Original Article
Host miR-146a promotes replication of human cytomegalovirus by suppressing type I IFN response in MRC-5 cells

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Abstract: Human cytomegalovirus (HCMV) is major cause of birth defects upon congenital infection as well as morbidity in immunosuppressed populations and has evolved several mechanisms to evade IFN-I responses. MiR-146a is found to regulate responses IFN-I in a variety of viral infections, but it is not clear whether the same function in HCMV. In this study, we study the effect of miR-146a on HCMV replication by miR-146a inhibitor and mimics. In addition, we used bioinformatics methods to find potential target genes of miR-146a. Effects of miR-146a on the immune response of type I interferon were also detected. Finally, regulation of miR-146a on the downstream IFN-stimulated genes (ISGs) including ISG15, ISG56, OAS1, and Mx1 in MRC-5 cells was detected by qRT-PCR analysis. We found that a host microRNA, miR-146a, was upregulated in HCMV infected cells, which obviously facilitated HCMV replication in host cells. Subsequently, we demonstrated that miR-146a was a potent negative regulator of IFN-I signaling pathway by targeting STAT1, resulting in the enhancement of HCMV infection. The last we found that miR-146a and ISGs expression levels were negatively correlated. In conclusion, these findings indicate that miR-146a suppression Type I IFN responses to human cytomegalovirus (HCMV) by targeting STAT1 in MRC-5 cells. These findings suggest that miR-146a may provide a potential strategy for anti-HCMV therapies.

Keywords: miR-146a, HCMV, replication, type I IFN response

Introduction

Human cytomegalovirus (HCMV), a member of the beta herpesvirus family, is a major cause of birth defects upon congenital infection as well as morbidity in immunosuppressed populations [1-3]. HCMV, also called human herpesvirus-5 (HHV-5), is one of the 8 human herpesviruses. HCMV is a common cause of congenital cytomegalovirus (cCMV) infections in developed as well as developing countries.

IFN-I have a variety of functions, mainly for antivirus, anti-parasitic, antibacterial, anti-tumor, which is one of the most important functions of IFN-I [4]. IFN-I also play an important role in the regulation of innate immune response and adaptive immune response [5, 6]. Receptor for IFN-I Interferon alpha receptor (receptors alpha, IFNAR), it is an α receptor protein located on the cell surface, which is composed of two subunits, IFNAR1 Composition with IFNAR2. IFNAR and Janus kinase 1 (Janus kinase Jak1) and tyrosine kinase (tyrosine kinase, Tyk) in combination with, and the latter can phosphorylation of signal transduction and transcription activation factor (signal transducers and activators of transcription (STAT) 1 and 2. These activated STAT molecules, together with IRF9, form a complex, IFN-stimulated gene (ISG) factor 3, and then translocate into the nucleus to bind the IFN-stimulated response element (ISRE) to initiate the transcription of ISGs, which are executors in the clearance of viral pathogens [7-10].

MicroRNAs (miRNAs) are endogenous, non-coding RNA of about 22 nucleotides that regulates gene expression by controlling target mRNA translation or degradation [11]. Studies have indicated that MiRNA has become a key transcriptional regulator of gene expression and is

actively involved in the regulation of innate and adaptive immune responses and host pathogen interactions in RNA [12-14]. There is evidence that the virus's miRNA and cellular origins can help the virus escape the immune response by targeting vital components in the host immune system [10, 12, 15]. A large number of studies have indicated that miR-146a regulates host cell type I IFN responses in a variety of diseases and infections, but it has not been reported to have the same regulation of type I interferon immune response in HCMV infection.

In this study, we identified that cellular miR-146a was up-regulated by HCMV infection, which obviously increase HCMV replication. Subsequently, we found that the promotion of viral replication effect of miR-146a is mainly dependent on targeted inhibition of STAT1. Additionally, we studied the mechanism of miR-146a participating in the I type interferon immune response, and found that it was mainly controlled by the interferon stimulating gene to regulate the host immune response.

Materials and methods

Cell culture and virus

Human fetal diploid lung fibroblasts MRC-5 cells were maintained in modified Eagle's medium (MEM) with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 units/ml streptomycin. HCMV clinical strain Han was isolated from a urine sample of a 5-month-old infant hospitalized in Shengjing Hospital of China Medical University. The virus was inoculated in MRC-5 cells maintained in MEM supplemented with 2% FBS and penicillin-streptomycin, then the cell lysate containing virus was harvested and stored at -80°C.

Patient samples

The serum of 10 patients undergoing Human cytomegalovirus were obtained from the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China) with written informed consent of patients according to the hospital's ethics committee.

microRNA-146a target prediction

The internet resource TargetScan (http://www.targetscan.org/) was used to predict potential microRNA-146a targets, using 'hsa-microRNA-146a' as a search term.

Luciferase reporter assay for survivin-3'-STAT1 targeting

Luciferase vectors including the 3'-UTR of survivin containing the STAT1-microRNA-146a response elements and the mutant were purchased from Genepharma. For the luciferase reporter experiment, HepG2 and Huh7 cells were seeded in 12-well plates and cotransfected with the luciferase reporter vectors and the microRNA-146a mimic, inhibitor or corresponding negative control. Luciferase activity was measured using the Dual-Light Chemiluminescent Reporter Gene Assay System (Applied Biosystems) and normalized to the β-galactosidase activity.

Real-time quantitative polymerase chain reaction (qRT-PCR)

For analysis of the expression of miR-146a, microRNAs were extracted from sample tissues and cells by miRVana kits (Ambion, Austin, TX, USA). The reverse transcription reaction was performed using the one-step primescript miRNA cDNA synthesis kit (Takara, Dalian, China). For mRNA analysis, total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) and cDNA was generated using M-MLV reverse transcriptase (Clontech, Palo Alto, CA, USA) according to the standard protocol. The mixture system containing cDNA templates, corresponding primers and SYBR Green qPCR Master Mix (Thermo Fisher, Shanghai, China) were subjected to RT-qPCR quantification. Relative expression levels were quantified and normalized to U6 SnRNA or β-actin (internal referee for miRNA or mRNA, respectively) using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Cells were harvested and lysed in cell lysis buffer (Beyotime, Haimen, China). Protein concentration was measured by Bradford method, and a total of 25 μg proteins was loaded and isolated using 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Then, proteins were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). After being blocked by 2.5% non-fat milk, the membranes were incubated with primary antibodies at 4°C overnight and secondary
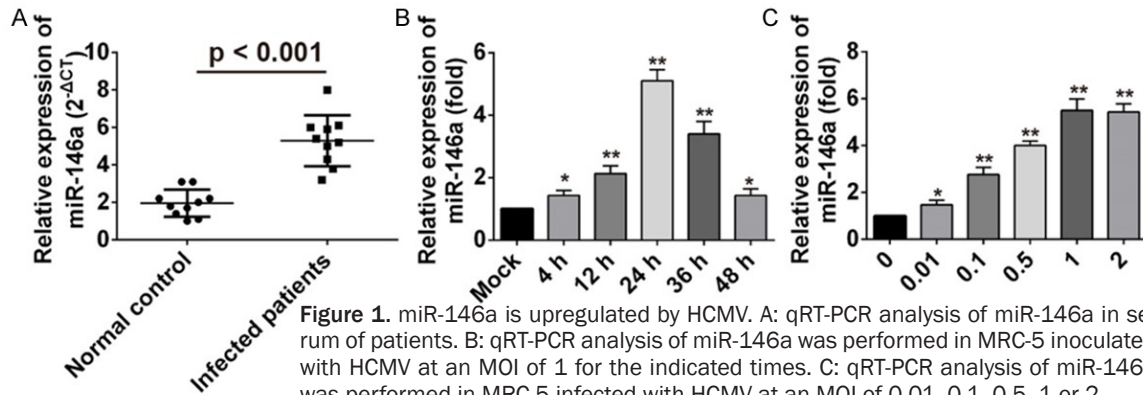
Figure 1. miR-146a is upregulated by HCMV. A: qRT-PCR analysis of miR-146a in serum of patients. B: qRT-PCR analysis of miR-146a was performed in MRC-5 inoculated with HCMV at an MOI of 1 for the indicated times. C: qRT-PCR analysis of miR-146a was performed in MRC-5 infected with HCMV at an MOI of 0.01, 0.1, 0.5, 1 or 2.

antibodies (Boster Corporation, Wuhan, China) for 1 h at room temperature. Finally, protein bands were visualized using an enhanced chemiluminescence kit (Amersham, Little Chalfont, UK). The following primary antibodies were used: anti-survivin (Abcam, Cambridge, UK); anti-Akt, anti-β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Quantification of IFN-α/β by ELISA

MRC-5 cells were seeded in a 6-well plate and transfected with the indicated miRNA (20 nM) for 24 h. The supernatants of treated cells were assayed for IFN-b protein release using the Human Interferon-α/β ELISA Kit (USCN Life Science, Wuhan, China) according to the manufacturer's instruction [22, 23]. Absorbance at 450 nm was read on microplate reader by using a Bio-Tek Synergy 2 microplate reader (Winooski, VT, USA).

Statistical analysis

Data were processed as mean ± SD. The differences were analyzed by the Student's t test or one-way analysis of variance and Student's t test. A *p* value of <0.05 was considered to be statistically significant.

Results

miR-146a is upregulated by HCMV

To investigate whether miR-146a can be regulated by HCMV, we detect the expression of miR-146a in serum of patients infected with HCMV by qRT-PCR. Our results showed that the expression of miR-146a in infected patients was significantly higher than that in normal control (**Figure 1A**). Then, a time-course assay was

performed to assess the expression of miR-146a in MRC-5 cells infected with HCMV. Our results showed that the level of miR-146a expression in MRC-5 was significantly increased in 24 h cells infected with HCMV, and then decreased (**Figure 1B**). It is suggested that miR-146a may be related to the early immune response. In order to investigate the relationship between miR-146a expression level and HCMV infection, we detected the expression level of miR-146a under different MOI of HCMV infection. We found that the expression level of miR-146a increased with the increase of HCMV concentration and reached the peak at MOI=1 (**Figure 1C**). These results indicate that HCMV can promote the production of miR-146a.

miR-146a enhances HCMV replication

First, we verified the over expression and interference efficiency of microRNA-146a, and found that over expression and interference efficiency of microRNA-146a in MRC-5 cells was very high (**Figure 2A**). To test whether miR-146a can affect HCMV replication, HCMV infection assays were performed in MRC-5 cells transfected with miR-146a mimics or miR-146a inhibitor. The culture supernatants were collected for quantification of virus titer. The amount of infectious virus production reduced significantly when miR-146a inhibitor transfected (**Figure 2B**). The amount of infectious virus production increased significantly when miR-146a mimics were transfected in MRC-5cells (**Figure 2C**). At the same time, we examined the viral protein gB and pp65 by Western blot analysis. Our results showed that interference miR-146a significantly inhibited HCMV viral protein gB and pp65 expression. Overexpression of miR-146a significantly promoted HCMV viral
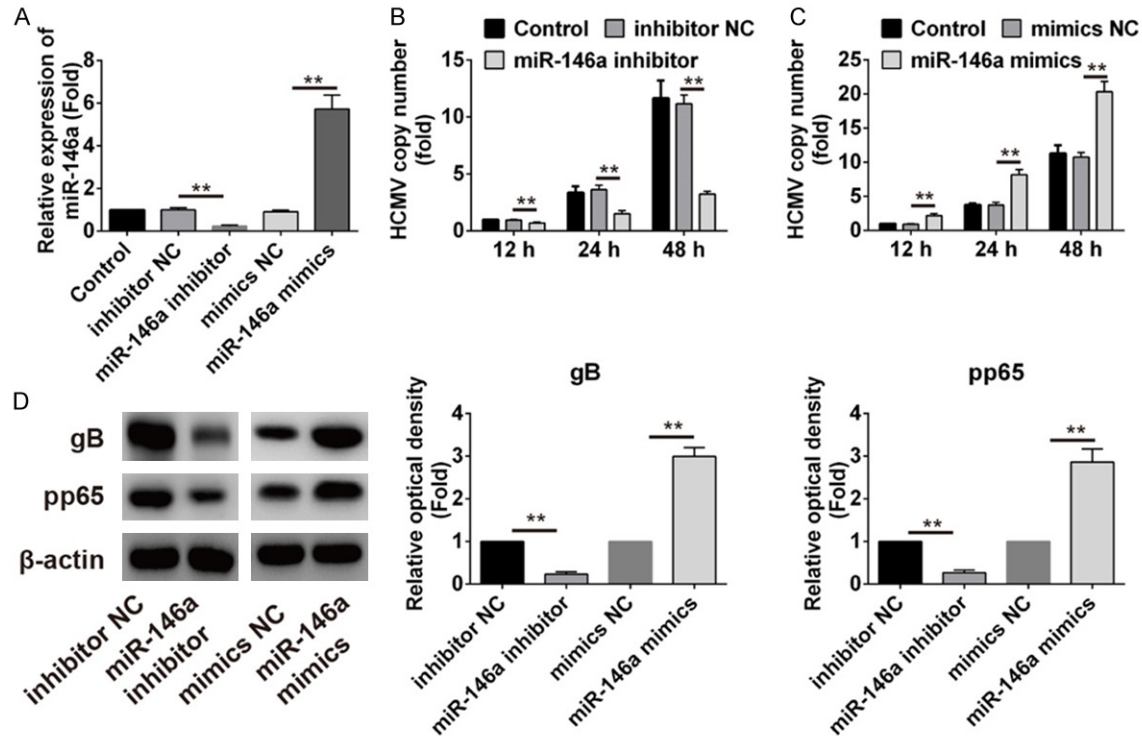
Figure 2. miR-146a enhances HCMV replication.MRC-5 were transfected with miR-146a mimics, NCs, miR-146a inhibitor, or negative control inhibitor at a final concentration of 60 nM for 24 h, followed by infection with HCMV. A: qRT-PCR analysis of miR-146a expression. B: MRC-5 were transfected with miR-146a inhibitor, control or inhibitor NC at a final concentration of 60 nM for 24 h and then infected with HCMV for 12, 24, or 48 h. Culture supernatants were collected to analyze virus titer. The results showed that interference miR-146a significantly inhibited HCMV replication. C: MRC-5 were transfected with miR-146a mimics, control or mimics NC at a final concentration of 60 nM for 24 h and then infected with HCMV for 12, 24, or 48 h. Culture supernatants were collected to analyze virus titer. The results showed that overexpression of miR-146a significantly promoted HCMV replication. D: Cells were then harvested for HCMV protein gB or pp65 detection using Western blot analysis.

protein gB and pp65 expression (**Figure 2D**). These results indicate that miR-146a can enhance HCMV replication.

STAT1 is a target of miR-146a

In order to understand the expression of miR-146a can enhance HCMV replication, we used bioinformatics methods to find potential target genes of it. We find that STAT1 is a putative target gene of miR-146a, which is regulate the immune system, cell differentiation, tumor suppression, cell growth inhibition and cell apoptosis. To confirm whether that STAT1 is the target gene of miR-146a, we investigated the miR-146a binding site in the STAT1 mRNA 3'-UTR. We construct a luciferase reporter plasmid containing wild type or mutant 3'-UTR STAT1 (**Figure 3A**). Then, the wild type or mutant plasmid with miR-146a inhibitor, miR-146a mimics, mimics NC and inhibitor NC transfected into

MRC-5 cells. The results showed that the overexpression of miR-146a was significantly reduced the luciferase activity of the wild type 3'-UTR STAT1, but there was no significant change in the mutant. In contrast, the inhibition of miR-146a significantly increased the luciferase activity of wild type 3'-UTR STAT1, while no significant changes in the mutant (**Figure 3B**). We detected the regulatory effect of miR-146a on STAT1, and the results showed that over expression of miR-146a inhibited the expression of STAT1, while inhibiting the expression of miR-146a to promote survivin expression (**Figure 3C**). These results indicate that STAT1 is a target of miR-146a.

Effect of MiR-146a on the immune response of type I interferon

In order to understand the effect of miR-146a on the immune response of type I interferon,

A

miR-146a   3' uuggguaccuuAAGUCAAGAGu 5'
                   | | | | | | | | | | |
STAT1 wt   5' acaauuauuuuUACAGUUCUCc 3'
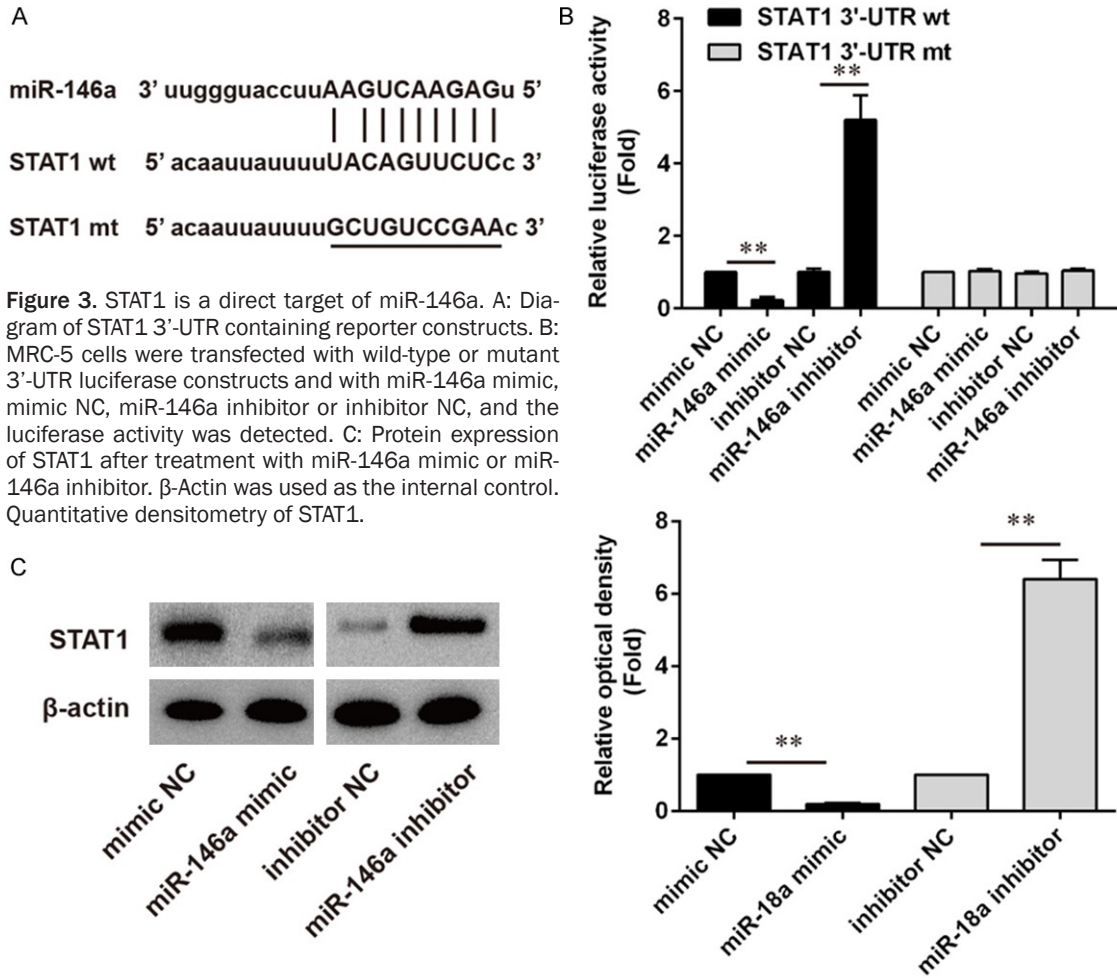
STAT1 mt   5' acaauuauuuuGCUGUCCGAAc 3'

Figure 3. STAT1 is a direct target of miR-146a. A: Diagram of STAT1 3'-UTR containing reporter constructs. B: MRC-5 cells were transfected with wild-type or mutant 3'-UTR luciferase constructs and with miR-146a mimic, mimic NC, miR-146a inhibitor or inhibitor NC, and the luciferase activity was detected. C: Protein expression of STAT1 after treatment with miR-146a mimic or miR-146a inhibitor. β-Actin was used as the internal control. Quantitative densitometry of STAT1.

C

we detected the expression of IFNα/β by qRT-PCR after overexpression or interference miR-146a. Our results showed that interference with miR-146a significantly increased the mRNA level of IFNα/β (**Figure 4A**), whereas over expression of miR-146a significantly decreased the mRNA level of IFNα/β (**Figure 4B**). Simultaneously, we detected the expression of IFNα/β in MRC-5 cells by ELISA. The results show that interfering MiR-146a significantly increased the concentration of IFNα/β, and over expression of MiR-146a significantly reduced the concentration of IFNα/β (**Figure 4C**, **4D**). These data suggested that MiR-146a suppression of HCMV infection was closely associated with IFNα/β production.

Effect of miR-146a on the expression of interferon induced antiviral gene

The MRC-5 cells were transfected with 20 nM of miR-146a inhibitor or negative control inhibi-

tor (NC) and then infected with HCMV at MOI of 1. Then, we detected the IFN-inducible genes, including ISG15, ISG56, OAS1, and Mx1 in MRC-5 cells by qRT-PCR analysis. The results showed that interfering with miR-146a increased the expression of IFN-inducible genes (ISGs) (**Figure 5A**). In contrast, over expression of miR-146a significantly reduced the expression of interferon stimulated genes, including ISG15, ISG56, OAS1, and Mx1 (**Figure 5B**). These results indicated that miR-146a and IFN-inducible genes expression levels were negatively correlated.

Discussion

MiRNA plays an important role in human physiological and pathological processes, such as embryonic development, organ formation, and tumor development [16-18]. MiR-146a is one of the most studied miRNAs, which plays an

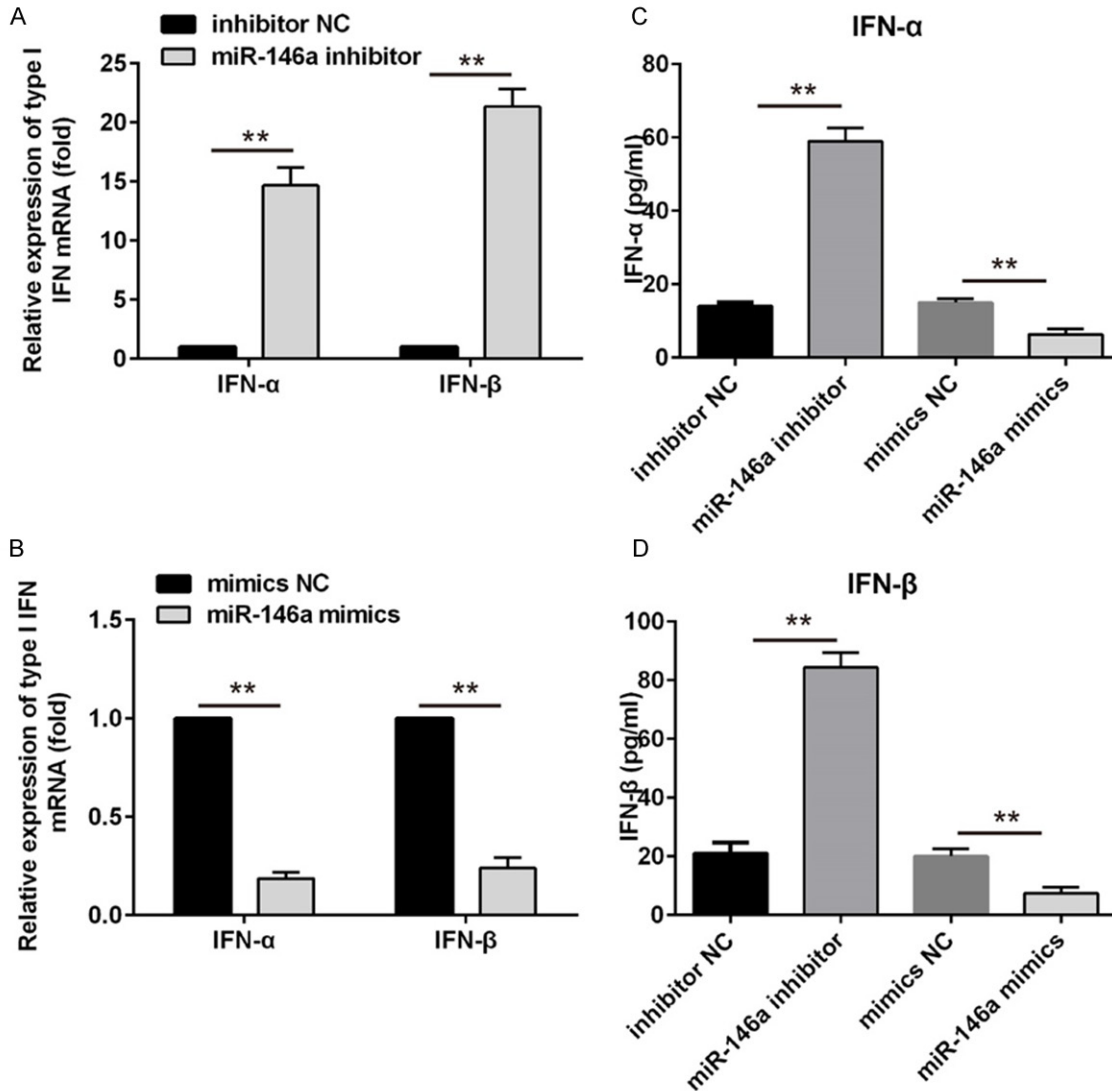**Figure 4.** Effect of MiR-146a on the immune response of type I interferon. A: qRT-PCR analysis of IFNα/β expression in HCMV-infected MRC-5 cells transfected with miR-146a inhibitor or inhibitor NC. B: The HCMV-infected MRC-5 cells were transfected with miR-146a mimcs or negative control mimcs (NC). Then, we detected the mRNA expression of IFNα/β by qRT-PCR analysis. C: The HCMV-infected MRC-5 cells were transfected with miR-146a mimic, mimic NC, miR-146a inhibitor or inhibitor NC. Then, we detected the concentration of IFNα by ELISA analysis. D: The HCMV-infected MRC-5 cells were transfected with miR-146a mimic, mimic NC, miR-146a inhibitor or inhibitor NC. Then, we detected the concentration of IFNβ by ELISA analysis.

important role in many physiological and pathological processes such as inflammation, autoimmune diseases, tumor and so on [19]. Some studies have indicated that miR-146a regulates host cell Type I IFN Responses in a variety of diseases and infections, but it has not been reported to have the same regulation of type I interferon immune response in HCMV infection. In this study, we found that MiR-146a can be upregulated by HCMV and enhanced HCMV replication.

STAT1 is a combination of a class containing and phosphorylation of tyrosine srchomology 2 (SH2) signal molecules, which can be in external signal promotes activation and from plasma cells directly into the nucleus caused by the transcription of multiple target genes. STAT1 promotes the transcription and expression of interferon stimulated genes [20-22]. Type I interferon is an important antiviral cytokine. Previous studies have indicated that activation of IFNα/β binds to the cell surface of
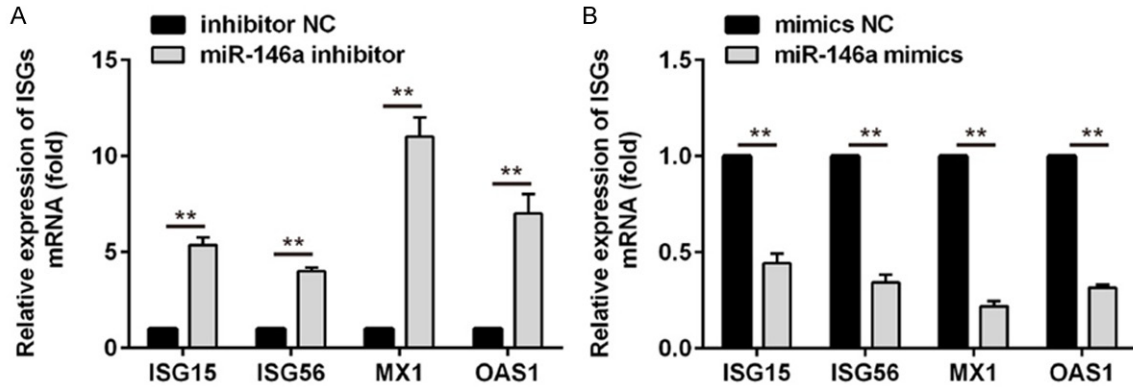
Figure 5. Effect of miR-146a on the expression of interferon induced antiviral gene. A: The HCMV-infected MRC-5 cells were transfected with, miR-146a inhibitor or inhibitor NC. Then, we detected the expression of ISGs by qRT-PCR analysis. B: The HCMV-infected MRC-5 cells were transfected with miR-146a mimic, mimic NC. Then, we detected the expression of ISGs by qRT-PCR analysis.

type I interferon receptor (IFNAR) through paracrine secretion or autocrine manner, which activation downstream of JAK2/STAT signaling pathway. Combination with the IFN α/β and IFNAR makes activation of protein tyrosine kinase Jak and phosphorylation of STAT1 and STAT2. Phosphorylated STAT1 two forms a transcription factor called AAF (IFN-alpha-activated factor); STAT1 and STAT2, IRF9 also forms another transcription factor ISGF3 (IFN-stimulated gene factor3). AAF and ISGF3 translocation into the nucleus and binds to contain DNA sequences of gas (IFN-gamma-activated sequence) in the and ISRE IFN-stimulated response element gene start codon, causing a large class of interferon inducible gene expression, including a number of antiviral gene [9, 23-27]. In this study, we found that STAT1 is the target gene of miR-146a, and miR-146a down regulated its expression. This indicates that miR-146a is involved in the immune response of I type interferon through its target gene STAT1.

Many viruses are very sensitive to type I interferon which play a role at any stage of viral replication [28]. Type I interferon antiviral function is induced by interferon protein factor to achieve, the more research including PKR, Adar, OAS (2', 5' 2 oligoadenylate synthetase), RNASEL and MX. OAS is a class of double stranded RNA dependent synthetase, which has been found three species in human cells [29]. After OAS is activated to produce 2-5A (5'-triphosphorylated, 2', 5'-phosphodiester-linkedoligoadenylates). 2-5A is the substrate of endogenous RNaseL, the activation of RNaseL induced degradation of viral single stranded

RNA [30]. Studies have indicated that miR-146a down regulated the expression of interferon stimulated genes by suppress the expression of interferon regulatory factor 5 (IRF5), signal transducer and activator of transcription 1 (STAT1) [28]. In the current study, we detected ISG15, ISG56, OAS1, and Mx1 protein in MRC-5 cells. The results show that miR-146a and IFN-inducible genes expression levels were negatively correlated. This indicated that miR-146a advanced HCMV replication by inhibition of type I IFN and the ISGs during HCMV infection.

In conclusion, we found that miR-146a is upregulated by HCMV. And miR-146a enhances HCMV replication. Then, we predicted and verified that the target gene of miR-146a is STAT1. Then, we demonstrate that miR-146a regulates I type immune responses and regulates the expression of interferon stimulated genes by target STAT1. Those data suggested that miR-146a could be used as a potential target for anti-HCMV infection.

Disclosure of conflict of interest

None.

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