Direct identification and quantification of host and viral miRNAs after influenza infection using the next generation ultra-high throughput DNA sequencer

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KEY MESSAGES
1. The host and viral whole transcriptomes (including miRNA and mRNA of pathogenic H5N1 and seasonal H1N1 influenza-virus-infected human macrophages) were quantified and compared.
2. A total of 43 new human miRNA candidates were discovered.
3. No miRNA was encoded by influenza viruses.
4. In response to H5N1 infection, the RIG-I-like receptor signalling pathway was the most significant pathway regulated by known human miRNAs.

Introduction
The subtype of avian influenza H5N1 virus is epizootic and panzootic, and can transmit zoonotically to humans. The fatality rate of H5N1 disease remains high at about 60%, but this rate may be over-estimated, as H5N1 viruses cause both severe and subclinical infection in humans, and these mild non-hospitalised H5N1 cases are not counted by the World Health Organization as confirmed cases.1

Cytokine dysregulation is one of the key contributory factors to H5N1 pathogenesis.2 The precise mechanisms by which the H5N1 virus elicits the differential host responses remain poorly understood.

MicroRNAs (miRNAs) are 21-23 nt RNA molecules that can modulate gene expression and play an essential role in the regulation of many diverse biological processes, including viral infection. Macrophages are key host immune cells that respond to viral infection and are a major source of many cytokines. The present study used the new generation ultra-high throughput Solexa sequencer to determine host- and viral-derived miRNAs (if any) induced by the highly pathogenic H5N1 and low virulent H1N1 viruses in human macrophages at early time points. Together with the mRNA expression profile, the possible regulating pathways/mechanisms were revealed by identifying miRNAs that may lead to influenza pathogenesis.

Methods
This study was conducted from May 2009 to October 2011.

Virus infection of macrophages
Human macrophages were infected with H1N1 (A/HK/54/98) and H5N1 (A/Viet/3212/04) at a multiplicity of infection (MOI) of two. Cells and viruses were prepared as described previously.1 After 30 minutes for virus adsorption, the inoculum was removed; cells were washed and incubated in macrophage serum-free medium (Invitrogen, CA, USA) supplemented with 0.6 mg/L penicillin and 60 mg/L streptomycin.

Total RNA isolation
Total RNA was extracted from cells 1, 3, and 6 hours post-infection using a mirVana miRNA Isolation Kit (Ambion, TX, USA) according to the manufacturer’s instructions. The Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) was used to assess RNA quality. All total RNA samples had a RNA Integrity Number (RIN) >9.0.

Ribosomal RNA depletion
Large 18S and 28S rRNAs were removed from 1 μg total RNA with the RiboMinus Transcriptome Isolation kit (Human/Mouse) (Invitrogen, CA, USA) according to the manufacturer’s instructions. The rRNA-depleted RNA was precipitated with Pellet Paint (Novagen, WI, USA), quality checked on the Agilent 2100 Bioanalyzer (Agilent Technologies).

Illumina mRNA library preparation
The rRNA-depleted RNA from 1 μg of total RNA was fragmented by incubation for 5 minutes at 94°C...
in 5 × Array Fragmentation Buffer (Ambion, TX, USA). The reaction was stopped by chilling the tube on ice and precipitated with Pellet Paint. Double strand cDNA was synthesised with the SuperScript Double Stranded cDNA synthesis kit (Invitrogen, CA, USA) using random hexamers according to the manufacturer’s instructions. The reaction was cleaned up on a QiAQuick PCR column (Qiagen, CA, USA). Double-stranded cDNA fragments were repaired with DNA Terminator End Repair Kit End (Lucigen, WI, USA) by incubation for 30 minutes at 30°C and then cleanup on a QiAQuick PCR column. The Klenow 3’ to 5’ exo- (NEB, MA, USA) was used to add a single ‘A’ base to the 3’end of blunt phosphorylated DNA fragments by incubation for 30 minutes at 30°C. After clean-up on a QiAQuick PCR column, Illumina PE Adapter was ligated to the end of DNA fragments with the Quick Ligation Kit (NEB, MA, USA) by incubation for 15 minutes at room temperature. The reaction was cleaned up on a QiAQuick PCR column. 180-200 bp fragments were excised from a 2% low range agarose gel. Fragments were enriched by 10 cycles using AccuPrime PfX DNA Polymerase (Invitrogen, CA, USA). The PCR product was run on Novex 8% TBE polyacrylamide gel and stained with SYBR Gold. The gel slice was cut off and cleaned up by QiAQuick Gel Extraction Kit. The concentration of gel purified DNA fragments was measured using a ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, DE, USA).

Illumina small RNA library preparation
A small RNA library was prepared from rRNA-depleted RNA from 1 µg of total RNA according to the Illumina manufacturer’s instructions. The 3’ adapter (5’–rAppAGATCCTGGAAGAGCGGC TTCAGCAGAAGGTCCGG/3ddC/-3’), 3ddC represented a 3’ OH blocking group) that had an adenylated 5’-end and a ddC 3’-end was ligated to the 3’of the RNA using T4 RNA Ligase 2, truncated. The 5’ adapter (5’–rArCrArCrUrCrUr UrUrCrCrCrUrArCrArCrGrArCrGrUrCrUrCrUr CrCrGrArUrCrU–3’) ligation was performed using T4 RNA Ligase 1. The ligation product served as a template for cDNA synthesis using SuperScript II Reverse Transcriptase and an oligonucleotide with the sequence complementary to 3’ adapter as RT primer (5’–CTCGGCATTCTCTGCTGAACCGCCTC–3’). The cDNA was amplified for 12 cycles using AccuPrime PfX DNA Polymerase. The PCR product was purified on Novex 8% TBE polyacrylamide gel and the gel slice in the range of 140-190 nt was extracted and cleaned up by QiAQuick Gel Extraction Kit. Adapters and RT primer used in this study were purchased from Integrated DNA technology.

Illumina sequencing
The amplified library was measured using quantitative PCR. RNA libraries were loaded into flow cell lanes at 8 pm concentration. mRNA and small RNA sequencing were carried out by running 38 and 76 cycles, respectively, on the Illumina IIG Genome Analyzer according to manufacturer’s instructions.

Illumina Sequencing data analysis
Sequences were extracted from image files using the Illumina pipeline based on the default parameters of the pipeline. Single read sequences with read length 38 bp for mRNA and 76 bp for small RNA with high quality were obtained.

mRNA sequencing reads were screened for polymer, primer sequences, and ribosomal RNA sequences The clean, high-quality sequencing reads were then mapped to human genome assembly (NCBI Build 37.1). The expression level was measured with reads per kilobase per million mapped reads (RPKM) by normalising the number of mapped reads to length of RNA and total number of mapped reads. The differential expression of genes was represented by fold change of RPKM of each gene in H1N1/ H5N1 infected samples in response to mock. The heatmap of gene expression was generated based on hierarchical clustering of Log2 (fold changes) using MeV viewer v4.6. Small RNA sequencing reads were trimmed with the low-quality ends (quality score=2) and primer adaptor sequences if more than 5 bp matched to the adaptor sequences. To quantify the trimmed sequencing reads, they were mapped to known miRNA database miRBase release 15. The abundance level of mature/mature* miRNA is represented by RPM (reads mapped per million mappable reads), where the mappable read counts normalised to the total number of mappable reads for each sample. In order to quantify the sequencing composition in the library, the remaining sequencing reads were aligned sequentially against known miRNA precursors, ribosomal RNA, tRNA (tRNA-SE), snRNA (snRNABase), and mRNA (NCBI RefSeq RNA release 39). Similar to the mRNA sequencing analysis, the relative abundance of miRNA is measured by fold change of RPM in H1N1/H5N1 infection samples in response to mock.

Pathway over-representation analysis
Pathway over-representation analysis was performed using the InnateDB platform (http://www.innatedb.ca). Over-representation analyses were performed using parameters (hypergeometric algorithm, Benjamini-Hochberg multiple testing correction). Results with corrected P values of ≤0.07 were considered statistically significant for mRNA pathways and ≤0.05 for miRNA target gene pathways. The choice of the P value was based on the conventional and empirical reasons such that the
significant and interested pathways were retained.

Results
Distinct cellular miRNA expression patterns in response to H1N1 and H5N1 influenza virus infection

The expression level of the known miRNAs in H1N1-, H5N1-, and mock-infected human macrophages at 1, 3, and 6 hours post-infection were compared. The proportion of sequence reads from each library that could be mapped to the miRBase 15, ranged from 19.13% to 32.08%. These mapped reads were from annotated known miRNAs as well as their precursor, mature, and mature star forms. The remaining sequence reads were derived from rRNAs, tRNAs, small snoRNAs, mRNAs, and the potential novel miRNA candidates. The relative abundance of miRNAs in each sample was measured with RPM by normalising against the different sequencing coverage depth of each sample. Compared with mock, the expression of miR-26b was apparently down-regulated in response to H1N1 and H5N1 infection at all time points. To investigate the differential expression of miRNAs in response to influenza A virus infection, the fold change of expression levels was calculated in influenza A virus infected cells and compared with mock-infected control cells. The miRNAs were ranked based on Z-statistics. The mature miRNAs with mappable reads >10 in at least one of nine sample datasets were included. Based on a fold change cut-off of 1.2, a total of 241 mature miRNAs were retained, and the differential expression pattern was revealed by the heat maps for H1N1- and H5N1-infected macrophages. From the mosaic structure of the heat maps, the altered expression trend in infection time and infection type was noted. Some miRNAs were up-regulated or down-regulated at all three time points and for both infection types. Many miRNAs had a similar expression pattern in response to H1N1 and H5N1 infection. However, several blocks of miRNAs behaved differently with time for H1N1 and H5N1 infection.

Based on a 1.2-fold cut-off, the differences in host response to H5N1 compared with H1N1 were determined. Most were co-regulated between H1N1 and H5N1 infection at 1, 3, and 6 hours. Respectively at 1, 3, and 6 hours after infection, 8, 4, and 5 mature miRNAs were up-regulated and 5, 7, and 22 mature miRNAs were down-regulated specifically in response to H1N1, whereas 6, 6, and 28 mature miRNAs were up-regulated and 14, 4, and 10 mature miRNAs were down-regulated specifically in response to H5N1. Notably, some were inversely regulated in response to H1N1 and H5N1 infection at the same time point. For example, compared with mock infection, miR-3123 was up-regulated (3.17 fold) after H1N1 infection and down-regulated (-1.84 fold) after H5N1 infection at 6 hours post-infection. It is believed that the differential expression for miRNAs correlates with that for their target genes.

The expression levels of selected miRNAs were verified using real-time PCR. Generally, all real-time PCR data correlated well with the RNA-Seq result.

Target genes of miRNAs and pathway analysis

The miRNAs with a fold-change of ≥1.2 and a Z-score of ≥3 were chosen for target prediction using DIANA-microT. Using the default setting of DIANA-microT, the target genes were obtained in response to H1N1 and H5N1 at each time point. The direction of expression (fold change ≥1.2) of target genes was inversely correlated with the expression of miRNAs. A list of miRNAs targeted genes was generated for pathway analysis using InnateDB.

Many pathways containing a number of genes targeted by differently expressed miRNAs were identified. For the ‘RIG-I like receptor signalling pathway’, there were 4 genes targeted by 7 up-regulated miRNAs and 9 genes targeted by 11 down-regulated miRNAs. Interestingly, in the 11 targeting down-regulated miRNAs, hsa-let-7 family has been verified to be tumour suppressor miRNAs responsible for regulating apoptosis in cell proliferation and developmental differentiation and involved in a feedback loop of MAPK signalling.3 hsa-miR-146a was recognised as innate immunity regulators acting as terminal transducers of TLR4 signalling and has been determined to target TRAF6 and IRAK1, whereas TRAF6 was also predicted to be targeted by hsa-miR-146a.4

Discovery of novel miRNA in human macrophages and influenza viruses

The sequencing reads falling into ‘other’ category, which could not be mapped to rRNA, tRNA, snoRNA, or mRNA, were used for computational detection of novel miRNAs for human mRNA-Seq. The unmappable reads from each library (an average of 20% of the total reads) were aligned against human genome reference (GRCh37) for searching and determination of the potential loci of the hairpin sequences using the package miRDeep.5 A total of 43 novel miRNAs were identified to be potentially novel across the nine samples falling into 33 non-redundant groups. This was because the identical or highly similar mature miRNAs may be located on distinct hairpin loci. Those miRNAs were the potential homologous miRNAs.

Among the 43 novel miRNA candidates, ‘AAAGAGCTTGGCTTCTTGGAGCC’ was represented in all nine libraries with a maximum score of 2.4. ‘CCTCAGAAACGCCTGTTCCTG’ and ‘GGCTCCACCTTCTCAGTGGTGG’ were represented in eight of the nine libraries with a maximum
score of 2.1. The three candidates were considered to be highly confident and will be further studied in future. Other novel miRNA candidates were represented in no more than five libraries with the score varying between 0~4.2.

**Discussion**

This study investigated the miRNA expression profile in macrophages infected with H1N1 and H5N1 at 1, 3, and 6 hours post-infection. The differences in expression level of miRNAs may contribute to the differential gene expression observed in influenza virus-infected human macrophages. Thus, the miRNA and mRNA datasets were overlaid to identify the key regulatory mechanisms/networks involved in influenza pathogenesis.

This study identified many pathways/genes that are targeted by differently expressed miRNAs in response to H5N1 or H1N1 virus infection. In response to H5N1 infection, the RIG-I like receptor signalling pathway was the most significantly enriched pathway regulated by miRNAs.

This study identified differentially expressed known and novel miRNA, mRNA, and signalling pathways in response to influenza infection. Further study should focus on these potential targets for drug development.

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**References**


