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# Human cytomegalovirus miR-US33-5p inhibits viral DNA synthesis and viral replication by down-regulating expression of the host Syntaxin3

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ABSTRACT

**During infection with human cytomegalovirus (HCMV), overexpression of hcmv-miR-US33 can inhibit the lytic viral replication and down-regulate US29 mRNA. However, it remains unknown whether inhibition of viral replication by miR-US33 is mediated by down-regulation of expression of *US29* or another host gene. Here, we identified the host gene Syntaxin3 (*STX3*) to be a direct target of hcmv-miR-US33-5p using Hybrid-PCR and luciferase-reporter assays. It was further demonstrated that the levels of STX3 protein were down-regulated in hcmv-miR-US33-5p-overexpressing cells. Experiments with STX3-specific siRNA, or with an inhibitor of hcmv-miR-US33-5p confirmed that hcmv-miR-US33-5p-mediated inhibition of HCMV DNA synthesis and of viral replication are specifically mediated by down-regulation of STX3 expression.**


## 1. Introduction

Human cytomegalovirus (HCMV) is a prevalent human pathogen that can cause significant morbidity and mortality in newborns and immunocompromised patients [1]. HCMV has a 230 kb genome and encodes at least 26 mature microRNAs (miRNA) from 16 precursors [2]. Post-transcriptional regulation of miRNAs on their target mRNAs is thought to be achieved via the bindings between nucleotides located at 2–7 at the 5′ end of miRNAs (named as seed region) and sequences at the 3′ untranslated region (3′-UTR) of target mRNAs [3]. Since the first virus expressed miRNAs were reported [4], many viral miRNAs have been discovered. Emerging evidences suggest that miRNAs may be associated with immune evasion, latency of herpes virus subfamily and regulation of viral replication [5–7]. It has been reported that over-expression of hcmv-miR-US33 can inhibit the lytic viral replication and down-regulate US29 mRNA in the context of HCMV infection [8]. Whether inhibition of HCMV replication by hcmv-miR-US33 is mediated by down-regulation of US29 expression or of other host gene expressions remains elusive.

In the present study, it was found that over expression of hcmv-miR-US33-5p, which is one miRNA derived from the precursor of hcmv-miR-US33, obviously reduce HCMV DNA synthesis. Syntaxin3 (STX3), a soluble N-ethylmaleimide sensitive factor attachment protein receptor protein, which participates in the cellular membrane fusion [9], was identified as a direct target of hcmv-miR-US33-5p. Inhibition of STX3 by hcmv-miR-US33-5p or its specific small interference RNA can reduce the synthesis of HCMV DNA.

## 2. Materials and methods

### 2.1. Cell culture and virus preparation

Human embryonic kidney cells (HEK 293) were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin and streptomycin. MRC-5 cells were maintained in minimal essential medium (MEM) supplemented with 15% FBS, 100 units/ml penicillin and streptomycin. A low passage HCMV isolate, Han, was isolated from a urine sample of a 5-month-old infant hospitalized in Shengjing Hospital of China Medical University. Han strain was passaged in MRC-5 cells maintained in MEM supplemented with 2% FBS,

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100 units/ml penicillin and streptomycin. A bacterial artificial chromosome of HCMV Han strain (HCMV Han BAC) carrying the enhanced green fluorescent protein coding sequence has been constructed by cooperation with professor Minhua Luo in Wuhan Institute of Virology, Chinese Academy of Sciences and doctor Hua Zhu in UMDNJ-New Jersey Medical School, USA (In express).

### 2.2. RNA isolation

Total RNAs was isolated from HCMV infected or uninfected MRC-5 cells using Trizol reagent (Invitrogen), chloroform and isopropanol according to the protocol. The extracted RNA was dissolved in 100 μl RNase free $H_2O$ and treated by TURBO DNA-free™ Kit (Ambion). The qualities and purities of the RNA preparations were estimated by electrophoresis on 1% agarose gel and the quantities were detected by ND-1000 spectrophotometer (Nanodrop Technologies).

### 2.3. Quantitative real-time PCR

To evaluate hcmv-miR-US33-5p expression in infected cells, MRC-5 cells growing in 60 mm plates were inoculated with HCMV Han strain at a multiplicity of infection (MOI) of 3 infectious particles per cell. Total RNAs were extracted from uninfected cells and infected cells at 24, 48, 72, 96 and 120 hours post infection (hpi). Reverse transcription (RT) was performed using hcmv-miR-US33-5p specific primer or primer of small nucleolus RNA (snRNA) U6 (Applied Biosystems) with TaqMan miRNA reverse transcription kit (Applied Biosystems). Quantitative PCR was carried out with Universal PCR Master Mix Kit (Applied Biosystems) and specific TaqMan probes of hcmv-miR-US33-5p and snRNA U6 (Applied Biosystems) following the manufacture's protocol using Applied Biosystems 7300 Fast Real-Time PCR System. The relative expression level of hcmv-miR-US33-5p was normalized to that of snRNA U6 in corresponding samples by $2^{-\Delta\Delta CT}$ method [10].

### 2.4. Hybrid-PCR and sequencing

Reverse transcription was performed using 1 μg total RNAs from Han infected MRC-5 cells and 3′-Full RACE Core Set kit (TaKaRa). Based on the principles described before [11], the compatible hcmv-miR-US33-5p hybrid-primer was designed and synthesized as follow: 5′-CGCCCRCGGTCCGGGCRCRRT-3′ (R = A or G). Hybrid-PCR was carried out using nested primers (outer primer: 5′-TACCGTCGTTCCACTAGTGATTT-3′ and inner primer: 5′-CGCGGATCCT CCACTAGTGATTTCACTATAGG-3′), which are homologous to the introduced sequence of the cDNA, and the hcmv-miR-US33-5P hybrid-primer. The first round amplification of hybrid-PCR was hot started at 72 °C, followed by 30-cycle amplification at an annealing temperature of 37 °C. 1 μl product of the first round amplification was used as templates in the second round PCR, in which the annealing temperature was increased to 55 °C. All products were gel-purified and cloned into PCR2.1 vectors (Invitrogen). Individual colony was randomly selected and the inserts of the selected colonies were sequenced on an ABI 3730 automated sequencer. mRNA specific sequences located between the corresponding sequence of the hcmv-miR-33-5p hybrid-primer and the polyA structure were intercepted and used to blast on line (http://www.ncbi.nlm.nih.gov/blast).

### 2.5. Plasmid construction

The 3′-untranslated region (3′-UTR) of STX3 was amplified from mRNA derived cDNA by STX3 specific primers of 5′-GGACTAG TCTGGCAGTCTCCTTGATT-3′ (sense) and 5′-CCCAAGCTTGGCCTT-TAGTTATTTTGTCT-3′ (antisense). After purification and digestion, the fragment was cloned into the *Spe* I and *Hind* III sites of pMIR-REPORT firefly luciferase (FFL) vector (Promega), named as pMIR-STX3-UTR. A mutant vector, pMIR-STX3-MUT, containing three point mutations of the putative binding site of hcmv-miR-US33-5p was generated from the pMIR-STX3-UTR using pyrobest DNA polymerase (TaKaRa) and a mutant primer of 5′-aggctacttat ggccggtcgcgcctccagcactcagacaga-3′ (Characters underlined present mutant nucleotides) according to the protocol of Site-directed Gene Mutagenesis Kit (Beyotime). Inserts in all the constructs were sequenced on an ABI 3730 automated sequencer.

### 2.6. Dual-luciferase reporter assays

To assess binding ability of hcmv-miR-US33-5p to STX3 3′-UTR, HEK293 cells were cultured in 24-well plates. The cells were co-transfected with 100 nM miRNA negative control (RiboBio), which has the lowest homology with any known miRNAs, or hcmv-miR-US33-5p mimics (RiboBio) together with 100 ng pRL-TK-Renilla-luciferase plasmid (Promega), and respectively with 200 ng pMIR-REPORT vector, pMIR-STX3-UTR or pMIR-STX3-MUT using Lipofectamine 2000 (Invitrogen) in triplicate wells.

Luciferase activities were measured using Dual Luciferase Reporter Assay System (Promega) at 48 hour post transfection according to the manufacturer's instructions. The transfection efficiencies were normalized by the renilla luciferase activities in corresponding wells.

### 2.7. Western blot

To analyze whether STX3 protein expression is affected by hcmv-miR-US33-5p during HCMV infection, MRC-5 cells cultured in 60 mm tissue culture plates were transfected respectively with miRNA negative control, hcmv-miR-US33-5p mimics and inhibitor for hcmv-miR-US33-5p (RiboBio). Six hours later, the cells were inoculated with HCMV Han strain at MOI of 0.5 PFU per cell. At 48 hpi, the infected cells were collected and lysed using mammalian protein extraction reagent (M-PER, Thermo). Proteins were separated on 12% SDS–PAGE gels and transferred onto polyvinylidene fluoride (PVDF) membranes. The protein levels of STX3 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were detected using specific antibodies of rabbit monoclonal anti-STX3 (Abcam) and mouse monoclonal anti-GAPDH (Santa Cruz), and horseradish peroxidase (HRP)-conjugated secondary antibodies (Zhong Shan), respectively. The blots were visualized via an electrochemiluminescence (ECL) detection system and were exposed using ChemiDocTM XRS+ (Bio RAD). Meanwhile, expressions of hcmv-miR-US33-5p in the MRC-5 cells were detected by quantitative real-time PCR as described above.

### 2.8. Viral DNA quantification

MRC-5 cells were transfected respectively with miRNA negative control, hcmv-miR-US33-5p mimics, inhibitor for hcmv-miR-US33-5p and small interference RNA (siRNA) for STX3 (Ambion). Six hours later, the cells were inoculated with HCMV Han strain at MOI of 0.5 PFU per cell and harvested at 48 hpi using phosphate buffered saline (PBS). DNA was extracted from the infected cells using General Allgen Kit (Cwbio). The DNA copies of HCMV and a cellular housekeeping gene β-Actin were quantified with a pair of HCMV UL83 gene specific primers (sense: 5′-GGGACACAACACCG-TAAAGC-3′ and antisense: 5′-GTCAGCGTTCGTGTTTCCCA-3′) [12] and a pair of β-Actin specific primers (sense: 5′-CGGAACCGCT-CATTGCC-3′ and antisense: 5′-ACCCACACTGTGCCCATCTA-3′), respectively, using Power SYBR® Green PCR Master Mix (Applied Biosystems). The relative viral DNA level was normalized to that of β-Actin in the corresponding sample by $2^{-\Delta\Delta CT}$ method.