MicroRNA-205-5p: A potential therapeutic target for influenza A

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Abstract
We are committed to finding host targets for influenza A therapeutics. The nucleoprotein (NP) plays an important role in influenza A virus replication and is an indispensable part of viral transcription and replication. Exploring endogenous substances that can modulate NP is critical for finding host targets. MicroRNAs (miRNAs, miR) are a novel class of powerful, endogenous gene expression regulators. Herein, we used miRanda to analyse the base complementarity between the NP gene and the 14 host miRNAs reported previously by us. MiRanda predicted that miR-431-5p, miR-744-3p and miR-205-5p could complement the NP gene. To understand the effect of these miRNAs on NP expression, we co-transfected 293 T cells with NP gene sequence containing above miRNAs binding site or full sequence of NP gene (transfected into pmirGlo or pcDNA3.1 vectors, respectively), and mimics of miR-205-5p, miR-431-5p and miR-744-3p. Dual luciferase reporter gene or Western blotting assays confirmed that miR-205-5p and miR-431-5p inhibit NP expression by binding with the miRNA binding site of NP gene. Further, we infected Mouse Lung Epithelial (MLE-12) cells overexpressing miR-205-5p and miR-431-5p with influenza A virus and performed Western blotting to examine NP expression. We found that NP expression was significantly reduced in MLE-12 cells overexpressing miR-205-5p and miR-431-5p during influenza A infection. The miR-205-5p overexpression-induced inhibition of influenza A replication could be attributed to the inhibition of NP expression. Further, we administered oseltamivir and Jinchai Antiviral Capsules (JC, an anti-influenza Chinese medicine) to influenza A virus-infected MLE-12 cells and mice. We found that miR-205-5p was significantly decreased increased in infected cells and lung tissues, and oseltamivir and JC could up-regulate miR-205-5p. In conclusion, we provide new evidence that miR-205-5p plays a role in regulating viral NP protein expression in combating influenza A and may be a potential target for influenza A therapy.

KEYWORDS
influenza a virus, MicroRNA-205-5p, nucleoprotein, therapeutic target
1 | INTRODUCTION

The influenza A virus has a wide host range, strong infectivity, rapid spread, and is prone to mutation. Due to the lack of completely effective prevention and treatment methods, Type A influenza is still a global health threat. Influenza A virus is a major cause of morbidity and a long-standing lurking virulent strain that can cause local outbreaks or global epidemics in a short time. Therefore, it is extensively researched by virologists. Due to the wide application of anti-influenza A virus drugs, the drug resistance of influenza A virus is increasing day by day. Therefore, it is urgent to develop new antiviral drugs and find new potential targets. The host target is not easy to mutate and is more conducive to the durability of the drug, which has become a research hotspot of researchers in recent years.

As a result, miR-205-5p stood out from the 14 candidate miRNAs for the first time that host miRNA exhibits antiviral function. Since miRNAs are negatively correlated with the expression of their target genes, the expression of viral NP in the host increases after influenza A virus infection; so, we screened miRNAs that were significantly reduced as the target of further research. Our previous studies showed that a 14 miRNAs cluster (Table 1) was the only significantly downregulated miRNA gene cluster in the lung tissue of mice with pneumonia caused by influenza A virus. Here, miRanda was used to analyse the base complementarity between the NP gene (Gene ID: 956531) and the 14 miRNAs mentioned above.

2.2 | Virus

The virus strain used in the study was A/Puerto Rico/8/34 (PR8, H1N1) (ATCC, USA), which is a well-characterized, mouse-adapted laboratory strain of influenza A virus. It is used as the genetic backbone for viruses from which inactivated influenza virus vaccines are generated. All experiments with live influenza viruses were performed in an animal biosafety level-2 (ABSL-2) laboratory or a biosafety level-2 (BSL-2) laboratory.

2.3 | Base complementation analysis

Since miRNAs are negatively correlated with the expression of their target genes, the expression of viral NP in the host increases after influenza A virus infection; so, we screened miRNAs that were significantly reduced as the target of further research. Our previous studies showed that a 14 miRNAs cluster (Table 1) was the only significantly downregulated miRNA gene cluster in the lung tissue of mice with pneumonia caused by influenza A virus. Here, miRanda was used to analyse the base complementarity between the NP gene (Gene ID: 956531) and the 14 miRNAs mentioned above.

2.4 | Dual luciferase reporter gene analysis

The NP gene sequence (5'-ggcagctcgggccaatcagcataacctacttgtctcagtagaatactctccttttgacagaacaccgtaTGGCAgAATT-3') is increasing day by day. Therefore, it is very important to explore the endogenous and became a powerful endogenous candidate for influenza A therapy.

The nucleoprotein (NP) plays an important role in influenza A virus replication; it is an indispensable part of viral transcription and replication. NP expression inhibition can block virus replication. Therefore, it is very important to explore the endogenous substances that can inhibit the NP for finding host targets.

MicroRNAs (miRNAs, miR) are a class of naturally occurring small non-coding RNA molecules. They bind to the 3′ untranslated regions (3′-UTR) of target mRNAs to either block the translation or initiate the transcript degradation. In 2005, scientists discovered for the first time that host miRNA exhibits antiviral function. Since then, host miRNA and viral gene interactions have become a new research hotspot in the field of miRNA and host antiviral defence. miR-323, miR-491 and miR-654 target influenza A virus base polymerase 1 gene (PB1) and inhibit the in vitro replication of H1N1 influenza virus in Madin-Darby Canine Kidney (MDCK) cells. Let-7c aberrantly expresses in influenza A virus infected human lung epithelial cells (A549) and targets the matrix protein 1 gene (M1) in influenza A virus and inhibits influenza A virus replication in A549 cells, playing a role in protecting host cells from the virus. However, thus far, miRNAs have not been systematically targeted for anti-influenza A viral therapy.

Based on the above reasons, host miRNAs are very likely to become novel drug therapeutic targets for influenza A. In this study, we aimed to mine host miRNAs that could not only inhibit NP expression in host cells but also be regulated by anti-influenza A drugs. As a result, miR-205-5p stood out from the 14 candidate miRNAs and became a powerful endogenous candidate for influenza A therapeutic target.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

This study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org). All procedures involving animals were approved by the Institutional Animal Care and Use Committee (China Academy of Chinese Medical Sciences, Beijing, China). The animal study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the China Academy of Chinese Medical Sciences (Beijing, China).
cactggagaatacagaggagaaatcagatagccgaaatcataaggatgatg- 3′) containing miR-205-5p, miR-431-5p and miR-744-3p binding sites was loaded into the pmirGlo vector. MiR-205-5p, miR-431-5p, miR-744-3p mimics and negative control (NC) were synthesized by Genepharma. The 293 T cells were co-transfected with NP gene sequence (using pmirGlo vector) and miR-205-5p, miR-431-5p, miR-744-3p mimics or the NC (using lipofectamine 2000 (Invitrogen)). 293 T cells transfected with pmirGlo vector containing NP gene sequence was used as control. The cells were harvested 24 h after co-transfection, and the luciferase activity was measured with a dual luciferase reporter assay system (Promega E1960) on a multi-label microplate detector (PerkinElmer EnSpire).

### 2.5 MicroRNA functional verification—inhibit the expression of NP synthesized in vitro

The NP gene (1525 bp) was loaded into the pcDNA3.1 vector. miR-205-5p, miR-431-5p, miR-744-3p and NC were synthesized by Genepharma. The 293 T cells were co-transfected with the pcDNA3.1 vector containing NP gene and miR-205-5p, miR-431-5p, miR-744-3p or NC (using lipofectamine 2000 (Invitrogen)). 293 T cells transfected with pmirGlo vector containing NP gene sequence was used as control. The cells were harvested 24 h after co-transfection, and the luciferase activity was measured with a dual luciferase reporter assay system (Promega E1960) on a multi-label microplate detector (PerkinElmer EnSpire).

### 2.6 MicroRNA functional verification—inhibit the expression of NP from influenza A virus

The mouse lung epithelial (MLE-12) cell lines were purchased from the BeNa Culture Collection. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), in a humidified atmosphere of 95% air- 5% CO\textsubscript{2} at 37°C. MLE-12 cells were cultured in 6 well culture plate, and then the cells were transfected with miR-205-5p mimic, miR-205-5p inhibitor, miR-431-5p mimic, miR-431-5p inhibitor, NC or inhibitor negative control (INC), with lipofectamine 2000 for 48 h. miR-205-5p inhibitor, miR-431-5p inhibitor, INC were synthesized by Genepharma. These miRNA mimic (or respective inhibitor) transfected MLE-12 cells were simultaneously infected with influenza A virus (MLE-12 Infection model). The cells cultured in complete medium were considered as normal control. The culture plate was cultured in a 37°C, 5% CO\textsubscript{2} incubator. The cells were harvested 48 h after transfection, and Western blot was carried out using the primary antibodies against NP (Abcam) and GAPDH (Cell Signalling) as an internal control.

### 2.7 Construction of influenza A virus pneumonia mouse model and drug treatment

Male and female ICR mice (SPF, 14 ± 1 g) were anaesthetized with isoflurane and intranasally administered with 15 times the lethal dose 50% (15 × LD\textsubscript{50}) of PR8 virus suspended in physiological saline. In addition, an uninfected group was administered intranasal physiological saline. On the day of infection, 27.5 mg/kg/d oseltamivir and 0.88g/kg/d Jinchai Antiviral Capsules (JC, an anti-influenza Chinese medicine) were given for four consecutive days. On the
TABLE 2  Cytopathic grade 6 criteria

| Grade | Cytopathic condition                      |
|-------|------------------------------------------|
| -     | Cells grow normally, no lesions appear   |
| ±     | Cytopathic < 10% of the entire monolayer |
| +     | Cytopathies account for <25% of the entire monolayer |
| ++    | Cytopathy accounts for <50% of the entire monolayer |
| +++   | Cytopathy accounts for <75% of the entire monolayer |
| ++++  | Cytopathies account for >75% of the entire monolayer |

5th day of the virus infection, the mice were sacrificed by cervical dislocation, and lung tissues were dissected. The doses administered to mice are converted from clinical doses.

2.8  Dose determination of oseltamivir and JC in MLE-12 cell experiments

Oseltamivir and JC were prepared into 5 and 50 mg/ml stock solutions with purified water, respectively, and then filtered with sterile membrane (Sartorius). Using the maintenance solution (DMEM containing 2% (v/v) FBS) to dilute the stock solution, 2-fold dilution to eight concentrations, and add to the 96-well culture plate where MLE-12 cells have grown into a monolayer (100 μl/well, four duplicate wells for each dilution). At the same time, a normal cell control was set. The culture plate was cultured in a 37°C, 5% CO₂ incubator, and the cytopathic conditions were observed under an inverted microscope every 24 h for 72 h. Determine the minimum dilution factor at which the cells do not have obvious lesions, calculate the maximum non-toxic concentration (TC₀), and calculate the 50% cytotoxic concentration (TC₅₀) according to the Reed-Muench method. 26

Cytopathy is judged according to six grades (Table 2).

A 96-well culture plate with MLE12 cells grown into a monolayer was taken, and the cell surface was washed three times with cell maintenance solution, then infected with 100 times 50% tissue culture infective dose (100 × TCID₅₀) of PR8 virus, and placed in a 37°C, 5% CO₂ incubator. After the virus was adsorbed for 1 h, the virus solution was discarded, and six dilutions of the drug solution (100 μl/well) below the TC₀ were sequentially added, and a normal cell control and a virus control were set at the same time. The cells were cultured in a 37°C, 5% CO₂ incubator, and the cytopathic conditions were observed under an inverted microscope every 24 h. After 72 h, the test results were recorded. The 50% inhibitory concentration (IC₅₀) was calculated according to Reed-Muench method, and the therapeutic index (TI) was calculated, TI = TC₅₀/IC₅₀ 24 Cytopathy is judged according to six grades (Table 2).

2.9  Cultured MLE-12 cell infection model and drug treatment

MLE-12 cells were cultured in culture medium (DMEM containing 10% (v/v) FBS), in a 37°C, 5% CO₂ incubator. These cultured MLE-12 cells were infected for 1 h with 100 × TCID₅₀ PR8 virus. Discard the virus solution 1 h after infection, and 20 μg/ml oseltamivir and 200 μg/ml JC were given for 48 h. Simultaneously, normal control cells and infected control cells were given equal amount of maintenance solution (DMED containing 2% (v/v) FBS). Oseltamivir and JC were formulated using maintenance solution and filtered through a 70 μm sterile filter (Sartorius).

2.10  Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

qRT-PCR was performed to verify the level of NP, miR-205-5p and miR-431-5p in the lung and the MLE-12 cells. The total RNA extracted (0.5 μg) with Trizol was reverse-transcribed using M-MLV reverse transcriptase (Thermo) with random primer for NP, and with a special stem-loop primer (Geneapherma) for miR-205-5p and miR-431-5p. qRT-PCR was performed on a real-time PCR system (ABI), using Power SYBR Green PCR Master Mix (ABI). All samples were analysed in fourfold, including the no-template control. The relative expression level was determined by 2ΔΔCT method and normalized to GAPDH or U6. The primer sequences are shown in Table 3.

2.11  Western blotting analysis

The protein samples were extracted from the lung tissues or MLE-12 cells with ExKine™ Total Protein Extraction Kit (abbkine). Protein samples (20 μg) were fractionated using SDS-PAGE using 10% polyacrylamide gels and then transferred onto a PVDF membrane. After blocking with non-fat milk, the blots were incubated with the primary antibodies: NP (1:10,000, Abcam) and GAPDH (1:2000, Cell Signalling), overnight at 4°C. Subsequently, the secondary antibody incubation was conducted with goat antimouse or goat antirabbit IgG antibody (1:10,000) for 3 h at room temperature. Blots were visualized by ECL (Millipore) and the density of bands was determined by Lane 1D software (Sage).

2.12  Histopathologic analysis

The lungs were fixed in 4% formalin, dehydrated in ascending ethanol concentrations, embedded in paraffin, sectioned into 4-μm-thick slices, and stained with haematoxylin and eosin (H&E). Histopathology photos were taken using a phase inverted microscope (Olympus).

2.13  Statistical analysis

All the results were expressed as mean ± standard deviation (SD). SPSS17.0 software was used for data analysis, analysis of variance
ANOVA was used to test for homogeneity of variance. Least significant difference (LSD) test was used when the variance was homogeneous; Dunnett T3 test was used when the variance was not significant difference (LSD) test was used when the variance was not homogeneous. Significance level was set at \( p < 0.05 \); \( p < 0.01 \) which represents highly significant differences.

### 3 | RESULTS

#### 3.1 | Three MiRNAs, miR-431-5p, miR-744-3p and miR-205-5p, complement the NP gene

Using miRanda we predicted that miR-431-5p, miR-744-3p and miR-205-5p were complementary to the NP gene, and using mirbase database, we found that the human miR-431-5p, miR-744-3p and miR-205-5p sequence is completely consistent with the mouse sequence, so the results of this study have direct clinical guiding value (Table 4, Figure 1).

#### 3.2 | miR-431-5p and miR-205-5p are capable of targeting NP gene

Dual luciferase reporter gene analysis was used to verify the binding of miR-431-5p, miR-744-3p, or miR-205-5p to NP gene. Compared with the control, the miR-205-5p and miR-431-5p mimics strongly suppressed the relative activity of the dual luciferase reporter fused with NP gene sequence (Figure 2). The inhibitory effect of miR-205-5p and miR-431-5p on downstream luciferase gene expression confirmed that miR-205-5p and miR-431-5p could target the binding site of NP gene.

#### 3.3 | miR-431-5p and miR-205-5p inhibit the expression of NP synthesized in vitro

The NP whole gene eukaryotic cell overexpression analysis was used to further verify the inhibitory effect of miR-205-5p, miR-744-3p or miR-431-5p on NP expression via NP gene binding. Western blotting was performed to examine the NP expression level. Compared with that of the control, miR-205-5p, miR-431-5p and miR-744-3p all can inhibit NP expression levels (Figure 3). However, the dual luciferase assay showed that miR-744-3p could not bind its specific site to inhibit downstream gene expression, so there may be other pathways for the inhibition of NP expression by miR-744-3p, the reasons for which will be discussed in detail later.

#### 3.4 | miR-205-5p inhibit the expression of NP from influenza A virus

MLE-12 cells over-expressing miR-205-5p or miR-431-5p were used to verify their inhibition on influenza A virus replication. Western blotting assay was carried out to check NP expression levels. NP expression was significantly decreased in influenza A virus infected MLE-12 cells overexpressing miR-205-5p (Figure 4). Therefore, in host cells, up-regulation of miR-205-5p levels is important for suppressing influenza A virus NP expression. Further, NP expression level was used to evaluate the influenza A virus replication ability, so up-regulation of host miR-205-5p expression can inhibit influenza A virus replication. In conclusion, miR-205-5p can be used as a therapeutic target for influenza A.

#### 3.5 | miR-205-5p: A potential target for drugs used to treat influenza A viral infection

We created a mouse pneumonia model using influenza A virus and treated with oseltamivir and JC. Influenza A virus infection causes a highly significant lung index increase. This lung index increase could be significantly reduced by oseltamivir and JC (Figure 5A). Detection of NP expression levels indicative of viral load in lung tissue. Influenza A virus infection causes a highly significant viral load increase. This viral load increase could be significantly reduced by oseltamivir and JC (Figure 5B). Influenza A virus infection caused several pathological changes: increased cell proliferation, inflammatory cell exudation, cell exfoliation and varied alveolar cavity size were observed in the lung tissue. These changes could be improved by oseltamivir and JC (Figure 5C). The above data show that oseltamivir and JC have a significant effect on influenza A virus pneumonia.

Differential expression of NP protein in influenza A virus infected lung tissue were verified by Western blot analysis. NP protein expression level was confirmed to be up-regulated in influenza A virus infected lung tissue and be down-regulated by oseltamivir and JC (Figure 6A). Differential expression of miR-205-5p in influenza A virus infected lung tissue were verified by qRT-PCR. miR-205-5p expression was confirmed to be significantly down-regulated in influenza A virus infected lung tissue and could be up-regulated by oseltamivir and JC (Figure 6B). The above results showed that the effects of oseltamivir and JC on host miR-205-5p expression were

| Table 3 Primer sequences in qPCR |
|------------------------------|
| **Gene** | **Primer type** | **Primer sequence** |
| NP | Forward | GTGTATGGACCTGCGTGAGC |
| GAPDH | Forward | AAGCCGATCTTTCACGAGCC |
| miR-431-5p | Forward | ACGCTTGTCTTGCAGGCC |
| miR-205-5p | Forward | CGCTTGCAGACGACATATA |
| miR-744-3p | Forward | TTCACGAATTTCGCTTCATC |
negatively correlated with their effects on viral NP expression in vivo.

The drug cytotoxicity test results show that the maximum non-toxic concentration can be obtained by diluting oseltamivir and JC original drug solution by 64 times. The original drug concentrations of oseltamivir and JC are 5 and 50 mg/ml respectively, so the TC<sub>0</sub> is respectively 0.078 and 0.78 mg/ml (Table 5). The TC<sub>50</sub> of oseltamivir and JC calculated by Reed-Muench method are 0.112 and 1.12 mg/ml respectively (Table 5). The results of the antiviral experiment of the drug on cells showed that oseltamivir still had a good antiviral effect at the lowest dilution (1024-fold dilution), while JC had a good antiviral effect at a 256-fold dilution (Table 6).

### TABLE 4 MiRNAs complementing the NP gene

| miRNA     | Gene symbol | Align score | Energy | miRNA start | miRNA end | Gene start | Gene end |
|-----------|-------------|-------------|--------|-------------|-----------|------------|----------|
| mmu-miR-205-5p | NC_002019.1 | 149         | -16.23 | 2           | 14        | 1434       | 1455     |
| mmu-miR-431-5p | NC_002019.1 | 157         | -24.56 | 2           | 19        | 1336       | 1358     |
| mmu-miR-744-3p | NC_002019.1 | 140         | -13.11 | 2           | 15        | 1265       | 1285     |

**FIGURE 1** Potential binding site between miR-744-5p, miR-205-5p or imR-431-5p and the NP gene. The complementary nucleotides between miR-744-5p, miR-205-5p or imR-431-5p and the target region of NP gene are connected with short vertical lines.

**FIGURE 2** NP is a target of imR-431-5p and miR-205-5p. (A) Dual luciferase activity assay was performed by cotransfected pmirGlo vector containing the NP gene sequence with miR-205-5p, miR-431-5p or miR-744-3p into 293T cells. The dual luciferase activity assay was performed 24 h later. (B) Transfection efficiency of 293T cells after 24 h (100×); (I) Light microscope of 293T cells transfected with LV5 labelled negative control miRNA after 24 h, (II) Fluorescence microscope of 293T cells transfected with LV5 labelled negative control miRNA after 24 h. n = 5, **p < 0.01. LV5, a fluorescent marker for labeling miRNA; NC, negative control miRNA.
due to its good efficacy at the lowest dilution (Table 6). Based on the above experimental results, the in vitro experimental doses of oseltamivir and JC were both selected at 256-fold dilution, that is, 0.02 and 0.2 mg/ml.

Differential expression of NP protein in influenza A virus infected MLE-12 cells was verified by Western blot analysis. NP protein expression level was confirmed to be up-regulated in influenza A virus infected MLE-12 cells and be down-regulated by oseltamivir.
**Figure 5** A mouse model of influenza A virus pneumonia and drugs treatment. (A) Influenza A virus infection causes a significant increase in the lung index, which could be significantly reduced by oseltamivir and JC. Lung index = wet lung weight (g)/body weight (g) × 100. (B) Influenza A virus infection causes a significant increase in the viral load of lung tissue, which could be significantly reduced by oseltamivir and JC. (C) In the uninfected group, there was no inflammatory infiltration in the lung tissue, and the structure was normal; in the infection group, there were a large number of cell proliferation, inflammatory cell exudation and exfoliated cells in the lung tissue, and the alveolar cavity size varies; in the Oseltamivir and JC group, inflammation and edema in the lung tissues were reduced. (H&E, 400×). (n = 10; **p < 0.01)

**Figure 6** In vivo the effect of oseltamivir and JC on the expression of host miR-205-5p was negatively correlated with the effect on the expression of viral NP. (A) NP protein up-regulate in influenza A virus infected lung tissue and down-regulated by Oseltamivir and JC; n = 6; **p < 0.01; JCH, twice the clinical dose of JC; JCL, half the clinical dose of JC; SF, Shufeng Jiedu capsule, an anti-influenza Chinese medicine. (B) miR-205-5p down-regulate in influenza A virus infected lung tissue and up-regulated by Oseltamivir and JC; n = 8; **p < 0.01; *p < 0.05
and JC (Figure 7A). Differential expression miR-205-5p in influenza A virus infected MLE-12 cells were verified by qRT-PCR. miR-205-5p expression was confirmed to be significantly downregulated in influenza A virus infected MLE-12 cells, and could be upregulated by oseltamivir and JC (Figure 7B). The above results showed that the effects of oseltamivir and JC on host miR-205-5p expression were also negatively correlated with their effects on viral NP expression in vitro.

In summary, In vitro and in vivo experiments, under the premise of ensuring that oseltamivir and JC have anti-influenza efficacy, both oseltamivir and JC can down-regulate the expression level of viral NP protein, while the expression level of miR-205-5p was up-regulated, suggesting that miR-205-5p is a therapeutic target of anti-influenza A drugs.

4 | DISCUSSION

The World Health Organization has always believed that influenza virus may be one of the most potential threats to humans. In recent years, with the increase in the scope of global species activities, the probability of influenza virus gene recombination among humans, poultry, pigs and other species has greatly increased, which has enhanced the variability of influenza viruses. At the same time, due to the wide application of anti-influenza virus drugs, the drug resistance of influenza virus is becoming more and more serious.

| Group | Dilution ratio | Lesion grade | TC50 | TC0 |
|-------|---------------|--------------|------|-----|
| Cell control | × | 0000 | × | × |
| Oseltamivir | 1:2 | 4444 | 0.112 mg/ml | 0.078 mg/ml |
| | 1:4 | 4444 |  |  |
| | 1:8 | 4444 |  |  |
| | 1:16 | 3333 |  |  |
| | 1:32 | 2222 |  |  |
| | 1:64 | 0000 |  |  |
| | 1:128 | 0000 |  |  |
| | 1:256 | 0000 |  |  |
| JC | 1:2 | 4444 | 1.120 mg/ml | 0.780 mg/ml |
| | 1:4 | 4444 |  |  |
| | 1:8 | 4444 |  |  |
| | 1:16 | 3333 |  |  |
| | 1:32 | 0000 |  |  |
| | 1:64 | 0000 |  |  |
| | 1:128 | 0000 |  |  |
| | 1:256 | 0000 |  |  |

Note: The primary concentration of oseltamivir and JC are 5 mg/ml and 50 mg/ml; cytopathic score: ‘+’ = 0, ‘+’ = 1, ‘++’ = 2, ‘+++’ = 3, ‘++++’ = 4, 4 duplicate holes were scored, respectively.

Therefore, it is urgent to develop new anti-influenza drugs and find new therapeutic targets.

The influenza A virus, belonging to the Orthomyxoviridae family, can cause severe lung infections and even death. The therapeutic targets of influenza A have been mainly viral proteins, such as ion channel protein (M2), neuraminidase (NA), hemagglutinin (HA), nucleoprotein (NP) and RNA-dependent RNA polymerase (RdRp). At present, most of the influenza A treatment drugs use these proteins as therapeutic targets; these anti-influenza A drugs will show good antiviral ability in the early stage of use, but due to the drug resistance of the virus, it is necessary to continuously develop new antiviral drugs. In recent years, in order to solve the problem of drug resistance of anti-influenza A drugs, scholars have been constantly looking for new antiviral targets that are not easily resistant to drug resistance to develop new antiviral drugs, among which host targets are the most studied. In this study, we took host factors as a breakthrough to screen and mine the target of anti-influenza A virus drugs, and miR-205-5p was successfully identified as an antiviral drug target candidate.

miRNAs participate in many biological host cell processes and are an important biological factor for host biological balance maintenance. miRNAs degrade target genes or blocks translation through complementary binding to target genes, thereby inhibiting protein expression. A viral infection represents interplay between two biological systems—the host cell and the virus. When these two biological systems are at interplay, host miRNAs could regulate the replication and pathogenicity of viruses. For example, simian foamy virus type 1 (PFV-1), vesicular stomatitis virus (VSV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV-1) affect the viral infection processes by modulating viral gene expression. While miR-323, miR-491 and miR-654 can target the same conserved region of the influenza A virus PB1 gene, let-7c can target the M gene of influenza A virus, they all can inhibit in vitro replication of influenza A virus. However, miRNA have not been systematically researched as the target for anti-influenza A viral therapy. We have been working to find novel host targets against influenza A virus, and in recent years have targeted host miRNAs. Earlier, we reported that a cluster of 14 miRNAs—viz. miR-30c-1-3p, miR-34b-3p, miR-92b-3p, miR-149-5p, miR-375-3p, miR-34c-3p, miR-449a-5p, miR-449c-5p, miR-411-3p, miR-431-5p, miR-744-3p, miR-205-5p, miR-208a-5p, and miR-299a-3p—is downregulated in the lung tissue of influenza A virus infected pneumoniae mice. In this study, miRanda was used to analyse the base complementarity between the influenza A virus gene and the 14 miRNAs mentioned above. MiR-431-5p, miR-744-3p, and miR-205-5p were predicted to be complementary to the NP gene by miRanda and could be further explored as candidate research targets.

The nucleoprotein (NP) is indispensable for the transcription and replication of influenza A virus. In the viral particles, the NP wraps the viral nucleic acid playing a protective role. The NP antigen structure is stable. Together with the matrix protein, it determines the virus type specificity and rarely undergoes mutation. It forms a ribonucleoprotein complex with RNA polymerases PB2, PB1, and
PA to aid viral transcription and replication. NP gene is not only closely associated with influenza A virus replication, but also has a large number of conserved sequences. Therefore, exploring biological mechanisms that can regulate the NP gene expression and inhibit viral replication has important research significance. In this study, it is important to confirm the candidate anti-influenza A virus target (miR-431-5p, miR-744-3p or miR-205-5p) inhibit the expression of NP protein by complementing the NP gene, and is valuable for clarifying their status of antiviral target. Dual-luciferase reporter gene analysis and miRNA functional validation experiments were performed to validate our ideas.

We co-transfected 293T cells with NP gene sequence containing special sites and miR-205-5p, miR-431-5p or miR-744-3p mimics. Dual luciferase reporter gene assays carried out on these co-transfected 293T cells confirmed that miR-205-5p and miR-431-5p can bind to NP gene. Similarly, 293T cells were co-transfected with NP gene and miR-205-5p, miR-431-5p or miR-744-3p mimics. Western blot assays on these co-transfected 293T cells showed that miR-205-5p, miR-431-5p and miR-744-3p can inhibit NP expression. Taken together, these results confirmed that miR-205-5p and miR-431-5p inhibited NP expression by binding its NP gene. Further, functional verification of miR-205-5p and miR-431-5p mediated inhibition of NP expression was performed using miRNA over-expression system. MLE-12 cells were simultaneously infected with influenza A virus and stimulated to over-express miRNA mimics (miR-205-5p or miR-431-5p) for 48 h. Western blot was performed to examine NP expression levels. We found that NP expression during influenza A virus infection is significantly reduced in MLE-12 cells over-expressing the miR-205-5p mimic. These findings suggest that host miRNA-205-5p can regulate the expression of influenza A virus NP protein by binding to the NP gene, which may be a candidate for drug therapy targets.

The conclusion that miR-205-5p can be used as a therapeutic target for anti-influenza A drugs needs to be confirmed by drug experiments. In this study, we used a mouse model of influenza A virus pneumonia to validate that miRNA-205-5p is a therapeutic target for anti-influenza A drug. Findings showed that pathological changes, including the lung index increase, viral load increase, tissue lesions

### TABLE 6  Antiviral effects of oseltamivir and JC on MLE-12 cells

| Group       | Dilution ratio | Lesion grade | IC_{50} | TI  |
|-------------|----------------|--------------|---------|-----|
| Cell control| ×              | 0000         | ×       | ×   |
| Viral control| ×            | 4444         | ×       | ×   |
| Oseltamivir | 1:64           | 2002         |         |     |
|             | 1:128          | 0000         | ×       | ×   |
|             | 1:256          | 0000         | ×       |     |
|             | 1:512          | 0000         |         |     |
|             | 1:1024         | 0000         |         |     |
| JC          | 1:64           | 4444         | 0.12mg/ml| 9.33|
|             | 1:128          | 2000         |         |     |
|             | 1:256          | 0000         |         |     |
|             | 1:512          | 4004         |         |     |
|             | 1:1024         | 4444         |         |     |

Note: The original concentration of oseltamivir and JC are 5 mg/ml and 50 mg/ml; cytopathic score: ‘–’ = 0, ‘+’ = 1, ‘++’ = 2, ‘+++’ = 3, ‘++++’ = 4, 4 duplicate holes were scored, respectively.

**FIGURE 7** In vitro the effect of oseltamivir and JC on the expression of host mir-205-5p was negatively correlated with the effect on the expression of viral NR. (A) NP protein up-regulate in influenza A virus infected MLE-12 cells and down-regulated by Oseltamivir and JC; n = 3; **p < 0.01; *p < 0.05. (B) miR-205-5p down-regulate in influenza A virus infected MLE-12 cells and up-regulated by Oseltamivir and JC; n = 4; **p < 0.01; *p < 0.05
enhance, and NP protein expression increase caused by influenza A virus infection can be alleviated by oseltamivir (a neuramidase inhibitor which inhibits viral budding) and JC (a traditional Chinese medicine which treat influenza A), and miR-205-5p expression decrease can be enhanced by oseltamivir and JC. The results suggest that miR-205-5p can be used as a therapeutic target for anti-influenza A drugs. The results suggest that miR-205-5p can be used as a therapeutic target for anti-influenza A drugs. Only two typical drugs were validated in this study, and there are many more drugs to be validated in the future.

5 | CONCLUSION

We provide new evidence that miR-205-5p plays an important regulatory role during influenza A virus infection. The regulation effect of miR-205-5p on influenza A virus replication could be attributed to the inhibition of NP expression. Simultaneously, we confirmed that the expression of miR-205-5p in vivo and in vitro can be up-regulated by oseltamivir and JC, suggesting that miR-205-5p is the target of oseltamivir and JC against influenza A virus. Moreover, here, we have studied effects of miRNA on segments of a single influenza A virus gene. Our future research will verify these findings for other gene segments. This study represents a major effort to elucidate antiviral molecular mechanisms dependent on host miRNAs to target influenza A virus infections. We provide a potential drug target—miR-205-5p. The results of this study provide new research ideas and approaches for the future research and development of new anti-influenza A virus drugs target and the in-depth study of antiviral mechanisms.

AUTHOR CONTRIBUTIONS

yan yan bao: Conceptualization (lead); data curation (lead); formal analysis (lead); funding acquisition (lead); investigation (lead); methodology (lead); project administration (lead). yu jing shi: Investigation (supporting). li run zhou: Investigation (supporting). shuangrong gao: Investigation (supporting). zhan gen geng: Investigation (supporting). lei bao: Investigation (supporting). ruo hua zha o: Investigation (supporting). xiao lan cui: Project administration (supporting).

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CONFLICT OF INTEREST

The authors confirm that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this published article.

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