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RESEARCH ARTICLE

Differential Expression Analysis of microRNA in the Central Nervous System of Mouse Infected with Rabies Virus

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ABSTRACT

Cellular microRNAs (miRNAs) play a key role in regulating the replication of rabies virus (RABV). To better understand the pathogenesis of RABV, we investigated the miRNAs expression in mice brain and spinal cord during RABV infection in the present study. The results showed the differential expression of 18 (12 up- and 6 down-regulation) and 5 (4 up- and 1 down-regulation) miRNAs with at least 3-fold change in the brain and spinal cord of GX01-infected mice, respectively. Eight miRNAs (7 up and 1 down-regulation) changed at least 3-fold in the brain of Flury-infected mice. Functional annotation of the differentially expressed miRNAs revealed that they are involved in immune response, biological process, apoptosis, and diseases (including cancer, long-term depression, vascular smooth muscle contraction, etc.). Three primary transcripts, miR-101c, miR-155-5p and miR-223-3p were selected for heterologous expression in BHK-21 cells after cloning into eukaryotic expression vector, pEGFP-C1, containing an eGFP marker. Our assays demonstrated that an increase in the N mRNA of RABV occurs in consistent with the up-regulated expression of the miRNAs, suggesting that these miRNAs are likely to be involved in promoting RABV transcription.

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INTRODUCTION

The rabies virus (RABV), a member of the Lyssavirus genus belonging to the family Rhabdoviridae, is a highly neurotropic virus that can infect all warm-blooded animals and cause a fatal infection of the central nervous systems (CNS) with 100% mortality rate (Schnell *et al*., 2010). The rabies epidemic is more prevalent in Asia and Africa. In China, rabies still remains as an important public health concern. In recent years, the number of humans who died of rabies in China was reported to be 2nd only to India (Knobel *et al*., 2005).

In the past two decades our research group has focused on investigating the epidemiology of dog rabies in the Guangxi province of Southern China (Liu *et al*., 2007; Tang *et al*., 2014). In animals, the duration of rabies infection from clinical onset is short with the infected animal dying within 1–3 days after the initial infection. Despite the short time period, the brains of the rabies infected animals have robust pathological lesions, which cause the death of the animal. Despite rabies being an old endemic problem with high incidence, the pathogenesis of RABV remains poorly understood.

Recent evidence has shown that microRNAs (miRNAs) play key regulatory roles in virus-host interactions (Cullen, 2010). miRNAs are endogenous, small, single-strand, non-coding RNA that contain 18-23 nucleotides and are crucial post-transcriptional regulators of gene expression (Xiao and Rajewsky 2009). To date, a number of 18-30 nt small RNAs have been identified as a crucial regulatory molecules in unicellular and multicellular organisms, and animal viruses (Ambros, 2004; Griffiths-Jones *et al*., 2008; Suryawanshi *et al*., 2015; Miskinyte *et al*., 2017; Yang *et al*., 2017).

To obtain insight into the pathogenesis of RABV and to understand whether miRNA is involved in pathogenesis we performed Solexa high-throughput sequencing (Illumina Genome Analyzer IIX) to investigate changes in

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miRNA expression in the brain and spinal cord of mice infected with a Guangxi street isolate, GX01 and a vaccine strain, Flury. Differential expression, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation and target genes of miRNAs were systematically analyzed.

MATERIALS AND METHODS

Viruses, cells and animals: A RABV street isolate, GX01, from a rabid dog in Guangxi province in southern China and a RABV vaccine strain, Flury, were used in this study. Viruses were grown in mouse neuroblastoma (N2a) cells maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Four-weeks-old BALB/c mice were challenged intracerebrally with a dose of 100 $TCID_{50}$ per 30 µL DMEM RABV GX01 and Flury strains. Mock-infected mice were injected intracerebrally with 30 µL DMEM.

Sample preparation and total RNA isolation: RABV challenged mice were euthanized after 7 days post-infection (dpi). Whole brains and spinal cords were harvested and stored in liquid nitrogen and sent to Beijing SinoGenoMax Co. for small RNA extraction and cloning. Briefly, total RNA samples were extracted from the full brains and spinal cords using miRNeasy Mini Kit (QIAGEN) and were used to construct the cDNA library; and sequencing was performed using a high-throughput Illumina Genome Analyzer IIX (Beijing SinoGenoMax Co. Led.).

Analysis of miRNA expression profiles: The brains and spinal cords of GX01-, Flury-, and mock-infected mice after 7 dpi were selected for miRNA microarray analysis. The expression level of miRNAs was estimated using a mouse miRNA expression profiling assay (Illumina, Inc., San Diego, CA, USA). Briefly, total RNA was extracted as detailed above and was constrcted small cDNA library was constructed and then high-throughout sequence analysis was performed by using IIIumina Genome Analyzer IIX.

Validation of the differentially expressed miRNAs by quantitative RT-PCR (qRT-PCR): qRT-PCR was performed to quantify miRNA expression levels in the brain and spinal cord samples from GX01-, Flury-, and mock-infected mice. First, reverse transcription was performed in 25 µL reactions containing 1 µL (1µg) total RNA, 1 µL Rtase mix, 1 µL (2.5U) polyA polymerase (GeneCopoeia Co., Ltd, Beijing), 5× reaction buffer and ddH_2O free from RNAse. The reaction mix was incubated for 60 min at 37°C at first and then 5 min at 85°C.

The qRT-PCR reaction was performed in 20 µL mixtures containing 10.0 µL 2× All-in-One qRT-PCR Mix, 2.0 µL All-in-One miRNA qPCR primer, 2.0 µL universal primer, 2.0 µL cDNA, and 4.0 µL ddH_2O. The qPCR reaction conditions were the following: initial denaturation at 95°C for 10 min, then 40 cycles of 95°C for 10 s and 60°C for 20 s followed by a melting curve analysis from 65 to 90°C with 0.5°C increase every 20 s.

KEGG pathway analysis of target genes: To understand biological functions of the target genes of differentially expressed miRNAs, we performed the KEGG functional annotation using DAVID Bioinformatics Resources 6.7 Soft (http://david.abcc.ncifcrf.gov/). The P-value threshold was set at <0.05.

Table 1: Differential expression of miRNA during RABV infection

Category	Tissue	Total	Fold change (≥3.0)		Fold change (2.0≤3.0)		Fold change (≤2.0)
			Up	Down	Up	Down	Up or down
GX01 vs mock	Brain	446	12	6	63	71	294
	Spinal cord	499	4	1	26	43	425
Flury vs mock	Brain	446	7	1	21	59	358
GX01 vs Flury	Brain		5				

Table 2: Differential expression of miRNAs in RABV infected mouse brain and spinal cord (Fold change≥3.0)

Category	Organ	Regulation	miRNA	Fold change	Position on chromosome
GX01 vs Mock	Brain	Up	miR-155-5p	5.58	16:84714385-84714449 [+]
			miR-1839-3p	3.44	7:88674802-88674874 [+]
			miR-21-3p	3.54	11:86397569-86397660 [−]
			miR-21-5p	3.22	11:86397569-86397660 [−]
			miR-223-3p	4.65	X:93438156-93438265 [+]
			miR-223-5p	5.29	X:93438156-93438265 [+]
			miR-466a-3p	3.12	2:10429545-10429617 [+]
			miR-466b-3p	3.12	2: 10395846-10395927 [+]
			miR-466c-3p	3.12	2:10403161-10403244 [+]
			miR-466e-3p	3.12	2:10400715-10400798 [+]
			miR-466p-3p	3.12	2:10427633-10427721 [+]
			miR-541-5p	3.12	12:110980619-110980708 [+]
		Down	miR-145-3p	−3.01	18:61807479-61807548 [−]
			miR-150-3p	−3.37	7:52377127-52377191 [+]
			miR-3068-3p	−3.01	12:88778629-88778707 [−]
			miR-383-3p	−3.53	8:39315187-39315256 [−]
			miR-673-3p	−3.20	12:110810200-110810290 [+]
			miR-676-5p	−3.01	X:97576436-97576524 [+]
	Spinal cord	Up	miR-155-5p,	4.46	16:84714385-84714449 [+]
			miR-223-3p,	3.22	X:93438156-93438265 [+]
			miR-223-5p,	3.24	X:93438156-93438265 [+]
			miR-34a-3p	3.08	4:149442563-149442664 [+]
		Down	miR-193b-5p	−3.05	16:13449616-13449694 [+]
Flury vs Mock	Brain	Up	miR-142-3p	3.73	11:87570366-87570429 [+]
			miR-155-5p	5.84	16:84714385-84714449 [+]
			miR-21-3p	4.02	11:86397569-86397660 [−]
			miR-21-5p	3.33	11:86397569-86397660 [−]
			miR-223-3p	3.69	X:93438156-93438265 [+]
			miR-223-5p	3.59	X:93438156-93438265 [+]
			miR-547-3p	3.23	X:65241549-65241626 [−]
		Down	miR-511-3p	−3.28	2:14182630-14182708 [+]
GX01 vs Flury	Brain	Up	miR-150-3p	3.80	7:52377127-52377191 [+]
			miR-451	3.47	11:77886672-77886743 [+]
			miR-676-5p	3.43	X:97576436-97576524 [+]

Constructing recombinant miRNA vectors: Genomic DNA was extracted from mice brain or spinal cord samples using TIANamp Genomic DNA kit (TIANGEN BIOTECH BEIJING CO., LTD). The primers specific to miRNA were used to amplify the miRNA transcripts by PCR using the following conditions: one cycle of 95°C for 5 minutes; 33 cycles of 94°C for 30 seconds, 54.5°C for 30 seconds, 72°C for 40 seconds (miR-155 and miR-223); 33 cycles of 94°C for 30 seconds, 57°C for 30 seconds, 72°C for 40 seconds (miR-101c) and one cycle of 72°C for 10 minutes. These primary transcripts were cloned into a cloning vector, pMD-18T, to confirm their sequences. Then, the miRNA primary transcripts were subcloned into a eukaryotic expression vector, pEGFP-C1, containing an eGFP marker using restriction enzymes Xho I and BamH I and cloning was confirmed by both Xho I/BamH I digestion and sequencing.

Expression of recombinant miRNAs in BHK-21 cells: Monolayers of BHK-21 cells (80% confluent; 5×10^5 cells) were prepared and transfected with the recombinant miRNAs containing eukaryotic expression vectors (pEGFP-C1-miR). Fluorescence of the transfected BHK-21 cells was observed through an inverted fluorescence microscope to determine the expression of miRNAs and transfection Transfection efficiency of miRNAs was determined by qRT-PCR.

Effect of recombinant miRNAs expression on RABV transcription: BHK-21 cells were transfected with recombinant miRNAs expressing eukaryotic expression vectors (pEGFP-C1-miR) and control pEGFP-C1 according to Jiang (Jiang *et al*., 2003). After 12 hours, these BHK-21 cells were infected with RABV vaccine Flury strain. The expression level of RABV N mRNA was measured by qRT-PCR.

RESULTS

Analysis of miRNA expression profile: The pre-miRNA and mature miRNA reads were downloaded from the updated version of miRBase, version Release18 (website: http://www.mirbase.org). Then, we calculated the mapped read count of each miRNA in the different samples using miRDeep (Friedlander *et al*., 2008), which revealed the differential expression profile of miRNA during RABV infection (Table 1). Compared with the mock samples, a total of 446 miRNAs were differentially expressed in the brains of mice infected with GX01 isolate and Flury strain. Among these, 12 and 6 miRNA demonstrated ≥3.0 fold change (up- or down-regulated) in a comparison of GX01 vs mock infected brains, respectively (Table 2); 63 and 71 miRNA were up- or down-regulated with 2.0–3.0-fold change, respectively; and 294 were up- or down-regulated with ≤2.0-fold change. In a comparison of brains from Flury vs mock infected mice, 7 and 1 miRNA were up- or down-regulated with ≥3.0 fold change, respectively; 21 and 59 were up- or down-regulated with 2.0-3.0-fold change, respectively; and 358 were up- or down-regulated with ≤2.0-fold change. In the spinal cord of mice infected with GX01, there were 499 differentially expressed miRNA. Among these, only 4 and 1 miRNA were up- and down-regulated with fold-change ≥3.0; 26 and 43 miRNAs were up- and down-regulated with 2.0-3.0-fold change; and most of the miRNA (425/499) were differentially expressed with a fold-change ≤2.0. These data suggested that intracerebral infection of mice with RABV results in significantly more changes in miRNA expression levels in the brain compared to the spinal cord.

Differential expression analysis of miRNAs: Further, we analyzed differentially expressed miRNAs using the miRDeep (Friedlander *et al*., 2008) in the following comparisons: GX01 spinal cord vs mock infected, GX01 brain vs mock infected brain, Flury brain vs mock infected brain, GX01 brain vs Flury brain **(**Fig. 1A**)**. Fig. 1B shows the differential expression of miR-155-5p, miR-223-3p and miR-223-5p in GX01-infected brain and up-regulation of miR-34a-3p in GX01-infected spinal cord compared with the mock infected brain. Additional data on differentially expressed miRNA with a fold-change ≥3.0 (up- or down-regulated) are shown in Table 2.

Validation of differentially expressed miRNAs expression level by qRT-PCR: To confirm the expression level of differentially expressed miRNAs identified through next generation sequencing, we chose three miRNAs for qRT-PCR analysis. As shown in the Fig. 2, the expression levels of miR-155-5p and miR-223-3p by Solexa were 4.462- and 3.216-fold higher than mock infected spinal cord, respectively, and were 8.169- and 10.489-fold higher by qRT-PCR. Thus, both Solexa and qRT-PCR showed consistent up-regulation of miR-155-5p and miR-223-3p. Another miRNA, miR-34a-3p, was also up-regulated in both Solexa and qRT-PCR analyses in the spinal cord of mice infected intracerebrally with the GX01 isolate (data not shown).

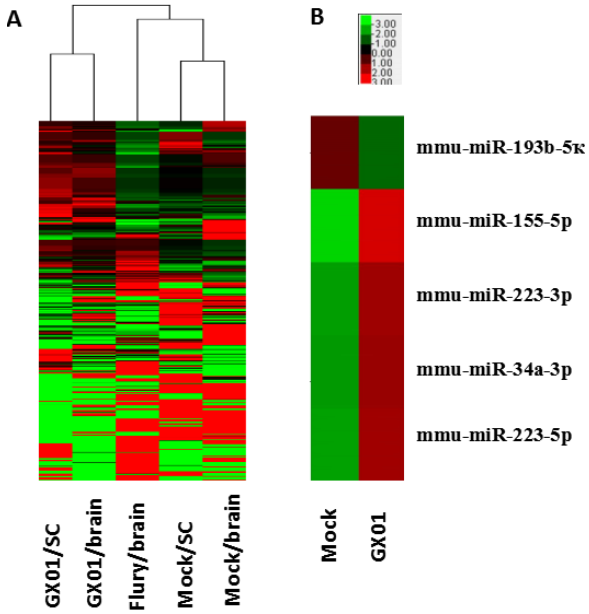

Fig. 1: Differential expression of miRNAs in mouse central nervous system infected with RABV A) Differential expression of miRNAs in the different mice samples. B) Five selected miRNA that were differentially expressed in the mouse brain infected with RABV GX01.

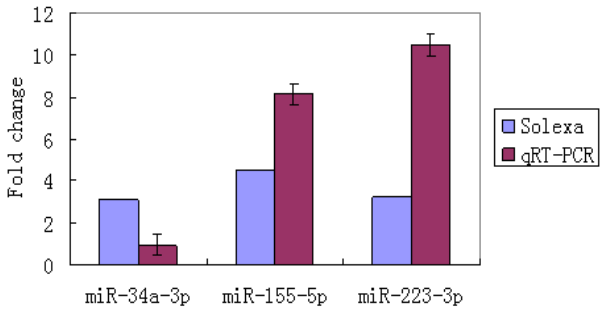

Fig. 2: Confirmation of differentially expressed miRNAs by qRT-PCR. Three miRNAs obtained from Solexa sequencing were confirmed to be differentially expressed by qRT-PCR.

KEGG pathway annotation of miRNA: To understand the biological functions of differentially expressed miRNAs and their target genes; and their regulation and importance in metabolic pathways, we performed the KEGG functional annotation of differentially expressed miRNAs using DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/) with the p-value threshold set at <0.05. Seven miRNAs derived from Flury-infected brains were involved in 23 pathways in biological process (mmu04810: regulation actin cytoskeleton), immune

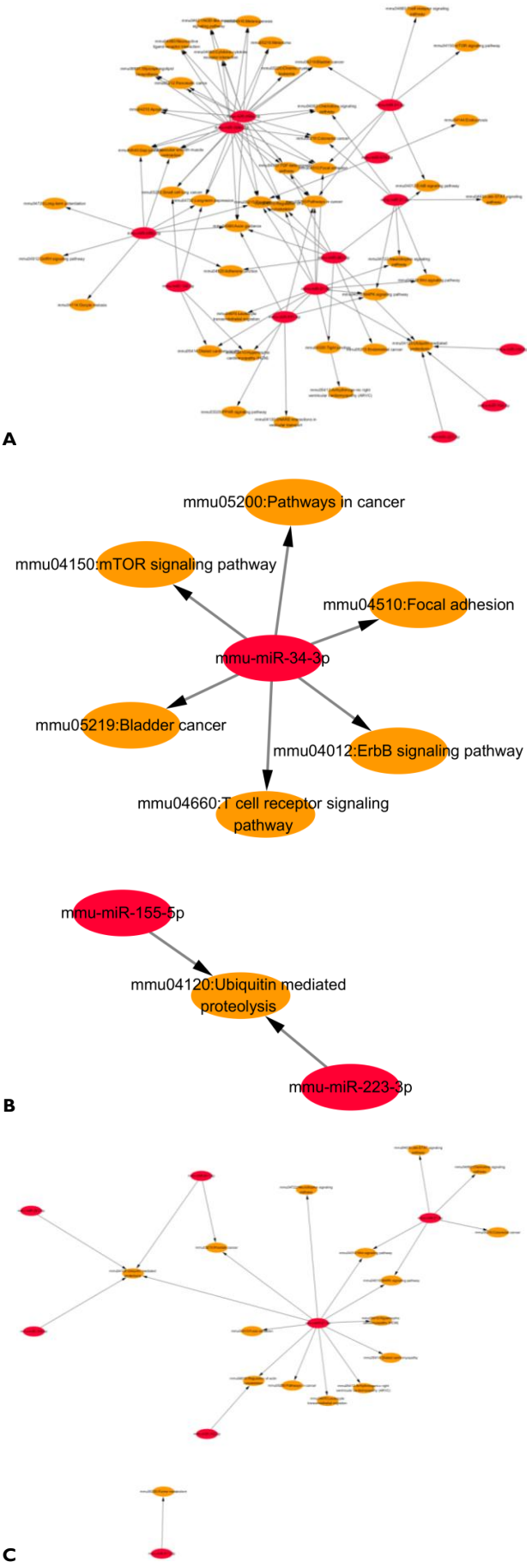

Fig. 3: KEGG pathway annotation of miRNA. A) GX01 brain vs Mock. B) GX01 spinal cord vs Mock. C) Flury brain vs Mock.

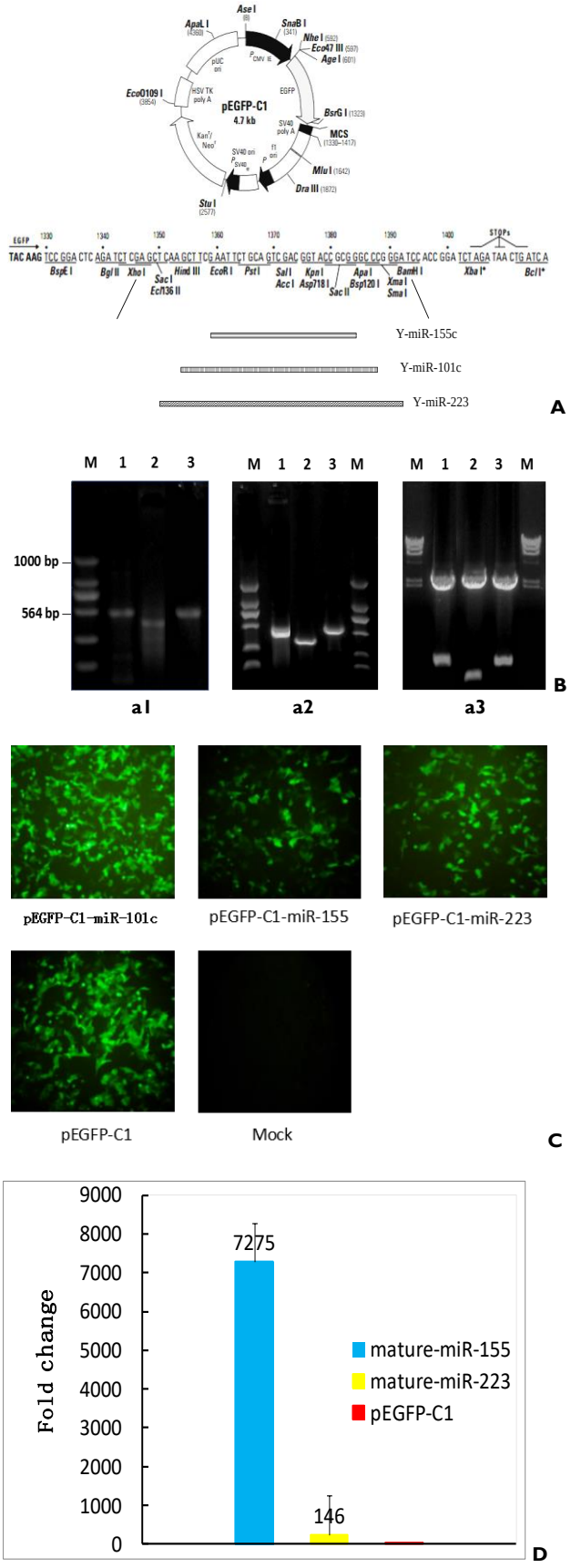

Fig. 4: Construction and expression of recombinant miRNAs in a eukaryotic expression system (BHK-21 cells). A) Construction and identification of recombinant miRNAs in an eukaryotic expression vector.B) Detection of eGFP tagged miRNAs via eukaryotic expression vector in BHK-21 cells.C) Detection of mature-miRNAs expression in BHK-21 cells.

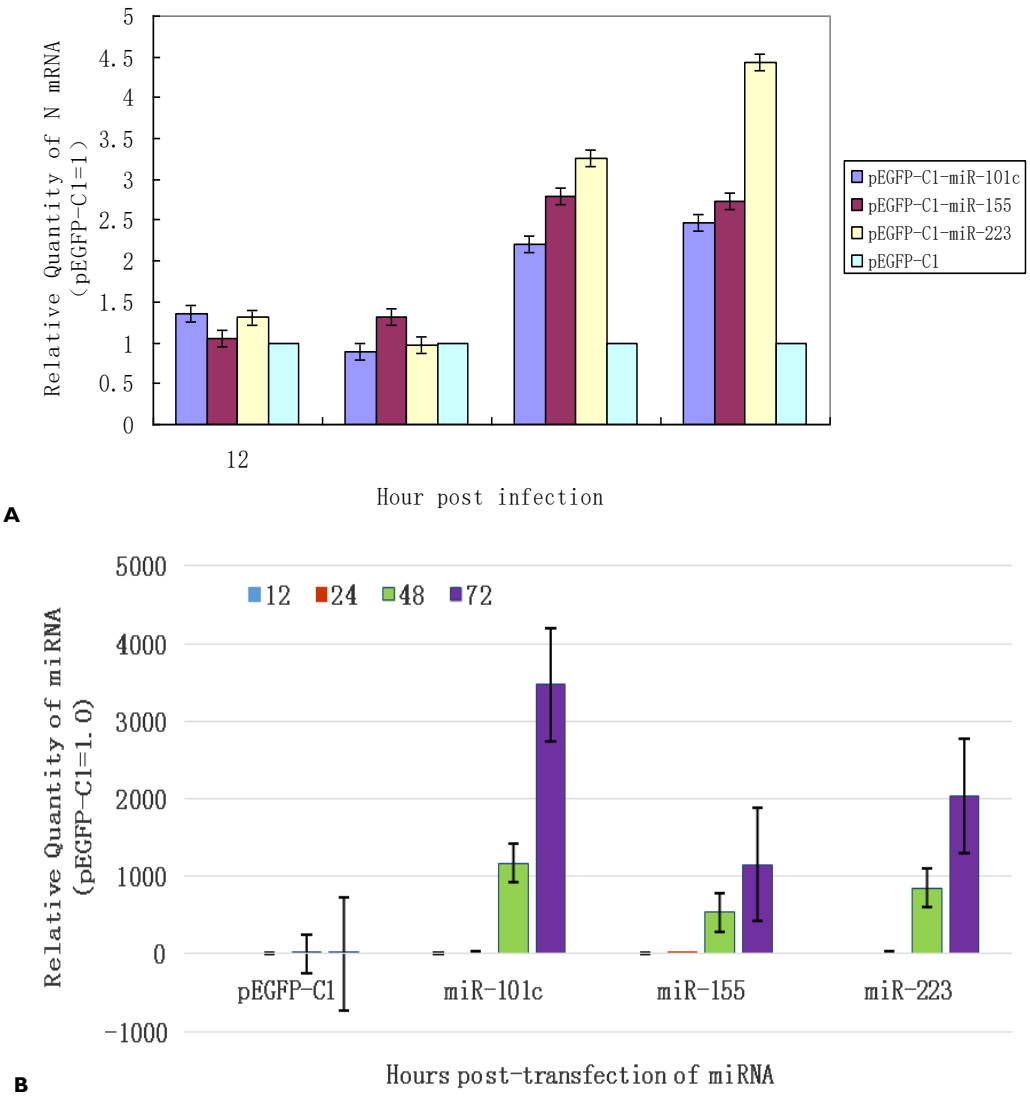

Fig. 5: Effect of miRNA expression levels on RABV replication *in vitro*. The BHK-21 cells were transfected with pEGFP-C1-miR-101c, pEGFP-C1-miR-155, pEGFP-C1-miR-223 or control pEGFP-C1 according to Lipofectamine™ 2000 Transfection Reagent. After 48 hours, these BHK-21 cells were infected with RABV Flury strain. A) Theexpression level of RABV N gene was measured by qRT-PCR. B) Expression level of mature miRNAs by qRT-PCR.

signaling transduction (mmu04010 MAPK signaling pathway), metabolism (mmu00230: purine metabolism), etc. (Fig. 3C). Eleven miRNAs from GX01-infected brain were involved in 90 pathways, including biological process (mmu04120: ubiquitin mediated proteolysis), pathogenesis (mmu04210: apoptosis); signaling transduction (mmu04722: neurotrophin signaling pathway, mmu04630: Jak-STAT signaling pathway etc.), diseases (mmu05215: prostate cancer, mmu05210: colorectal cancer, mmu04730: long-term depression, mmu04270: vascular smooth muscle contraction), etc. (Fig. 3A). Representative results are shown in Fig. 3B and are derived from the spinal cord of mice infected with GX01.

Construction of miRNAs eukaryotic expression vector: Three primary transcripts, miR-101c, miR-155-5p and miR-223-3p, were amplified from the genomic DNA of mice infected with RABV (Fig. 4A a1). The amplicons were then cloned into pMD-18T to obtain pMD-18T-miR-101c, pMD-18T-miR-155 and pMD-18T-miR-223 (Fig. 4A a2). After confirming the sequences of the cloned plasmids they were subcloned into a eukaryotic expression vector, pEGFP-C1, containing an eGFP marker. The resulting recombinant eukaryotic expression vectors were pEGFP-C1-miR-101c, pEGFP-C1-miR-155 and pEGFP-C1-miR-223 that were verified by Xho I/BamH I digestion (Fig. 3A, 4A) and sequencing.

Expression of recombinant miRNAs in BHK-21 cells: BHK-21 cells were transfected with the three recombinant eukaryotic expression vectors, pEGFP-C1-miR-101c, pEGFP-C1-miR-155 and pEGFP-C1-miR-223 and fluorescence was observed through an inverted fluorescence microscope. Transfection efficiency determined using qRT-PCR showed that BHK-21 cells transfected with pEGFP-C1-miR-101c, pEGFP-C1-miR-155 and pEGFP-C1-miR-223, as well as the control pEGFP-C1 vector expressed green fluorescence at 48 hours post-transfection (hpt). The fluorescence was distributed in the cytoplasm and nucleus but was not evident in control BHK-21 cells that were not transfected (Fig. 4B). These data demonstrated that the three miRNAs, miR-101c, miR-155-5p and miR-223-3p, could be expressed in BHK-21 cells.

Moreover, transfection efficiency of pEGFP-C1-miR-155 and pEGFP-C1-miR-223 determined by qRT-PCR showed high efficiency with miR-155-5p and miR-223-3p showing 7275 and 148 fold increase in eGFP expression, respectively, compared with the empty pEGFP-C1 vector (Fig. 4C).

Effect of recombinant miRNA expression on RABV transcription: BHK-21 cells were transfected with pEGFP-C1-miR-101c, pEGFP-C1-miR-155, pEGFP-C1-miR-223 and control pEGFP-C1 according to Lipofectamine™ 2000 Transfection Reagent (Thermo Fisher Scientific Inc., MA, USA). After 12 hours, the plasmids containing media were removed and the cells were infected with the Flury strain RABV. Then, the expression of RABV N gene was measured by qRT-PCR. As shown in Fig. 5, the expression level of N gene remained stable at 12 and 24 hpi. In contrast, the expression level increased at 48 and 72 hpi. Compared to the empty vector, relative level of RABV N mRNA at 48 hpi increased 2.2, 2.8 and 3.3 fold in cells transfected with pEGFP-C1-miR-101c, pEGFP-C1-miR-155, pEGFP-C1-miR-223, respectively. At 72 hpi, relative level of RABV N mRNA increased 2.5, 2.7 and 4.4 fold in cells transfected with pEGFP-C1-miR-101c, pEGFP-C1-miR-155, pEGFP-C1-miR-223, respectively (Fig. 5A). And the expression level of mature miRNAs of miR-101c, miR-155-5p and miR-223-3p by qRT-PCR were presented in Fig. 5B. Consistently, the expression level of three miRNAs, miR-101c, miR-155-5p and miR-223-3p, also did not increase at 12 and 24 hpi. However, the levels of the three miRNAs compared to pEGFP-C1 increased 1167, 532 and 851 fold at 48 hpi, and 3469, 1148 and 2037 fold at 72 hpi. These data indicated that the recombinant miRNA vectors can expressed eGFP with high efficiency in BHK-21 cells and that the increase in the levels of N mRNA was consistent with the up-regulation of the miRNAs, suggesting that an increase in N mRNA of the RABV could induce the expression of miRNA.

DISCUSSION

miRNA play a critical role in mammals and it has been reported that about 30% of mammalian genes are regulated by miRNA (Filipowicz *et al*., 2008). To date, thousands of miRNAs have been discovered from a variety of species. These miRNAs are complementary to the 3' non-coding region of mRNA and regulate transcription of hundreds of genes. Many miRNAs are associated with host immune response, such as development and differentiation of B and T cells, growth of neutrophils and mononuclear cells, production of inflammatory factors and antibodies (Lindsay 2008; Pedersen and David 2008; Tsitsiou and Lindsay 2009; Xiao and Rajewsky, 2009). Moreover, miRNAs also play key roles in the regulation of viral infection and virulence (Ghosh *et al*., 2009; Zhao *et al*., 2012a; 2012b). Applying artificial microRNA (amiRNA) for targeting a single target, the N gene of the RABV resulted in more than 90% reduction of the viral genome in Neuro2A cells 72 hpi; however, multiple amiRNAs aimed at single or multiple N mRNA targets yielded comparable inhibitory outcomes similar to the single amiRNA against perfectly matched targets (Israsena *et al*., 2009). RABV is a neurotrophic virus that can infect the neurons of all warm-blooded animals. Compared with the vaccine strains, the histopathological changes of the nervous system caused by the street RABV strain were typical. The clinical symptoms and histopathological changes are dependent on the RABV virulence. Therefore, we explored the changes in miRNA expression in mice brain infected with RABVs of different virulences.

In this study, we used a vaccine strain called Flury and a street strain, GX01 isolate (from a rabid dog) to challenge mice intracerebrally. The brain and spinal cord of the infected mice were used to create mRNA libraries that were sequenced using Solexa to identify differentially expressed miRNAs.

Zhao *et al*. (2012a) reported that in mice brains infected with an ERA vaccine strain 10 miRNAs were significantly up-regulated and 6 miRNAs were significantly down-regulated. However, mice brains infected with the Fujian street strain had 9 miRNAs that were significantly up-regulated and 1 miRNA that was significantly down-regulated (Zhao *et al*., 2012b). More importantly, these differentially expressed miRNAs (up- or down-regulation) differed completely between the two strains. Functional analysis showed that these differentially expressed miRNAs could be involved in many immune-related signaling pathways, such as Jak-STAT, MAPK, cytokine-cytokine receptor interactions and Fc gamma R-mediated phagocytosis (Zhao *et al*., 2012a; 2012b). In addition, a total of 53 miRNAs were found to be differentially expressed in RABV-infected samples compared with mock samples **(**Ghosh *et al*., 2009; Shi *et al*., 2014).

In this study, we performed an experiment to analyze the differential expression profile of miRNAs in mice brains and spinal cords infected with a vaccine strain called Flury and a street isolate termed GX01. Our results confirmed that the expressed profile of miRNAs differed greatly between the two strains. A large number of miRNAs were identified to be up- or down-regulated with a fold change ≥2.0 in brain and spinal cord post-infection by Flury or GX01 (Table 1). Of the miRNAs with fold change ≥3.0, 5 (miR-155-5p, miR-21-3p, miR-21-5p, miR-223-3p and miR-223-5p) were induced in the brain by both Flury and GX01; 3 (miR-155-5p, miR-223-3p and miR-223-5p) were simultaneously induced in brain and spinal cord by GX01 (Table 2). Our findings on miRNAs differed completely from Zhao and Shi's reports (Zhao *et al*., 2012a; 2012b; Shi *et al*., 2014), these miRNAs no one the same with Zhao and Shi's findings, suggesting that cause by different virus strain. The data obtained would be very helpful to further explore the relationship between the miRNAs and viral virulence.

In this study, the KEGG annotation of the target genes of differentially expressed miRNAs found that these miRNAs were not only involved in immune-regulated pathways, but also involved in many other biological processes, including development, metabolism, apoptosis, neuroactive processes, and diseases such as cancer. (Fig. 3). The target genes of differentially expressed miRNAs were determined using the miRNA target prediction database, miRDB. These analyses provide important

insights into the mechanisms of interaction between the RABV and the host.

Sreekumar found that miR-221 was down-regulated in human bronchial epithelial cells infected with respiratory syncytial virus (RSV). miR-221 was also shown to suppress the replication of RSV (Sreekumar *et al*., 2012). Neurotrophin and its receptor tropomyosin-related kinase A (TrkA) were inhibited following miR-221 expression during RSV infection. Another neurotrophin receptor, p75NTR, is also a receptor for RABV. The present study shows that target genes of modulated miRNAs were involved in the neurotrophin signaling pathway. NTRK2 is a common target of miR-21-3p and miR-21-5p (Table 1), suggesting that both miRNAs might regulate neurotrophin signaling pathway during RABV infection.

In our study three miRNAs, miRNA-155-5p and miR-223-3p with fold change ≥3.0 (by Solexa and by qRT-PCR), and miR-101c with fold change ≤2.0 (by Solexa only), were selected for heterologous expression in a eukaryotic expression vector, pEGFP-C1. Surprisingly, all three miRNAs increased the yield of RABV N mRNA, indicating that these miRNAs could promote transcription level of RABV.

This study has identified a few novel miRNAs specific for RABV. Our evidence suggests that the host miRNAs could play crucial role in regulating gene expression in response to RABV infection in the CNS. The miRNAs obtained from RABV -infected brain and spinal cord would be useful for further analyzing the interaction mechanism between host and RABV infection.

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Authors contribution: TRL conceived and designed the study. YP and LJX performed the experiments. XKW, XNL and JJL analyzed and interpreted the data. YP wrote the manuscript. All authors read and approved the final manuscript.

REFERENCES

Ambros V, 2004. The functions of animal microRNAs. Nature 431:350-5.

Cullen BR, 2010. Five questions about virus and microRNAs. PLoS Pathog 6:e1000787.

Filipowicz, Bhattacharyya SN and Sonenberg N, 2008. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 9:102-4.

Friedlander MR, Chem, Adamidi C, *et al*., 2008. Discovering microRNAs from deep sequencing data using miRDeep. Nature Biotechnol 26:407-15.

Ghosh Z, Mallick B and Chakrabarti J, 2009. Cellular versus viral micro-RNAs in host-virus interaction. Nucleic Acids Res 37:1035-48.

Griffiths-Jones S, Saini HK, van Dongen S, *et al*., 2008. miRBase: tools for microRNA genomics. Nucleic Acids Res 36: D154-58.

Israsena N, Supavonwong P, Ratanasetyuth N, *et al*., 2009. Inhibition of rabies virus replication by multiple artificial microRNAs. Antiviral Res 84:76-83.

Jiang GF, Zhuang ZX, Liu QZ, *et al*., 2003. Construction of eukaryotic expression vector of hMTH1 gene antisense RNA. Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi 21:57-60.

Knobel DL, Cleaveland S, Coleman PG, *et al*., 2005. Re-evaluating the burden of rabies in Africa and Asia. Bull World Health Organ 83:360-8.

Lindsay MA, 2008. microRNAs and the immune response. Trends Immunol 29:343-51.

Liu Q, Xiong Y, Luo T, *et al*., 2007. Molecular epidemiology of rabies in Guangxi Province, south of China. J Clin Virol 39:295-303.

Miskinyte G, Devaraju K, Grønning Hansen M, *et al*., 2017. Direct conversion of human fibroblasts to functional excitatory cortical neurons integrating into human neural networks. Stem Cell Res Ther 8:207.

Pedersen I and David M, 2008. MicroRNAs in the immune response. Cytokine 43: 391-4.

Schnell MJ, McGettigan JP, Wirblich C, *et al*., 2010. The cell biology of rabies virus: using stealth to reach the brain. Nat Rev Microbiol 8: 51-61.

Shi N, Zhang XY, Dong CY, *et al*., 2014. Alterations in microRNA expression profile in rabies virus-infected mouse neurons. Acta Virol 58:120-7.

Sreekumar O, Cheryl W and Giovanni P, 2012. MicroRNA-221 Modulates RSV Replication in Human Bronchial Epithelium by Targeting NGF Expression. PloS One 7:e30030.

Suryawanshi H, Sarangdhar MA, Vij M, *et al*.,2015. A simple alternative to stereotactic injection for brain specific knockdown of miRNA. J Vis Exp 26:e53307.

Tang H, Pan Y, Wei X, *et al*., 2014. Re-emergence of rabies in the Guangxi province of Southern China. PLoS Negl Trop Dis 8:e3114.

Tsitsiou E and Lindsay MA, 2009. microRNAs and the immune response. Curr Opin Pharmacol 9:514-20.

Xiao C and Rajewsky K, 2009. MicroRNA control in the immune system: basic principles. Cell 136:26-36.

Yang J, Zhang X, Chen X, *et al*., 2017. Exosome mediated delivery of miR-124 promotes neurogenesis after Ischemia. Mol Ther Nucleic Acids 7:278-87.

Zhao P, Zhang K, Feng H, *et al*., 2012. Infection with street strain rabies virus inducesmodulation of the microRNA profile of the mouse brain. Virol J 9:159.

Zhao P, Zhao L, Zhang T, *et al*., 2012. Changes in microRNA expression induced by rabies virus infection in mouse brains. Microb Pathog 52:47-54.