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ORIGINAL PAPER

Dynamic expression of miR-132, miR-212, and miR-146 in the brain of different hosts infected with *Angiostrongylus cantonensis*

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Abstract Increasing evidence shows that microRNAs (miRNAs) are a family of regulatory molecules involved in many physiological processes, including the inflammation in central nervous system (CNS) and neurological disorders. *Angiostrongylus cantonensis* (*A. cantonensis*) is the major cause of human infectious eosinophilic meningitis and can induce CNS injury. In the present study, we investigated the expression of miRNAs involved in neuronal functions such as miR-132-3p/212-3p, and miR-146a-5p, inflammation-related miRNA, in the modulation of inflammation of CNS of mice and rats induced by *A. cantonensis*. The functions of differentially expressed miRNAs were analyzed through bioinformatics methods, and the expression of chosen target genes were investigated by quantitative reverse transcription polymerase chain reaction. The results showed that miR-146a-5p upregulated in the brain of rats after 21 days; *A. cantonensis* infection and the expression of miR-132-3p and miR-146a-5p upregulated in the brain of mice model infected by *A. cantonensis*. The expression of the target genes of mmu-miR-146a-5p such as Irak1 and Traf6 downregulated in 14 days and 21 days after *A. cantonensis* infection. Our results supply more information about the involvement of the miRNAs in the regulation of inflammation of CNS induced by *A. cantonensis*.

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Introduction

MicroRNAs (miRNAs) are small, noncoding RNAs that play critical roles in the regulation of gene expression at the post-transcriptional level (Lim et al. 2005). There are abundant miRNAs in the nervous system, and a highly conserved pathway of miRNA biogenesis is closely linked to the transport and translatability of mRNAs in neurons (Kosik 2006). Research shows that miRNA-146a is an important modulator of the innate immune response and inflammatory signaling in specific immunological and brain cell types (Lukiw et al. 2011). And the changes in the expression of miR-146a have been implicated in both the development of multiple cancers and in the negative regulation of inflammation induced via the innate immune response (Williams et al. 2008). There is evidence to approve that miR-132 is an important molecule regulating embryonic stem cell differentiation into dopamine neurons by directly targeting Nurr1 gene expression (Yang et al. 2012). The former report also reveals that the miR-212/132 cluster contained the miRNAs most responsive to brain-derived neurotrophic factor treatment (Remenyi et al. 2010). These miRNAs may be involved in the pathogenesis of inflammation-related disease and may help promote the diagnosis and treatment for these diseases.

The rat lungworm, *Angiostrongylus cantonensis* (*A. cantonensis*), as the causative agent of eosinophilic meningitis (EM), can induce central nervous system (CNS) injury. Rats are the principal permissive host, and mice are nonpermissive host (Alicata 1991; Alto 2001; Lee et al. 2006). Research shows that larval development is retardant, and the CNS injuries are more

serious in nonpermissive host (OuYang et al. 2012). Humans are incidentally infected either by eating uncooked intermediate hosts or by consuming vegetables containing the living third-stage larvae (Tsai et al. 2012; Chen et al. 2011; Lv et al. 2009). Central nervous system manifestations included headache, fever, stiff neck, hyperesthesia, cranial nerve palsy, diplopia, and ataxia. Laboratory findings included peripheral eosinophilia and cerebrospinal fluid eosinophilia, elevated immunoglobulin E levels, and transient increases in white blood cell count and in serum levels of creatine kinase, transaminase, and lactate dehydrogenase (Alto 2001; Slom et al. 2002; Tsai et al. 2001). Infected nonpermissive host such as mice suffered more serious injuries and provoked more intense inflammatory response as compared to infected permissive host such as rats. The adjustment mechanism of the pathogenesis of angiostrongyliasis cantonensis is complicated and needs more investigation.

The objective of this present study was to explore the expression change of some brain enriched miRNAs during the inflammation of CNS of different hosts and their function in EM induced by *A. cantonensis* through analysis of some of their target genes. Some miRNAs such as miR-132-3p, miR-212-3p, and miR-146a-5p were included in this study.

Materials and methods

Experimental animals

Thirty-eight male Balb/c mice and 38 healthy male SD rats aged 6 weeks were purchased from the Center of Experimental Animals of Sun Yat-Sen University. All animals were kept in germ-free circumstance with free access to standard pellet diet and clean water. Animals were cared for in accordance with the guidelines developed by the China Council on Animal care, and all procedures were performed according to the regulations approved by the Animal Care and Use Committee of Guangdong Province, China. Two species were separately and randomly divided into these groups as follow: 0 day with no infection (5), 7 days infection (8), and control (3), 14 days infection (8), and control (3), 21 days infection (8), and control (3) (all these controls with no infection).

Parasite preparation and animal infection

The third-stage larvae of *A. cantonensis* were obtained from naturally infected giant African snails. The shell were crashed, and the tissues were homogenized and digested in artificial gastric juice (0.3 % pepsin, 0.7 % HCL, and 10 ml/g tissue) at 37 °C for 2 h. Then the third-stage larvae were collected and counted under a microscope after washed by the distilled water. Mice and rats were each orally infected with 30 and 50 *A. cantonensis* L3 larvae separately.

Histochemistry examination of animal model and tissue preparation for RNA isolation

The mice were sacrificed by cervical dislocation, and the rats were sacrificed under deep anesthesia by ether inhalation at 7, 14 and 21 days after infected by *A. cantonensis*; 0 day with no infection group was deal with the first day, and all other control groups were also included at the same day with infection groups. After decapitation, the cerebrum tissue was removed quickly using RNase-free instruments and frozen in liquid nitrogen and stored at −80 °C until use. And the specimens of the brain tissue of these infection group and control were fixed in 4 % paraformaldehyde for 2 days. After embedded in paraffin, they were serially sectioned and stained with hematoxylin eosin (HE) according to the conventional staining methods.

RNA isolation

Total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. They were stored at −80 °C until further use after total RNA was resuspended in DEPC-treated water. The quantity and integrity of the total RNA was assessed with a NanoDrop 2000 ultramicrospectrophotometer (Thermo Scientific, USA).

Quantitative reverse transcription polymerase chain reaction

First-strand cDNA synthesis of the miRNA was prepared using First-Strand cDNA Synthesis Kit (Invitrogen) following the manufacturer's protocols. Then the RT-PCR for detecting miRNAs expression was performed with SYBR Green Supermix (Bio-Rad) according to the protocol as follows: (1) 95 °C for 30 s; (2) 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 70° C for 10 s; and (3) all reactions were run in triplicate (Chen et al. 2005). All these primers of miRNAs were bought from Ruibo Company, and the U6 gene was used as an endogenous control. The RT-PCR for chosen mRNA targets was performed as follows: (1) 95 °C for 2 min; (2) 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °Cfor 15 s; (3) melting curve analysis; (4) 50 °C for 30s; and (5) all reactions were run in triplicate. The primers of mRNA targets (Traf6 and Paip2) used in quantitative reverse transcription polymerase chain reaction (qRT-PCR) were chosen from PrimerBank (Wang et al. 2012). These primers were synthetised by Life Technologies Company, and the actin beta gene was used as control.

Statistical analysis

Relative quantification method was used and the relative expression level was calculated with the equation $2^{-\Delta\Delta Ct}$.

Data were analyzed with independent samples *T* test or one-way ANOVA by using SPSS 16.0, and the value of $P<0.05$ was considered as statistically significant.

Target genes analysis

The prediction of target genes for differentially expressed miRNA was performed using online database Mirecords (http://mirecords.biolead.org/) (Xiao et al. 2009). And the target genes predicted in at least four databases integrated in Mirecords were chosen. To describe the properties of target genes and gene products, gene ontology (GO) was used for annotation and enrichment analysis (Harris et al. 2004). At the same time, the target genes with experimental support for differentially expressed miRNA were performed using TarBase 5.0 (http://diana.cslab.ece.ntua.gr/tarbase/) (Vergoulis et al. 2012). Then the intersection of predicted target genes in at least five databases integrated in Mirecords and validated target genes in TarBase were pick out for further qRT-PCR investigation.

Results

Construction of animal model of *A. cantonensis* infection and pathological changes in the brain tissue

The larval of *A. cantonensis* were found in the brain of both mice and rats in 7 days after *A. cantonensis* infection. These proved the successful construction of animal model of *A. cantonensis* infection. And the pathological change in the

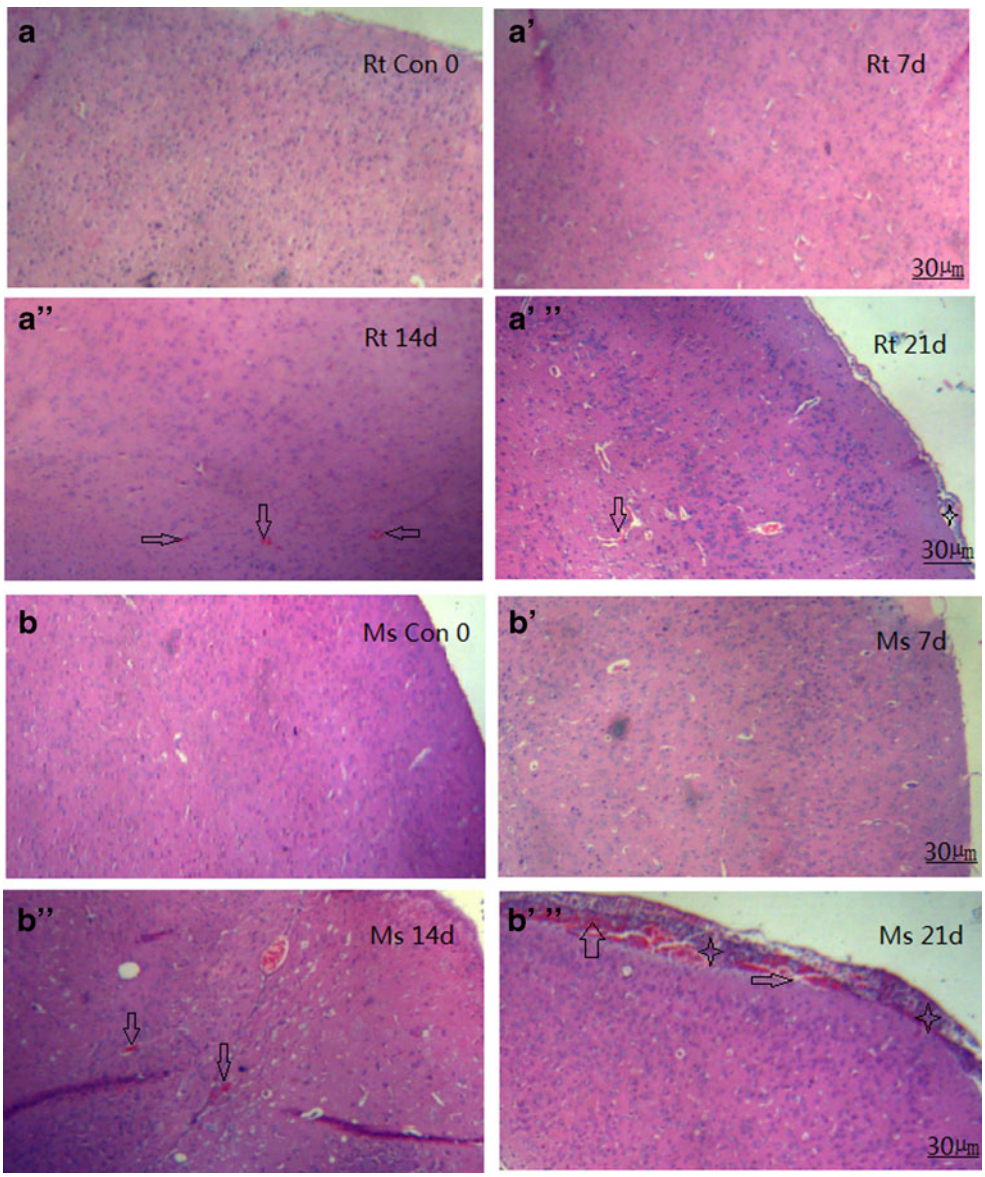

Fig. 1 HE staining of the brain tissues taken from *A. cantonensis*-infected rodents and control. **a** Rat model and **b** mice model. *Asterisk* showed infiltration of inflammatory cells and *arrow* showed hemorrhages

brain of infected animal in different infection time was observed through histological examination.

For mice infection model, no obviously abnormal brain tissue was observed in 7 days. While meningeal thickening and inflammatory reaction could been observed at 14 days and aggravated significantly in 21 days. Histological examination revealed mechanical damages in the brain tissue caused by *A. cantonensis* larval invasion. Cavities, hemorrhages, and infiltration of large amount of inflammatory cells were observed in the brain parenchyma. And worms were seen in the subarachnoid space (Fig. 1).

In rat infection models, *A. cantonensis* larval were found in the brain of 7-day group but brain tissue showed no significant difference with control. Hemorrhages were found in the brain parenchyma in 14 days, and more dilated vessels and infiltration of inflammatory cells were observed in the brain tissue in 21 days after *A. cantonensis* infection (Fig. 1).

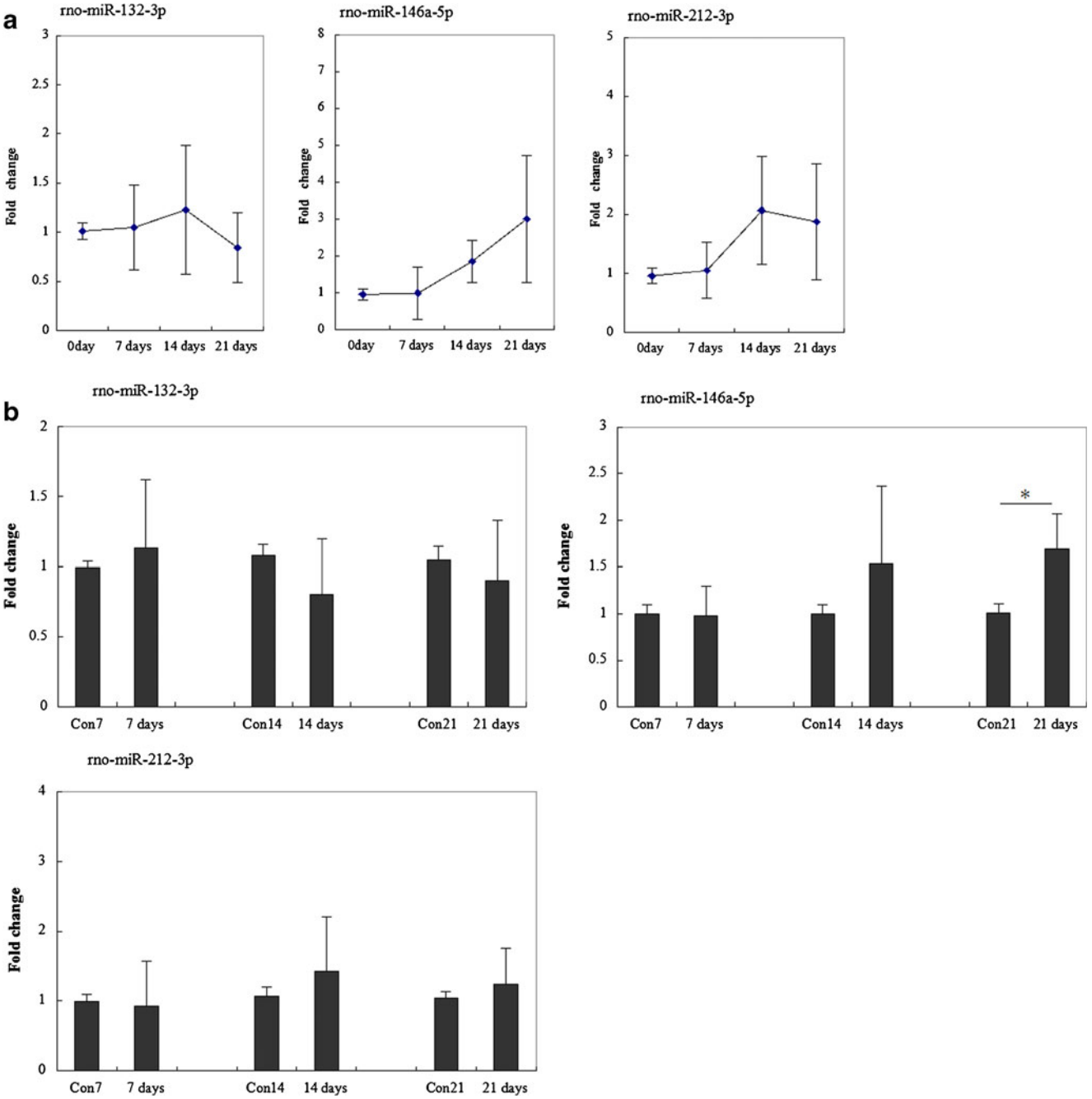

Fig. 2 Dynamic relative expression of miR-132-3p, miR-146a-5p, and miR-212-3p of the cerebrum tissue in the rat models infected with *A. cantonensis*. **a** Values were presented as levels of $2^{-\Delta\Delta Ct}$ compared to controls (0 day with no infection), $n=5$/group and **b** values were presented as levels of $2^{-\Delta\Delta Ct}$ compared to controls with the same days, $n=3$/group. The *asterisk* represents $p<0.05$

Dynamic relative expression of miR-132-3p/212-3p and miR-146a-5p in the cerebrum tissue of rats and mice model infected with *A. cantonensis*

During the period of *A. cantonensis* infection, miR-146a-5p and miR-132-3p/212-3p showed dynamic fluctuation in both rats and mice model compared with 0-day group with no infection (Figs. 2a and 3a). In rat models, the results showed that miR-146a-5p upregulated in the cerebrum tissue of rat models after 21 days of *A. cantonensis* infection compared to the tissues from the control group of the same time (Fig. 2b). However, there was no significant difference to be found in the expressions of miR-132-3p and miR-212-3p (Fig. 2b). In mice model, the expression of miR-146a-5p showed significant up-regulation in the 14 and 21 days infection with *A. cantonensis* compared with control group of same age (Fig. 3b). The level

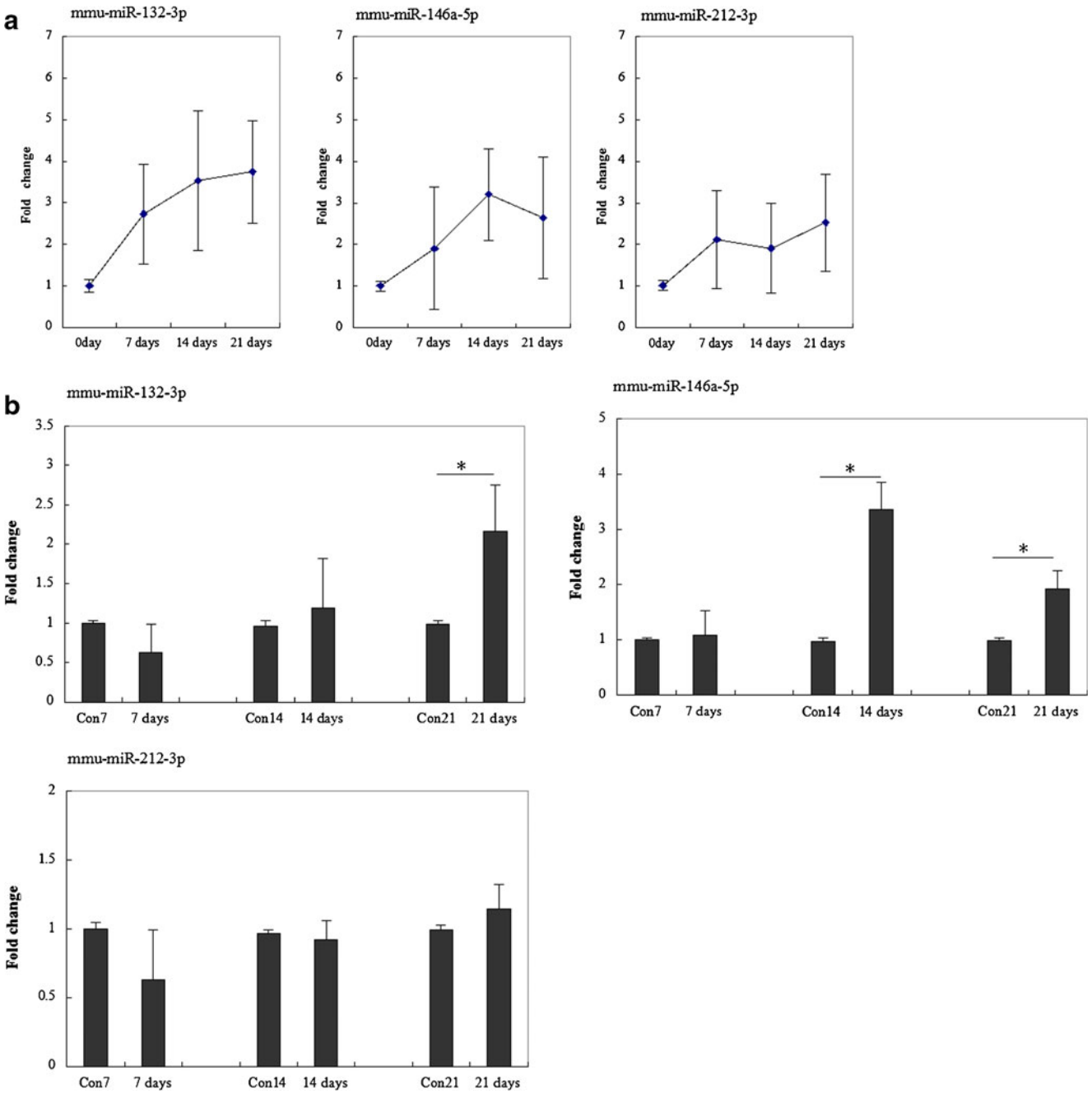

Fig. 3 Dynamic relative expression of miR-132-3p, miR-146a-5p, and miR-212-3p of the cerebrum tissue in the mice model infected with *A. cantonensis*. **a** Values were presented as levels of relative quantification $2^{-\Delta\Delta Ct}$ compared to controls (0 day with no infection), $n=5$/group and **b** values were presented as levels of $2^{-\Delta\Delta Ct}$ compared to controls with the same days, $n=3$/group. The *asterisk* represents $p<0.05$

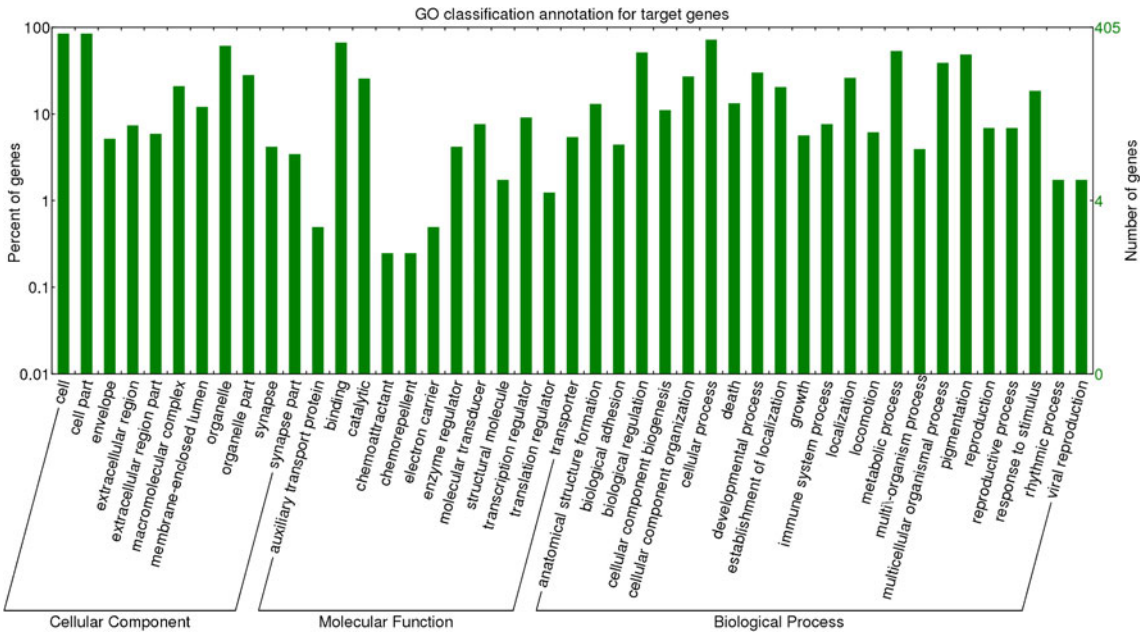

Fig. 4 Partial gene ontology classification annotated for the predicted target genes

of miR-132-3p expression upregulated significantly in 21 days infection with *A. cantonensis* compared with control group of same age (Fig. 3b), while the expression of miR-212-3p showed no difference compared with the same time control (Fig. 3b).

Target genes analysis for differentially expressed miRNAs

The chosen predicted target genes of mmu-miR-146a and mmu-miR-132 include 203 and 255 mRNA genes separately (Table S1), while there are no predicted target genes of rno-miR-146 in accordance with the former screening conditions. GO was used for annotation and enrichment analysis of the target genes and gene products of differentially expressed miRNAs such as miR-132 and miR-146a in mice model. The analysis showed that the predicted target genes appeared to be involved in cellular process, metabolic process, biological regulation, and others biological process (Fig. 4). Among these target genes, Hnrnpd, Bivm, Sfrs6, Med1, Eif4g2, Runx1t1, Cask, Traf6, Syt1, Irak1, and Hmbox1 are the predicted target genes of mmu-miR-146a in at least five databases integrated in Mirecords. To the same standard, Arhgef11, Bnip2, Calu, Daam1, Dcun1d3, Dusp9, Hn1, Osbpl8, Pde7a, Pten, Rfx3, Sema6a, Setd5, Sox5, Spry1, Timm9, Tjap1,Brca1, D15Ertd621e, Paip2, Tmeff1 Usp9x, and Zcchc11 are the predicted target genes of mmu-miR-132 (Table 1).

The validated target gene of rno-miR-146a is KLF4, and those of mmu-miR-146a-5p include NM_008363 (Irak1), NM_172161.4 (Irak2), Nos2, Ifng, and Traf6. With the same standard, MMP-9, Kdm5a, Ep300, Btg2, Mecp2, Rfx4, and Paip2 are the validated target genes of mmu-miR-132-3p (Table 2). The intersection of predicted target genes in at least five databases integrated in Mirecords and validated target genes in TarBase include Irak1, Traf6, and Paip2 (Tables 1 and 2). The expression of Irak1, Traf6, and Paip2 were analyzed using qRT-PCR. The primers for these chosen mRNA targets were shown in Table 3. The results showed that Irak1 and Traf6 downregulated in 14 and 21 days after *A. cantonensis* infection (Fig. 5).

Table 1 Predicted target genes in at least five databases for differentially expressed miRNAs

miRNA	Target genes
mmu-miR-146a	Hnrnpd, Bivm, Sfrs6, Med1, Eif4g2, Runx1t1, Cask, Traf6, Syt1, Irak1(NM_008363), Hmbox1
mmu-miR-132	Osbpl8 (NM_001003717, NM_175489), Tjap1, Dusp9, Arhgef11, Timm9 (NM_001024853, NM_001024854, NM_013896), Calu (NM_007594, NM_184053), Hn1, Pde7a, Pten, Usp9x, Brca1, Rfx3, Sox5, Spry1, Bnip2, Sema6a, Tmeff1, Daam1(NM_026102, NM_172464), Paip2, Setd5, D15Ertd621e, Dcun1d3, Zcchc11

Table 2 Validated target genes for differentially expressed miRNAs

miRNA	Target genes
rno-miR-146a	KLF4
mmu-miR-146a-5p	Irak1(NM_008363), Irak2(NM_172161.4), Nos2, Ifng, Traf6
mmu-miR-132-3p	MMP-9, Kdm5a, Ep300, Btg2, Mecp2, Rfx4, Paip2

Table 3 Summary of chosen mRNA targets primers used in qRT-PCR

Gene name	Primer	Sequence (5′–3′)
Irak1	Forward primer	CCCTGGATCAACCGCAACG
	Reverse primer	TGGGTCTGGGAGCCTGGAA
Traf6	Forward primer	ATGCAGAGGAATCACTTGGCA
	Reverse primer	ACGGACGCAAAGCAAGGTT
Paip2	Forward primer	GAACGCTGTTTCCAAGAAATGC
	Reverse primer	CCAGAGAAGAGCCATCACTGATA

Discussion

A. cantonensis is a parasite that causes EM and has been reported to be present on most Pacific islands (Tsai et al. 2004; Sugaya et al. 1997; Jitpimolmard et al. 2007). The parasitic worms are neurotropic in man, and the infection presents with severe unrelenting headache, paresthesias, or a cranial nerve palsy (Alto 2001; Lo Re and Gluckman 2001). The larvae in nonpermissive host such as mice and human cannot undergo normal development and fail to complete the pulmonary migration which induces more intense inflammatory response as compared to infected rats (Yoshimura et al. 1994). miRNAs are single-stranded noncoding regions of approximately 21 nucleotides that regulate protein synthesis by targeting mRNAs for translational repression or degradation at the posttranscriptional level. In recent years, a large amount of research interest in studies on miRNA-mediated modulation of gene functions during neuroinflammation (Thounaojam et al. 2013).

The miR-146a expression was confirmed to be present in reactive astrocytes. In human temporal lobe epilepsy with hippocampal sclerosis, increased astroglial expression of miR-146a was observed mainly in regions where neuronal cell loss and reactive gliosis occurred (Aronica et al. 2010). Experimental data showed that a large amount of nodular lesions with infiltration of numerous inflammatory cells were found in the brain parenchyma of mice or rat models infected with *A. cantonensis*. The immunohistochemical staining revealed a severe loss of neurons in the core of these focal lesions, and only a few injured neurons survived in the peripheral regions of the focal lesion (OuYang et al. 2012). In this study, the expression level of miR-146a-5p upregulated significantly and separately in rat models of 21 days infection and mice model of 14 and 21 days infection with *A. cantonensis* compared with control group of same age. These suggested the higher frequency of lesions and inflammation situation. Report showed that targeted deletion of this gene results in several immune defects. In response to inflammatory cues, miR-146a was induced as a negative-feedback regulator of the astrocyte-mediated inflammatory response (Zhang et al. 2010). miR-146a may play an important role in neurological disorders associated with chronic inflammation (Iyer et al. 2012).

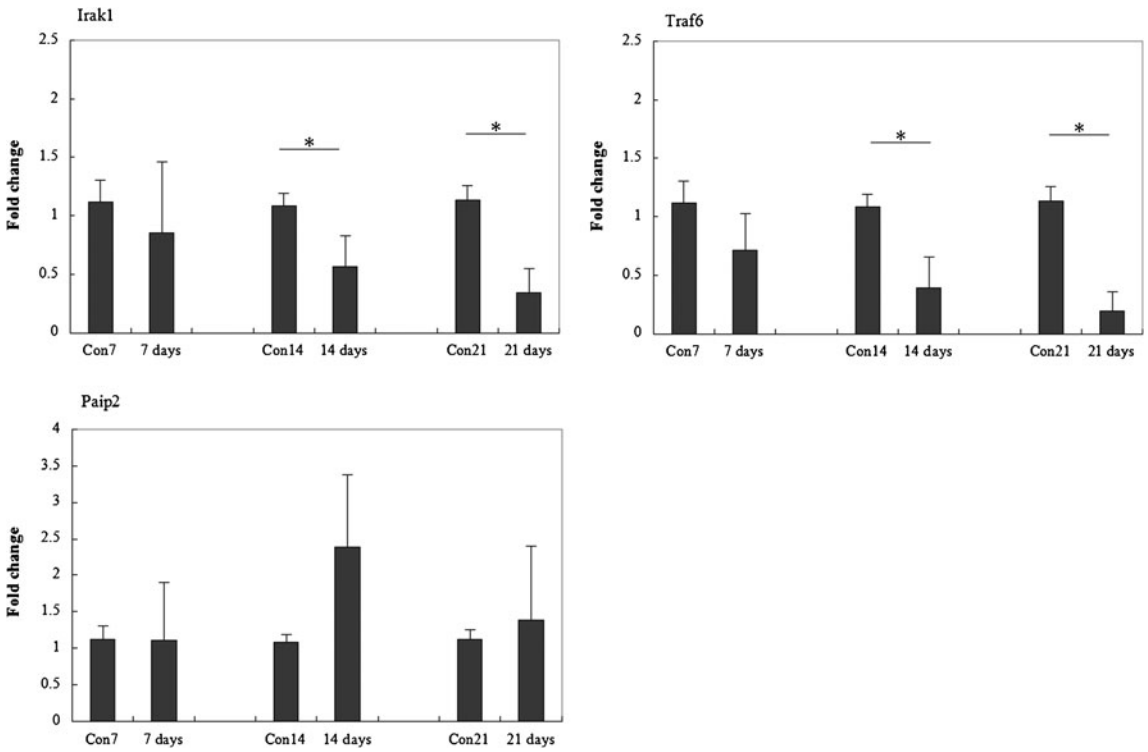

Fig. 5 Dynamic relative expression of Irak1, Traf6374, and Paip2. The values were presented as levels of $2^{-\Delta\Delta Ct}$ compared to controls with the same days, $n=3$/group. The *asterisk* represents $p<0.05$

miR-132 and miR-212 are tandem miRNAs whose expression is necessary for the proper development, maturation, and function of neurons and whose deregulation is associated with several neurological disorders, such as Alzheimer's disease and tauopathies (Wanet et al. 2012). Former research demonstrated that miR-132and miR-212 have been associated with brain-related disorders (Wong et al. 2013). In the present study, the expression of miR-132-3p in mice model showed significant upregulation in the 21 days of infection with *A. cantonensis* compared with the same age control group. Research showed that retroviral knockdown of miR-132 using a specific "sponge" containing multiple target sequences impaired the integration of newborn neurons into the excitatory synaptic circuitry of the adult brain (Luikart et al. 2011). miR-132 may function as a regulator of inflammation institution of the brain (Shaked et al. 2009).

The GO enrichment analysis revealed that the predicted target genes were most involved in many biological processes such as cellular process, metabolic process, and biological regulation. The targeted genes analysis showed that Irak1 and Traf6 downregulated in 14 and 21 days after *A. cantonensis* infection. Recent research proved that miRNA-146a and miRNA-146b-5p directly bound to the IRAK1 3′-UTR and regulated immune and inflammatory responses (Xie et al. 2013). Experiment study showed that TRAF6 was an essential adaptor protein for the NF-kappaB signaling pathway and played an important role in inflammation and immune response (Peng et al. 2013). The result of the present study indicated that miRNA-146 might play a regulated functional role in inflammation through IRAK1 and Traf6.

In conclusion, our study showed that brain-specific miRNAs, such as miR-132-3p and miR-146a-5p, a regulator of the innate immune response, upregulated in the cerebrum tissue of mice infected with *A. cantonensis*. And the target genes of mmu-miR-146a-5p such as Irak1 and Traf6 downregulated. The expression of miR-146a-5p also upregulated in the rat model. The present study suggested the involvement of the miRNAs in the regulation of inflammation of CNS induced by *A. cantonensis*.

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