Original Research

Hsa-miR-27b is up-regulated in cytomegalovirus-infected human glioma cells, targets engrailed-2 and inhibits its expression

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Impact statement

Our study is the first to demonstrate that the HCMV infection could alter the expression of cellular microRNAs of the host glioma cells, which may develop an understanding of the pathogenesis of the HCMV infection in the microRNA level. Recently, HCMV infection and engrailed-2 have been reported to be related to the autism spectrum disorder (ASD). In this study, we confirmed that engrailed-2 is the target of hsa-miR-27b. As far as we know, our findings of the hsa-miR-27b up-regulation in the HCMV-infected glioma cells, targeting engrailed-2 and inhibiting its expression have never been reported or documented. Our data indicate that miR-27b may be related to the development of neurological disorders with the HCMV infection. The newly identified miR-27b/EN2 signal pathway may provide new insights into the glioma pathogenesis and a novel target for glioma therapy.

Abstract

Human cytomegalovirus (HCMV) dormant infection can alter the expression of the hosts' microRNAs (miRNAs) and impact on the regulation of target genes. To investigate the differentially expressed miRNAs induced by HCMV in human glioma U251 cells, a comprehensive miRNA screen was performed. As a result, 19 up-regulated and 14 down-regulated miRNAs were determined. Of these, hsa-miR-27b (miR-27b) attracted our attention. MiR-27b levels in U251 cells increased 7.70-fold, 8.64-fold, and 4.78-fold, respectively, post 24 h, 48 h, and 72 h HCMV infection, compared to those in the mimic-infected cells, and this up-regulation was further confirmed by quantitative RT-PCR. The bioinformatic analyses show that miR-27b targets engrailed-2 (EN2) gene; however, the effect of miR-27b on EN2 is rarely encountered. In this study, we initially conducted dual luciferase assay to validate the target function of miR-27b on EN2. The results manifested that EN2 is a novel target of miR-27b, which could directly target the 3′ untranslated region (3′-UTR) of the gene. We further found that the miR-27b transfected glioma U251 cells exhibited longer cell bodies with more synapses and multiple-angle shapes; moreover, Western blot detection revealed that the EN2 protein levels in these cells were significantly low. In conclusion, our study originally reports the up-regulation of miR-27b in HCMV-infected glioma cells. Our study also provides the first experimental evidence that miR-27b could affect glioma cells' growth, target EN2 and inhibit its expression in glioma cells. Our data indicate that miR-27b may be related to the development of neurological disorders with HCMV infection. The newly identified miR-27b/EN2 signal pathway may provide new insights into the glioma pathogenesis and a novel target for glioma therapy.

Keywords: Hsa-miR-27b, engrailed-2, micro-RNA, HCMV infection, glioma, inhibition

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Introduction

Human cytomegalovirus (HCMV) is a major cause for intrauterine infections. Congenital HCMV infection can cause a variety of neurodevelopmental sequelae.[1] The pathogenesis through which CMV impairs the central nervous system (CNS) remains unknown. The virus has developed a variety of mechanisms to adapt the cellular environment, including genes and non-coding RNAs, to assist its proliferation and transmission.[2] It is becoming increasingly clear that microRNAs (miRNAs), as small non-coding RNAs containing ~22 nucleotides, are of vital importance in regulating the expression of target genes at the post-transcriptional stage. HCMV latent infection can alter the expression of the hosts' cellular miRNAs and affect the target genes' function.[3–5]

To investigate the pathogenic mechanism of HCMV-induced abnormality on the nervous system via miRNAs, we performed a comprehensive miRNA screening of human glioma U251 cells post HCMV infection. As a result, 19 up-regulated and 14 down-regulated miRNAs were determined. Of these, miR-27b attracted our attention because it was identified as a tumor depressor in some

cancers.[6,7] The bioinformatic analyses show that miR-27b targets engrailed-2 (EN2) gene; however, no previous study has been carried out to validate this correlation. EN2 falls into the homeobox (HOX) gene family, which encodes homeodomain-containing transcription factors that function in CNS and early embryonic development.[8] EN2 is essential in some cell lineages in the cerebellum, coordinating fissure development and accelerating cerebellum growth.[9] In healthy adults, the known expression site of EN2 is in the nucleus of Purkinje neurones within the nervous system.[10] EN2 is required in maintaining the 5-HT levels during perinatal and postnatal periods and stimulating the development of the dorsal raphe nucleus.[11] EN2 plays not only key roles in developing mesencephalic dopaminergic (mDA) neurons but also impacts on the adult mDA neurons' biological function.[12]

In this study, we have explored whether miR-27b directly aims at the 3′ untranslated region (3′-UTR) of EN2 gene and regulates its expression in human glioma U251 cells. By doing this, we hope to provide experimental basis to help elucidate the mechanism of miRNA regulation induced by HCMV infection in the development of neurological abnormalities and improve understanding of the glioma pathogenesis triggered by miR-27b/EN2.

Materials and methods

Cell cultures and treatments

Human glioma cell line U251 was supplied by the Shanghai Cell Resource Center of the Chinese Academy of Sciences. HCMV AD169 strain and human embryonic lung fibroblast (HELF) cells were preserved by our laboratory. Glioma U251 cells were proliferated in RPMI 1640 medium with 10% fetal bovine serum (FBS) (Hyclone, Utah, USA) and remained under sub-confluent state at 37°C under 5% CO_2. HCMV AD169 was propagated in HELF cells in DMEM medium (Hyclone, Utah, USA) and tested by plaque titration. Then, the U251 cells were exposed to HCMV at a multiplicity of infection (m.o.i.) of 5. Viral-infected cells were collected at 24 h, 48 h, and 72 h post-infection. A mimic infection was introduced into virus-free U251 cells as control.

MiRNA microarray and data analysis

The collected cells then underwent total RNA extraction and concentration. MiRNA was isolated from 50 to 100 μg of the total RNA using the miRNeasy Mini Kit (QIAGEN, Duesseldorf, Germany) in accordance with the manufacturer's protocol. The miRNA was labeled by the miRCURY™ Hy3™/Hy5™ Power labeling kit (Exiqon, Vedbaek, Denmark). The labeled miRNA was then hybridized with the miRCURY™ LNA array (v.16.0) (Exiqon, Vedbaek, Denmark). The array contains human miRNA probes available at the miRNA Registry of http://www.mirbase.org/accessed miRBase R16.0, Sept 2010. After that, the fluorescent intensities on each slide were scanned by GenePix 4000B (Molecular Devices, CA, USA) and computed with a built-in GenePix Pro 6.0 software. Median values of four spots for each miRNA were determined by conducting duplicate probes in each chip and running the hybridization twice for each sample on two separate days. The criterion used for differentially expressed miRNAs analysis was an absolute fold-change of at least greater than 1-fold, comparing with the control. Microarray chip analysis was implemented by KangChen Bio-tech (Shanghai, China).

Quantitative RT-PCR and target gene prediction

The miRNA microarray data revealed that miR-27b was significantly up-regulated in HCMV-infected U251 cells. To quantitatively confirm the high expression of miR-27b, real-time PCR was implemented in triplicate for each sample. Following total RNA extraction and reverse transcription, the All-in-One™ Human qPCR Kit (Gene Copoeia, MD, USA) was carried out to amplify the cDNA of miR-27b. The forward primer (5′-CAGTGGCTAA GTTCTGCAAA-3′) for miR-27b and the universal adaptor reverse primer were provided by GeneCopoeia. The qPCR was performed in 20 μl reaction volumes, detected by SYBR Green on the ABI 7900 analyzer (Applied Biosystems, CA, USA), according to the manufacturer's instructions. The assay was initiated at 95°C for 10 min, and then was passed through 40 cycles of 95°C for 10 s, 64.5°C for 20 s, and 72°C for 10 s, and finally extended at 72°C for 6 min. U6 was employed as an internal control. Data were evaluated by comparative threshold cycle (Ct) method and analyzed by $2^{-\Delta\Delta Ct}$.[13]

To predict putative gene targets of miR-27b, three commonly used databases were executed. These are miRanda (http://www.microrna.org/microrna/home.do/), PicTar (http://www.pictar.org/), and TargetScan (http://www.targetscan.org/). The gene identified by at least two of the databases was considered to be a potential target of miR-27b.

Construction of LV3-HmiR-27b

The LV-3 (pGLVH1/GFP + Puro) vector was supplied by GenePharma (Shanghai, China), which contains the H1 and CMV promoters, an anti-puromysin gene, a green fluorescent protein (GFP) reporter gene, and the *BamH*I/*EcoR*I cleavages. The hsa-miR-27b precursor was directly synthesized and inserted into the LV-3 vector by GenePharma to construct the recombinant LV3-HmiR-27b in *E. coli* $DH_5\alpha$. A scrambled sequence of 5′- TTCTCCG AACGTGTCACGT -3′, sharing no homology with the human gene, was adopted to establish the recombinant LV3-shNC as a negative control. To generate a mature miR-27b, the recombinant LV3-HmiR-27b was transfected into the HEK-293T cells. Briefly, first, 50,000 to 100,000 HEK-293T cells in 500 μl DMEM medium with 10% FBS per well were inoculated in a 24-well plate, exposed to 5% CO_2 at 37°C for 12–18 h until 70–80% of the cells were confluent at the time of transfection. Second, 4 μg of the plasmid LV3-HmiR-27b or LV3-shNC were isolated and mixed with 15 μl transfection reagent of RNAi-Mate (GenePharma, Shanghai, China). Following immediate vortex for 10 s and incubation for 15 min at ambient temperature, the mixture was added into the prepared HEK-293T cells for

transfection. After culturing for 24–48 h at 37°C in 5% CO_2, the cells were collected for further use.

Construction of Psi-EN2-3′UTR-S/AS

The 3′-UTR of EN2, which contains the potential binding sites of miR-27b (position 400–407, 1056–1062, and 1304–1309), was amplified by PCR. The templates used in the PCR were directly from genomic DNA of U251 cells, since the EN2 3′ region contains no introns and shares the same sequence as the 3′-UTR of its mRNA. The primers for 3′-UTR sense were F, 5′-CGC*CTCGAG*CTCCATTATATGA CAT-3′ and R, 5′-TAT*GCGGCCGC*TAGATCCTGGAGGAT T-3′; for antisense, F, 5′-TTA*GCGGCCGC*CTCCATTATATG ACAT-3′ and R, 5′-GCT*CTCGAG*TAGATCCTGGAGGAT T-3′. The italicized nucleotides were enzyme cleavages for *Xho*I or *Not*I. A 50-μl PCR sample was subjected to 94°C for 5 min, 35 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 2 min, and then extended at 72°C for 10 min. The PCR products were then ligated into the psiCHECK™-2 vector (Promega, Wisconsin, USA), containing *Xho*I/*Not*I cleavages, to construct the recombinant vector Psi-EN2-3′UTR-S or Psi-EN2-3′UTR-AS, followed by transformation into *E. coli* $DH_5\alpha$.

Dual luciferase assay

The endotoxin-free plasmid DNA of each above recombinant LV3-shNC, LV3-HmiR-27b, Psi-EN2-3′UTR-S, Psi-EN2-3′UTR-AS, or the blank psiCHECK-2 in *E. coli* $DH_5\alpha$ was isolated by the E.Z.N.A.® Endo-Free Plasmid Maxi Kits (Omega, Georgia, USA). For co-transfection, the plasmid DNA of Psi-EN2-3′UTR-S (or Psi-EN2-3′UTR-AS) was mixed with LV3-HmiR-27b (or LV3-shNC) at the ratio of 1 μg:3 μg and divided into four groups: Psi-CHECK-2 control, LV3-HmiR-27b + Psi-EN2-3′UTR-S, LV3-HmiR-27b + Psi-EN2-3′UTR-AS, and LV3-shNC + Psi-EN2-3′UTR-S. Co-transfection was conducted with polyethylenimine (PEI) (Sigma-Aldrich, MO, USA). In short, 1 μg of the mixed-DNA was diluted in 10 μl ddH_2O and then added into 10 μl, 0.1 μg/μl PEI solution. The mixture was promptly vortexed for 15 s and maintained at room temperature for 15 min to form the DNA–PEI complex. The complex was further subjected to transfecting the HEK-293T cells. The preparation of the HEK-293T cells was the same as we stated previously. After transfecting for 48 h, the luciferase detection was performed following a Dual-Luciferase Reporter Assay System (Promega, Wisconsin, USA). The luciferase activity values of firefly and renilla were measured on a GloMax® 20/20 Luminometer (Promega, Wisconsin, USA).

Morphological observation and Western blotting

To investigate the impact of hsa-miR-27b on EN2 expression, as well as the morphological alteration of the glioma cells, the plasmid LV3-HmiR-27b or LV3-shNC was transfected into U251 cells. Initially, 300,000 to 800,000 U251 cells were plated in 2 ml RPMI 1640 medium containing 10% FBS per well in a six-well plate, cultured for 12–18 h at 37°C in an incubator with 5% CO_2 until 70–80% of the cells were in the state of confluence. Next, 3 μg of the endotoxin-free LV3-HmiR-27b or LV3-shNC plasmid DNA were added into 4.5 μl of the Lipofectamine™ 2000 (Invitrogen, CA, USA), vortexed at once and incubated for 20 min at room temperature. The combined samples were then transfected into the prepared U251 cells. Following cultivation for 24–48 h, puromycin was added into the medium at a final concentration of 0.5 μg/ml to screen stably transfected cell line with puromycin resistance. After that, the positive cells were subcultured with puromycin for another 48 h for morphological observation and then harvested to detect the expression of EN2 protein by Western blotting. The primary antibody of anti-EN2 was purchased from GeneTex CA, USA and the 34 kDa EN2 proteins were determined by enhanced chemiluminescence (Bio-Rad, CA, USA) with reference to our previous study.[14] The EN2 levels in different groups were reported as relative intensity units normalized to β-actin.

Statistical analysis

The statistical software IBM SPSS 20.0 (IL, USA) was employed in this study. One-way analysis of variance (ANOVA) was performed in comparing the mean values of the studied groups. A two-tailed $P < 0.05$ was considered significant.

Results

Differentially expressed miRNAs and quantitative RT-PCR validation of hsa-miR-27b

The high-throughput miRNA microarray determined 19 up-regulated and 14 down-regulated miRNAs in human glioma U251 cells post 24 h, 48 h, and 72 h HCMV infection, compared to those in the mimic-infected cells, as shown in Table 1. Of these, hsa-miR-27b levels increased 7.70-fold, 8.64-fold, and 4.78-fold at the three differently infected stages, respectively. The over-expression of hsa-miR-27b in HCMV-infected U251 glioma cells was further validated by quantitative RT-PCR. The q-PCR results were reported as relative ratios in comparison with U6 in U251 cells. The mean value of the triplicate validations for each sample was obtained. Figure 1 illustrates the comparison of hsa-miR-27b relative expression between the HCMV-infected cells and the mimic group.

Prediction of hsa-miR-27b targeting EN2

All the three databases of miRanda, PicTar, and TargetScan predict that EN2 is the putative gene targeted by hsa-miR-27b. The seed sequence of EN2 and the potential binding sites to hsa-miR-27b are shown in Figure 2.

Results of dual luciferase experiment

As expected, the luciferase activity value in HEK-293T cells decreased significantly ($P < 0.05$) when co-transfected with LV3-HmiR-27b + Psi-EN2-3′UTR-S, compared to any of the other three groups, where cells were transfected with LV3-HmiR-27b + psi-EN2-3′UTR-AS, or LV3-shNC + Psi-EN2-3′UTR-S, or psi-CHECK-2 alone, whereas

Table 1 The differentially expressed cellular miRNAs and their fold-changes in HCMV-infected U251 cells, compared with the mimic-infected cells

	Fold-changes of each miRNA		
miRNA name	**24 h. p.i**	**48 h. p.i**	**72 h. p.i**
Up-regulated miRNAs			
hsa-miR-1275	17.31	34.30	30.74
hsa-miR-296-3p	14.40	4.19	5.30
hsa-miR-1246	10.78	4.34	8.20
hsa-miR-3174	9.24	5.48	2.06
hsa-miR-409-3p	8.87	23.41	20.50
hsa-miR-622	8.79	3.11	3.39
hsa-miR-27b	7.70	8.64	4.78
hsa-miR-379	7.61	6.07	2.99
hsa-miR-499-5p	6.63	14.15	19.26
hsa-miR-92a	6.58	10.57	3.72
hsa-miR-381	5.68	13.44	14.24
hsa-miR-25	5.67	5.78	2.49
hsa-miR-221	5.42	9.96	2.74
hsa-miR-152	5.22	7.38	5.16
hsa-miR-146a	4.77	5.81	3.49
hsa-miR-411	3.58	9.97	5.91
hsa-miR-154	3.22	2.10	5.87
hsa-miR-409-5p	2.69	7.11	8.85
hsa-miR-192	2.60	4.99	4.88
Down-regulated miRNAs			
hsa-miR-634	0.02	0.06	0.30
hsa-miR-122	0.04	0.02	0.43
hsa-miR-129-3p	0.06	0.26	0.50
hsa-miR-340	0.07	0.02	0.44
hsa-miR-18b	0.07	0.07	0.39
hsa-miR-769-5p	0.08	0.03	0.05
hsa-miR-133b	0.09	0.38	0.37
hsa-miR-200c	0.10	0.03	0.46
hsa-miR-767-5p	0.15	0.07	0.43
hsa-miR-574-3p	0.23	0.12	0.21
hsa-miR-4301	0.23	0.23	0.44
hsa-miR-4286	0.29	0.27	0.46
hsa-miR-196a	0.30	0.43	0.28
hsa-miR-944	0.36	0.08	0.33

p.i: post-infection.

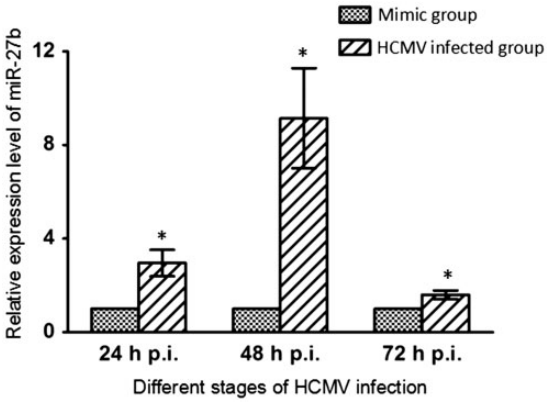

Figure 1 Quantitative RT-PCR validation of hsa-miR-27b expression in HCMV-infected U251 glioma cells, compared with the mimic group.
*refers to $P < 0.05$
p.i: post-infection

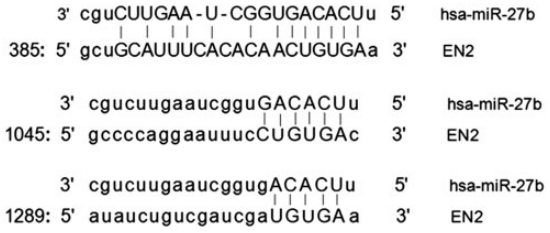

Figure 2 The seed sequence of EN2 and the predicted target positions to hsa-miR-27b
EN2: engrailed-2
Note: The binding sites are shown in capitals

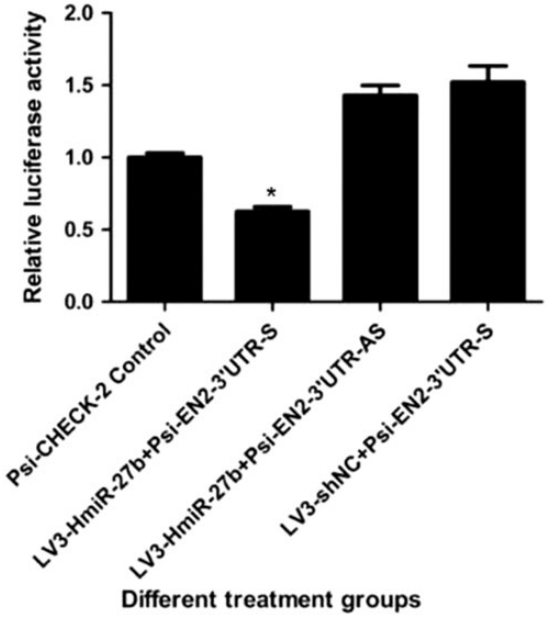

Figure 3 The relative values of luciferase activity in different groups. * refers to $P < 0.05$ when cells were transfected with LV3-HmiR-27b + Psi-EN2-3′UTR-S, compared to any of the other three groups, where cells were transfected with psi-CHECK-2 alone, or LV3-HmiR-27b + psi-EN2-3′UTR-AS, or LV3-shNC + Psi-EN2-3′UTR-S
3′-UTR: 3′ untranslated region

no significant differences were observed between them (Figure 3).

Inhibition of EN2 expression triggered by hsa-miR-27b

One-way ANOVA statistical results demonstrated that the EN2 protein level in the U251 cells transfected with LV3-HmiR-27b significantly decreased ($P < 0.05$) in comparison with those in the LV3-shNC transfected cells or the mock control. Figure 4 compares the Western blot data of relative gray ratios in groups.

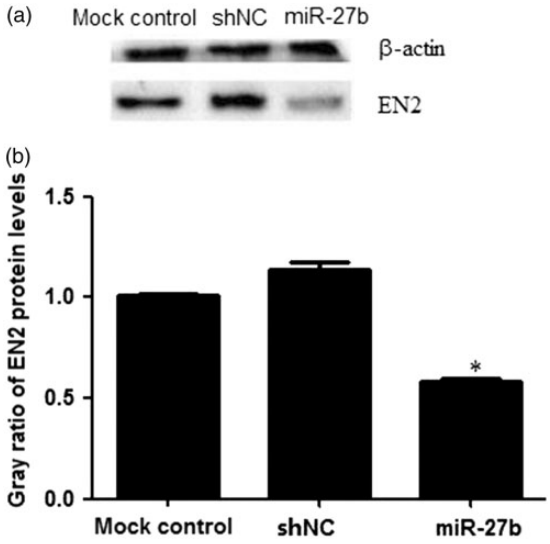

Figure 4 The EN2 protein expression levels in groups subjected to different treatments. (a) Western blot detection of EN2 in different groups. β-actin was used as an internal control of each sample and (b) comparison of the relative expression levels of EN2 in different groups. Mock control: The group of untreated U251 cells; shNC: The negative control group of U251 cells transfected with LV3-shNC; miR-27b: The treatment group of U251 cells transfected with LV3-HmiR-27b
EN2: engrailed-2
Note: *refers to $P < 0.05$ in comparison to either of the two controls

Morphological changes in glioma U251 cells

As shown in Figure 5(c), the LV3-HmiR-27b-transfected glioma U251 cells cultured for 48 h exhibited longer cell bodies with more synapses and multiple-angle shapes, compared to the non-transfection cells in Figure 5(a), while no obvious changes were observed in cells transfected with LV3-shNC in Figure 5(b).

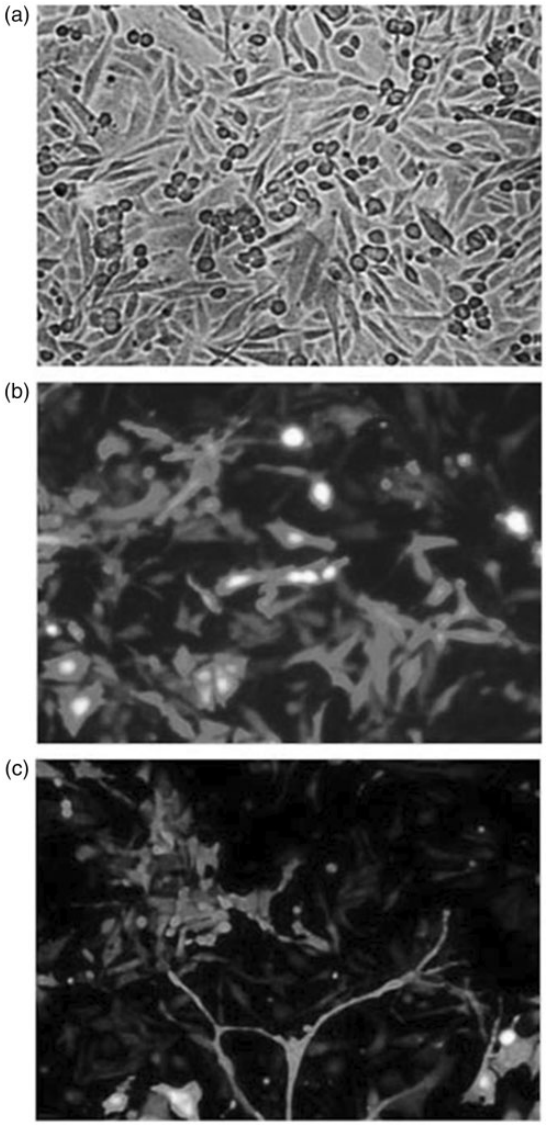

Figure 5 Morphological changes of glioma U251 cells 48 h post treatment, observed under inverted microscope (magnification 100 T). (a) Untreated U251 cells. (b) LV3-shNC-transfected U251 cells. (c) LV3-HmiR-27b-transfected U251 cells, representing the morphological changes

Discussion

There have been reported findings of HCMV infection resulting in changes in host-cellular miRNA expression, biogenesis, or activity.[3,4] HCMV-induced changes in cellular miRNAs and the relationship between miRNAs and their targeting genes should be taken with caution. In the present study, we identified that hsa-miR-27b in human glioma U251 cells was highly expressed during HCMV latency. MiR-27b is known as a tumor repressor in a couple of cancers, regulating miRNA levels of Sp1 or LIM kinase 1 in non-small cell lung cancer (NSCLC), or vitamin D receptor in melanoma.[15–17] In particular, miR-27b has emerged as a regulatory hub to block growth and tumor progression of neuroblastoma cells via directly inhibiting the 3′-UTR of peroxisome proliferator-activated receptor γ (PPARγ).[18] Bioinformatic analysis indicates that EN2 is a putative gene targeted by hsa-miR-27b; however, to date, there are no experimental data to support this. Our investigation initially confirmed that miR-27b targeted EN2 and inhibited its expression in glioma U251 cells.

About 5%–10% of the embryos congenitally infected with HCMV could develop neuro-developmental abnormalities at birth.[19] Accumulating evidence indicated that HCMV infection may cause autism spectrum disorder (ASD) after birth.[20,21] Interestingly, EN2 was identified as a possible ASD susceptibility gene. Genetic studies reported that the EN2 knockout mice displayed neurobehavioral or neuropathological impairment relevant to ASD.[22,23] In our study, miR-27b levels in glioma U251 cells were significantly elevated after HCMV infection; the expression of EN2 in the miR-27b-transfected U251 cells was significantly decreased. Our findings implicate that miR-27b may be related to the development of HCMV-infected neurological disorders by impacting on EN2. Therapeutically, attenuating miR-27b expression might benefit patients with HCMV infection.

EN2 has been proven to be over-expressed in several cancers and associated with tumor development. It is an ideal biomarker for prostate cancer because EN2 is not produced by benign hyperplastic prostate cells or normal

prostate tissue.[24] Pandha et al.[25] confirmed that the urinary EN2 levels in pre-surgical prostate tumor patients were closely related to the tumor volume and stage. Significantly expressed EN2 was found in bladder cancer cells and repression of EN2 inhibited the proliferation and invasion of the cancer.[26] Decreased EN2 expression was determined in the clear cell renal cell carcinoma (CCRCC) tissue or in CCRCC cell lines; and the EN2 levels were in negative relation to the tumor's histological grade.[27] A latest research identified EN2 as a new regulator functioning in accelerating chronic myeloid leukemia towards acute T-lympho blast.[28] Here, we provide experimental evidence that EN2 expression in human glioma 251 cells could be hindered by miR-27b. There is a considerable amount of literature describing the role of miR-27b. Many of the surveys focus on its impact on cancers as an important regulator as we discussed previously. Nevertheless, as far as we know, the effect of miR-27b on EN2 is rarely encountered. Our study revealed that EN2 is a novel target of miR-27b; miR-27b transfection in glioma cells could restrain EN2 expression in the cells and alternate the cell morphology as well. The goal of this preliminary work focuses on screening the differentially expressed miRNAs in HCMV-infected U251 cells, validating EN2 as a target of miR-27b and its inhibition on EN2. Continued work will be required to compare the EN2 expression in HCMV-infected U251 cells with those in the non-infected cells, transfect an miR-27b antagomir into U251 cells, super-infect them with HCMV, and then detect the expression alternation of EN2. Whether miR-27b affects proliferation and invasion of glioma cell via targeting EN2 requires further investigation as well.

In summary, we first identified a significant up-regulation of hsa-miR-27b in HCMV-infected glioma U251 cells. Moreover, we validated that EN2 is a target of hsa-miR-27b. Lastly, we demonstrated that miR-27b transfection inhibited EN2 expression in glioma cells and affected the growth of glioma cells. Our data imply that miR-27b may be related to the development of neurological disorders with HCMV infection. The newly identified miR-27b/EN2 signal pathway may provide new insights into the glioma pathogenesis and a novel target for glioma therapy.

Authors' contributions: LW, MY, SL conducted most of the laboratory procedure and prepared the manuscript. WL took part in the miRNA screening and data analysis. GD and GW helped to improve the experiment and guided the young researchers in details. LC participated in designing the study, data analysis and revision of the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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