MicroRNA expression profile in RAW264·7 macrophage cells exposed to *Echinococcus multilocularis* metacestodes

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SUMMARY

MicroRNAs (miRNAs) are short noncoding RNAs, involved in the regulation of parasite diseases. However, a role of miRNAs in *Echinococcus multilocularis* infection remains largely unknown. In this study, we first found the expression levels of key genes involved in miRNA biogenesis and function, including *Ago2*, *Xpo5*, *Tarbp2* and *DgcR8*, were obviously altered in the macrophage RAW264·7 cells exposed to *E. multilocularis* metacestodes. Compared with the control, 18 and 32 known miRNAs were found to be differentially expressed ($P < 0{\cdot}05$ and fold change >2) in the macrophages exposed to *E. multilocularis* metacestodes for 6 and 12 h, respectively. Among these, several are known to be involved in regulating cytokine activities and immune responses. Quantitative real-time polymerase chain reaction results showed that the expression of nine selected miRNAs was consistent with the sequencing data at each treatment time points. Moreover, there were statistically significant correlations between the expression levels of miRNAs and their corresponding targeted genes. Our data give us some clues to pinpoint a role of miRNAs in the course of infection and immunity of *E. multilocularis*.

Key words: microRNAs, macrophage cells, *Echinococcus multilocularis*, metacestodes.

INTRODUCTION

Alveolar echinococcosis (AE) is caused by a parasitic tapeworm *Echinococcus multilocularis* (Deplazes and Eckert, 2001) and is primarily characterized by a tumour-like growth of metacestodes in the liver of rodents or, occasionally, human beings (Zhang *et al*. 2012). AE is one of the most dangerous human parasitic zoonosis worldwide (Craig *et al*. 2017). Although a high effective governmental measure has been taken, *E. multilocularis* infection remains wide-spread and cannot be effectively controlled in some mountainous and pastoral areas in China (Wang *et al*. 2008). Many Studies on *Echinococcus* spp., especially the whole genome sequencing of *Echinococcus* spp. and *in vitro* culture of *E. multilocularis* protoscoleces, have provided a possibility for pinpointing miRNA roles in the development and growth and host–parasite interactions, and a potential as diagnostic targets (Tsai *et al*. 2013; Zheng *et al*. 2013*a*).

MicroRNAs (miRNAs), a class of small noncoding RNA molecules, mainly execute regulatory functions *via* repressing translation or triggering degradation of the targeted mRNA (Guo *et al*. 2010). Increasing evidence showed that most miRNAs are involved in many pathologic processes and diseases (Cai *et al*. 2016). It was shown that host miRNAs were dysregulated in response to pathogen infection (Judice *et al*. 2016). On the other hand, pathogens including parasitic helminths may deliver their proteins and miRNAs into host cells to modulate host cell functions to benefit infection (Carriere *et al*. 2016). Several potential approaches have been proposed for regulation of macrophage functions by *E. multilocularis*. Previous studies demonstrated that excretory/secretory products of *E. multilocularis* could downregulate macrophage functions (Rakha *et al*. 1991). Nono *et al*. (2012) found that excretory/secretory products of *E. multilocularis* specifically influence dendritic cells to modulate immune responses. Recently, our study found that exosome-like vesicles secreted by *E. multilocularis* metacestodes could modulate gene expression in macrophages (Zheng *et al*. 2017). Above results suggested some active molecules released by *E. multilocularis* metacestodes probably have important roles in regulating the host response. Macrophages play a crucial role in innate and adaptive immune responses against *E. multilocularis* infection (Vuitton and Gottstein, 2010). To date, no studies have examined the effects of *E. multilocularis* on the RNA-induced silencing complex (RISC)

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pathway in macrophages. In this study, the expression level of key RISC components and miRNA expression profile in macrophage RAW264·7 cells exposed to *E. multilocularis* metacestodes were determined. The result will help us further understand the interactions between host and *E. multilocularis*.

MATERIALS AND METHODS

Ethics statement

Animal experiments were approved by Animal Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. All animal experiments in the study were handled in strict accordance with good animal practice according to the Animal Ethics Procedures and Guidelines of the People's Republic of China.

Parasites and RAW264·7 cell treatment

Echinococcus multilocularis Qinghai isolate was maintained in 6-month-old DBA/2 mice in our laboratory. Larval cysts were collected from infected mice, washed in sterile phosphate buffered saline (PBS), minced and filtered through a 400 μm pore size metal mesh to obtain the protoscoleces. *Echinococcus multilocularis* protoscoleces were cultured in 25 mL flask contained RPMI 1640 medium and 20% fetal bovine serum (FBS) as previously described (Wang *et al*. 2016).

2×10^6 macrophage RAW 264·7 cells were cultured in RPMI 1640 medium supplemented with 10% FBS at 37 °C, 5% CO2, until the confluency reached 70–80%. 6×10^3 *E. multilocularis* metacestodes were added into each flask. After treatment for 6 and 12 h, the cells were washed for three times in PBS, respectively. Mock-treated cells were used as control by addition of the same volume of RPMI 1640 medium.

Isolation of total RNA and high-throughput sequencing

Total RNA was extracted from the mock- and protoscolex-treated cells using TRIzol reagent (Invitrogen), respectively. The RNA concentration and integrity was determined using Agilent 2100. High-throughput small RNA sequencing was carried out in Novogene (Beijing, China). After RNA sequencing, we performed the following bioinformatics analysis. Briefly, after removing low quality reads and adaptor sequences, only sequences perfectly matching the mouse genome (http://www.ncbi.nlm.nih.gov/genome/genomes/52) were used for the further analysis. Known miRNAs were identified by BLAST searching against the MirGeneDB database (http://www.mirgenedb.org) and the relative miRNA expression levels were analysed using DEGseq as previously described (Wang *et al*. 2010). miRNAs with a fold change >2 and a *t*-test *P* value < 0·05 were regarded as differentially expressed.

miRNA target gene prediction and bioinformatics analysis

Targets genes of differentially expressed miRNAs were predicted using three databases: TargetScan (http://www.targetscan.org/vert_71) (Agarwal *et al*. 2015), miRanda (http://www.microrna.org/microrna) (Betel *et al*. 2008) and RNAhybrid (Lai and Meyer, 2016). Gene Ontology (GO) term analysis was performed using GOseq based Wallenius non-central hyper-geometric distribution (Young *et al*. 2010). KEGG pathway enrichment analysis of DEGSeq was performed by KOBAS software. Hierarchical clustering was performed to distinguish miRNA expression patterns among samples.

Quantitative real-time polymerase chain reaction (qRT–PCR)

Differentially expressed miRNAs were validated using quantitative reverse transcription RT–PCR (qPCR) with SYBR green and specific primers (GeneCopoia). U6 RNA was selected as a reference for normalization. Poly A polymerase was used to added poly A to the 3′ ends of miRNAs. The 20 μL reverse transcription reaction contained 2 μL total RNA (approximate 1 μg), 5 μL 5 × PAP/RT Buffer, 1 μL Polymerase, 1 μL reverse transcriptase mix and 11 μL RNase-free water. The reaction protocol was as following: 60 min at 37 °C and 5 min at 85 °C. RT–PCR was performed using All-in-One™ miRNA qRT–PCR mix containing SYBR Green (GeneCopoia). The 20 μL PCR reaction included 2 μL of cDNA (1:5 dilution), 10 μL 2 × All-in-One qPCR Mix, 2 μL Universal Adaptor PCR Primer, 2 μL 10 × miRNA qPCR Primer, 0·4 μL ROX Dye and 3·6 μL ddH_2O. The reactions were incubated in a 96-well plate at 95 °C for 15 min, followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 70 °C for 30 s. Relative fold changes were calculated by $2^{-\Delta\Delta Ct}$.

Expression levels of nine genes that encode key RISC components were analysed. First-strand cDNA synthesis was conducted using 1 μg total RNA with the ThermoScript™ RT–PCR System (Invitrogen). The reaction mixture was incubated at 42 °C for 1 h and 75 °C for 5 min. The reaction mixture was diluted 10-fold with nuclease-free water. RT–PCR was performed using All-in-One™ qPCR Mix (GeneCopoeia, Washington, USA) with the following thermocycling conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 15 s. *Gapdh* were screened to identify an appropriate reference gene. Validated primers for all of the above genes were

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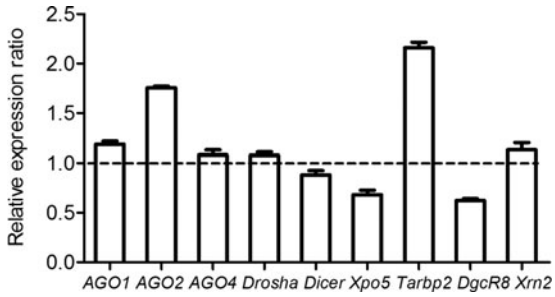


Fig. 1. Effects of *E. multilocularis* metacestodes on the expression of nine genes involved in miRNA biogenesis in RAW264·7 macrophage cells. The expression levels of nine genes were normalized to that of β-actin and data were expressed as means ± S.E.M.

purchased from GeneCopoeia. The relative expression levels of nine genes were calculated using the $2^{-\Delta\Delta Ct}$ formula. Data used in the final analysis were collected from triplicate independent experiments.

Statistical analysis

Data analyses were performed using GraphPad Prism 5 software and a one-tailed unpaired *t*-test was used for comparison of two groups, with a *P* value <0·05 being considered to be significantly different.

RESULTS

The expression of RISC components in the RAW264·7 cells exposed to E. multilocularis protoscoleces

To detect whether protoscoleces affect miRNA biogenesis and function, the expression levels of nine genes including *Ago1*, *Ago2*, *Ago4*, *Tarbp2*, *Xpo5*, *DgcR8*, *Dicer*, *Drosha* and *Xrn2* were examined by qPCR in the RAW264·7 cells exposed to *E. multilocularis* protoscoleces. Compared with untreated RAW264·7 cells, the mRNA levels of *Ago2* and *Tarbp2* increased up to 1·76-folds and 2·16-folds, whereas *Xpo5* and *DgcR8* were reduced by 34 and 43%, respectively. The rest were not changed (Fig. 1).

Data description of three small RNA sequencing libraries

Considering the ectopic expression of *Ago2*, *Tarbp2*, *Xpo5* and *DgcR8*, we then examined the miRNA profiles of RAW264·7 cells after protoscolex treatment for 6 and 12 h, respectively. In this study, 13 792 508, 12 090 305 and 14 921 792 total reads were obtained from the mock-infected cells, emu-treated 6 h-cells and emu-treated 12 h-cells, respectively (Table S1). After data cleaning, approximately 95% reads from all the three samples were ultimately obtained and used for downstream analysis, and

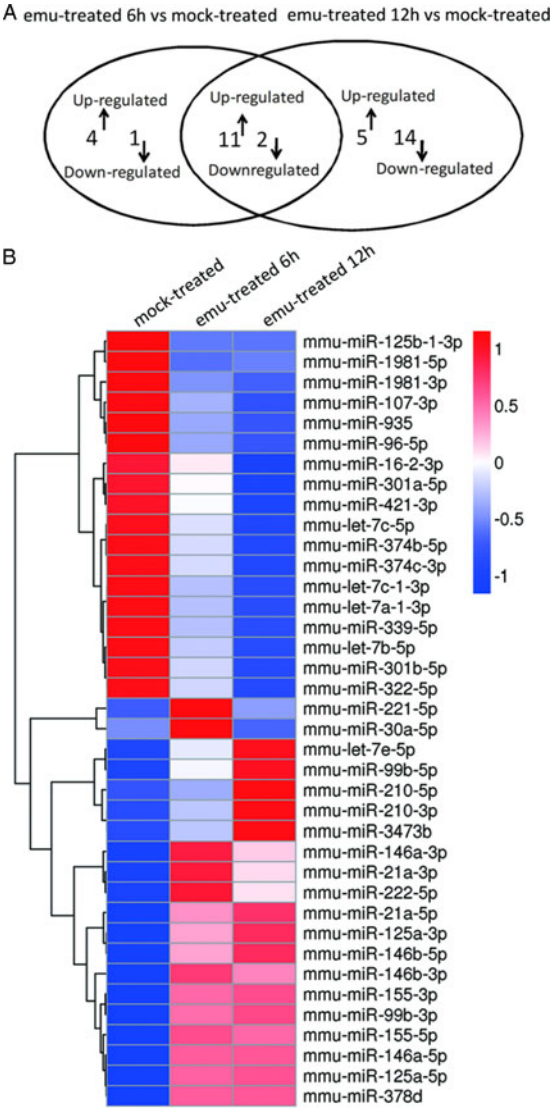


Fig. 2. Venn diagram showing the unique and overlapping miRNAs (A) and heatmap diagram of differential miRNA expression among mock-treated-, emu-treat 6 h- and emu-treat 12 h-libraries (B). (A) Each row represents a miRNA and each column represents a sample. The colour scale illustrates the relative level of miRNA expression: Red, increased expression; blue, decreased expression; and white, mean value. (B) 18 and 32 known miRNAs were differentially expressed in the emu-treated 6 h- and emu-treated 12 h-libraries compared with the mock-infected library, respectively. 13 commonly shared miRNAs (11 miRNAs upregulated and two miRNAs downregulated) were found.

over 81% of clean reads were mapped to the mouse genome (Table S1). The size distribution of small RNAs was similar in the three libraries, and the majority of these miRNAs were 21–24 nt in length.

Identification of differentially expressed known miRNAs

In total, 661 distinct known miRNAs were non-redundantly identified from three libraries, 82·9%

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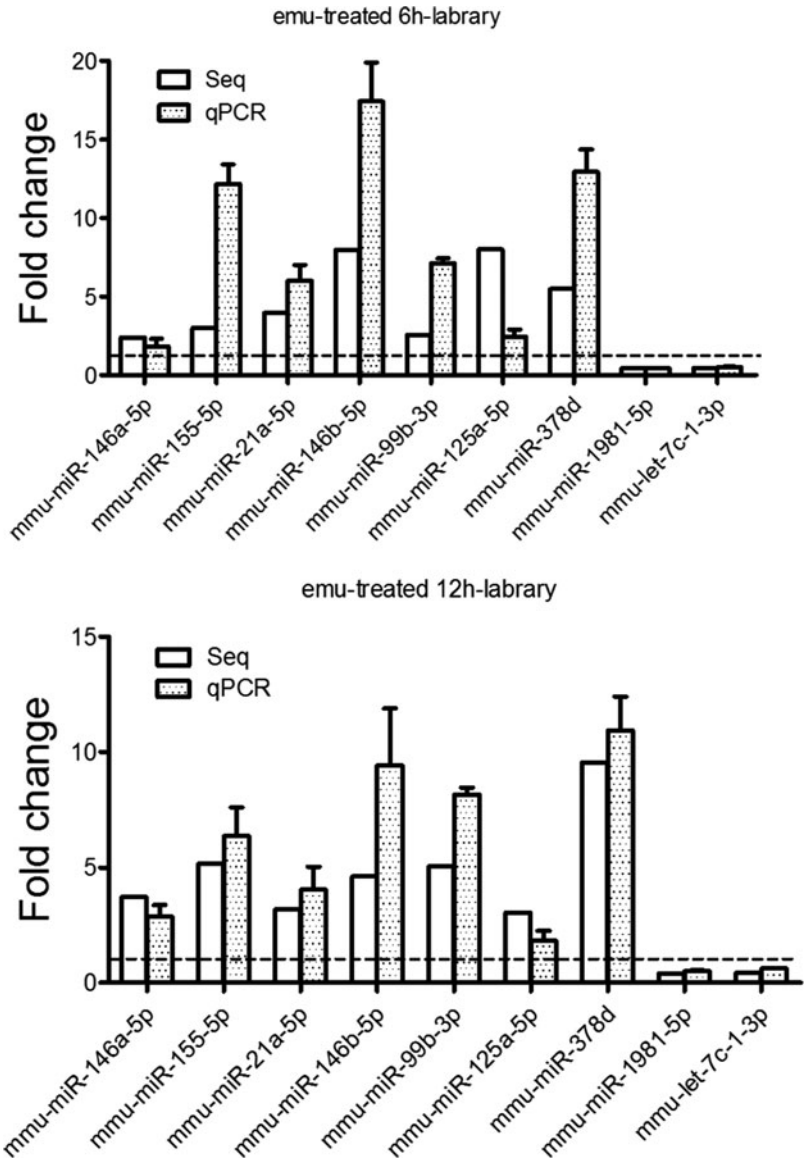


Fig. 3. Validation of miRNA expression in the RAW264·7 cells exposed to *E. multilocularis* protoscoleces for 6 h (A) and 12 h (B) by qPCR. qPCR data were expressed as means ± S.E.M. Every sample was tested in triplicate and data used for the final analysis were from three independent experiments.

(548/661) of which were commonly shared by all samples. Compared with mock-treated library, 18 and 32 known miRNAs were found to be differentially expressed in the emu-treated 6 h- and emu-treated 12 h-libraries, respectively (Fig. 2; Tables S2 and S3). Of them, 13 known miRNAs were commonly shared in the two emu-treated libraries, with 11 miRNAs being upregulated and two downregulated (Fig. 2).

Validation of miRNA sequencing data by qPCR analysis

To further validate the expression of miRNAs identified, qRT–PCR was carried out for nine selected miRNAs, of which seven were up-regulated and two downregulated (mmu-miR-1981-5p and mmu-let-7c-1-3p). Consistent with the sequencing results, there was an increase in mmu-miR-378d, mmu-miR-155-5p, mmu-miR-146a-5p, mmu-miR-21a-5p and mmu-miR-125a-5p expression, and a decrease in mmu-miR-1981-5p and mmu-let-7c-1-3p expression in two emu-treated libraries compared with the mock-treated cells (Fig. 3).

Analysis of potential targets of differentially expressed miRNAs

A total of 2719 putative target genes were obtained for these differentially expressed miRNAs. According to *P* values of the enrichment analysis of target genes, the top 15 GO terms of biological process, molecular function and cellular component were shown in Fig. 4, respectively. Most of target genes were highly enriched in biological process (e.g. response to stimulus, immune response, signal transduction),

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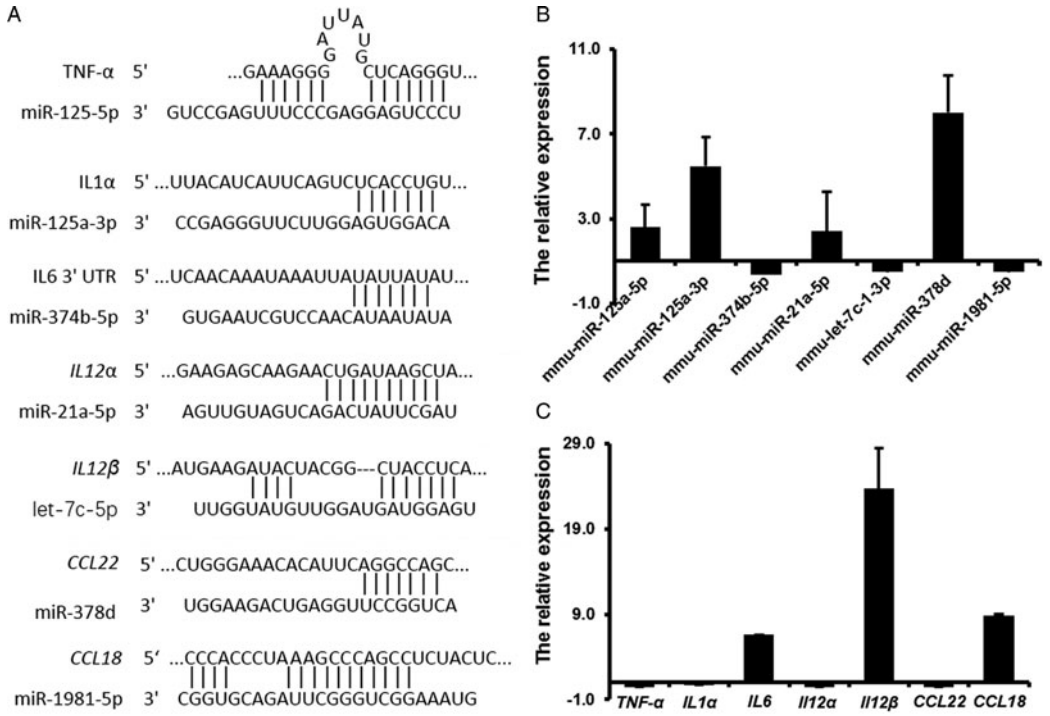


Fig. 4. GO molecular function annotations of the target genes of differentially expressed miRNAs. According to *P* value, top15 GO terms of biological process, molecular function and cellular component were shown in the emu-treated 6 h- (A) and 12 h-libraries (B), respectively.

molecular function (e.g. protein binding, cytokine receptor binding and cytokine activity) and cellular component (e.g. cell part, organelle). In contrast, Inflammatory response genes were highly enriched in inflammatory response in emu-treated 6 h-library, but apoptotic and cell death genes were highly enriched in emu-treated 12 h- library. KEGG pathway analyses showed that significantly enriched pathways included tumour necrosis factor (TNF) signalling pathway, F-kappa B signalling pathway, apoptosis (Table S4). However, Toll-like receptor signalling pathway was only enriched in emu-treated 6 h- library.

Correlation of expression between microRNAs and their potential targets

It was interesting to note that among the target genes, several belonged to cytokine families. In order to check if these cytokines were correlated with the corresponding miRNAs in expression, we measured their expression levels using qRT–PCR. Fig. 5 showed the relative expression levels of mmu-miR-125a-5p, mmu-miR-21a-5p, mmu-miR-374d-5p, mmu-miR-125a-3p, mmu-miR-378d, mmu-miR-1981-5p and mmu-let-7c-1-3p and of seven cytokine genes (*TNF-α*, *IL1α*, *IL6*, *IL12α*, *IL12β*, *CCL22* and *CCL18*,) of their predicted targets at 24 h (Fig. 5). These results indicate that there is a statistically significant correlation ($r = -0{\cdot}802$; $P = 0{\cdot}049$) between the expression levels of seven selected miRNAs and their corresponding cytokine targets (Fig. S1).

DISCUSSION

As the important post-transcriptional regulators of gene expression, miRNAs play important roles in intricate host–pathogen interactions (Zheng *et al*. 2013*b*). Through the use of high-throughput methods (e.g. deep sequencing and miRNA microarray), changes of host miRNA profiles have been explored during parasite infection (Britton *et al*. 2015). Increasing evidence shows that host miRNAs in the relevant cells or tissues are dysregulated during helminthic infection, hinting that miRNAs are crucial mediators in regulating parasite–host interplay (Cai *et al*. 2016). For instance, our previous study found that *E. multilocularis* infection disturbed miRNA expression in mouse liver (Jin *et al*. 2017). In recently, we found that 58 host circulating miRNAs were dysregulated in the sera of *E. multilocularis*-infected mouse (Guo and Zheng, 2017). To date, no studies have examined the expression profile of miRNAs in immune cells in response to *E. multilocularis* exposure. Therefore, we herein investigated miRNA expression profiles in the macrophage cells exposed to *E. multilocularis* protoscoleces, providing clues to further pinpoint a role of miRNAs in regulation of immune responses during infection.

During host responses to infection, different active molecules released by *Echinococcus* species at distinct infectious phages modulate expression of a variety of host genes (Lin *et al*. 2011). Our recent study demonstrated that exosome-like vesicles secreted by *E. multilocularis* metacestodes could

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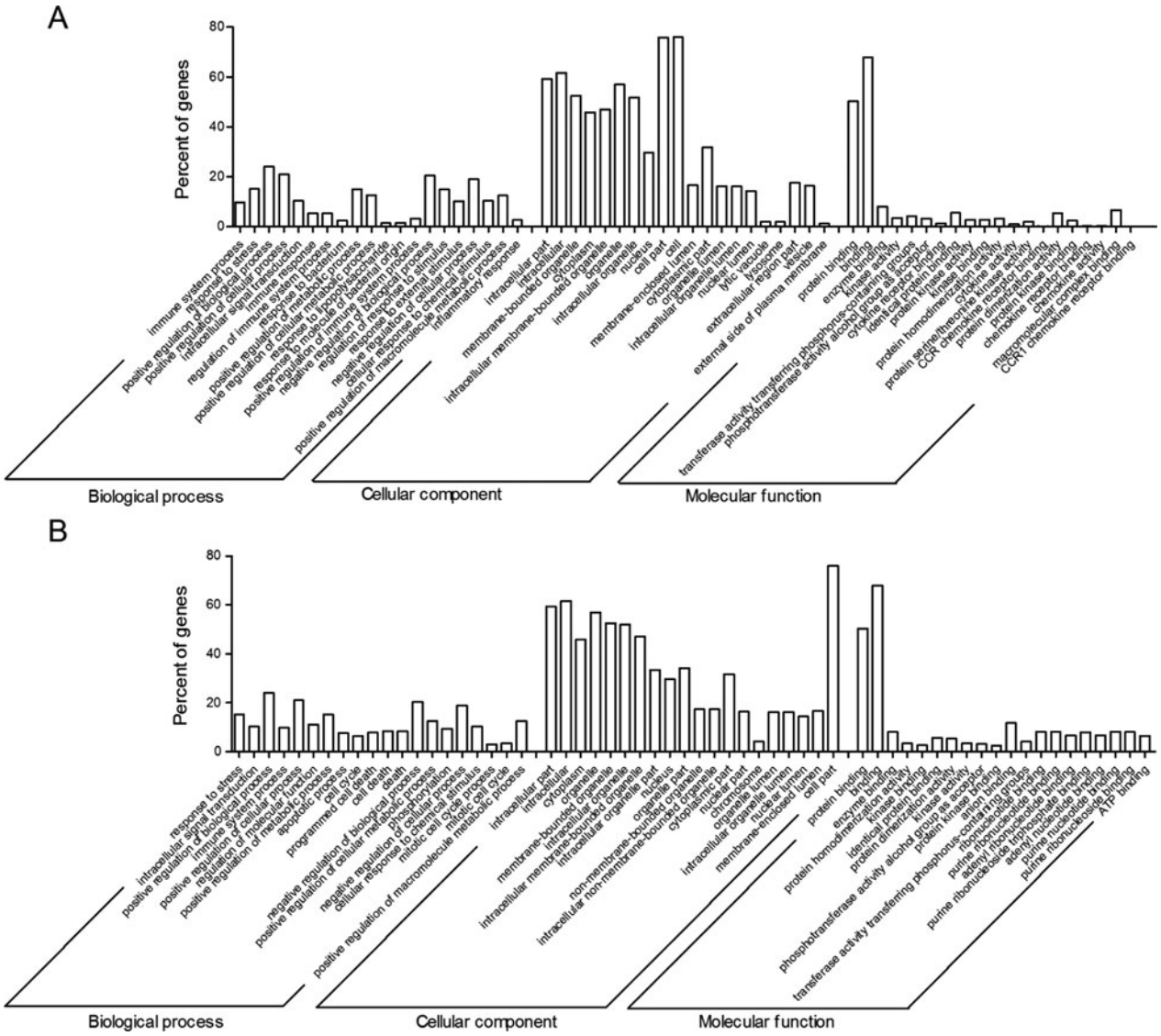


Fig. 5. Correlation between the miRNA expression and the corresponding targeting mRNA of cytokines and chemokines. (A) Shown is the predicted interaction between miRNAs and its corresponding target genes. (Band C) Expression means of mmu-miR-125a-5p, mmu-miR-125a-3p, mmu-miR-374d-5p, mmu-miR-21a-5p, mmu-let-7c-1-3p, mmu-miR-378d and mmu-miR-1981-5p (B) is negatively correlated with *TNF-α*, *IL1α*, *IL6*, *IL12α*, *CXCL22* and *CCL18* (C) in the emu-treated cells at 12 h post infection.

convey protein and miRNA cargoes into host cells and modulate host gene expressions (Zheng *et al*. 2017). Moreover, it was also found that *E. multilocularis* infection could disturb the expression of four key genes involved in miRNA biogenesis in the mouse liver (Jin *et al*. 2017). In this study, we found the transcript levels of *Ago2*, *Tarbp2*, *Xpo5* and *DgcR8* were significantly altered in the RAW264·7 cells exposed to *E. multilocularis* protoscoleces compared with the control. Downregulation expression of *Ago2* or *Dicer1* could decrease the expression of selected apoptosis- and development-related miRNAs in mouse preimplantation embryos (Shen *et al*. 2010). Further study found Ago2 and Dicer1 may regulate GW182 (glycine-tryptophan protein of 182 kDa) protein expression to affected miRNA biogenesis (Shen *et al*. 2010). Therefore, although the detailed regulation mechanisms are not yet clear, the abnormal expression of these key genes may affect the action of RISC, subsequently altering the miRNA biogenesis and lifespan.

Exposure to any pathogen leads to significant changes in the expression of specific miRNAs in immune cells (Buck *et al*. 2014; Carriere *et al*. 2016). In agreement with this idea, significant alterations in miRNA expression profile were observed in the RAW264·7 cells exposed to *E. multilocularis* protoscoleces. It was found that 18 and 32 known miRNAs were differently expressed in emu-treated 6 h-library and 12 h-library, respectively. The number of downregulated miRNAs was increased with increasing treatment time. It is well known that *Echinococcus* species dynamically secrete active molecules and induce distinct immune response profiles at different infectious stages. The rapid changes of miRNAs at different time points are in agreement with this idea. From this point, these differentially expressed miRNAs can be used as a potential target to decipher the pathogenesis. Interestingly, some immune-related miRNAs (e.g. miR-146a/b, miR-155a and miR-125a-5p) were predominantly upregulated in the treated RAW264·7 cells. It was shown that the expression levels of miR-146a and miR-155 were increased in response to *Toxoplasma Gondii* infection (Cannella *et al*. 2014). Several studies revealed that mmu-miR-146a regulated immune and inflammatory response genes *via* target IRAK1, IRAK2 and TRAF6 (Taganov *et al*. 2006). Early studies showed that miR-155 was upregulated following lipopolysaccharide-stimulation in macrophages (Hung *et al*. 2013). miR-155 promotes inflammation *via* suppression of inhibitors of inflammation, such as the suppressor of cytokine signaling 1 (SOCS-1) and Src homology-2 domain-containing inositol 5-phosphatase 1 (SHIP1) (O'Connell *et al*. 2009; Zhao *et al*. 2017). These data imply that miR-146a-5p and miR-155a might play important roles in the regulation of host immune response modulation during *E. multilocularis* infection. The regulatory roles of differentially expressed miRNAs were explored by predicting the potential target genes of those miRNAs. The most enriched GOs were involved in cell communication, signal transduction, signalling receptor activity, cytokine activity and immune response. The expression levels of cytokine mRNAs such as *TNF-α*, *IL1α*, *IL6*, *IL12α*, *IL12β*, *CCL22* and *CCL18* were alerted in the macrophages exposed to *E. multilocularis* protoscoleces. Cytokines are involved in many aspects of inflammation and immunity, especially to parasite infection (Scales *et al*. 2007; Amri *et al*. 2009). Previous studies showed that IL-12a, IFN-γ and IL-4 were significantly expressed in the hepatic parasitic lesion at the early stage of *E. multilocularis* infection, CXCL9, IL-4, IL-5, CCL17 upregulated at the middle stage, and IL-10 and TGF-b permanently expressed at the late stage (Wang *et al*. 2014). There were statistically significant correlations between the expression levels of cytokines and their corresponding miRNAs, implying a role for these miRNAs in regulating host immune responses against *E. multilocularis*. Further studies will be needed to verify whether the miRNA changes lead to altered expression of cytokine gene, either directly (by acting as miRNA targets) or indirectly (e.g. by affecting transcription factors).

Concluding remarks

Previous study showed that RAW 264·7 cells most closely mimic primary bone marrow-derived macrophages in terms of cell surface receptors and responses to microbial ligands that initiate cellular activation *via* Toll-like receptors 3 and 4 (Berghaus *et al*. 2010). However, caution must be taken when extrapolating the findings obtained with RAW 264·7 cells to other primary macrophage-lineage cells, primarily because phenotype and function of the former cells may change with continuous culture. In conclusion, we report for the first time that the miRNA expression profile of macrophages exposed to *E. multilocularis* metacestodes is significantly and rapidly altered. It is suggested that the alterations in macrophage miRNA levels likely reflect the remarkable capacity of parasites in modulation of host immune responses.

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SUPPLEMENTARY MATERIAL

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