Original Paper

The Novel H7N9 Influenza A Virus NS1 Induces p53-Mediated Apoptosis of A549 Cells

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Key Words

Avian influenza A virus • H7N9 • NS1 • Apoptosis • p53

Abstract

Background: H7N9, emerged as an avian influenza virus outbreak in Eastern China in early 2013, and represented another major threat to global health. Roles of its NS1 protein, an essential viral factor, in regulating apoptosis remain unknown. **Methods:** Apoptotic effect and features of H7N9/NS1 in the human A549 alveolar basal epithelial cell line were examined by caspase 3/7 activity assay and western blotting of apoptotic associated proteins. Effects of H7N9/NS1 on mitochondrial membrane potential were investigated by flow cytometry. **Results:** The expression of H7N9/NS1 in A549 cells activated caspase 3/7 and increased the protein levels of cleaved caspase 7 and cleaved poly (ADP-ribose) polymerase (PARP). H7N9/NS1-expressing A549 cells displayed a decrease in mitochondrial membrane potential. In addition, H7N9/NS1 increased the protein levels of total p53, p53 phosphorylated at Ser46 and Ser37, activated caspase 9, and the Bax/Bcl-2 ratio. **Conclusion:** Our results suggest that H7N9/NS1 protein causes the accumulation of p53 by increasing phosphorylation levels of p53 and the induction of mitochondrial dysfunction, which may contribute to H7N9/NS1-induced apoptosis in A549 cells.

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Introduction

Since February 2013, an outbreak of a novel influenza A virus, denoted H7N9, in Eastern China has caused at least 695 human infections with a mortality rate greater than 30% [1]. Because H7N9 is of avian origin, this challenges the concept that influenza A viruses with low pathogenicity in birds rarely infect humans and do not cause severe disease [2]. The significantly increased cytokine and chemokine levels, in particular type I IFN gene expression, and interaction of different viral factors with host cellular molecules contribute

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to the pathogenesis of the infection [3-5]. The severity of influenza A virus infection is also closely related to dysfunction of apoptosis regulation [6]. Previous studies found that influenza A virus infection causes apoptosis in many types of cell lines [7]. The ability to induce apoptosis of influenza A virus is strain-specific and host cell-specific, which may be due to the fact that different viral factors from different influenza A virus strains may vary in their activities to modulate the host cell apoptotic response [8].

Influenza A virus is an enveloped virus with eight segments of negative-sense RNA encoding genes for as many as 16 proteins [9, 10]. Genome sequencing indicates that the H7N9 is a novel reassortant virus, and that all of its genes are of avian origin, with six "internal genes", including the NS1 gene, being derived from avian influenza A viruses H9N2 while the HA and NA originate from duck and wild-bird viruses [11, 12]. The NS1 protein of influenza A virus is a multifunctional virulence factor that play a pivotal role in viral infection. The role of NS1 in apoptosis has not been fully established, as NS1 is reported to have both pro- and anti-apoptotic functions. It has been shown previously that NS1 protein derived from H5N9 or H5N1 virus induces apoptosis in MDCK, Hela, NCI-H292, A549 and HEK293 cells via caspase-dependent pathway [13-16]. However, other studies have reported NS1 from H1N1 or H3N2 recombinant viruses activates the PI3K/Akt pathway to down-regulate apoptosis in MDCK and Vero cells [17, 18]. Such conflicting observations may also be a consequence of differences in influenza virus subtypes and strains, as well as the host cell types being used in the experiments.

It has been previously reported that NS1 derived from low pathogenicity H9N2 virus can induce apoptosis in chicken macrophages via the Fas/(FasL)-mediated pathway [19], while in human alveolar epithelial cells, H9N2 virus-induced apoptosis proceeds via the death receptor and mitochondrial pathways in parallel [20]. Although the NS1 gene of the novel H7N9 influenza A virus is derived from the closely-related avian H9N2 virus, several amino acids in NS1 protein of this novel H7N9 influenza A virus has changed (Fig. 1). The apoptotic effect of NS1 protein from H7N9 influenza A virus in human cells has yet to be explored. Therefore, the goal of this research was to investigate whether H7N9/NS1 could induce apoptosis in human alveolar basal epithelial cells A549 and the possible mechanisms and pathways involved in the action.

Material and Methods

Cell lines and virus strains

Human lung adenocarcinoma cells A549 and Madin-Darby canine kidney (MDCK) cells purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) were cultured at 37°C with 5% CO₂ in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin.

A/chicken/Zhejiang/DTID-ZJU01/2013 (H7N9) virus was initially isolated from a patient with a fatal disease. The virus isolate was passaged twice through MDCK cells and once in the allantoic cavity of embryonated eggs and was the virus used in our experiments.

Construction of recombinant plasmids

Viral RNA was purified using the NucleoSpin® RNA Virus (Macherey-Nagel) according to the manufacturer's protocol, and then, 0.5 µg purified RNA was used for reverse transcription, using the PrimeScript® RT reagent Kit (Takara) with primer Uni12 as the specific reverse primer. The cDNAs (about 200 ng) from the viruses were amplified by PCR, using the specific primers listed in Table 1. PCR products were digested with specific restriction enzymes and inserted into a pcDNA3-Flag vector (a modified pcDNA3 vector providing an N-terminal Flag tag), and the open reading frame of cDNA in the expression plasmids was verified by sequencing (BGI, China).

Immunofluorescence microscopy

Transfected A549 cells were analyzed for the expression of H7N9/NS1 protein by immunofluorescence. In briefly, 24 h after transfection, A549 cells were washed twice with PBS, fixed in 4% paraformaldehyde

Fig. 1. Sequence alignment of NS1 proteins derived from different avian influenza A viruses. An alignment comparing NS1 protein of A/chicken/Zhejiang/DTID-ZJU01/2013(H7N9) (ZJ2013_H7N9) (GI: 479280303) to A/chicken/Beijing/1/94(H9N2) (BJ94_H9N2) (GI: 5817835), A/chicken/Iran/SS2/2008(H9N2) (Ir2008_H9N2) (GI: 398324881), A/HongKong/483/97(H5N1) (HK1997_H5N1) (GI: 8307819) and A/chicken/Jilin/hq/2003(H5N1) (JL2003_H5N1) (GI: 116583054) was generated using the Clustal X program.

Table 1. Primers used in construction of recombinant plasmids and qPCR analysis

Primer Name	Sequence (5'-3')	Gene
NS79-F	CGGGGTACCATGGATTCCAATACGTG <i>KpnI</i>	H7N9/NS1 (Flag-tagged)
NS79-R	CCGGAATTCTACTTTGTAGAGAGT <i>BamHI</i>	
Uni 12	AGCAAAAGCAGG	Specific reverse primer
p53-F	CCTCAGCATCTTATCCGAGTGG	p53
p53-R	TGGATGGTGGTACAGTCAGAGC	
ATCB-F	ATGGGTCAGAAGGATTCCTATGT	β -actin
ACTB-R	GGTCATCTTCTCGCGGTT	

in PBS for 5 min and permeabilized in 0.2% Triton X-100, 0.04% SDS in PBS for 3 min. After blocking in normal goat serum (Boster, Wuhan, China) for 30 min, cells were incubated overnight at 4 °C with primary monoclonal antibody against the Flag epitope (1:300 dilution, Sigma). Secondary antibody IgG-FITC (Sigma) was applied at a dilution of 1:500. Before mounting the coverslips, cells were counterstained with 300 nM 6-diamidino-2-phenylindole (DAPI, Sigma) in PBS was used for 5 min. Cells were analyzed under an Olympus FV1000 laser confocal microscope (Olympus, Japan).

RNA preparation, RT-PCR, and real-time Quantitative PCR (qPCR)

H7N9/NS1 and the empty vector were transfected into A549 cells for 24 h, using Lipofectamine 2000 reagent, according to the protocol of the manufacturer (Invitrogen). Total RNA was extracted from cells using TRIzol reagent (Invitrogen). RNA (1 μ g) from each sample was reverse-transcribed into cDNA using the PrimeScript® RT reagent kit (Takara). QPCR was carried out in an Applied Biosystems PRISM 7500 qPCR (Life Technology) using SYBR® Select Master Mix (Invitrogen). qPCR primers used are listed in Table 1.

Transient transfection and protein expression system

Approximately 10^6 A549 cells were transfected with 4 μ g of H7N9/NS1 or control plasmids in a 6-well plate. Cells were then collected 0, 6, 12, 18, 24, 30, and 48 h post-transfection, washed with phosphate-buffered saline (PBS), and trypsinized. The cell pellet was then resuspended in 100 μ l of lysis buffer (2% sodium dodecyl sulfate, 10% glycerol, 0.0625 M Tris-HCl, pH 8.0), and the suspension was incubated on ice

for 30 min. After boiling for 10 min and centrifugation at 12,000 g for 20 min, the supernatant was collected for western blot analysis. For the pifithrin- α pre-treatment experiment, A549 cells were incubated with pifithrin- α (10 μ M) for 6 h before transfection with H7N9/NS1 plasmid or control plasmid.

Trypan blue assay

Following transfection, cells were harvested at different time intervals (0, 6, 12, 18, 24 and 30 h). Cell viability was evaluated by Trypan blue dye exclusion and expressed as % of control culture as described previously [21].

LDH assay

Cell lysate of H7N9/NS1- and empty vector-transfected cells was determined by measuring the release of lactate dehydrogenase in the medium using the LDH Cytotoxicity Assay kit (Cayman Chemical, USA) and following the manufacturer's instructions. Each treatment was performed in triplicate.

Apoptosis assay

The Caspase-Glo[®]3/7 Assay provides a homogeneous luminescent assay that measures caspase-3/7 activity. A549 cells were inoculated at a density of 10^4 cells/well (100 μ l of DMEM with 10% FBS) in 96-well plates 24 h before transfection. Caspase-Glo 3/7 reagent (Promega Corporation, Madison, WI) was then added to each well in a 1:1 ratio 24 h after transfection. The contents of the plate were gently mixed using a plate shaker at 300~500 rpm for 30 s. The cells were then lysed at room temperature for 2 h. Luminescence activities (relative caspase 3 and 7 activities) were measured as relative light units (RLUs) with a GloMax Multi+ Luminometer (Promega, Madison, WI).

Western blot analysis

Monolayers of cells transfected with DNA or untransfected cells were lysed, and the total protein concentration was determined by the bicinchoninic acid assay (Thermo Scientific, Rockford, IL). Proteins with equivalent concentrations were heated for 5 min at 100°C in sample buffer and were then analyzed by 10% SDS-PAGE and detected using the following antibodies: anti-Flag (Sigma; 1:3,000) for Flag-tagged NS1 protein, 1C12 (Cell Signaling Technology; 1:1,000) for p53, pho-Ser³⁷ p53 and pho-Ser⁴⁶ p53 (Cell Signaling Technology; 1:1,000) for phosphorylated p53 proteins, anti-PARP (Cell Signaling Technology; 1:2,000) for poly (ADP ribose) polymerase, anti-caspase-7, 8 and 9 (Cell Signaling Technology; 1:1,000) for activated caspases, anti-Bax (ABCam Biotechnology; 1:2,000) for Bax protein, anti- Bcl-2 (ABCam Biotechnology; 1:1,000) for Bcl-2 protein, and for the loading control anti- β -actin monoclonal antibody (Sigma; 1:5,000) was used. Each experiment was repeated at least three times and comparable results were obtained each time.

Measurement of mitochondrial membrane potential

Changes in mitochondrial membrane potential were assessed by a JC-1 Assay kit (Beyotime, China). Briefly, cells were inoculated at a density of 10^5 cells per well onto 6-well plates 24 h before transfection. Cells were collected and washed with PBS 24 h post-transfection, incubated with the medium containing 20 μ g/ml JC-1 at 37°C for 20 min, then analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA).

Results

Expression of H7N9/NS1 in A549 cells

To assess the expression of NS1 protein in A549 cells after transfection with the pcDNA3-Flag-NS1 plasmid, the cells were collected at 0, 6, 12, 18, 24, 30, and 48 h post-transfection. Total protein from the cell lysates was subjected to western blot analysis. NS1 protein expression was detectable as early as 6 h and peaked by 24 h post-transfection (Fig. 2A). The expression of NS1 protein was further confirmed by indirect immunofluorescence at 24 h post-transfection (Fig. 2B). Confocal microscopy revealed prominent nuclear localization of NS1 protein at 24 h post-transfection in A549 cells. In summary, the constructs were successfully transfected and found to express NS1 in A549 cells.

Fig. 2. (A) Time course of H7N9/NS1 protein expression in A549 cells. Anti-Flag antibodies were used to detect the Flag-NS1 fusion protein as a single protein band with a size of approximately 25 kD in the western blot analysis of A549 cells transfected with H7N9/NS1 plasmid. Approximately 20 μ g of total cell lysate was loaded into each lane. β -actin was used as a loading control. (B) Confocal analysis of expression of NS1 protein in A549 cells. Cells were inoculated on a cover slip, fixed with 4% PFA 24 h after transfection and immunostained with mouse monoclonal anti-Flag antibody and with anti-mouse FITC-conjugated antibodies. Cells were counterstained with DAPI for nuclear staining.

Fig. 3. (A) Analysis of cell viability. Transfected cells at different time intervals (0, 6, 12, 18, 24 and 30 h) were harvested and cell viability was determined by trypan blue exclusion. (B) LDH assay. Cell lysis measured by LDH release at different time post-transfection. Average values and standard deviation bars are shown from three independent experiments performed in triplicate.

Expression of H7N9/NS1 is cytotoxic to cells

Trypan blue dye exclusion test and LDH assay were done to determine the cell viability and lysis following NS1 expression with respect to empty vector at different intervals. The percentage of live cells in vector control (mock) and NS1-transfected cells at 24 h was found to be 86.6 ± 0.84 and 61.9 ± 1.16 , respectively. The LDH assay data indicated a significant increase in cell lysis in NS1 transfected cells (16.8 ± 0.92), as compared with vector control (7.9 ± 0.43), at 24 h post-transfection, concurrent with a decrease in cell viability (Fig. 3A, B). These data showed that the expression of NS1 protein results in cellular cytotoxicity and eventual cell death.

Fig. 4. Caspase pathway activation in A549 cells induced by the H7N9/NS1 protein. Cells were harvested at both 0 h and 24 h post-transfection and assayed for caspase 3/7 activity and western blot analysis. (A) Caspase 3/7 activity of NS1-transfected A549 cells. Each treatment was performed in triplicate. Triple asterisk indicates $p < 0.001$ versus mock. (B) Western blot analysis of caspase 7 and PARP cleavage were used to assess apoptosis in NS1 transfected A549 cells. Total proteins were isolated and separated by 10% SDS-PAGE. Samples were subjected to western blotting using the indicated antibodies: anti-human PARP antibody, anti-caspase 7 antibody, and anti-Flag antibody. β -actin was used as a loading control. Mock, cells transfected with pcDNA3-Flag empty vector; H7N9/NS1, cells transfected with pcDNA3-Flag-H7N9/NS1.

Fig. 5. Expression of H7N9/NS1 increases the p53, at the protein level, but not the mRNA level in transfected A549 cells. (A) Cells at 0 h and 24 h post-transfection were collected for western blot analysis to detect the expression of total p53 protein. Mock, cells transfected with pcDNA3-Flag empty vector; H7N9/NS1, cells transfected with pcDNA3-Flag-H7N9/NS1. The results shown are representatives of triplicate experiments. The level of induction (n-fold) was calculated from density values for protein bands relative to the corresponding β -actin results. Double asterisk indicates $p < 0.01$ (B) The mRNA level of p53 in H7N9/NS1-expressing cells and mock-transfected cells as detected by qPCR. Total RNA was extracted from H7N9/NS1-expressing cells and mock-transfected cells at 0 h and 24 h post-transfection. Results show the mean of 3 experiments $\pm$ SD and are normalized to the β -actin gene. Fold change was calculated relative to respective controls.

Expression of H7N9/NS1-induced apoptosis in transfected A549 cells

After establishing that H7N9/NS1 protein is cytotoxic to A549 cells, we investigated whether the novel avian influenza A virus H7N9 NS1 was able to modulate host cell apoptosis like NS1 protein of other reported influenza A virus subtypes or strains. Normally, apoptosis is accompanied by activation of caspases. We evaluated caspase 3/7 activity at 24 h post-transfection (Fig. 4A). This time point was selected based on the observation that

Fig. 6. Up-regulation of p53 phosphorylation at Ser37 and Ser46 in H7N9/NS1-expressing A549 cells. The p53 phosphorylation level of H7N9/NS1-expressing A549 cells was measured by western blot and corresponding densitometry. Cell lysates harvested 24 h post-transfection were processed for western blot with pho-Ser37 p53, pho-Ser46 p53 and p53 antibodies. β -actin was used as the loading control. The results shown are representative of three independent experiments. Double asterisk indicates $p < 0.01$ versus mock.

NS1 protein was highly expressed 24 h post-transfection. The results showed that the cells transfected with H7N9/NS1 exhibited significant caspase 3/7 activity, in contrast to mock cells. To confirm that the caspase pathways were involved in apoptosis induced by H7N9/NS1 protein, we further tested the activation of caspase-7. Our results showed that activated caspase-7 was detectable in H7N9/NS1-expressing cells 24 h post-transfection (Fig. 4B, the upper panel). In addition, PARP cleavage was in H7N9/NS1-transfected A549 cells 24 h post-transfection (Fig. 4B, the middle panel). Collectively, all these data indicated that apoptosis is initiated in H7N9/NS1-expressing A549 cells.

Up-regulation of p53 at the protein, but not mRNA level in H7N9/NS1-expressing A549 cells

The promoter region of caspase 7 contains a binding site for p53, a well-known apoptotic mediator [22, 23]. To investigate whether p53 played a role in the H7N9/NS1-induced apoptosis of A549 cells, we assessed the expression of p53 at both the mRNA and protein levels by qPCR and western blot analyses. Total p53 in H7N9/NS1-transfected A549 cells increased by about 2.0-fold, 24 h post-transfection, compared with the mock (Fig. 5A). Surprisingly, it could be seen that p53 mRNAs remained constant, in both H7N9/NS1- and mock-transfected cells (Fig. 5B). These data suggest that p53 was not transcriptionally stimulated, and the detectable up-regulation of p53 by H7N9/NS1 expression is partly due to posttranslational modification.

Expression of H7N9/NS1 increases p53 phosphorylation on serine 46 and serine 37

A number of studies have indicated that numerous serines on p53 are phosphorylated, in response to a stress signal, leading to p53 protein stabilization, and thereby enhance its function and/or affect the binding specificity of p53 to target sequences in the genome [24]. Immunoblotting of p53 and phosphorylated p53, Ser37-pho-p53 and Ser46-pho-p53 showed clear up-regulation for both total p53 and phosphorylated p53 (Fig. 6A). To quantify alterations in p53 phosphorylation level, we performed corresponding densitometry of bands of phosphorylated p53 following normalization to total p53. The ratio of phosphorylated p53 to total p53 indicated that the phosphorylation at Ser37 and Ser46 was up-regulated by

Fig. 7. Apoptosis induced by H7N9/NS1 in A549 cells is p53-dependent. A549 cells were incubated for 6 h in the presence or absence of pifithrin- α (10 μ M) and then transfected with either empty vector or an H7N9/NS1-expressing plasmid for 24 h. (A) Caspase 3/7 activity was significantly decreased in pifithrin- α -pretreated, H7N9/NS1-transfected A549 cells compared with H7N9/NS1-transfected cells ($p < 0.01$). (B) Expression of cleaved PARP was decreased in pifithrin- α -pretreated, H7N9/NS1-transfected A549 cells compared with H7N9/NS1-transfected cells.

2.89-fold and 3.05-fold, respectively (Fig. 6B), suggesting phosphorylation at these two sites contributes to p53 accumulation.

The apoptosis induced by H7N9/NS1 in A549 cells is p53-dependent

We further confirmed the role of p53 by pretreating A549 cells with the p53 inhibitor, pifithrin- α . Assay for caspase 3/7 activity and western blot for PARP analysis showed that pifithrin- α pre-treatment of A549 cells, prior to H7N9/NS1 transfection, reduced the induction of apoptosis (Fig. 7), indicating that the apoptosis induced by H7N9/NS1 protein in A549 cells might occur partly in a p53-dependent manner.

Expression of H7N9/NS1 causes the mitochondrial membrane depolarization and changes the expression of apoptosis-associated proteins

To further delineate the caspase cascade pathway by which H7N9/NS1 protein might be involved in the induction of apoptosis, we next examined the activation of the intrinsic apoptotic pathway marker, caspase 9 and extrinsic apoptotic pathway marker, caspase 8. We detected a significant activation of caspase 9, but failed to detect activation of caspase 8 in H7N9/NS1-transfected cells (Fig. 8A). Accumulation of p53 has been reported to disrupt the interactions between pro- and anti-apoptotic Bcl-2 family proteins [25]. We next examined the expression of Bcl-2 and Bax protein in A549 cells by western blot analysis. H7N9/NS1 expression increased the expression of Bax and decreased the expression of Bcl-2 (Fig. 8B), causing a 2.3-fold increase in the Bax/Bcl-2 ratio in H7N9/NS1-expressing cells compared with mock transfected cells (Fig. 8C). Because Bcl-2 family members play an important role in maintaining mitochondrial integrity, we investigated the effect of H7N9/NS1 protein on the mitochondrial membrane potential following H7N9/NS1 transfection. Cells were collected at 24 h post-transfection and mitochondrial membrane potential was detected with the fluorescent probe JC-1, using a flow cytometer. JC-1 exhibits potential-dependent accumulation in mitochondria, which results in the fluorescence emission shift from green to red. A decrease in the red/green fluorescence intensity ratio indicates mitochondrial depolarization. Flow cytometry analysis showed that 24 h after transfection, there was a significant decrease in the JC-1 red/green fluorescence ratio of H7N9/NS1-transfected A549 cells compared with both mock-transfected and untreated A549 cells (Fig. 8D). These results indicate that expression of H7N9/NS1 in A549 cells causes mitochondria membrane depolarization.

Fig. 8. Effects of H7N9/NS1 on apoptosis-associated proteins and mitochondrial membrane potential in A549 cells. (A) Western blot analyses of caspase 9 and caspase 8 activation. (B) Western blot analyses of the expression levels of Bax and Bcl-2. (C) Quantified results of the Bax/Bcl-2 ratio. (D) Mitochondrial membrane potential detected by flow cytometry. Data are expressed as the means $\pm$ SD of three experiments. A single asterisk indicates $p < 0.05$, and double asterisk indicates $p < 0.01$. UT, untreated cells; Mock, cells transfected with pcDNA3-Flag empty vector; H7N9/NS1, cells transfected with pcDNA3-Flag-H7N9/NS1.

Discussion

Apoptosis is an important physiological mechanism for maintaining cellular homeostasis. It is a multifactorial and strictly regulated process, which is generally induced by two major pathways: the death receptor pathway, and the mitochondrial pathway. Apoptosis through these two major pathways is closely related to various aspects of influenza A virus infection. The host utilizes apoptosis to eliminate influenza A virus by removing virus-infected cells, while viruses can modulate apoptosis for their effective replication and survival [26-29]. Previous studies have shown that the H9N2 virus induces apoptosis mainly via the mitochondrial pathway in human alveolar epithelial cells [20], and NS1 from H9N2 virus can induce death receptor-mediated apoptosis in chicken macrophages [19]. This prompted us to investigate if the NS1 protein of the novel avian H7N9 influenza A virus can also induce apoptosis in human cells, like its closely-related H9N2 virus, or the NS1 protein of H9N2 in chicken cells. Therefore, in the present study, we investigated the apoptotic effect of H7N9/NS1 protein in A549 cells and the mechanism and pathway involved in this action.

We determined that the NS1 gene, from the novel avian influenza virus A/Zhejiang/DTID-ZJU01/2013 (H7N9), is able to induce apoptosis in A549 cells. We performed western blot analysis and immunofluorescence to show the construct was being expressed (Fig. 2A and B) and able to induce apoptosis in A549 cells. The cells undergoing later stage of apoptosis and released their contents. Both Trypan blue exclusion and LDH release assays showed that H7N9/NS1 protein is cytotoxic to the cells and the cytotoxicity increases with the time and the expression level of NS1 protein (Fig. 3A and B).

Generally, apoptosis induction is accompanied by activation of caspases [30]. The death receptor pathway and mitochondria pathway are crucial for the activation of caspase-8 and caspase-9, respectively. Both activated caspase-8 and caspase-9 activate common downstream caspases, mainly caspase-3 and caspase-7, and then apoptosis takes place [31]. PARP is reported to be one of the main cleavage targets of caspase-3 *in vivo* [32]. It was also shown that caspase 7, which shares the same substrate preference as caspase 3, can cleave PARP more efficiently [33]. Cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis [34]. In the present study, Results of our caspase

3/7 activity assay confirmed that H7N9/NS1 induces apoptosis in A549 cells. In addition, western blotting showed that the expression of H7N9/NS1 could activate caspase-7 and the cleavage of PARP at 24 h post-transfection (Fig. 4). Taken together, these studies confirm that H7N9/NS1 induces apoptosis in A549 cells.

It was previously reported that p53 protein, a well-known apoptotic mediator, was implicated in the apoptotic response in influenza virus-infected cells [35, 36]. The p53 protein is classified as a stress response protein and is broadly activated in response to DNA damage, oxidative stress, and inappropriate proliferation [37, 38]. We showed that the protein level of p53 is dramatically increased in H7N9/NS1-expressing A549 cells (Fig. 5A), indicating that apoptosis induced by H7N9/NS1 protein may in part depend on the activation of p53. In addition, pretreatment of A549 cells with the p53 inhibitor (pifithrin- α), prior to NS1 transfection, reduced the apoptotic activity (Fig. 7), which suggests that apoptosis induced by H7N9/NS1 protein in A549 cells occurs in a p53-dependent manner. Regulation of p53 activities depends on a complex network of posttranslational modifications and protein interactions [39]. A previous study has also reported that the phosphorylation level of recombinant A/PR8/34 virus expressing defective NS1 protein infected cells is greatly decreased compared with wild-type virus-infected cells [40]. In the present study, we found that the phosphorylation of p53 on both Ser37 and Ser46 is triggered by the expression of H7N9/NS1 protein in A549 cells. Phosphorylation of serine 37 by kinases markedly reduces the ability of p53 to bind to MDM2 and causes a significant accumulation of p53 [41]. Phosphorylation of serine 46 by p38 kinase was involved in regulating the expression of p53AIP1, and inducing p53-dependent apoptosis [42]. However, the relationship between the expression of H7N9/NS1 protein and the enhanced phosphorylation level of p53 has yet to be explored. Nevertheless, our findings suggest that the increased phosphorylation level may be in part responsible for the accumulation of p53 during H7N9/NS1-induced apoptosis in A549 cells.

The Bcl-2 protein family is important to maintain mitochondrial integrity and mitochondria membrane potential [43]. Under normal conditions, the anti-apoptotic protein Bcl-2 binds to and sequesters the pro-apoptotic protein Bax. Thus, the ratio of Bax/Bcl-2 plays an important role in determining cell apoptosis or survival [44]. Western blotting of caspase 9, Bcl-2 and Bax, and flow cytometry analysis showed that expression of H7N9/NS1 activates caspase 9 (Fig. 8A), increases the Bax/Bcl-2 ratio (Fig. 8C), and decreases mitochondrial membrane potential (Fig. 8D), indicating that mitochondrial damage might be involved in H7N9/NS1-induced apoptosis in A549 cells. Of note, avian influenza virus A/HK/483/97 (H5N1) NS1 protein has been reported to activate caspase-8 in human airway epithelial cells to induce apoptosis via the death receptor pathway [14]. However, NS1 protein derived from another H5N1 virus strain has been shown to activate caspase-9 in A549 cells [15], indicating a mitochondrial-mediated apoptosis, and suggesting that H5N1/NS1 might either interacts with Fas to recruit and activate Fas-associated death domain-containing protein (FADD) or influences downstream signaling.

In conclusion, the present study shows that NS1 protein derived from a novel avian influenza A virus is cytotoxic and induces caspase-dependent apoptosis in A549 cells. The H7N9/NS1-induced apoptosis proceeds at least in part through the mitochondria-mediated pathway by modulating Bcl-2 family members. In addition, increased levels of total p53 and phosphorylated p53 might be the upstream signaling molecules for initiating the apoptotic effect in human A549 cells.

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Disclosure Statement

The authors declare that they have no conflict of interest.

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