

## Original Article

# Cellular microRNA-214 contributes to H1N1 influenza A virus-mediated apoptosis through repression of anti-apoptotic factors Livin in A549 cells

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**Abstract:** Influenza A virus (IAV) infection results in host cell death and major tissue damage. MicroRNAs (miRNAs) play an important role in the regulation of gene expression and are involved in many cellular processes including inhibition of viral replication in infected cells. Recent studies have revealed that miR-214 is involved in a variety of biological processes, including apoptosis. However, the effects and mechanisms of miR-214 on influenza A virus mediated apoptosis have not been well understood. In this study, we found that several apoptosis-associated miRNAs were stimulated in influenza A virus-infected A549 cells by microRNA PCR array analysis and validated using qPCR in serum samples from patients with H1N1 infection. Because miR-214 was significantly upregulated among them, we investigated its function. We identified anti-apoptotic factors Livin as a direct target of miR-214 by bioinformatics analysis and confirmed by using luciferase activity assay and Western blot. Functional overexpression of miR-214 promoted A549 cells apoptosis induced by influenza A virus, while knockdown of miR-214 inhibited A549 cells apoptosis. Moreover, we demonstrated that silencing of Livin rescued the inhibition of cell apoptosis induced by miR-214 inhibitor in influenza A virus-infected A549 cells, whereas Livin overexpression could reverse the promotion of cell apoptosis induced by miR-214 mimic. Taken together, our findings indicate that miR-214 is involved in H1N1 influenza virus-mediated apoptosis through repression of anti-apoptotic factor Livin.

**Keywords:** MicroRNA, influenza virus, miR-214, Livin

## Introduction

Influenza A virus is a kind of single negative-stranded RNA virus which cause worldwide epidemic in a short time for its great contagiousity, fast spread speed and a wide range of host primarily [1]. Even in non-pandemic years, influenza A viruses infect 5-15% of the global population and result in > 500,000 deaths per year [2]. Although antiviral drugs are used to prevent and treat influenza A virus infection, these drugs have some side effects for the human nervous system, and because of the fast mutation rate of influenza A virus, it is easy to produce drug-resistant strains with the extensive use of drugs. Therefore, understanding the pathogenesis of influenza virus infection is essential to preventing and controlling future outbreaks.

Apoptosis is considered to be one of the host cell responses against influenza virus infection

[3-5]. Influenza virus is a cytolytic virus that damages the epithelium cells in the human respiratory tract and cause death of infected cells. Previous studies have showed that influenza virus induces cell death through activation of the apoptotic pathway during infection [4]. Recent observations showed that a number of viruses, including influenza viruses, may manipulate the cell death signaling pathway to promote viral replication [6-8]. However, the mechanism of virus-induced cellular damage that eventually leads to cell death is not well understood.

MicroRNAs (miRNAs) are a class of endogenous small noncoding RNAs with 18-22 nt in length. Mature miRNAs specifically bind semi-complementarily to target mRNA and induce target mRNA degradation [9, 10]. MiRNAs have been identified in various organisms and involved in various biological processes, such as develop-

ment, cancer, viral pathogenesis and so on [11-13]. Increasing evidence supports that microRNAs have important functions in viral replication and may be used by host cells to inhibit or promote viral infections. And, expression of microRNAs has been reported for various viruses, such as human immunodeficiency virus [14], hepatitis B virus [15], hepatitis C virus [16] and Epstein-Barr virus [17]. For influenza viruses, kinds of miRNAs have been found both in cultured cells and in animal models. A recent study from Guan et al. showed that miR-29c was significantly up-regulated in influenza A virus-infected A549 cells and influenza virus infection suppressed expression of BCL2L2 by inducing the expression of miR-29c [18]. However, it is still unclear about the role of microRNA during influenza A virus infection.

In the current study, we used both microRNA PCR array and quantitative reverse transcription polymerase chain reactions (qRT-PCR) to assess miRNA expression in influenza A virus-infected A549 cells and found miR-214 was significantly up-regulated. Moreover, our study demonstrated that anti-apoptotic factors Livin was a direct and functional target for miR-214. Mechanistically, we experimentally confirmed that induction of the cellular miR-214 by influenza virus contributed to virus-mediated apoptosis through repression of anti-apoptotic factor Livin.

## Materials and methods

### *Cell and virus*

A549, a human pulmonary epithelial cell line (ATCC CCL-185), and Madin-Darby canine kidney (MDCK) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, and 2 mM L-glutamine at 37°C in a CO<sub>2</sub> incubator.

Influenza viruses, A/Puerto Rico/8/34 (H1N1) were grown in the allantoic cavities of 10-day-old embryonated chicken eggs as previously described, and the titers of the virus strains were determined on MDCK cells.

### *Viral infections*

The infection assay involved 1 h incubation at 37°C with A/Puerto Rico/8/34 (H1N1) at the indicated multiplicity of infection (MOI). Then,

cells were washed twice with phosphate buffer saline (PBS) and replenished with fresh media without virus. Cells used to study A/Puerto Rico/8/34 (H1N1) were cultured and infected in DMEM supplemented with 5% FBS. Supernatants were harvested at the indicated time after infection.

### *Sample collection*

A total of 10 healthy volunteers and 10 H1N1 virus-infected patients from the People's Hospital of Jiaozuo were enrolled in our study. Serum samples within a period of 14 days from the onset of the infection were then collected and stored at -70°C until use. Healthy controls were recruited randomly from people who underwent a regular health check-up without clinical symptoms of any infectious disease. This study was approved by the Ethics Committee of the People's Hospital of Jiaozuo and written informed consent was obtained from all participants.

### *RNA extraction*

Total RNA was extracted from A549 cells infected with Influenza viruses, A/Puerto Rico/8/34 (H1N1) at 4 h, 12 h and 24 h post-infection using an RNeasy® mini kit (QIAGEN GmbH, Hilden, Germany). Potential genomic DNA contamination was removed from the samples by treatment with RNase-free DNase (QIAGEN) for 15 min at room temperature. Concentration and purity were determined using a NanoDrop 1000™ spectrophotometer (Thermo Fisher, Dubuque, IA, USA), while the integrity of miRNA was further assessed using an Agilent 2100 Bioanalyzer (Agilent Tech, Palo Alto, CA).

### *PCR array*

A549 cells were infected with influenza virus (5 MOI) for 3 h and RNA was extracted from infected and control cells using the RNeasy® mini kit (QIAGEN GmbH, Hilden, Germany) and then sent to QIAGEN for miRNA PCR array analysis. Data were analyzed using ANOVA and t tests. Normalization of expression was performed using a cyclic Lowess method [19]. TargetScan (<http://www.targetscan.org>) was used for miRNA target prediction.

### *Real-time PCR*

For miRNAs analysis, total RNA was isolated from serums of patients infected with influenza

virus using the MicroRNA Extraction and Purification Kit (Novland, Shanghai, China). Real-time PCR was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems). Specific primers and probes for mature microRNAs and snRNA RNU6B were obtained from Ambion. All reactions were conducted in triplicate. Quantitative normalization was performed on U6 for miRNA detection.

### *Cell transfection*

MiR-214 mimic, miR-214 inhibitor and miR negative control (NC) were synthesized by Qiagen. A549 cells were plated in six-well plates ( $1.5 \times 10^5$  cells per well) for 24 h and transfected with miRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells transfected with miRNA were harvested 48 h post-transfection.

### *Luciferase assays*

Luciferase reporter assay was performed as described previously. Dual luciferase assays were conducted in a 24 well plate format. pGL3-Livin 3'UTR report/pGL3-Livin 3'UTR Mutant report + TK100 Renilla report were transfected into 70% confluent HEK293 cells, along with miR-214 mimic, miR-214 inhibitor or each control. After 48-h transfection, firefly and renilla luciferase were quantified sequentially using the Dual Luciferase Assay kit (Promega, USA) following the manufacturer's recommendations.

### *Western blot assay*

After transfection, cells were lysed by using mammalian protein extraction reagent RIPA (Beyotime, Haimen, China) supplemented with protease inhibitor cocktail (Roche) and transferred to PVDF membrane, after blocking in 5% skimmed milk for 30 mins at room temperature. The member was incubated with antibodies against Livin (goat polyclonal, 1:3,000) and  $\beta$ -actin (Sigma) for 2 h at room temperature, followed by incubating with horseradish peroxidase-linked secondary antibody for 1 h at room temperature and visualized with ECL.

### *Apoptosis assay*

To detect the effects of miR-214 on A549 cell apoptosis, the cells (50-60% confluent) were

transfected with miR-214 mimics, inhibitor or negative control and co-transfected with miR-214 mimics and pcDNA3.1-Livin or miR-214 inhibitor and si-Livin. After treatment, cells were resuspended in binding buffer containing Annexin V-FITC and propidium iodide (PI) according to manufacturer's instructions (KeyGEN) and assessed by flow cytometry. Results were expressed as percentage of the vehicle control levels set at 100%. Each sample was run in triplicate.

### *Plasmid construction*

The Livin sequence was subcloned into the pcDNA3.1 vector (Invitrogen, USA). Livin ectopic expression was achieved through pcDNA3.1-Livin transfection using lipofectamine 2000 (Invitrogen, USA), with an empty pcDNA3.1 vector used as a control. Plasmid vectors (pcDNA3.1-Livin and pcDNA3.1) for transfection were extracted using Midiprep kits (Qiagen, Germany), and transfected into A549 cells. After 48 h the initial transfection, the cells were infected with virus.

### *RNA interference (RNAi) knockdown*

Non-specific siRNA (si-NC) and si-Livin were purchased from Invitrogen. Typically, cells were seeded in six-well plates and then transfected the next day with specific siRNA (100 nM) and control siRNA (100 nM) by using Lipofectamine RNAi MAX, according to the manufacturer's protocol (Invitrogen). After 48 h the initial transfection, the cells were infected with virus.

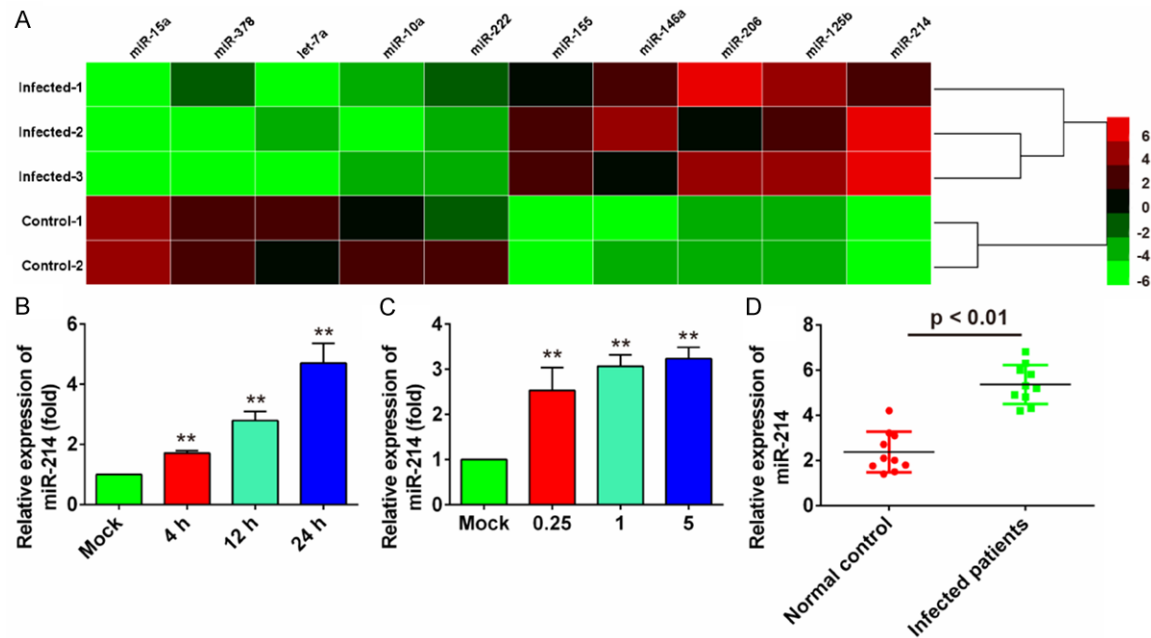
### *Statistical analysis*

Statistical analyses were performed with SPSS 13.0 software. The results were evaluated by  $\chi^2$  test and the other data were evaluated by Student's t-test and expressed as the mean  $\pm$  SD from three independent experiments. A *P*-value of less than 0.05 was considered statistically significant.

## **Results**

### *MiR-214 is significantly up-regulated in H1N1 influenza virus-infected A549 cells*

Recently, an increasing number of reports suggest that that miRNAs played an important role in the control of apoptosis by regulating post-transcriptional processes [20]. Therefore, we



**Figure 1.** Apoptosis-associated miRNA expression in H1N1 influenza virus-infected A549 cells. A: PCR array analysis for miRNA was performed with RNA extracts from influenza virus-infected A549 cells. Heat map represented the significantly upregulated apoptosis-associated miRNAs. B: A549 cells were infected with influenza virus and samples were withdrawn at the indicated time points. miRNAs extracted and then analyzed by qPCR. C: A549 cells were infected with increasing MOIs of influenza virus, Total RNA was extracted and then miR-214 expression was analyzed by qPCR. D: miR-214 is up-regulated in serum samples of patients infected with influenza virus compared with normal samples, as measured by real-time PCR. All data represent the mean  $\pm$  SD results of three independent experiments. \*\*P < 0.01 vs mock.

utilized microRNA PCR assay to detected host miRNAs expression profiling in A549 cells infected with influenza A virus. Among 10 apoptosis-associated miRNAs on the arrays, miR-214 was the most up-regulated (**Figure 1A**). Subsequently, we compared miR-214 expression in A549 infected with influenza A virus (H1N1) at different concentration for 4 h, 12 h and 24 h to control by qPCR. As shown in **Figure 1B**, **1C**, the expression of miR-214 was increased in a dose- and time-dependent manner. Consistent with the results obtained with the cell lines, miR-214 was also significantly up-regulated in patients infected with H1N1 influenza A virus (**Figure 1D**). Taken together, these data suggest that miR-214 was up-regulated in A549 cell lines and clinical samples, suggesting broad and essential roles of miR-214 during influenza virus infection.

#### *miR-214 targets and inhibits Livin protein expression*

Using algorithms for target gene prediction, TargetScan, Livin was identified as one of the

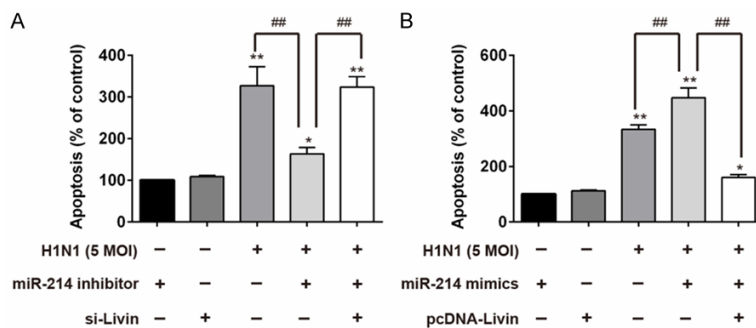
potential targets of miR-214. The predicted binding of miR-214 with Livin 3'UTR was illustrated in **Figure 2A**. Livin has been implicated in apoptosis in tumor cells [21]. Here, we test whether Livin was a direct target gene of miR-214 using the dual-luciferase activity assay. Results showed that miR-214 significantly suppressed the luciferase activity of the wildtype (WT) 3'UTR of Livin, without effect on its mutant (Mut) (**Figure 2B**). In addition, western blot analysis showed that miR-214 overexpression markedly decreased the protein level of Livin, whereas miR-214 inhibition increased the protein expression of Livin (**Figure 2C**). Moreover, we found that influenza virus infection decreased the levels of Livin protein in A549 cells in a time-dependent manner (**Figure 2D**). All these results suggested that miR-214 played a role in regulating the Livin expression in A549 cells.

#### *Effects of miR-214 on apoptosis induced by influenza virus in A549 cells*

MiR-214 overexpression has been shown to promote cell apoptosis in several human can-







**Figure 4.** Upregulated miR-214 promotes apoptosis induced by influenza A virus infection by regulating Livin expression. A549 cells were first treated with miR-214 inhibitor, miR-214 mimic, si-Livin or pcDNA-Livin for 24 h. After being washed, cells were challenged with influenza virus for 48 h. Cell apoptosis was measured by annexin V-FITC/PI double staining with the use of flow cytometry analysis. A: Silence of Livin rescued the inhibition of apoptosis induced by miR-214 inhibitor in A549 cells infected with influenza virus. B: Overexpression of Livin reversed the promotion of apoptosis induced by miR-214 mimic in A549 cells infected with influenza virus. \* $P < 0.05$ , \*\* $P < 0.01$  vs inhibitor or mimic NC group, ### $P < 0.01$  vs miR-214 inhibitor or miR-214 mimic.

direct target of miR-214. Functional analyses showed that overexpression of miR-214 promoted A549 cells apoptosis induced by influenza A virus, and the cell apoptosis was reversed by pcDNA-Livin. These findings indicate that miR-214-mediated Livin suppression is involved in influenza virus-induced cell apoptosis in A549 cells.

Recently, an increasing number of reports suggest that cellular miRNAs play an important role in the control of cell apoptosis induced by virus infection [28]. For example, Fu Q et al. found that miR-27b attenuated apoptosis induced by transmissible gastroenteritis virus (TGEV) infection via targeting runt-related transcription factor 1 (RUNX1) [29]. A study from Smith et al. showed that miR-154 promoted West Nile virus (WNV)-mediated apoptosis via inhibiting the expression of CCCTC-binding factor (CTCF) and the epidermal growth factor receptor (EGFR) [30]. However, the involvement of miRNAs during influenza A virus infection is still unclear. Recent studies have suggested miRNA involvement in influenza A virus infection and replication [31-33], we hypothesized that one or more cellular miRNAs could be involved in the H1N1 IAV infection. In this study, we identify one cellular miRNA (miR-214) that is induced in A549 cells after influenza A virus infection and demonstrate that miR-214 is in-

creased in a dose- and time-dependent manner in the infected cell.

A variety of studies have demonstrated that miR-214 induced apoptosis in various tumors [22, 34, 35]. For example, Heishima K et al. found that miR-214 promoted apoptosis in canine hemangiosarcoma by targeting the COP1-p53 Axis. Recently, several studies demonstrated miR-214 not only mediated differentiation, senescence, angiogenesis, cell migration but also virus replication. For functional assessment of miR-214, we transfected A549 cell lines with this miRNA. Consistent with our hypothe-

sis, transfection of these cells with miR-214 positively regulates cell apoptosis after influenza virus infection. However, the mechanism still remains unclear.

It is well known that miRNAs regulate posttranscriptional processes by binding to the 3'UTR of target genes. Yang X et al. found that miR-214 decreased the apoptosis of myocardial cells through PTEN [36]. In this study, we confirmed that the anti-apoptotic factor Livin was the target of miR-214 during influenza A virus infection. Previous studies demonstrated Livin-overexpressing cells were more resistant to apoptotic stimuli than normal cells [37, 38]. Thus, Livin intervention contributes to the apoptosis of cancer cells, while whether miR-214 regulates apoptosis of A549 cell infected with influenza A virus via Livin is unclear. Our dates showed that Livin overexpression reversed A549 cell apoptosis induced by influenza A virus infection. This suggests that miR-214 regulates influenza A virus-induced apoptosis, at least in part via targeting Livin, which is an important supplement of Livin function.

In summary, our finding suggests miR-214 is involved in influenza virus-mediated apoptosis through repression of antiapoptotic factors Livin, which may have potential for therapeutic strategies.

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## Disclosure of conflict of interest

None.

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