centrifuge at 14,000 x g (~14,000 RPM) for 1 minute.
11. Discard the flowthrough and reassemble the spin column with its collection tube. Then repeat the step 10-11 twice.
12. Spin the column for 2 minutes in order to thoroughly dry the resin. Discard the collection tube.
13. Place the column into a fresh 1.7 mL Elution tube provided with the kit. Then add 40 µl of Elution Solution A to the column.
14. Centrifuge for 2 minutes at 200 x g (~2,000 RPM), followed by 1 minute at 14,000 x g (~14,000 RPM).

Library preparation

Purified small RNA in the amount of 0.2 µg was used to construct 4 libraries of uninfected cells and influenza-B infected cells at 24 hours post infection, and were pooled together after they were tagged with different indexes according to the TruSeq Small RNA sample preparation kit protocol (Illumina) as described below.

Ligate 3' Adapter

1. Combine the following volumes in a new 200 µl PCR tube on ice:

   - RA3 (1 µl)

   - 1 µg total RNA in nuclease-free water (5 µl)

   The total volume is 6 µl.
2. Pipette to mix, and then centrifuge briefly.
3. Place on the preheated thermal cycler.
4. Incubate at 70°C for 2 minutes.
5. Remove from the thermal cycler and place on ice.
6. Preheat the thermal cycler to 28°C.
7. Combine the following volumes in a new 200 µl PCR tube on ice.
8. Pipette to mix, and then centrifuge briefly.
9. Add 4 µl to the tube of RA3/total RNA mixture.

   The total volume is 10 µl.
10. Pipette to mix.
11. Place on the preheated thermal cycler.
12. Incubate at 28°C for 1 hour.
13. Add 1 µl STP and pipette to mix.
14. Continue incubating at 28°C for 15 minutes.
15. Remove from the thermal cycler and place on ice.

Ligate 5' Adapter

1. Preheat the thermal cycler to 70°C.
2. Add 1.1 × N µl RA5 to a new 200 µl PCR tube.
3. Place on the preheated thermal cycler.
4. Incubate at 70°C for 2 minutes.
5. Remove from the thermal cycler and place on ice.
6. Preheat the thermal cycler to 28°C.
7. Add 1.1 × N µl 10mM ATP to the tube of RA5.
8. Pipette to mix.
9. Add 1.1 × N µl T4 RNA Ligase to the RA5/ATP mixture
10. Pipette to mix.
11. Add 3 µl to the tube of RA3 mixture.

    The total volume is 14 µl.
12. Pipette to mix.
13. Place on the preheated thermal cycler.
14. Incubate at 28°C for 1 hour.
15. Remove from the thermal cycler and place on ice.

Reverse Transcription

1. Add 6 µl each adapter-ligated RNA library to a new 200 µl PCR tube.
2. Add 1 µl RNA RT Primer to the tube of adapter-ligated RNA.
3. Pipette to mix, and then centrifuge briefly.
4. Place on the preheated thermal cycler.
5. Incubate at 70°C for 2 minutes.
6. Remove from the thermal cycler and place on ice.
7. Preheat the thermal cycler to 50°C.
8. Combine the following volumes in a new 200 µl PCR tube on ice.

   5X First Strand Buffer (2 µl)

   12.5 mM dNTP Mix (0.5 µl)

   100 mM DTT (1 µl)

   RNase Inhibitor (1 µl)

   SuperScript II Reverse Transcriptase (1 µl)

   The total volume per library is 5.5 µl.
9. Pipette to mix, and then centrifuge briefly.
10. Add 5.5 µl to the tube of adapter-ligated RNA/primer mix.
11. Pipette to mix, and then centrifuge briefly.

    The total volume is 12.5 µl.
12. Place on the preheated thermal cycler.
13. Incubate at 50°C for 1 hour.
14. Remove from the thermal cycler and place on ice.

Amplify Libraries

1. Combine the following reagents in a new 200 µl PCR tube on ice to prepare the PCR master mix.

   Ultrapure water (8.5 µl)

   PML (25 µl)

   RP1 (2 µl)

   RPIX (2 µl)

   The total volume per library is 37.5 µl.

2. Pipette to mix, and then centrifuge briefly.
3. Place on ice.
4. Add 37.5 µl PCR master mix to the adapter-ligated RNA mixture. The total volume is 50 µl.
5. Pipette to mix, and then centrifuge briefly.
6. Place on ice.
7. Place on the preheated thermal cycler.
8. Incubate using the following program on the thermal cycler:

   Choose the preheat lid option and set to 100°C.

   98°C for 30 seconds

   11 cycles of: 98°C for 10 seconds

   60°C for 30 seconds

   72°C for 15 seconds

   72°C for 10 minutes

   4°C hold

9. Run each library on a High Sensitivity DNA chip.

Run Gel Electrophoresis

1. Combine 2 µl CRL and 2 µl DNA loading dye in a new 1.5 ml microcentrifuge tube.
2. Pipette to mix.
3. Combine 1 µl HRL and 1 µl DNA loading dye in a new 1.5 ml microcentrifuge tube.
4. Pipette to mix.
5. Combine all amplified cDNA construct (typically 48–50 µl) and 10 µl DNA Loading Dye in a new 1.5 ml microcentrifuge tube.
6. Pipette to mix.
7. Load 2 gel lanes with 2 µl CRL/loading dye mixture.
8. Load 1 gel lane with 2 µl HRL/loading dye mixture.
9. Load 2 gel lanes with 25 µl each of amplified cDNA construct/loading dye mixture.

   The total load volume is 50 µl.
10. Run the gel for 60 minutes at 145 V or until the blue front dye leaves the gel.
11. Remove the gel from the unit.

Recover Purified Construct

1. Open the cassette according to manufacturer instructions and stain the gel with ethidium bromide in a clean container for 2–3 minutes.
2. Place the gel breaker tube into a 2 ml microcentrifuge tube.
3. View the gel on a Dark Reader transilluminator or a UV transilluminator.

Individual bands or pooled bands can be sequenced. The 147 nt band primarily contains mature miRNA generated from ~22 nt small RNA fragments. The 157 nt band contains piwi-interacting RNAs, some miRNAs, and other regulatory small RNA molecules. It is generated from ~30 nt RNA fragments.

4. Using a razor blade, cut out the bands from the 2 lanes that correspond approximately to the adapter-ligated constructs derived from the 22 nt and 30 nt small RNA fragments. The band containing the 22 nt small RNA fragment with both adapters is 147 nt long. The band containing the 30 nt RNA fragment with both adapters is 157 nt long.
5. Place the band of interest into the 0.5 ml gel breaker tube.
6. Centrifuge the nested tubes at 20,000 × g for 2 minutes to move the gel through the holes into the 2 ml tube.
7. Add 200 µl ultrapure water to the gel debris in the 2 ml tube.
8. Rotate or shake for at least 2 hours to elute the DNA.
9. Transfer the eluate and gel debris to the top of a 5 µm filter.
10. Centrifuge at 10 seconds at 600 × g.

The Agilent 2100 Bioanalyzer system (Agilent) was used to evaluate the DNA libraries with a high sensitivity DNA chip (Agilent). The quantification of the DNA libraries' concentrations was performed by using Qubit fluorometer (Invitrogen) with a Quant-iT™ DNA Assay kit (Invitrogen). All DNA libraries were mixed with equal concentrations, followed by single read sequencing for 50 cycles using a MiSeq platform (Illumina).

NGS Data analysis

The fundamental analysis of sequencing data was operated by Miseq reporter software version 2.4. Low-quality DNA reads (defined by Q-score < 30) and adaptors were discarded for reliable analysis. The passing-filtered DNA contigs (defined by Q-score > 30) were mapped against the human genome (hg19), all known mature & precursor human miRNA (from miRbase) and potential contaminant RNA (classified as human tRNA, rRNA and mRNA) by using the Bowtie Software [http://bowtiebio.sourceforge.net] algorithm. The clusters of reads that could be mapped to human genomic DNA and contaminant RNAs were removed, while clusters of contigs which could be mapped to the human genomic DNA and the miRNA were considered as candidate miRNAs to be profiled later. The miRNAs were categorized and counted based on the frequency of reads that matched the database of miRbase (www.mirbase.org) [13-17]. Differential expression of miRNA was calculated in terms of fold changes as previously described [12]. Briefly, fold change between cases and control was calculated by log2 of normalized expression in case divided by normalized expression in control. The normalized expression was calculated by the number of miRNA reads divided by the number of total miRNA reads in each group. The calculated fold changes higher than 1 were considered as a significant difference. In addition, the miRNAs with fold changes higher than 2 were considered as candidate miRNAs for confirmation by qPCR analysis.

Transfection of mimic, negative control and inhibitor of interested miRNAs

To study the effect of interested human miRNAs whose expression is changed due to the infection of influenza B virus, the miRNA mimics, miRNA inhibitors and mimic negative control were used in *in vitro* study. The miRNA mimics, miRNA inhibitors and mimic negative control were individually transfected into the $3x10^5$ A549 cells per well in 6 wells-plate by Lipofectamine2000 (Invitrogen) transfection reagent. After 24 hours post transfection, the cells were harvested and the small RNA was extracted. The expression of interested miRNA was quantified by poly-U polymerization followed by the stem-loop RT-PCR as described later. The amount of interested miRNA was optimized to reach the miRNA expression level comparable to the expression level that responded to influenza B virus infection in previously step with no toxicity to the cells. Cell viability was examined by using MTT assay.

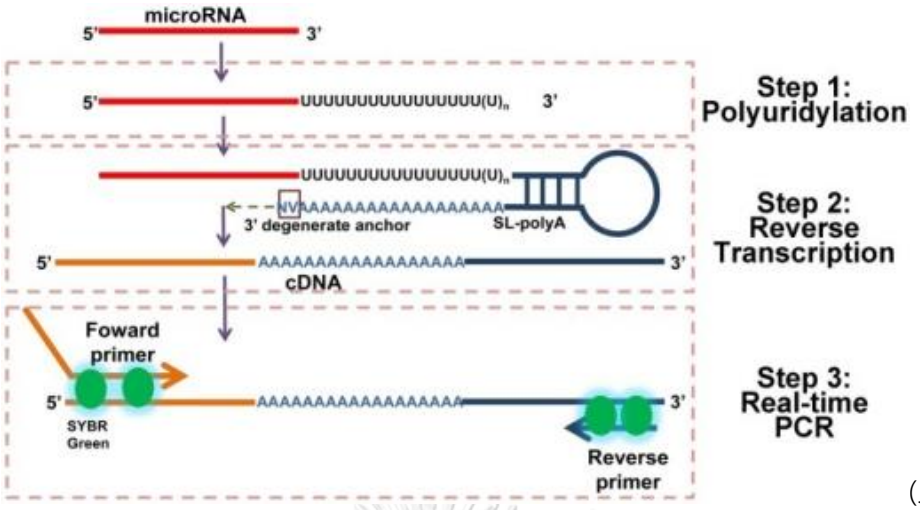
Quantification of candidate miRNAs expression by stem-loop RT-qPCR

The RT-qPCR of interested miRNAs was performed to quantify the expression in transfected groups. Total small RNAs extracted from the experiment were poly-U polymerized by poly-U polymerase (Figure 5). Then the stem-loop primer with poly-A tail was used for reverse transcription. And the expression of interested miRNAs were determined by real-time PCR using miRNA-specific forward primer as the forward primer and stem-loop specific primer as the reverse primer with SYBR qPCR master mix. The non-coding small nuclear RNA which called U6 was used as reference gene. The expression ratio of miRNA was calculated by $\Delta\Delta C_T$ method as described below.

$$\Delta C_T = C_{T\,(miRNA)} - C_{T\,(U6)}$$

$$\Delta\Delta C_T = \Delta C_{T\,(infected)} - \Delta C_{T\,(uninfected)}$$

$$Ratio = 2^{-\Delta\Delta C_T}$$

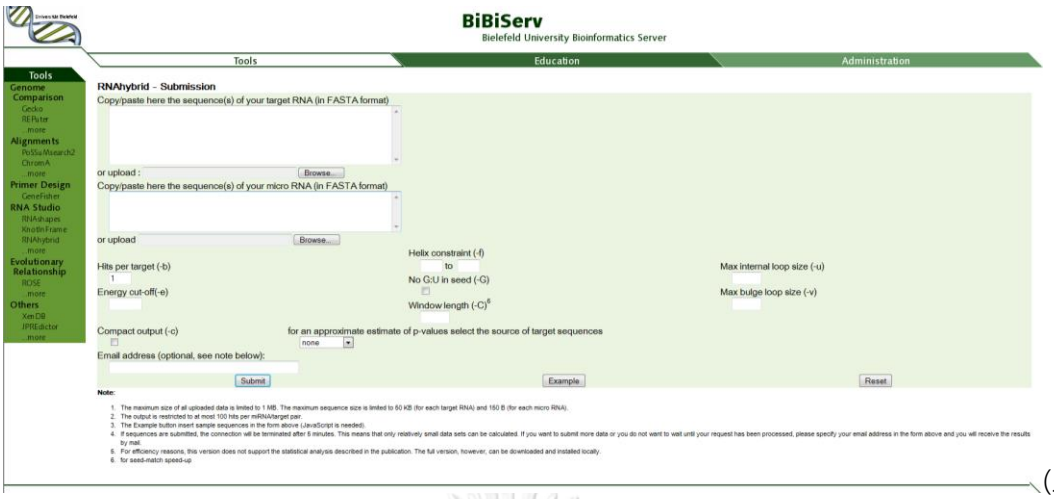


(50)

Figure 5 The stem-loop RT-qPCR assay for miRNA quantification

In silico prediction of viral-targeted gene

Influenza B virus (B/Thailand/CU-B5522/2011) genome was used for miRNA targeted genes prediction. The seed region binding rule was used as criteria for target gene prediction. Hybridization pattern and binding energy between candidate miRNA and its viral-targeted gene were predicted by RNAHybrid (51) as shown in figure 6. The results were characterized in terms of hybridization pattern and pairing energy (mfe). The hybridization patterns obtained from RNAhybrid were classified into 4 categories including 5'canonical, 5'seed, 3'compensatory and ineffective hybridization. Only the target viral genome with pattern hybridization were selected as candidate target for miRNAs.



(51)

Figure 6 RNAHybrid program

Vector construction

To validate the influenza B virus genes which can be targeted by interested miRNAs, the 3′-UTR reporter assay was performed. The reporter target vectors were constructed by ligating miRNA target site of viral gene into the 3′-UTR of reporter gene: firefly luciferase (Luc). Oligonucleotides (Table 1) were designed containing miRNA-target site region of viral genes connecting with restriction sites in both 5' and 3' similar to the reporter vector.

Table 1 Sequences of oligo nucleotides for vector construction

Oligo name	sequence (5'-3')
Backbone vector: pGL3MS2/Basic	
NEP-428-top	CTAGCATGTTGATGGCCCAACTGAAATC
NEP-449-bottom	TCGAGATTTCAGTTGGGCCATCAACATG
NB&NA-1123-top	CTAGCCGATGTCTAAAACTGAAAGGC
NB&NA-1142-bottom	TCGAGCCTTTCAGTTTTAGACATCGG
NP-23-top	CTAGCAGTAAAAGAACTGAAAAC
NP-39-bottom	TCGAGTTTTCAGTTCTTTTACTG
PB2-1406-top	CTAGCTGAATGCATCTGACTATACACTGAAAGGC
PB2-1433-bottom	TCGAGCCTTTCAGTGTATAGTCAGATGCATTCAG
PB2-1451-top	CTAGCATGTAATTGACGACTTTAGCTCTACTGAAACC
PB2-1481-bottom	TCGAGGTTTCAGTAGAGCTAAAGTCGTCAATTACATG
PB1-956-top	CTAGCTGGCTATGACTGAAAGAC
PB1-972-bottom	TCGAGTCTTTCAGTCATAGCCAG
Backbone vector: pSilencer3.0-H1	
siLuc/Luc2-TS	GATCCCACCCCAACATCTTCGACGTTCAAGAGACGTCGAAGATGT TGGGGTGTTTTTTGGAAA
siLuc/Luc2-BS	AGCTTTTCCAAAAAACACCCCAACATCTTCGACGTCTCTTGAACG TCGAAGATGTTGGGGTGG

Backbone vector digestion

1. Prepare the restriction digestion reaction;

   1 µg DNA (2ul if ~> 500ng/ul)

   2 µl Buffer

   1 µl Restriction enzyme

   H_2O to final volume of 20 µl

2. Incubate at 37°C for 1 h

3. Heat inactivation

4. Prepare dephosphorilation reaction by adding 2.2 µl Antartic Phosphatase Buffer and 1 µl of Antartic phosphatase
5. Incubate at 37°C for 30 min
6. Run digestion on a 1% agarose gel electrophoresis
7. Cut the selected band for purification

DNA purification by using HiYield Gel/PCR Fragments Extractation Kit (RBC) as following protocol;

1. Excise the agarose gel slice containing relevant DNA Fragments and remove extra agarose to minimize gel slice.
2. Transfer up to 300mg of the gel slice into a microcentrifuge tube.
3. Add 500 µl of DF Buffer to the sample and mix by vortexing.
4. Incubate at 55°C for 10-15 minutes until the gel slice has been completely dissolved. During incubation, invert the tube every 2-3 mins.
5. Place a DF Column into a Collection Tube.
6. Apply 800 µl of the sample mixture from previous step into the DF Column.
7. Centrifuge at max. 10,000 x *g* (13,000 rpm) for 30 seconds.
8. Discard the flow-through and place the DF Column back in the Collection Tube.
9. Add 600 µl Wash Buffer (ethanol added) to the DF Column.
10. Centrifuge at max. 10,000 x *g* (13,000 rpm) for 30 seconds.
11. Discard the flow-through and place the DF Column back into the Collection Tube.

12. Centrifuge again for 2 minutes at max 10,000 x *g* (13,000 rpm) to dry the column matrix.
13. Transfer dried DF Column into a new microcentrifuge tube.
14. Add 20-50 µl Elution Buffer or water to the center of the column matrix.
15. Allow to stand for 2 minutes until Elution Buffer or water is absorbed by the matrix.
16. Centrifuge for 2 minutes at full speed to elute purified DNA.

Insert oligo DNA preparation

1. Do 1x PCR amplification (ON ICE) with the following protocol

    6.5 µl H_2O

    1 µl (100uM) Top strand oligo

    1 µl (100uM) Bottom strand oligo

    1 µl T4 DNA ligase buffer

    0.5 µl PNK enzyme

2. Mix by pipetting
3. Annealing in thermocycler

    95°C 5 min

    60°C 2 min

    37°C 30 min

Ligation

1. Preparing ligation reaction;

   5.5 µl H_2O

   2 µl purified digested backbone vector

   1 µl annealed insert oligo

   1 µl T4 DNA ligase buffer

   0.5 µl T4 DNA ligase

2. Incubate at room temperature for 1 h.
3. Heat inactivate at 65°C for 10 min

Transformation

1. Gently load 10 µl (ligation) on 50 µl *E. coli* bacteria
2. Incubate 30 min on ice
3. heat shock 42°C for 30 sec
4. Incubate 5 min on ice
5. Add 150 µl SOC media
6. Incubate at 37°C for 1 h with shanking
7. Spread on plate with appropriate antibiotics
8. Incubate at 37°C overnight (16-18 h)
9. Select the colonies and pick into LB broth for liquid culture
10. Incubate at 37°C with shanking overnight (16-18 h)

Plasmid purification

Plasmids were purified from culture media by using HiYield Plasmid Kit (RBC) as following protocol;

1. Transfer 1.5ml of bacterial culture to a microcentrifuge tube
2. Centrifuge for 1 min at full speed (13,000 rpm) in a microcentrifuge and discard supernatant.
3. Add 200 µl of PD1 Buffer (RNase A added) and resuspend the cell pellet by vortexing or pipetting.
4. Add 200 µl of PD2 Buffer and mix gently by inverting the tube 10 times. Do not vortex, avoid shearing genomic DNA.
5. Allow mixture to stand for 2 minutes at room temperature until lysate clears.
6. Add 300 µl of PD3 Buffer and mix immediately by inverting the tube 10 times. Do not vortex.
7. Centrifuge for 2 min at full speed (13,000 rpm).
8. Place a PD Column in a 2ml Collection Tube.
9. Apply the clear lysate (supernatant) from Step 7 to the PD Column.
10. Centrifuge at full speed (13,000 rpm) for 30 seconds.
11. Discard the flow-through and return the PD Column back to the 2ml Collection Tube.
12. Add 400 µl of W1 Buffer in the PD Column.
13. Centrifuge at full speed (13,000 rpm) for 30 seconds.
14. Discard the flow-through and return the PD Column to the 2ml Collection Tube.
15. Add 600 µl of Wash Buffer (ethanol added) to PD Column.
16. Centrifuge at full speed (13,000 rpm) for 30 Seconds.

17. Discard the flow-through and return the PD Column to the 2ml Collection Tube.
18. Centrifuge again for 3 min at full speed to dry the column matrix.
19. Transfer the dried PD Column to a clean 1.5ml microcentrifuge tube.
20. Add 50 µl of Elution Buffer or ddH2O (pH 8.0-8.5) directly onto the center of the membrane.
21. Avoid residual buffer adhering to the wall of the column.
22. Allow to stand for 2 min until the liquid is absorbed.
23. Centrifuge for 2 min at full speed (13,000 rpm) to elute plasmid DNA.

3'-UTR reporter assay

The A549 cells were divided into three groups. The first group was transfected with miRNA expression vector. The second group was transfected with pSilencersiLuc/Luc2. Third group was transfected with pSilencer-scramble. Then all three groups were transfected with reporter target vector (pmiRGLO). All transfections were used Lipofectamine2000 as transfection reagent. After24 hours post transfection, cells were harvested and the relative luciferase activity was analyzed to determine the expression of reporter gene as following protocol;

1. Remove growth media from cultured cells.
2. Rinse cultured cells in 1X PBS. Remove all rinse solution.
3. Dispense the recommended volume (below) of 1X PLB into each culture vessel.
4. Gently rock/shake the culture vessel for 15 minutes at room temperature.
5. Transfer lysate to a microcentrifuge tube.

6. Plate 96-wells pate with ≤20µl of PLB Lysate/well.
7. Dispense 50 µl of LAR II.
8. Measure firefly luciferase activity.
9. Dispense 50 µl of Stop & Glo® Reagent.
10. Measure *Renilla* luciferase activity.
11. Calculate the relative luciferase activity.

$$\text{Relative Luciferase activity} = \frac{RLuc\ expression}{Luc\ expression}$$

RNA-Seq

Total RNAs were extracted from the cells by GenUP Total RNA Kit (BiotechRabbit). The protocol was described below;

1. Transfer cells to an appropriate reaction tube and pellet by centrifugation.
2. Discard the supernatant.
3. Resuspend the cells in 400 µl Buffer LYSIS LR.
4. Incubate at room temperature for 2 min.
5. Resuspend by carefully pipetting up and down, and incubate at room temperature for an additional 3 min.
6. Transfer the lysate to a Mini Filter DNA placed in a collection tube.
7. Discard the reaction tube.
8. Centrifuge at 10,000 x *g* (~12,000 rpm) for 2 min.
9. Discard the Mini Filter DNA and keep the filtrate.
10. Add an equal volume of 70% ethanol (400 µl) to the filtrate and mix by pipetting.
11. Transfer the sample to a Mini Filter RNA placed in a new Collection Tube.

12. Centrifuge at 10,000 x *g* (~12,000 rpm) for 2 min.
13. Discard the Collection Tube with the filtrate.
14. Place the Mini Filter RNA into a new Collection Tube.
15. Add 500 µl Buffer WASH A to the Mini Filter.
16. Centrifuge at 10,000 x *g* (~12,000 rpm) for 1 min.
17. Discard the Collection Tube with the filtrate.
18. Place the Mini Filter RNA into a new Collection Tube.
19. Add 700 µl Buffer WASH B.
20. Centrifuge at 10,000 x *g* (~12,000 rpm) for 1 min.
21. Discard the Collection Tube with the filtrate.
22. Place the Mini Filter RNA into a new Collection Tube.
23. Centrifuge at maximum speed for 2 min to remove residual ethanol.
24. Discard the Collection Tube.
25. Place the Mini Filter into an Elution Tube.
26. Add 30-80 µl Water, RNase-free to the center of the Mini Filter RNA.
27. Incubate at room temperature for 1 min.
28. Centrifuge at 6,000 x *g* (~8,000 rpm) for 1 min.
29. Discard the Mini Filter RNA.

Total large RNAs were used for mRNAs profiling and lncRNAs profiling by RNA-seq. The total extracted RNAs from Victoria lineage-infected cells, Yamagata lineage-infected cells and uninfected cells were sent to Vishuo Biomedical which providing RNA-seq service. The quantity and quality of RNAs sample were analyzed by Agilent 2100 Bioanalyzer (Agilent Technologies), NanoDrop (Thermo Fisher Scientific Inc.) and 1% agarose gel. Then the next generation sequencing library preparations were

constructed by using NEBNext® Ultra™ RNA Library Prep Kit for Illumina®. The poly(A) mRNA isolation were performed by using NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB). Followed by fragmentation and priming using NEBNext First Strand Synthesis Reaction Buffer and NEBNext Random Primers. After the library preparation, the RNAs sequencing was performed on illumine® HiSeq system.

The raw sequencing reads were received in FASTQ formatted files. The samples were sequenced in paired-end (PE) mode with length 150 bases. The quality of the raw sequencing reads were assessed with the FastQC v.0.11.5 software. To ensure only good quality bases are used in the downstream analysis, the raw sequencing reads were subjected to trimming process with Trimmomatic v.0.32 software. In the trimming process, the sequencing adapters, leading and trailing bases below Q3 were first trimmed. The reads were then scanned from both ends, using a 4bp-wide sliding window, within which the low quality (lower than Q20) bases were trimmed. Finally, the resulting reads of length at least 50 bases were selected for further analysis.

The surviving paired-end reads were aligned to the Homo sapiens reference genome (HISAT2 index : Ensembl GRCh37 genome_tran) with HISAT2 v.2.1.0 software. HISAT2 index provides hierarchical graph FM index (HGFM) of the human reference genome plus transcripts. The resulted sequence alignment files (BAM formatted file) were then sorted by reference position as required by next analysis software. The alignment files were analyzed individually with the Cufflinks v.2.2.1 software to detect and quantify previously known transcripts and to discover new transcripts or isoform(s). Cufflinks v.2.2.1 infer the splicing structure of each gene by referring to the splicing of known transcript annotated in the reference genome as well as from the aligned sequencing reads.

The 'transfrags' reported by the algorithmn were saved individually in a annotation file (GTF formatted file) which will be merged in the downstream analysis. After transcripts assembly, Cufflinks v.2.2.1 quantifies the expression level of each 'transfrag' in the sample. With the merged annotation file, the expression level of each annotated 'transfrags' were calculated using Cuffdiff. The expression unit calculated is FPKM, Fragments Per Kilobase Million. Besides, differential expression (DE) analysis was performed according to the given comparison groups. The expression fold change between each comparison group were calculated and log-transformed. Multiple output files were generated by Cuffdiff. Significant DE genes or transcripts (q-value < 0.05) were extracted for each comparison groups.

lncRNA profiling

The RNA-seq results from previous step were also aligned with human long noncoding RNAs in the NONCODE database (version 5.0) (http://www.noncode.org) using the TopHat program. To identify the differential expression lncRNAs, data from both lineage of influenza B infected cells and uninfected cells were used for differential gene expression analysis by Cufflinks tool calculating the abundances of transcripts in term of fragments per kilobase of exon per million fragments mapped (FPKM).

Protein profiling

The proteins from cells were used for mass spectrometry for protein profiling. The cells were washed with PBS twice and pelleted by centrifuge at 4,000 rpm for 5 min. Then the cells were lysed by using follow protocol;

1. Add 100-300 ul of lysis buffer (5% deoxycholate in TEAB 25 mM)
2. Sonicate the lysate (pulse 15 sec, off 5 sec for 5 min.)
3. Centrifuge at 13,000 rpm for 5 minutes at 4°C.
4. Collect the supernatant into new microtubes.

After that the protein concentration was quantified with bicinchoninic acid method (BCA) assay by using Pierce™ BCA Protein Assay Kit (ThermoScientific);

1. Pipette 25µL of each standard or unknown sample replicate into a microplate well.
2. Add 200 µL of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds.
3. Cover plate and incubate at 37°C for 30 minutes.
4. Cool plate to RT. Measure the absorbance at or near 562nm on a plate reader.

The proteins from each sample were diluted to the similar concentration (100 µg) following by trypsin digestion as described below;

1. Adjust volume with 20 mM TEAB to 100 ul
2. Add 100 mM DTT (in 20 mM TEAB)10 ul (DTT 0.015 g.)
3. Mix and spin down, Incubate at 37 oC, 30 min.
4. Add 100 mM IA (in 20 mM TEAB) 40 ul (IA 0.018 g.)
5. Mix and spin down , Incubate at RT, 30 min. in the dark
6. Add 100 mM DTT 56 ul
7. Mix and spin down, Incubate at RT, 15 min.

8. Dilute sample by 20 mM TEAB 10 times (total volume of 100 ug protein)
9. Add trypsin 1:50 ug protein (stock 20 ug) : add 10 ul trypsin (dissolve in 50 mM acetic acid) , 37oC overnight 16 Hr.
10. Stop reaction by 100 % TFA (final conc. 0.5%), centrifuge 15,000 rpm, 5 min
11. Dry the samples by using SpeedVac.

To perform the protein profiling for protein differential expression comparing between infected and uninfected cells, proteins from each sample were labeled with different isotope by using dimethyl labeling method.

1. Reconstitute the digested samples (around 500 µg) in 100 µL of 100mM TEAB.
2. Add 15 µL of 4% (v/v) each formaldehylde isotope to each sample tube for the light, medium and heavy labeling samples. Mix briefly and spin down.
3. Add 15µL of 0.6 M NaBH3CN to light and medium labeling samples, and 15 µL of 0.6 M NaD3CN to heavy labeling sample.
4. Incubate at RT in fume hood for 1 hour
5. Quench the labeling by adding 30 µL of 1% (v/v) ammonia solution. Mix and spin down.
6. Add 15 µL of FA to further quench the reaction and acidify the samples for desalting and LC-MS/MS analysis.
7. Mix the labeled samples preparing for desalting

The desalting of each sample were performed following this protocol;

1. Condition the column with 1 ml of 100% CAN
2. Equilibrate the column with 1 ml of 0.1% FA
3. Load sample slowly (1drop/3sec.), save unbound fraction
4. Wash column tree times with 1 ml 0.1 FA

   (** vacuum the column untill dry before elute peptides)
5. Elute peptides slowry (1 drop/3sec.) twice with 500 ul of 0.1% FA / 50% CAN.
6. Dry the samples by using SpeedVac.

Then, the proteins profiling was analyzed by nano Liquid-Chromatography (nLC) (Ultimate 3000, Dionex) coupled to a mass spectrometer (Q Exactive, Thermo Fisher Scientific) through an EASY-Spray nano-electrospray ion source (Thermo Scientific). Mass spectrometry was constituted of full scans (m/z 300–1800) ata resolution of 70,000 (at m/z 200) followed by up to 10 data dependent MS/MS scans at a resolution of 17,500.

RAW data files from the mass spectrometer were used to search against a human protein database using the SEQUEST algorithm embedded in Proteome Discoverer (PD) software (Thermo Scientific). False discovery rate (FDR) was calculated. Quantification was carried out by PD. Benjamini-Hochberg (BH) FDR estimation was used for statistical evaluation of the MS derived ratios of unique peptides between biological replicates. A BH FDR threshold of 10% was used as the confidence cutoff.

Statistical analysis

Data obtained from differential expression and relative luciferase activity between each group was analyzed by Student's t-test. For the RNA-Seq results the q-value which is the FDR adjusted p-value was used instead of p-value. A p-value or q-value ≤ 0.05 and was considered statistically significant.

Chapter 4 Results

microRNAs profile from human cells infected with influenza B virus.

According to the result of miRNA profiles, 106 miRNAs were detected at 24 hours post infection. These miRNAs could be divided into three groups, 13 miRNAs were downregulated (>1-fold decrease), 21 miRNAs were up-regulated (>1-fold increase) and 72 miRNAs were unchanged (Figure 7). The miRNAs hsa-miR-320a, hsa-miR-421, hsamiR-455-5p, hsa-miR-361-5p, hsa-miR-376c, hsa-miR-345, hsa-miR-425, hsa-miR486-5p, hsa-miR-582-5p, hsa-miR-889, hsa-miR-125b, hsa-miR-484 and hsa-miR183 were down-regulated, while the miRNAs hsa-miR-769-5p, hsa-miR-301a, hsamiR-30e*, hsa-miR-532-5p, hsa-miR-30c, hsa-miR-410, hsa-miR-20a, hsa-miR-151-5p, hsa-miR-15b, hsa-miR-194, hsa-miR-423-5p, hsa-miR-127-3p, hsa-miR-221, hsamiR-30a*, hsa-miR-5480, hsa-miR-411, hsa-miR-374a, hsa-miR-128, hsa-miR-181c, hsa-miR-193b and hsa-miR-23b were up-regulated.

To predict the human gene targets of the miRNAs in response to influenza B virus, the targeted genes were selected from the miRTarbase version 4.5 database, and these were validated by other strong evidence (Reporter assay, Western blot and qPCR). Targeted genes of differential expressed miRNAs at 24 and 48 hours post infection and their hybridization patterns with minimum free energy (MFE) are shown in table 2.

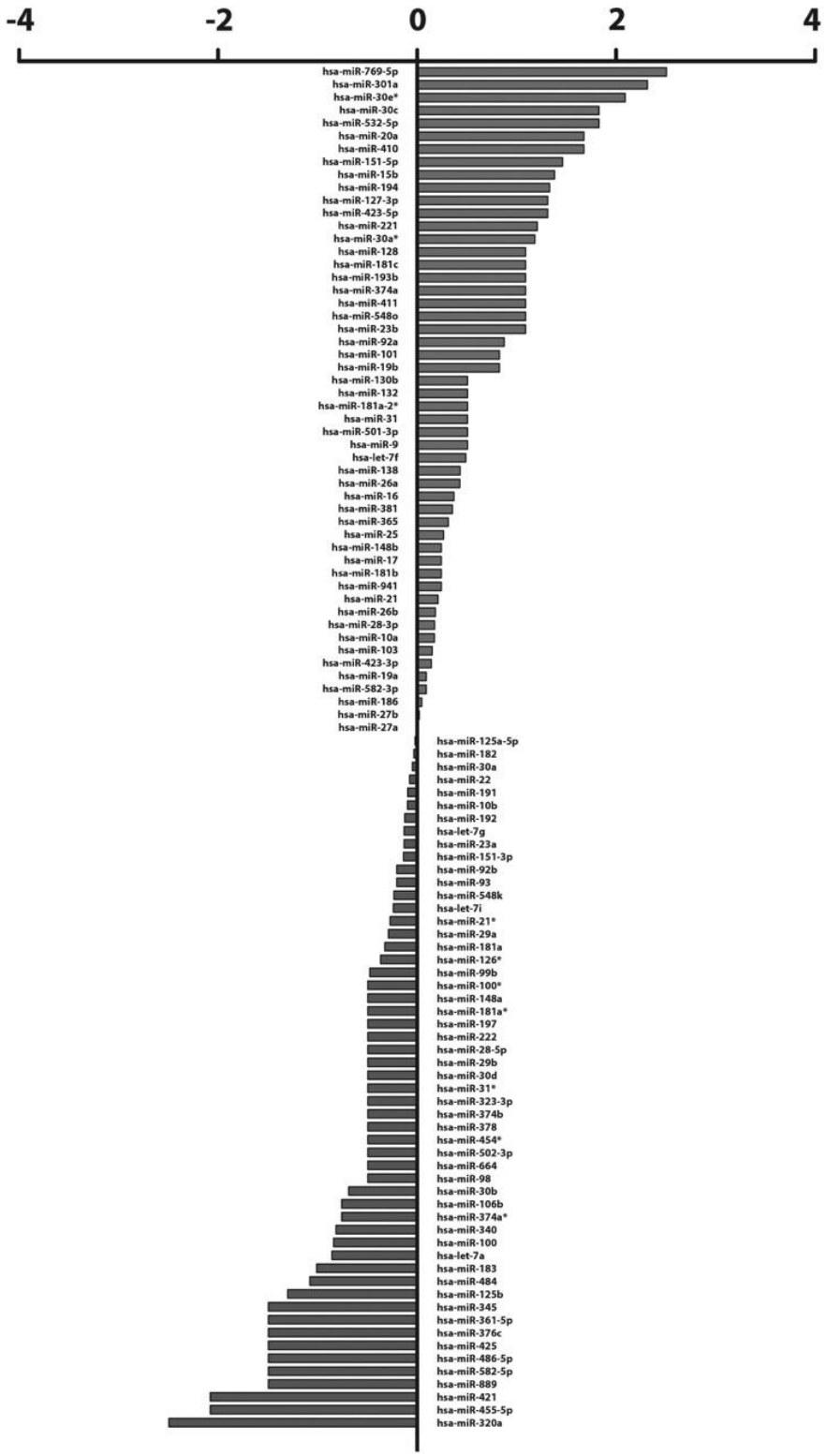


Figure 7 Differential expression of miRNA profiles of influenza B virus infected cells at 24 hours post infection.

Table 2 Validated target genes of miRNAs responded to influenza B infection

miRNAs	Target genes*	Hybridization pattern	MFE (kcal/ mol)	Validation Method**
miR-769-5p	*TRAPPC2B*	target 5' UAGCCUGGAAAUCUUU 3' miRNA 3' UCGAGUCUUGGGUCUCCAGAGU 5'	-16.9	R
miR-301a-3p	*SMAD4*	target 5' UCUUUUGCACUU 3' miRNA 3' CGAAACUGUUAUGAUAACGUGAC 5'	-14.2	R, W, Q
miR-30e-3p	*NFKBIA*	target 5' GUUAUGAGCG-CAAAGGGGCUGAAAG 3' miRNA 3' CGACAUUUGUAGG----CUGACUUUC 5'	-19.3	R, W, Q
miR-421	*CBX7*	target 5' UGCAGCCCUUUCUGCAUGGGGUGCUCUGUUGAC 3' miRNA 3' CG-CGGGUUAA-------UUAC-AGACAACUA 5'	-25.5	W, Q
miR-320a	*MCL1*	target 5' ACCUGUUCUUA---CAGCUUUU 3' miRNA 3' AGCGGG--AGAGUUGGGUCGAAAA 5'	-19.5	R, W, Q
	AQP4	target 5' UUGCCCCAUAAGAGCAGUAGCUUUU 3' miRNA 3' AGCGGGAGAG--UUGG-GUCGAAAA 5'	-21.7	R
	NPR1	target 5' ACUCAUGUGCAGUCAGCUUUU 3' miRNA 3' AGCGGGAGAGUUGGG-----UCGAAAA 5'	-19.1	R, W
	MAPK1	target 5' CUCGCAUGACUGUUA---CAGCUUUC 3' miRNA 3' AGCGG---GAGAGUUGGGUCGAAAA 5'	-16.4	R

*trafficking protein particle complex 2B (*TRAPPC2B*); SMAD family member 4 (*SMAD4*); NFKB inhibitor alpha (*NFKBIA*); chromobox 7 (*CBX7*); BCL2 family apoptosis regulator (*MCL1*); aquaporin 4 (*AQP4*); natriuretic peptide receptor 1 (*NPR1*); mitogen-activated protein kinase 1 (*MAPK1)*

**Validation methods; R-Reporter assay, W-Western blot and Q-qPCR

Transcriptome of influenza B virus-infected cells

To investigate the human cells responses to influenza B virus infection, the A549 cells were infected with two lineages of influenza B virus; Victoria (B/Thailand/CU-B5522/2011) and Yamagata (B/Massachusetts/2/2012). At 24 hpi, the total RNAs was collected from the infected cells. Large RNAs was used to performed mRNAs profiling by RNA-seq. The raw sequencing reads were received in FASTQ formatted files. The samples were sequenced in paired-end (PE) mode with length 150 bases. To ensure that only good quality bases were used in the downstream analysis, the raw sequencing reads were subjected to trimming process. The surviving paired-end reads were aligned to the Homo sapiens reference genome (HISAT2 index : Ensembl GRCh37 genome_tran. The following table 3 summarized the alignment rates for each samples.

Table 3 Summary of trimmed reads.

Sample Name	Number of Trimmed Reads	Number of Mapped Reads	Mapping Rate (%)
B/Thailand/CU-B5522/2011 rep.1	61,871,664	54,393,314	87.9
B/Thailand/CU-B5522/2011 rep.2	61,406,880	54,035,009	88.0
B/Massachusetts/2/2012 rep.1	58,536,512	52,435,581	89.6
B/Massachusetts/2/2012 rep.2	56,964,152	50,928,207	89.4
Uninfected rep.1	56,877,004	48,881,048	85.9
Uninfected rep.2	58,365,868	50,188,133	86.0

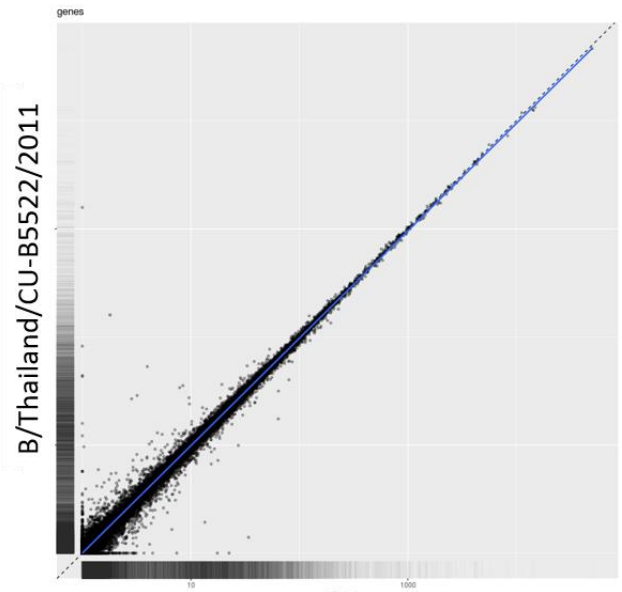
For differential expression analysis (DE), the significant DE genes or transcripts (q-value < 0.05) were extracted for each comparison groups. The overall expression profiles of the Victoria lineage (B/Thailand/CU-B5522/2011) and Yamagata lineage (B/Massachusetts/2/2012) infected cells compared to the uninfected cells were shown in scatter plot (Figure 8). Figure 9 shows the volcano plot and Figure 10 shows the heatmap of significant DE genes (q-value < 0.05) for each comparison groups.

There were 16 genes upregulated in Victoria lineage infection while 13 genes in Yamagata infection (Figure 11). Among these genes, 7 genes were found to be significantly upregulated in both lineages including AL162151.3 (pseudogene), MX1 (MX dynamin like GTPase 1), OASL (2'-5'-oligoadenylate synthetase like), ISG15 (ISG15 ubiquitin-like modifier), IFI6 (interferon alpha inducible protein 6), IRF7 (interferon regulatory factor 7) and IFIH1 (interferon induced with helicase C domain 1).

Nine genes were found to be specifically upregulated in Victoria lineage infection including CRCT1 (cysteine rich C-terminal 1), RP11-529H20.6, C5orf60 (chromosome 5 open reading frame 60), RNVU1-15 (RNA, variant U1 small nuclear 15), NRG4 (neuregulin 4), ZRANB2-AS2 (ZRANB2 antisense RNA 2), CMPK2 (cytidine/uridine monophosphate kinase 2), HERC5 (HECT and RLD domain containing E3 ubiquitin protein ligase 5), and SAMD9 (sterile alpha motif domain containing 9). In addition, 6 genes were specifically upregulated to Yamagata infection including IFNL2 (interferon lambda 2), IFNL1 (interferon lambda 1), IFNL3 (interferon lambda 3), TMEM40 (transmembrane protein 40), IFNB1 (interferon beta 1) and DSCR8 (Down syndrome critical region 8).

There was no similar downregulated in both Victoria and Yamagata lineages infection. There were 4 downregulated genes in Victoria lineage infection including LAMC2 (laminin subunit gamma 2), EGR1 (early growth response 1), MLLT10 (histone lysine methyltransferase DOT1L cofactor) and GMFG (glia maturation factor gamma). In addition, 2 genes were downregulated in Yamagata lineage infection including SIRPB1 (signal regulatory protein beta 1) and PCBP3 (poly(rC) binding protein 3).

A



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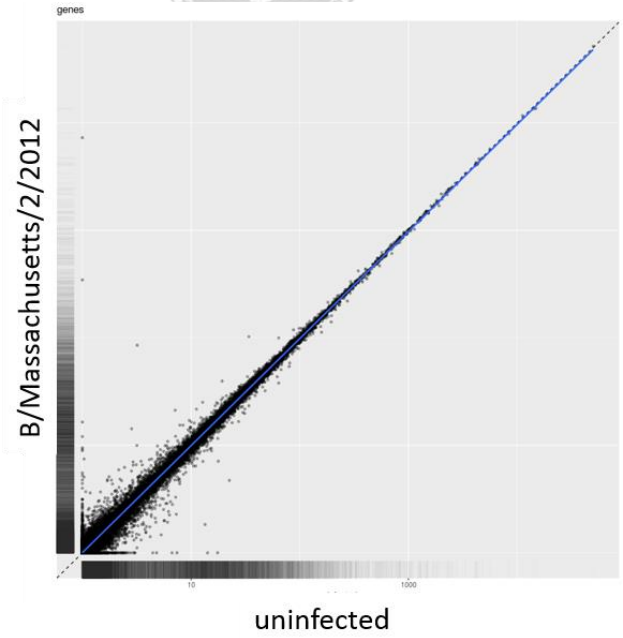
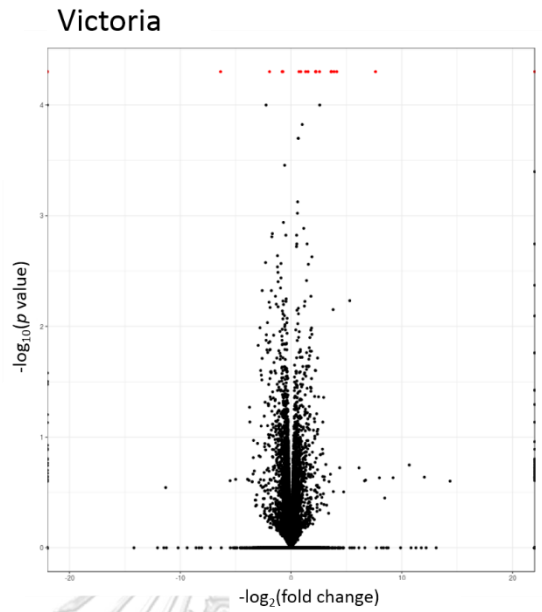


Figure 8 Scatter plot of pairwise comparisons between infected and uninfected cells (A) Victoria lineage and (B) Yamagata lineage.

A



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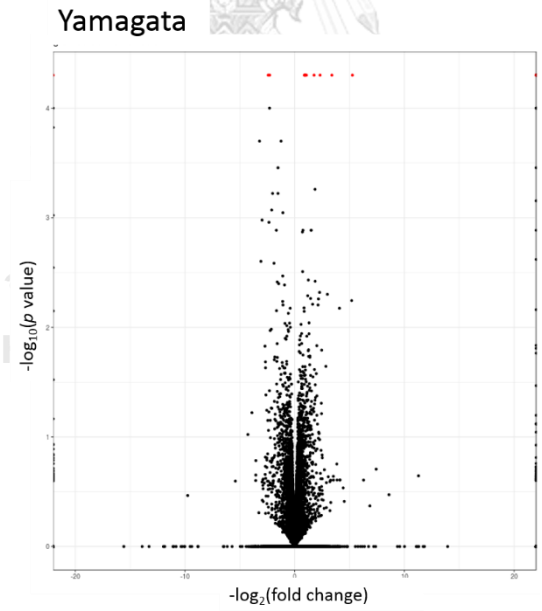


Figure 9 Volcano plot of RNAs expressed in infected and uninfected cells

Volcano plot with negative log of the p-value on the y-axis (base 10) and log fold change (base 2) on x-axis of comparisons between RNAs expressed in infected and uninfected cells; (A) Victoria lineage and (B) Yamagata lineage. Significant DE genes (*p*-value ≤ 0.05) are colored in red.

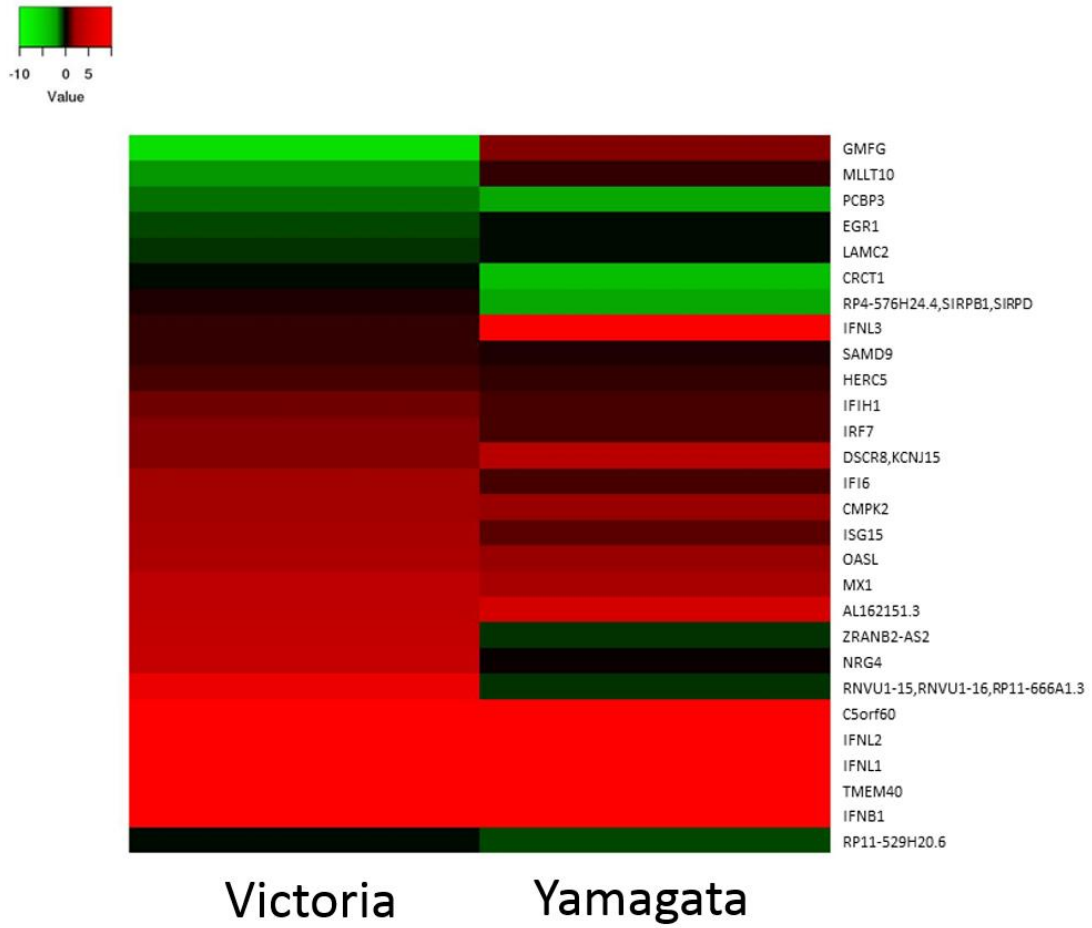
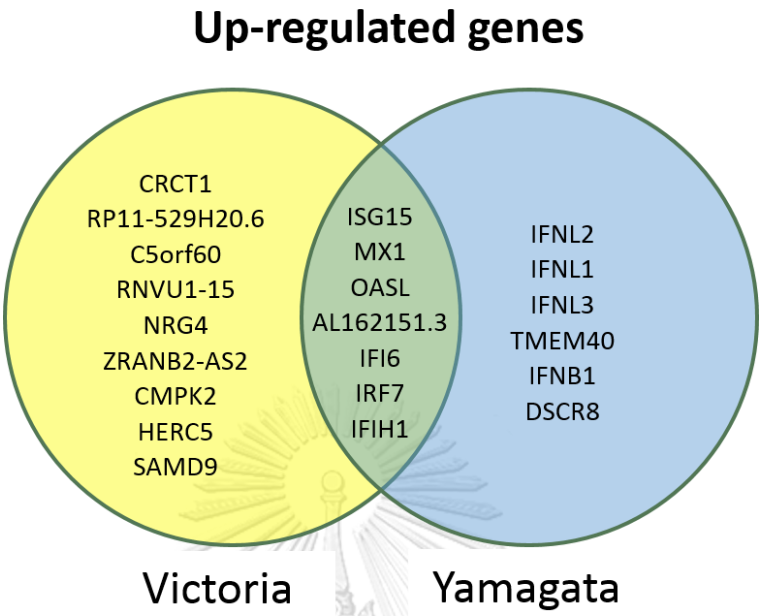


Figure 10 Heatmap of significantly different expression genes between influenza B virus infected cells and uninfected cells.

A



B

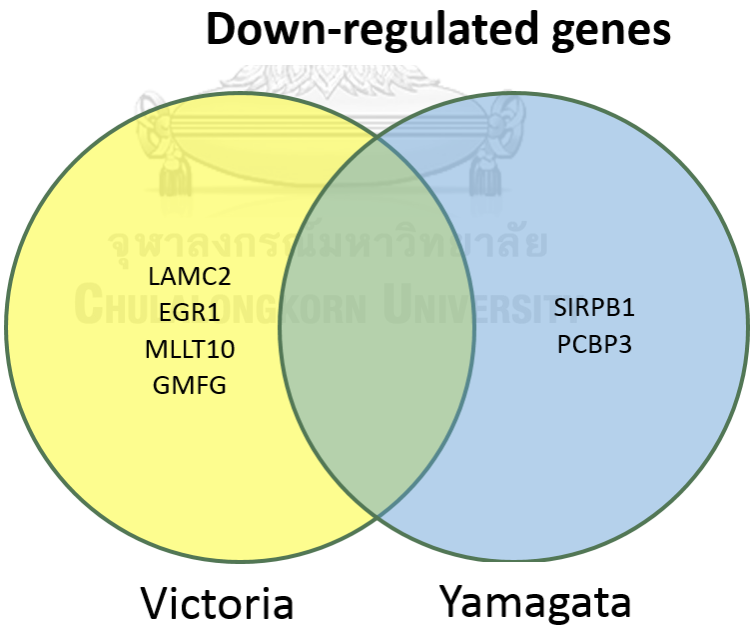


Figure 11 Venn diagrams represented the significant differential expression of genes that responded to influenza B virus infection.

(A) up-regulated genes (B) down-regulated genes

Differential expression of lncRNAs in cells infected with influenza B virus.

Many studies suggested that the intergenic regions of the human genome exhibit tissue-specific transcription resulting in the production of a variety of non-coding RNA species, including long non-coding RNA (lncRNA) (more than 200 bp) and microRNAs (~22 bp) which are involved in many cellular processes. In this study we also focused on the lncRNAs that responded to influenza B virus infection.

The RNA-seq result was used to analyzed for lncRNAs profiling by mapping sequences to the annotation database for long non-coding RNAs; NONCODEV5. And the differential expression of each lncRNA comparing between each lineage of influenza B infection and the uninfected cells were analyzed. The heatmap represented the significantly different expression of lncRNAs in influenza B virus infected cells (Figure 12).

There were 5 lncRNAs up-regulated in Victoria lineage influenza B virus infection including; NONHSAT002168.2:12-4867, NONHSAT028794.2:220-538, NONHSAT151746.1:537-912, NONHSAT189042.1:20-372 and NONHSAT225001.1:22-566. While 4 lncRNAs were up-regulated in Yamagata infection (NONHSAT097594.2:284-1349, NONHSAT173724.1:2-643, NONHSAT248121.1:18-638 and NONHSAT257967.1:14-1298) (Figure 13 A).

Twelve lncRNAs were found to be down-regulated in Victoria lineage infection (NONHSAT015510.2:0-1139, NONHSAT056749.2:70-307, NONHSAT082259.2:3-248, NONHSAT119380.2:143-619, NONHSAT151317.1:0-784, NONHSAT155666.1:36-272, NONHSAT158685.1:0-599, NONHSAT159980.1:0-1288, NONHSAT209353.1:5-540, NONHSAT229323.1:681-1139, NONHSAT239154.1:14-525 and NONHSAT239199.1:813-

1324) and 6 gene were specific to Yamagata infection (NONHSAT015510.2:0-1154, NONHSAT029530.2:59-614, NONHSAT075872.2:145-460, NONHSAT159222.1:2-540, NONHSAT169541.1:14-644, NONHSAT174775.1:660-1205 and NONHSAT254964.1:39-1811). Interestingly, there was no similar responded between Victoria lineage and Yamagata lineage influenza B viruses infection (Figure 13 B).

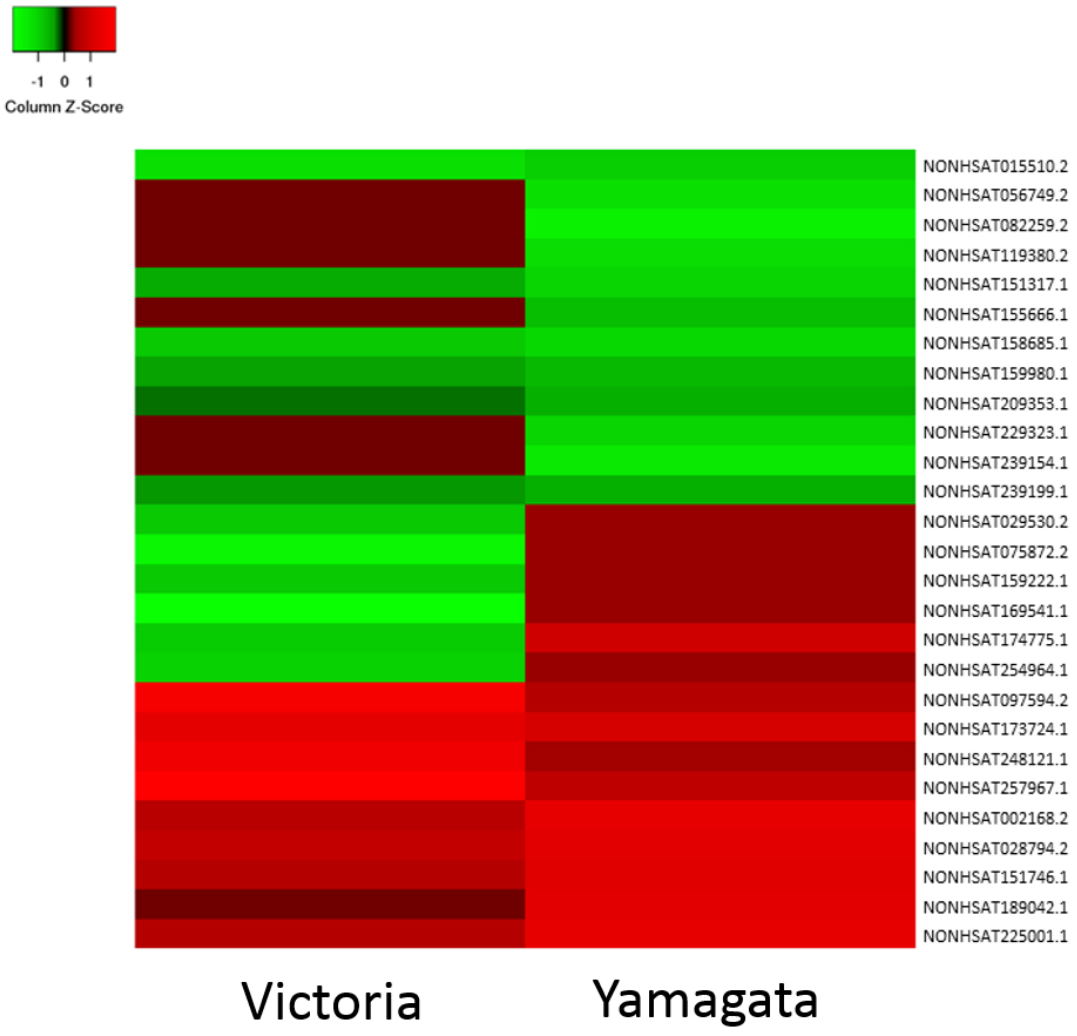
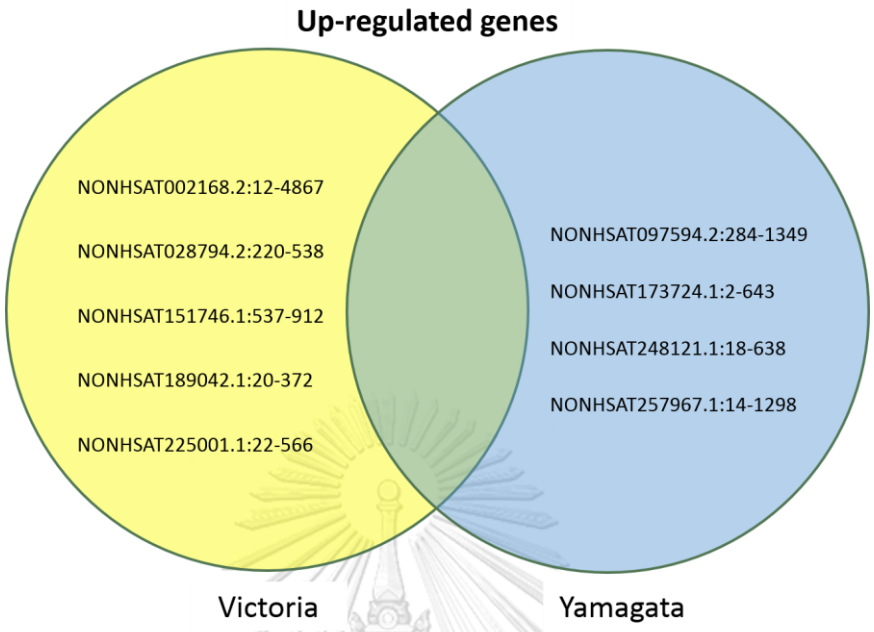


Figure 12 Heatmap of significantly different expression lncRNAs between influenza B viruses infected cells and uninfected cells.

A



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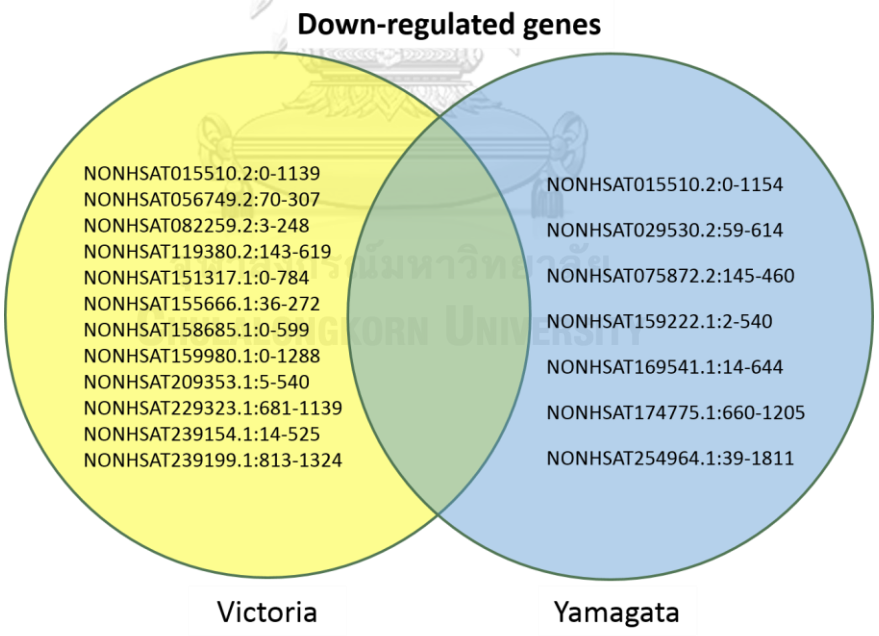


Figure 13 Venn diagrams represented the significant differential expression of lncRNAs that responded to influenza B virus infection.

(A) up-regulated genes (B) down-regulated genes

Differential expression of proteins in human cells infected with influenza B virus

The overall protein expression profiles of the Victoria lineage (B/Thailand/CU-B5522/2011) and Yamagata lineage (B/Massachusetts/2/2012) infected cells compared to the uninfected cells were shown in the volcano plot (Figure 14). There were 2 proteins found to be up-regulated including MX1 (MX dynamin like GTPase 1) and ATPA (ATP synthase subunit alpha). And 2 proteins were down-regulated in Victoria lineage infected cells; RL36A (60S ribosomal protein L36a) and LAMC2 (Laminin subunit gamma-2) (Figure 15). Whereas only 1 protein found to be up-regulated; MX1 and 2 proteins including CTAB and ERP29 (Endoplasmic reticulum resident protein 29) were down-regulated in Yamagata lineage infected cells.

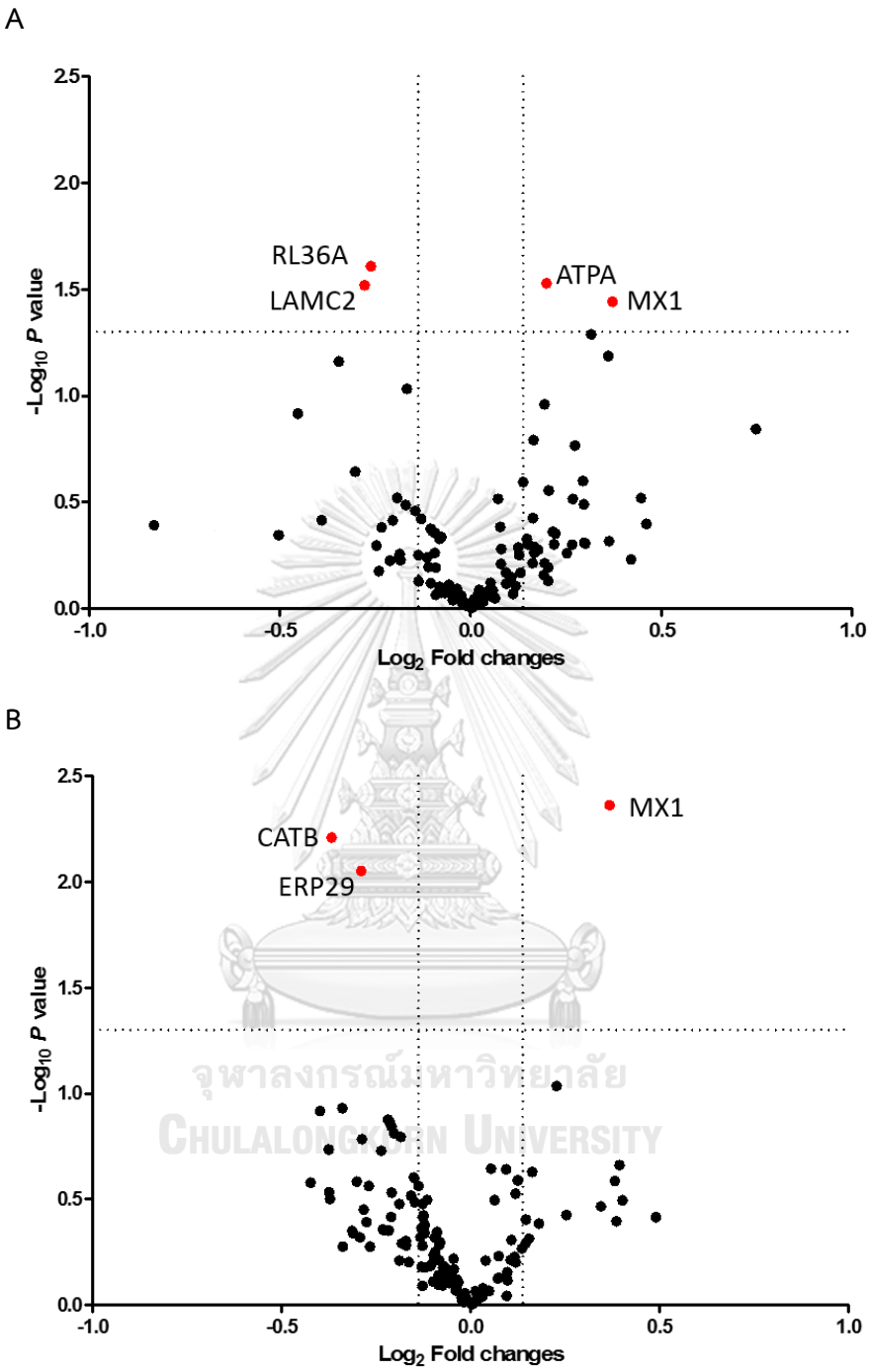
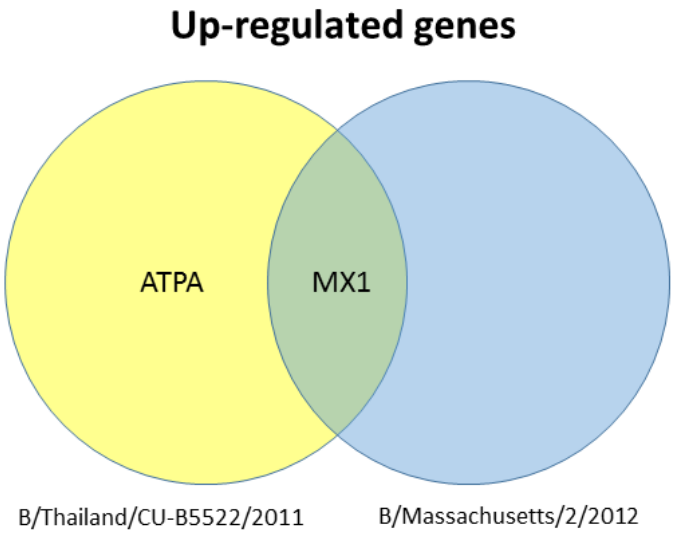


Figure 14 Volcano plot between proteins expressed in infected and uninfected cells. The volcano plot with negative log of the p-value on the y-axis (base 10) and log fold change (base 2) on x-axis; (A) Victoria lineage and (B) Yamagata lineage. Significant DE genes (*p*-value ≤ 0.05) are colored in red.

A



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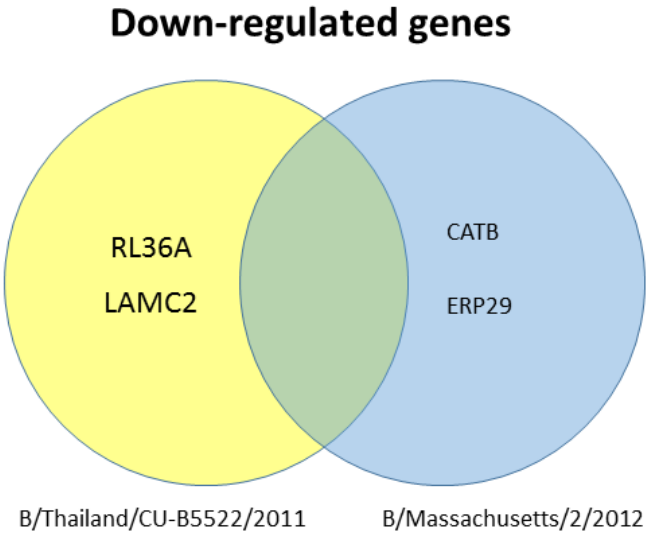

Figure 15 Venn diagrams of significant differential expression proteins.

This diagrams represents the differential expression of proteins responded to influenza B virus infection (A) up-regulated genes and (B) down-regulated genes.

Multi-omics integration

The miRNAs profile, mRNAs profile and protein profile were integrated to predict the interaction network among responded genes in influenza B virus infected cells. The predicted targets of responded mRNAs were not significantly changed in both transcriptome and proteome. As shown in table 4, the candidate target genes from the *in silico* prediction of each miRNAs and their fold change from RNA-seq result shows that the fold change of the target gene is around 1. It can imply to be unchanged when compare with the uninfected cells. This might because the effect of a single time point collection. Another possibility is the lncRNAs which can function as miRNA sponge and compete with the miRNA target genes. We further analyzed the *in silico* prediction of hybridization between responded lncRNAs and responded miRNAs. The result shows in table 5 below. When the lncRNAs act as miRNA sponge, the miRNA that can bind to the lncRNAs may be inhibited and cannot function.

Table 4 Summary of responded miRNAs and their target RNAs with fold change

miRNA	Fold change of miRNA	Target RNA	Fold change of target RNA
miR-301a-3p	2.314	SMAD4	1.000
miR-30e-3p	2.091	NFKBIA	0.919
miR-421	-2.078	CBX7	1.289
miR-320a	-2.494	MCL1	0.951
		NPR1	0.947
		MAPK1	0.962

Table 5 Hybridization prediction between miRNAs and lncRNAs

miRNAs	lncRNAs (FluB lineage)	Hybridization pattern	MFE (kcal/ mol)
miR-769-5p	*NONHSAT 239199.1* (Victoria)	lncRNA 5' ACUCAG-AUCCAUGGGUCUCU 3' miRNA 3' UCGAGUCUUGGGUCUCCAGAGU 5'	-30.5
miR-30e-3p	*NONHSAT 0155510.2* (Victoria)	lncRNA 5' GGUGAACUCUCUGUAUACUUGCUGAAAGU 3' miRNA 3' CGACAUUUGU-AGGC-------UGACUUUC 5'	-22.1
	NONHSAT 056749.2 (Victoria)	lncRNA 5' UCUGUUCUGCCUUCUGCUGAGGGG 3' miRNA 3' CGACAUU-UGUAGGCUGACUUUC 5'	-21.5
	NONHSAT 151317.1 (Victoria)	lncRNA 5' AGCUC-GGACAGCGGCUCCGGGUUCUGGAAGG 3' miRNA 3' CGACAUUUGU-----AGGCU---GACUUUC 5'	-21.8
	NONHSAT 159980.1 (Victoria)	lncRNA 5' ACUGCGGAGCGCGCCGGCUGGAAA 3' miRNA 3' CGACA-UUUGUA-GGCUGACUUUC 5'	-26.6
	NONHSAT 209353.1 (Victoria)	lncRNA 5' AGUUGCUAAACAGCUGGCUAACCAAACAGGAGC 3' miRNA 3' CGAC-AUUUGUAGGCUGA---------CUUUC 5'	-21.1
	NONHSAT 229323.1 (Victoria)	lncRNA 5' CCUGGGAGGACAUCCUUCCCCAUCCUCCAGGCUGAAAA 3' miRNA 3' CGACA--UUUGUAGG--------------CUGACUUUC 5'	-23.9
	NONHSAT 239199.1 (Victoria)	lncRNA 5' AGCUGGGGAGAUGGUCUGGUGAAUGGUAGGCUGAGAC 3' miRNA 3' CGACA---UUUGUAGG-----------CUGACUUUC 5'	-22.9
	NONHSAT 015510.2 (Yamagata)	lncRNA 5' GGUGAACUCUCUGUAUACUUGCUGAAAGU 3' miRNA 3' CGACAUUUGU-AGGC--------UGACUUUC 5'	-20.8
	NONHSAT 029530.2 (Yamagata)	lncRNA 5′ UCUGUGGAUCCAAUCUGUAAUCUGCAGAGC3′ miRNA 3' CGACAUUUG---UAGGCU---GAC-UUUC 5'	-26.6
miR-532-5p	*NONHSAT 229323.1* (Victoria)	lncRNA 5' CGCGGUGCGCCCCCUUCCCACCUGCGCG-AGGGCAUC 3' miRNA 3' UGCCA--------------GGAUGUGAGUUCCGUAC 5'	-29.6
	NONHSAT 254964.1 (Yamagata)	lncRNA 5' CUCCUUGAAACCAGAGGGCAAGGCAUGA 3' miRNA 3' UGCCAGGA--UGU-GA-----GUUCCGUAC 5'	-25.5
miR-410	*NONHSAT 158685.1* (Victoria)	lncRNA 5' CGGC--UCUGUGACAGUCUACCAUAUUAUGUUA 3' miRNA 3' UGUCCGGUAGACAC-------------AAUAUAA 5'	-18.4
	NONHSAT 075872.2 (Yamagata)	lncRNA 5' UAGUGGCCGUUUAGUGUUGA 3' miRNA 3' UGUCCGGUAGA-CACAAUAUAA 5'	-24.8
miR-511	*NONHSAT 254964.1* (Victoria)	lncRNA 5' CACUAGCCUGCCAGCACCUUCUUCUUUCCUUGAA 3' miRNA 3' UGAUCUGACACUCG-----------AGGAGCU 5'	-24.7

Function of miR-30e-3p in respond to influenza B virus infection

In addition, from the literature review suggested that miR-30e-3p was also upregulated in human cells infected with influenza A virus and Dengue virus. Therefore, the miR-30e-3p was selected as the candidate to study the effect of miRNA to influenza B virus production. The mimic miRNA and miRNA inhibitor were used in this study to upregulated and downregulated the expression of miR-30e-3p, respectively. The transfection of miR-30e-3p mimic significantly increased the amount of miR-30e-3p in the A549 cells (Figure 16) while the cell viability of transfected cells was not different from untreated and mock (Figure 17).

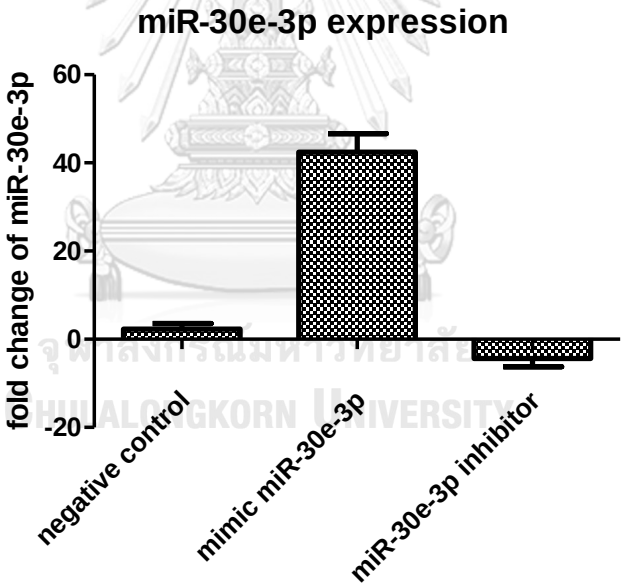

Figure 16 The miR-30e-3p expression levels in A549 cells transfected with miR-30e-3p mimic, miR-30e-3p inhibitor and negative control.

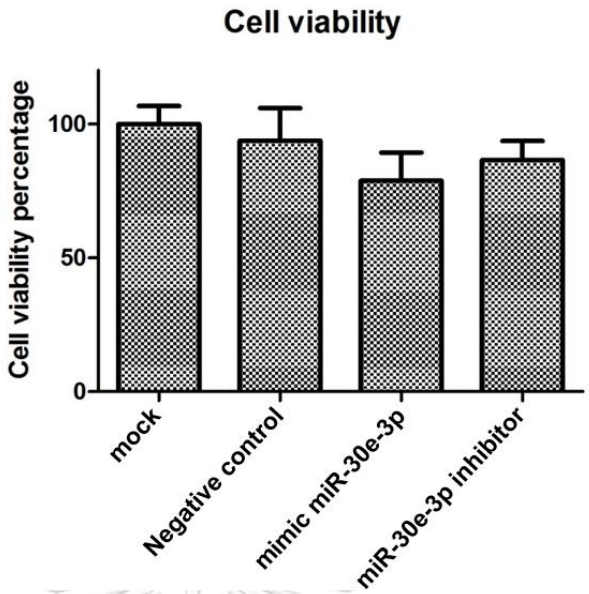

Figure 17 Cell viability of transfected cells.

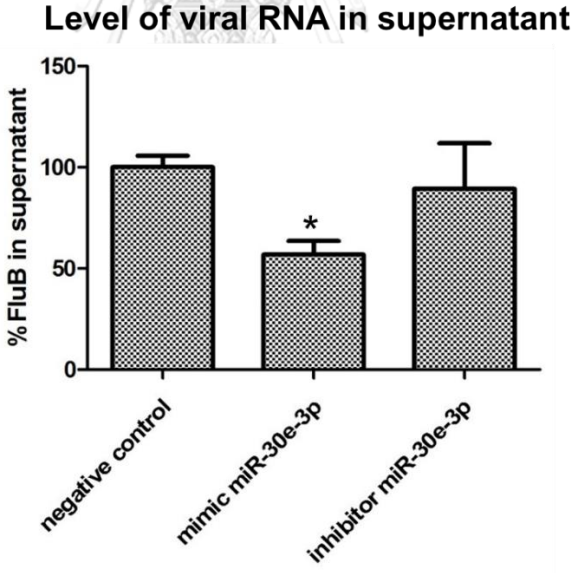

Figure 18 Level of influenza B virus RNA in supernatant from A549 cells transfected with miR-30e-3p mimic, miR-30e-3p inhibitor and negative control.

Effect of miR-30e-3p and influenza B virus production.

The effect of miR-30e-3p to influenza B virus production was studied by using the transfection of miR-30e-3p mimic, miR-30e-3p inhibitor and negative control to the A549 cells. At 24 hours post-transfection, cells were infected with influenza B virus at MOI = 0.5. After 24 hours post-infection, supernatant was collected and extracted for total viral RNAs. Reverse transcription was performed by using random hexamer and the cDNA of influenza B virus was determined by using TaqMan probe. The result as shown in Figure 18 found that the upregulated miR-30e-3p significantly reduced influenza B virus production.

Influenza B viral targets of miR-30e-3p.

In silico prediction for viral targets of miR-30e-3p was shown in Figure 19. To validate the effectiveness of miR-30e-3p to silence the expression of these viral genes, 3'-URT reporter assay was performed. The sequences of predicted viral target sites were used to construct reporter vector by using pmiRGLO as backbone. For miRNA expression vector, the sequence of duplex miR-30e was used with the backbone, pSilencer3.0-H1. For silencing positive control, pSilencer_siLuc2 was constructed to silence the expression of Luc2 gene which is the reporter gene in pmiRGLO. All constructed vectors were confirmed by Sanger sequencing. Luciferase assay was performed after 48 hours post-transfection and the relative luciferase activity was calculated. As shown in Figure 20, the relative luciferase activity of both NA and NP were significantly decreased in the cells cotransfected with target reporter vector and miR-30e-3p expression vector. This indicated that the miR-30e-3p can directly target to NA and NP of influenza B virus and silence their expressions.

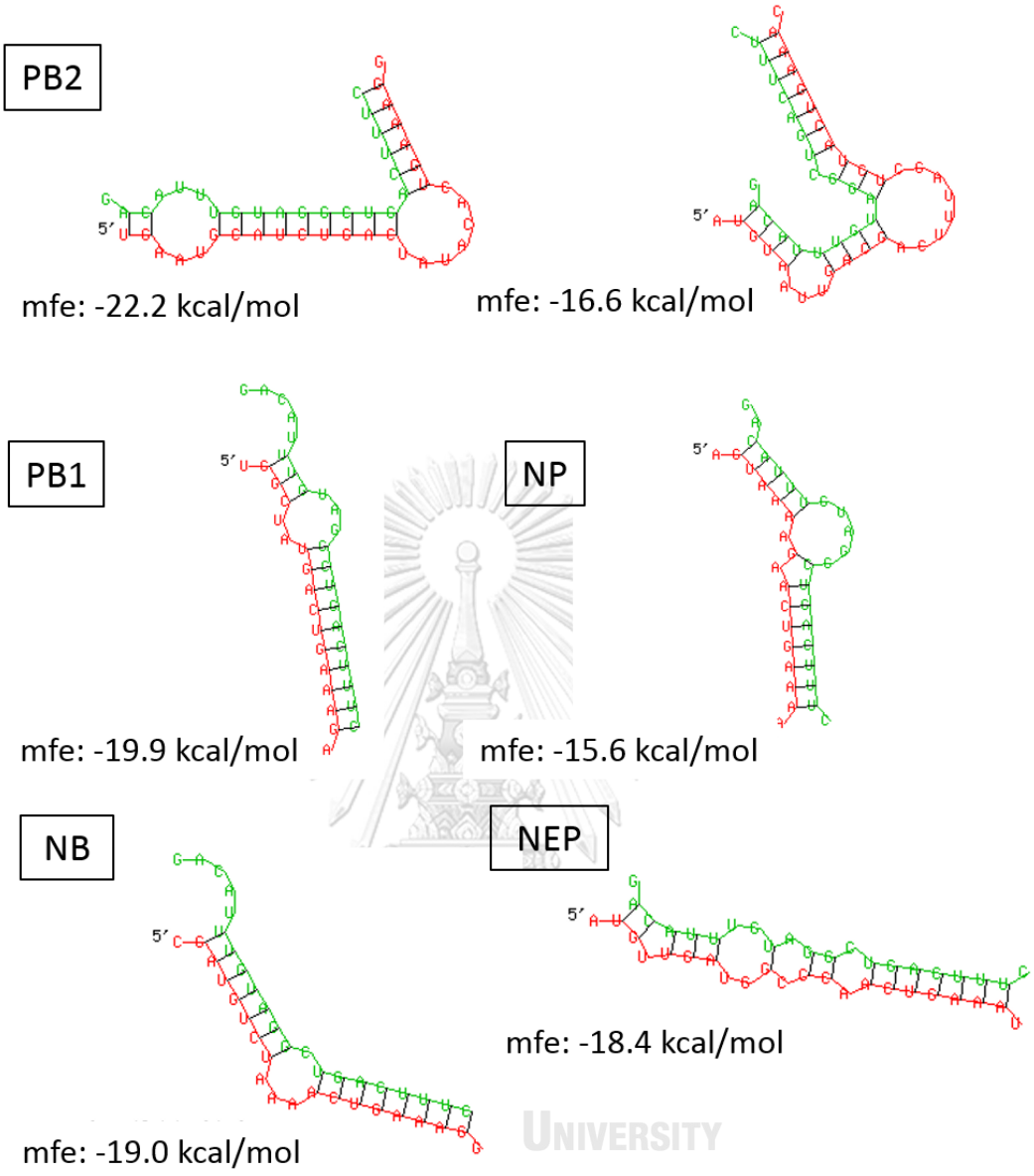

Figure 19 *In silico* prediction of influenza B viral target of miR-30e-3p.

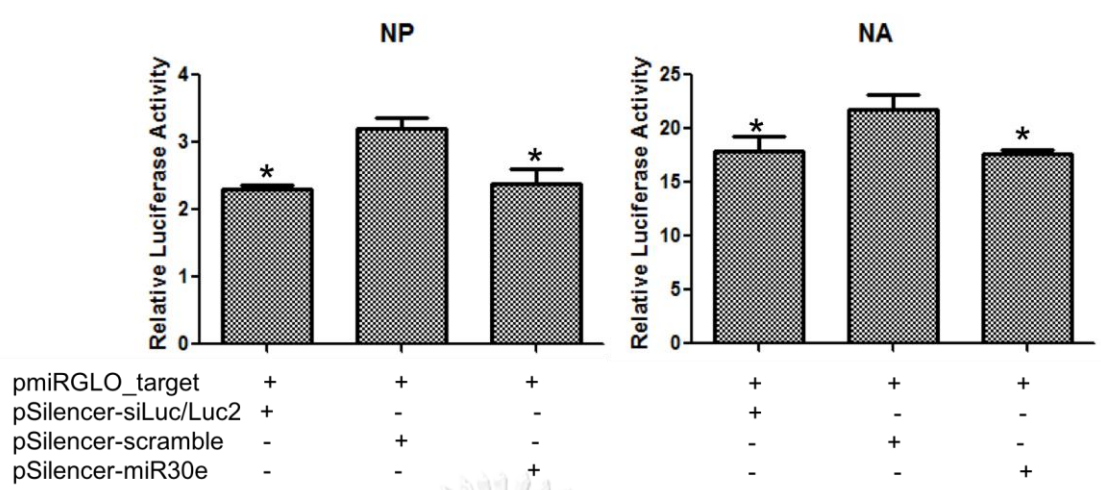

Figure 20 Luciferase assay determined the direct target of miR-30e-3p to viral genes of influenza B virus.

Chapter 5 Discussion and Conclusion

Discussion

This study is the first report of systems biology study in influenza B virus infection. Using the multi-omics study to investigate human cellular responses to the infection of influenza B virus in both lineage; Victoria and Yamagata integrated the data from several parts of the responses. It is a holistic approach to interpreting the complexity of cellular responses to influenza B virus infection that starts from the understanding that the networks form the whole human cells more than the sum of their parts. And the A549 cells were used in this study to represent human epithelial lung cells. The A549 cells are cancerous cell line, the transcriptome and proteome of this cell might be different from normal human epithelial lung cells. But these cells have been used for various influenza researches e.g. host genes responded to influenza virus infection (52, 53). The candidate of responded genes in this study might be useful for the further host-viral interaction study.

All the samples in this study including total RNAs and total proteins were collected at 24 hours post infection (hpi). Even they were collected at the single time point but at 24 hpi was used in many viral-host interaction studies to represent the early stage of the viral infection. The limitation of single time point collection might affect the outcome of data integration. For example the interaction between the responded miRNAs and their targeted genes which may not be significantly decreased when collect the miRNAs and their targets genes at the same time. And for 24 hours after the infection, the protein profiling compared between infected and uninfected cells might be not much changed due to the half-life of the proteins.

According to the results of miRNAs profiling, various miRNAs responding to infection of influenza B virus were both up-regulated and down-regulated. MiRNAs can inhibit gene expression by translational repression or mRNA degradation. This may play an important role in response to influenza B virus infection. The up-regulated miRNAs may lead to the repression of some host genes that are advantageous to the virus during viral replication. Alternatively, some miRNAs were upregulated during influenza B virus infection and the repression of those genes lead to an antiviral response e.g. the up-regulation of miR-30e-3p which targets NFKBIA promoting NF-**K**B dependent IFN production. The production of IFN can trigger the innate immune response and may suppress viral replication (54). MiR-376c which was found to be down-regulated in this study was reported to be up-regulated in the serum pool of influenza A virus (subtype H7N9) infected patients (55). However, the function of hsa-miR-376c and its target genes in influenza virus infection remains unclear.

Both miR-889 and miR-484 were shown to be down-regulated due to the influenza B virus infection. Hsa-miR-889 has been suggested to be associated with respiratory development and lung development, whilst hsa-miR-484 has been reported to target mitochondrial fission protein, Fis1 (56). Previous studies have suggested that hsa-miR-484 may be downregulated during apoptosis (57). Thus, the down-regulation of hsa-miR-484 may result from apoptosis due to influenza B virus infection.

The hsa-miR-30 family has been suggested to be relevant in lung repair (58). According to the results hsa-miR-30a, hsa-miR-30c and hsa-miR-30e* were upregulated at 24 hours post infection. This may indicate the role of miRNAs in the host response to viral infection.

According to the RNA-seq results, the differential expression analysis showed the responded genes of Victoria and Yamagata lineages of influenza B viruses. For the genes responded in both lineages, most of them are innate immune response genes or interferon-related genes; MX1, OASL, IFI6, IRF7 and IFIH1. The IRF7 encodes interferon regulatory factor 7 playing a role in the transcriptional activation of virus-inducible cellular genes including the type I interferon genes (59). MDA5 is a part of the RIG-I-like receptor (RLR) family functions as a pattern recognition receptor that is a sensor for viruses (60). The MX1, OASL and IFI6 are interferon-inducible genes (61, 62) which also play roles in viral infection response.

For lineage specific responded gene, the Victoria specific upregulated CMPK2 which interferon stimulated gene reported to be involved in maintenance of intracellular UTP/CTP. This function of CMPK2 was reported to restrict viral replication including PRRSV and HIV virus (63, 64). The HERC5 which upregulated in Victoria lineage infection is a member of the HERC family of ubiquitin ligases and encodes a protein with a HECT domain and five RCC1 repeats. Pro-inflammatory cytokines upregulate expression of this gene in endothelial cells. The protein localizes to the cytoplasm and perinuclear region and functions as an interferon-induced E3 protein ligase that mediates ISGylation of protein targets (65). And the functional analyses showed that the human small HERCs exhibit different degrees of antiviral activity (66). The Victoria infection upregulated SAMD9 encodes a sterile alpha motif domain-containing protein. The encoded protein localizes to the cytoplasm and may play a role in regulating cell proliferation and apoptosis (67).

For the Yamagata lineage infection, many upregulated genes are related to cellular immune response. Including IFNL2, IFNL1, IFNL3 and IFNB1. The IFNL2 which also known as IL28A encodes a cytokine distantly related to type I interferons and the IL-10 family. Expression of the cytokines encoded by this family genes can be induced by viral infection (68). IFNL1 or IL29 encodes a cytokine distantly related to type I interferons and the IL-10 family that function related to IFNL2 and IFNL3 (69).

Interestingly, the LAMC2 which was downregulated in Victoria lineage infection for both mRNA and protein level as shown in the mRNAs profile and proteins profile. This gene encodes subunit of Laminins which are extracellular matrix glycoproteins that connect epithelial cells to the basement membrane. They have been implicated in a wide variety of biological processes including cell adhesion and differentiation (70). In contrast to this study, LAMC2 was reported to be upregulated when cells infected with rhinovirus (71).

This study is also the first study investigating the lncRNAs responding to influenza B virus infection. According to the lncRNAs profiling result, there were several lncRNAs responded to the infection of influenza B virus. The lncRNAs profiling in this study was performed after the poly(A) enrichment so the result was gathered less lncRNAs than using total RNA to prepare the library. Even though, the lncRNA profile of poly(A) enriched library was also reliable (72). Interestingly, there was no similar responded lncRNA in both Victoria and Yamagata lineages infection. Due to the limitation of lncRNAs study that the information for lncRNA function is limited. Even the functions of these lncRNAs are still unknown and the interaction between these lncRNAs and viral infection is unclear but the responded lncRNAs found in this study may be useful when integrated with the up-coming lncRNAs study.

Moreover, the MX1 gene was found to be up-regulated in both Victoria and Yamagata lineages of influenza B virus infection. And it was also detected in both transcriptome and proteome. This finding indicated that the MX1 gene may play important role in early response to the influenza B virus infection. It was also reported that Mx1 protein can inhibit influenza virus replication by interfering the viral ribonucleoprotein complex assembly (73-75).

Conclusion

In summary as shown in Figure 21 when human cells infected with influenza B virus, cells will respond to the infection by changing the transcription of host genes including mRNAs, lncRNAs and miRNAs. The host miRNAs can directly target to viral RNA and inhibit viral replication. Moreover, the responded miRNAs can also target to host mRNAs and regulate responded gene expression via mRNA degradation or translation repression. The lncRNAs may function as miRNA sponge and inhibit miRNA function. These regulation may lead to the protein expression level of responded genes.

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PUBLICATION	1: Jampoka K, Muangpaisarn P, Khongnomnan K, Treeprasertsuk S, Tangkijvanich P, Payungporn S. Serum miR-29a and miR-122 as Potential Biomarkers for Non-Alcoholic Fatty Liver Disease (NAFLD). Microrna. 2018;7(3):215-222. 2: Khongnomnan K, Poomipak W, Praianantathavorn K, Saengchoowong S, Pisitkun T, Poovorawan Y, Payungporn S. Human MicroRNAs Expression Profiles in Influenza B Virus-Infected Cells based on Illumina MiSeq Platform. Microrna. 2018;7(3):204-214. 3: Makkoch J, Poomipak W, Saengchoowong S, Khongnomnan K, Praianantathavorn K, Jinato T, Poovorawan Y, Payungporn S. Human microRNAs profiling in response to influenza A viruses (subtypes pH1N1, H3N2, and H5N1). Exp Biol Med (Maywood). 2016 Feb;241(4):409-20. 4: Khongnomnan K, Makkoch J, Poomipak W, Poovorawan

Y, Payungporn S. Human miR-3145 inhibits influenza A viruses replication by targeting and silencing viral PB1 gene. Exp Biol Med (Maywood). 2015 Dec;240(12):1630-9.

5: Wacharapluesadee S, Sintunawa C, Kaewpom T, Khongnomnan K, Olival KJ, Epstein JH, Rodpan A, Sangsri P, Intarut N, Chindamporn A, Suksawa K, Hemachudha T. Group

C betacoronavirus in bat guano fertilizer, Thailand. Emerg Infect Dis. 2013 Aug;19(8):1349-51.

AWARD RECEIVED

"Very Good" award in the category of MSc/PhD students at the Graduate Research Competition 2018, Faculty of Medicine, Chulalongkorn University