

# Avian influenza A H5N1 infection on human cellular microRNA profile: identification of gene regulatory pathway

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## KEY MESSAGES

1. Based on the broad-catching microRNA (miRNA) microarray approach, dysregulation of miRNA expression is mainly observed in highly pathogenic avian influenza A (H5N1) virus infection.
2. miRNA 141 (miR-141) was induced shortly after influenza A (H1N1) virus infection. Induction was greater in H5N1 infection than in seasonal H1N1 infection.
3. A cytokine, transforming growth factor- $\beta$ 2, which plays an important role in regulating inflammatory processes, was identified as a target of miR-141 binding. This suggests that influenza

A virus infection, in particular highly pathogenic H5N1, can affect the inflammatory processes via miR-141 induction.

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## Introduction

Avian influenza remains a threat to poultry and human health. From December 2003 to March 2011, more than 534 human infections and 316 deaths were reported to the World Health Organization. Outbreaks of avian influenza A (H5N1) virus infection in poultry have swept Southeast Asia and many other parts of the world. Cytokine storm and reactive haemophagocytic syndrome are the key features to distinguish H5N1 infection from seasonal influenza A (H1N1) virus infection.<sup>1-3</sup>

MicroRNAs (miRNAs) are a new class of 18-23 nucleotide non-coding RNAs that play critical roles in a wide spectrum of biological processes. MiRNA is one of the major gene regulatory families in eukaryotic cells.<sup>4,5</sup> However, the functions of most of the identified miRNAs remains unknown. The miRNA pathway exists in viral species, indicating that the host miRNAs may also have a direct or indirect regulatory role on viral replication.

This study was conducted from December 2008 to November 2010 and aimed to elucidate how H5N1 infection disturbs the human gene regulatory pathways leading to adverse pathological events. We hypothesised that miRNAs could be involved in the influenza virus infection response.

## Materials and methods

### Infection of cell culture with influenza A viruses

The H5N1 virus (A/Hong Kong/483/1997) was

isolated from a patient with fatal infection in Hong Kong in 1997, and the H5N1 virus (A/Thai/KAN1/2004) was isolated from a patient with fatal infection in Thailand in 2004. For comparison, a human H1N1 strain isolated in 2002 (A/HongKong/ CUHK-13003/2002) was included. NCI-H292 cells were grown to confluence in sterile T75 tissue culture flasks for the inoculation of virus isolate at a multiplicity of infection of one.

### RNA extraction and miRNA expression profiling

Total RNA was extracted from normal and infected NCI-H292 cells using Trizol reagent (Invitrogen; Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol. MiRNAs were labelled using miRNA labelling reagent (Agilent Technologies, Santa Clara, CA, USA) and hybridised to human miRNA arrays (Agilent Technologies) according to the manufacturer's protocol. Each miRNA array facilitated us to interrogate 866 human miRNAs. The results were analysed using Genespring GX 10.0.2 software (Agilent Technologies).

### TaqMan real-time RT-PCR for quantification of miRNAs

Total RNA was reversely transcribed with looped miRNA-specific real-time primers provided in the TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA, USA). Each cDNA was amplified with sequence-specific TaqMan MiRNA Assays. All

samples were tested in triplicate and were compared with the threshold cycle obtained from 18S rRNA assay (Applied Biosystems) for the normalisation of total RNA input.

**qRT-PCR for quantification of TGF-β2 miRNA level and enzyme-linked immunosorbent assay measurement of TGF-β2 protein level**

Total RNA extracted from cell cultures was reversely transcribed to cDNA using the poly(dT) primers and SuperScript III Reverse Transcriptase (Invitrogen), and quantified by real-time PCR. Expression of the β-actin gene was also quantified in a similar way for normalisation. The comparative delta-delta C<sub>T</sub> method was used to analyse the results. Cell culture supernatant was collected at 24 hours post-infection for the analysis of TGF-β2 expression using enzyme-linked immunosorbent assay (Emax ImmunoAssay Systems, Promega, Madison, WI, USA).

**Reverse transfection of a mimic and an inhibitor of miRNA-141**

The cells were transfected in suspension after trypsinisation with 60 nM anti-miR, pre-miR, or a negative control (Applied Biosystems). At 24 hours post-transfection, the cells were lysed for qRT-PCR analysis or subjected to either H1N1 or H5N1 virus infection.

**Results**

A list of differentially expressed miRNA was identified for H1N1 and H5N1 subtypes, and the temporal pattern of expression was delineated. Among the listed profiles of differentially regulated miRNA, miR-1246, miR-663, and miR-574-3p were up-regulated (>3-fold) at some time points during the course of infection with H5N1, compared with the non-infected control cells. Moreover, miR-100\*, miR-21\*, miR-141, miR-1274a, and miR1274b were highly down-regulated (>3-fold) in infection with H5N1, particularly at 18 or 24 hours post-infection, compared with the non-infected control cells. Similar miRNA profiles were also observed in H1N1 infection, but the magnitude of changes (<2-fold) was much lower than that in H5N1 infection.

The targets were predicted using the TargetScan computer software (<http://www.targetscan.org/>). There was a 3'UTR binding site on TGF-β2 for miR-141. miR-141 was initially up-regulated at 3 hours post-infection, which was higher in H5N1 infection (5- to 14-fold) than in H1N1 infection (2- to 3-fold) [Fig 1].

Using ectopic expression of miR-141, the level of TGF-β2 mRNA was significantly decreased in miR-141 transfected cells but not in negative-control miRNA-mimicking transfected cells (Fig 2).

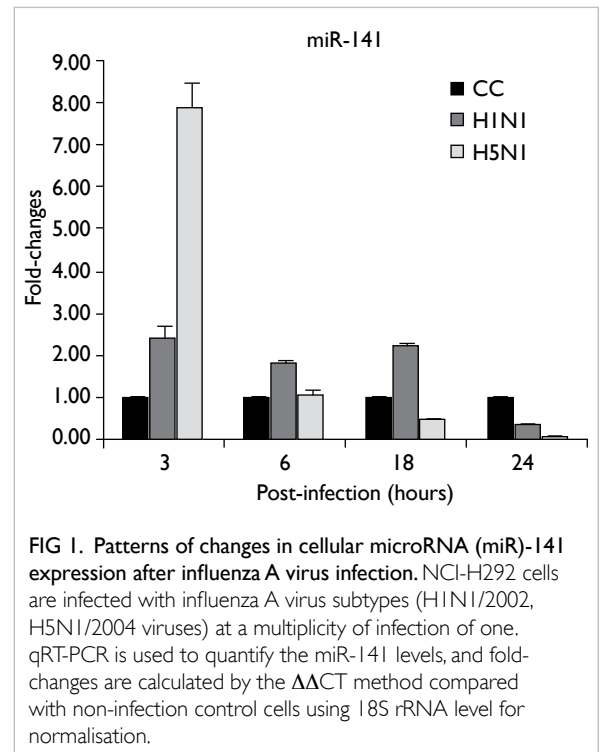


FIG 1. Patterns of changes in cellular microRNA (miR)-141 expression after influenza A virus infection. NCI-H292 cells are infected with influenza A virus subtypes (H1N1/2002, H5N1/2004 viruses) at a multiplicity of infection of one. qRT-PCR is used to quantify the miR-141 levels, and fold-changes are calculated by the ΔΔCT method compared with non-infection control cells using 18S rRNA level for normalisation.

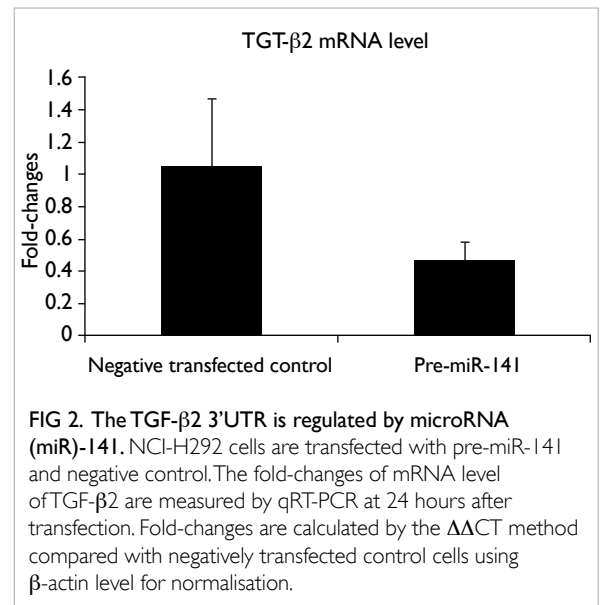
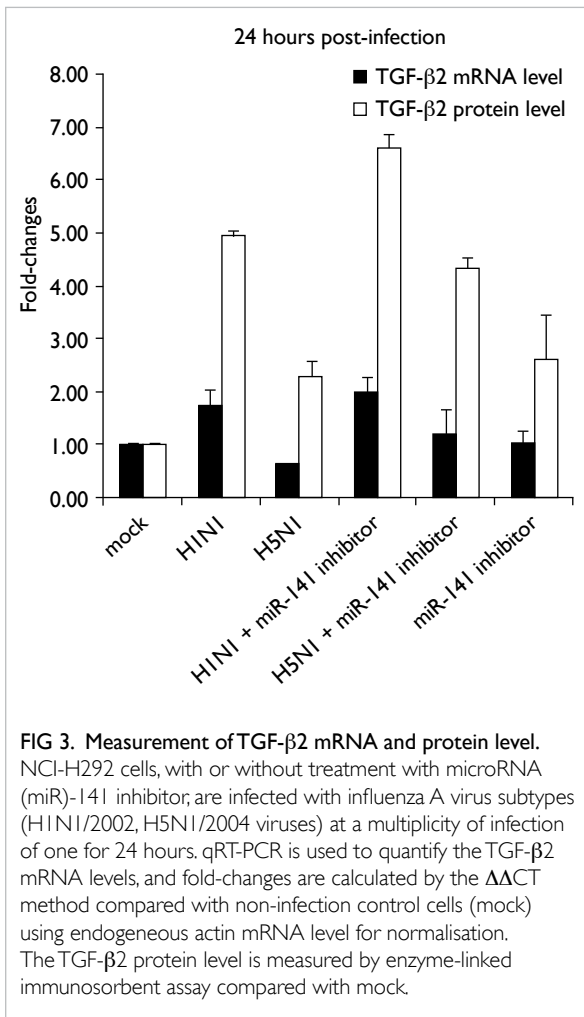


FIG 2. The TGF-β2 3'UTR is regulated by microRNA (miR)-141. NCI-H292 cells are transfected with pre-miR-141 and negative control. The fold-changes of mRNA level of TGF-β2 are measured by qRT-PCR at 24 hours after transfection. Fold-changes are calculated by the ΔΔCT method compared with negatively transfected control cells using β-actin level for normalisation.

The functional relevance of changes in miRNA-141 expression during influenza A virus infection was then assessed using anti-miR miRNA inhibitors. The anti-miR miR-141 inhibitor could cause an increase in TGF-β2 protein expression in H1N1 or H5N1 infected cells (Fig 3), compared with cells only infected with H1N1 or H5N1 without anti-miR miR-141 inhibitor treatment.



**FIG 3. Measurement of TGF-β2 mRNA and protein level.** NCI-H292 cells, with or without treatment with microRNA (miR)-141 inhibitor, are infected with influenza A virus subtypes (H1N1/2002, H5N1/2004 viruses) at a multiplicity of infection of one for 24 hours. qRT-PCR is used to quantify the TGF-β2 mRNA levels, and fold-changes are calculated by the  $\Delta\Delta CT$  method compared with non-infection control cells (mock) using endogenous actin mRNA level for normalisation. The TGF-β2 protein level is measured by enzyme-linked immunosorbent assay compared with mock.

## Discussion

In this study, influenza A virus infection altered the regulation of cellular miRNAs; the extent was greater in H5N1 infection than in H1N1 infection. The expression of miR-141 was affected by influenza A virus infection. The altered miR-141 expression then affected the expression of the cytokine TGF-β2. In fact, the miR-141 is a member of the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429). Previous studies of miR-141 mainly involved its role in cancer; miR-141 was markedly down-regulated in cells that had undergone epithelial to mesenchymal transition in response to TGF-β, and was overexpressed in ovarian and colorectal cancers and down-regulated in prostate, hepatocellular, renal cell, and gastric cancer tissues. This raises a controversy about the role of miR-141 in cancer progression. Furthermore, the miR-200 family members have roles in maintaining the epithelial phenotype of cancer cells. A member of this family—miR-200a—was differentially expressed in response to influenza virus infection in another study. The targets of miR-200a are associated with viral gene

replication and the JAK-STAT signalling pathway, which is closely related to the type 1 interferon-mediated innate immune response. However, the effect of miR-141 on virus infection is not known, except that enterovirus can induce miR-141 and contribute to the shut-off of host protein translation by targeting the translation initiation factor eIF4E.

In addition, influenza A virus infection reduces or promotes the expression of the host miR-141 in a time-dependent manner. In this study, TGF-β2 mRNA was suppressed in miR-141 overexpressed cells. This is in line with another study showing that the 3'UTR of TGF-β2 mRNA contains a target site for miR-141/200a, and the expression of TGF-β2 was significantly decreased in miR-141/200a transfected cells. Furthermore, miR-141 may not only work as translational repressors of target mRNAs, but also cause a decrease in TGF-β2 mRNA levels. These findings are similar to recent data demonstrating that some miRNAs can alter the mRNA levels of target genes. This ability is probably independent of the ability of these miRNAs to regulate the translation of target mRNAs.

In this study, antagomiR-141 moderately increased the accumulation of TGF-β2 protein during influenza A virus infection. This might be because, by the use of anti-miR miR-141 inhibitor that decreases the cellular pool of miR-141, the translation control of the TGF-β2 mRNA was subsequently released and caused the TGF-β2 protein to express and accumulate during influenza A virus infection. H1N1 was the only subtype that could induce a sustained increase in TGF-β2 at the protein level. H1N1 infection induced a small amount of miR-141 expression, whereas H5N1 infection induced a higher amount of miR-141 expression in the early phase of infection. As a consequence of the higher amount of miR-141 in H5N1 infection, TGF-β2 expression might be more greatly reduced than in H1N1 infection. TGF-β2 plays a vital role in T-cell inhibition, as it can act as both an immunosuppressive agent and a potent proinflammatory molecule through its ability to attract and regulate inflammatory molecules. Furthermore, TGF-β2 inhibits T helper 1 cytokine-mediated induction of CCL-2/MCP-1, CCL-3/MIP-1α, CCL-4/MIP-1β, CCL-5/RANTES, CCL-9/MIP-1γ, CXCL-2/MIP-2, and CXCL-10/IP-10. Moreover, the pro-inflammatory responses during influenza A virus infection are tightly controlled by anti-inflammatory mediators such as TGF-β2 to protect the easily damaged lung tissue from destructive side effects of virus-induced inflammation. Therefore, the down-regulation of TGF-β2 protein by miR-141 may be an important step in the excessive inflammation progression during influenza A virus infection, particularly in H5N1 infection. However, whether the recovery of TGF-β2 expression by anti-miR miR-141

inhibitor could resolve the hypercytokinaemic stage of H5N1 infection needs to be further studied.

One limitation of this study was that the roles of other miRNAs whose expression was also altered after infection by influenza A virus were not assessed. The miRNA microarrays used did not contain probes for every known miRNA; thus it is possible that influenza A virus infection affects the expression of some other miRNAs not yet studied. The virus may interact with miRNA regulatory pathways differently in other cell or tissue types, or in other physiological states.

## Conclusion

Based on the broad-catching miRNA microarray results, dysregulation of miRNA expression was mainly observed in highly pathogenic H5N1 infection. miR-141 was induced at an early phase of influenza A virus infection; the induction was higher in H5N1 infection than in H1N1 infection. Moreover, TGF- $\beta$ 2, which plays an important role in regulating inflammatory processes, was identified as

a target of miR-141 binding. As a result, influenza A virus infection could affect the inflammatory processes via miR-141 induction.

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