Study on the mechanism of miRNAs on liver injury in the condition of protoscocephalus alveolarus transhepatic portal vein infection

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Abstract

Echinococcus multiloculasis is a zoonotic parasitic disease caused by Echinococcus multilocularis, which can cause liver injury, but the mechanism of liver injury is still unclear. Here, Echinococcus multilocularis was injected via the hepatic portal vein to establish a mouse model of infection, and high-throughput RNA sequencing was performed for detecting the expression of miRNAs in the liver of mice infected with 2000 Echinococcus multilocularis after 3 months infection, in order to understand the potential molecular mechanism of liver injury caused by Echinococcus multilocularis infection. Overall, 71 differentially expressed miRNAs were found in liver in comparison with control and a total of 36 mouse miRNAs with |FC|>0.585 were screened out, respectively. In addition, Targetscan (V5.0) and miRanda (v3.3a) software were used to predict differential miRNAs target genes and functional enrichment of target genes. Functional annotation showed that "cytokine-cytokine interaction", " positive regulation of cytokine production", " inflammatory respose", " leukocute activation" were enriched in the liver of Echinococcus multilocularis-infected mice. Moreover, the pathways "human cytomegalovirus infection", "cysteine and methonine metabolism", "Notch signaling pathway" and "ferroptosis" were involved in liver disease. Furthermore, 4 miRNAs (mmu-miR-30e-3p, mmu-miR-203-3p, mmu-miR-125b-5p and mmu-miR-30c-2-3p) related to liver injury were screened and verified. This study revealed that the expression profiling of miRNAs in the livers was changed after Echinococcus multilocularis infection, and improved our understanding of the transcriptomic landscape of hepatic echinococcosis in mice.

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Abstrat

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RNA sequencing was performed for detecting the expression of miRNAs in the liver of mice infected with 2000 *Echinococcus multilocularis* after 3 months infection, in order to understand the potential molecular mechanism of liver injury caused by *Echinococcus multilocularis* infection. Overall, 71 differentially expressed miRNAs were found in liver in comparison with control and a total of 36 mouse miRNAs with |FC|>0.585 were screened out, respectively. In addition, Targetscan (V5.0) and miRanda (v3.3a) software were used to predict differential miRNAs target genes and functional enrichment of target genes. Functional annotation showed that "cytokine-cytokine interaction", " positive regulation of cytokine production", " inflammatory respose", " leukocute activation" were enriched in the liver of *Echinococcus multilocularis* -infected mice. Moreover, the pathways "human cytomegalovirus infection", "cysteine and methonine metabolism", "Notch signaling pathway" and "ferroptosis" were involved in liver disease. Furthermore, 4 miRNAs (mmu-miR-30e-3p, mmu-miR-203-3p, mmu-miR-125b-5p and mmu-miR-30c-2-3p) related to liver injury were screened and verified. This study revealed that the expression profiling of miRNAs in the livers was changed after Echinococcus multilocularis infection, and improved our understanding of the transcriptomic landscape of hepatic echinococcosis in mice.

Key words: Echinococcus multilocularis, miRNA, liver injury

Introduction

Echinococcosis is a parasitic disease caused by the larvae of Echinococcus tapeworms parasitic on the human body[1-3]. The disease is mainly distributed in the developed areas of animal husbandry in the western of China. Ningxia is also the main epidemic area of echinococcosis[4]. Echinococcosis is mainly divided into two categories: cystic echinococcosis(CE) and alveolar echinococcosis(AE). the most common site of disease is mainly in the liver, and a few can also be found in the lungs and other organs[5, 6]. The prevalence and incidence of CE is higher than AE[7, 8]. But the damage of CE to the body is significantly higher than that of AE. CE is relatively easy to diagnose by B-ultrasound imaging and it can be removed by surgery[9, 10]. AE showed infiltrative growth and the early clinical symptoms were not obvious, so it is not easy to be discovered. Once discovered, it is often in the late stage and cause damage to organs. AE is very serious, which is also called "worm cancer". The operation on AE is extremely difficult, which is necessary to resect the part of the liver (including pathogens) damaged by infiltration and growth. In the most severe cases, liver transplantation is required[11-13]. However, the molecular mechanism of liver injury after Echinococcus multilocularis (*E. multiloculari* s) infection is still unclear.

MicroRNAs (miRNAs) are small non-coding RNAs with the capability of modulating gene expression at the post-transcriptional level[14, 15]. In recent years, miRNAs have been widely involved in the regulation of cancer[16], infectious diseases[17]and other diseases[18]. Altered expression of miRNAs is associated with liver metabolism dysregulation, liver injury, liver fibrosis and tumour development, making miRNAs attractive therapeutic strategies for the diagnosis and treatment of liver diseases[19]. Here, We injected *E. multiloculari* s through the hepatic portal vein to establish a mouse model of infection, after 3 months of infection, transcriptome sequencing was performed, and miRNAs related to liver injury were screened out. These miRNAs may become the potential targets for the prevention and treatment of AE.

Results

Establishment of a mouse model of hepatic portal vein infection

The cysts were removed from the gerbils, and the protoscoleiae were isolated(Fig 1A). 1% eosin staining was used to identify the viability of protoscolmus, and the viability exceeded 90%, which can be used for infection. A mouse model of secondary infection was established by injecting the protoscolmus via the hepatic portal vein. B-ultrasound was used to detect the liver lesion of mice after 3 mouths of infection, as is shown in Fig 1B, there were obvious lesions in the liver tissue of the mice in the infection group, compared with the control group. The entry of pathogens into the body can induce the body to generate an immune response. So the *E. multiloculari* s -specific antibodies in the serum was detected by Enzyme-linked immunosorbent assay (ELISA). The results showed that the levels of *E. multiloculari* s-specific IgG, IgM, IgA and IgE antibodies in the serum of mice in the infection group increased significantly(Fig 1C-F). And the total IgG,

IgM, IgA, and IgE antibodies in serum were also significantly increased(Fig 1G-J). This suggested that the mouse model of infection through the hepatic portal vein was successfully constructed and *E. multilocularis* infection can induce high levels of antibody responses.

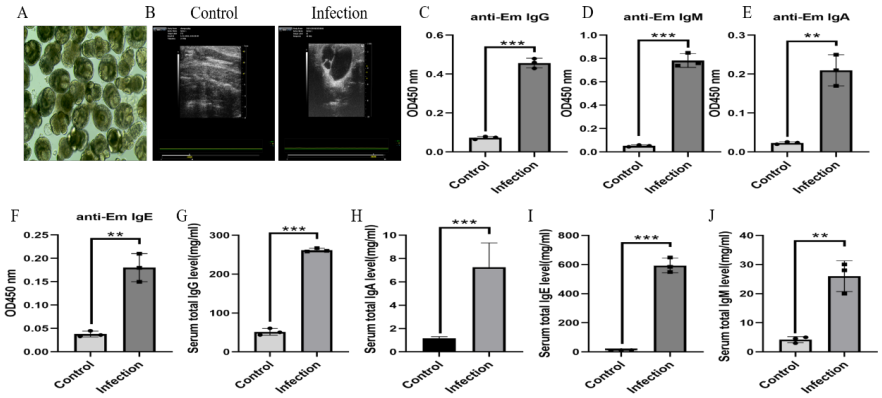

Fig1 Identification of mouse models of infection. A. Imaging of *E. multiloculari* s. B. B-ultrasound detection of liver in mice. C-F. Changes of *E. multiloculari* s-specific IgG, IgM, IgA and IgE antibodies. G-J. Changes of total IgG, IgM, IgA and IgE antibodies. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$.

E. multilocularis infection can cause liver injury.

In order to determine whether *E. multiloculari* s infection can cause liver damage. we used HE staining method to detect the liver pathological damage. The results showed that the mice in the infection group had obvious lesions in the liver and granulocytes gathered around the lesions compared with control group(Fig 2A). The results of Masson staining showed that there were a large amount of collagen fiber deposition in the liver tissue of mice in the infection group(Fig 2B). In addition, The levels of AST and ALT in the serum of mice in the infection group were significantly higher than those in the control group(Table2 and Fig 2C). This results suggested that*E. multiloculari* s parasitism can cause damage to liver tissue.

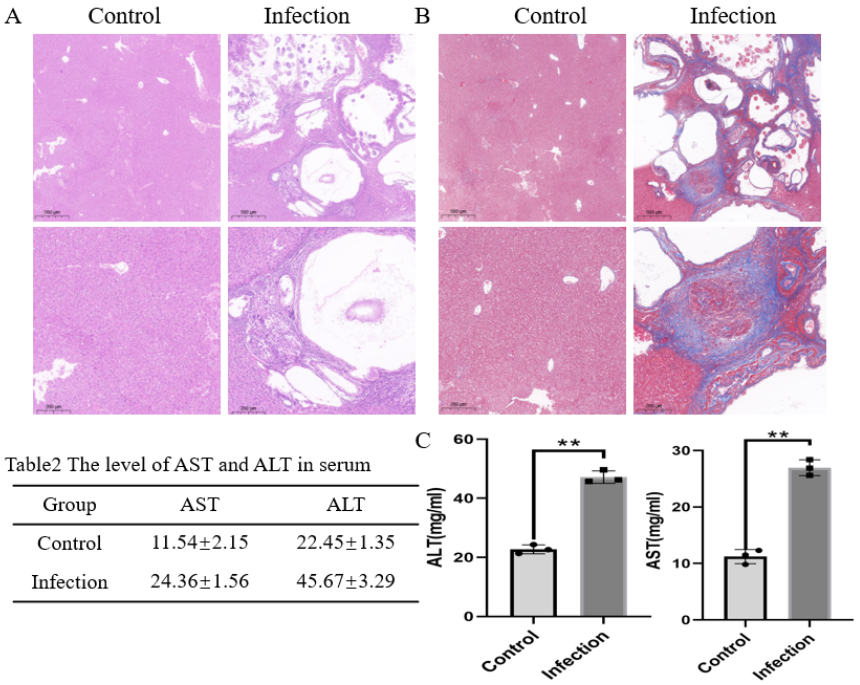

Table2 The level of AST and ALT in serum

Group	AST	ALT
Control	11.54±2.15	22.45±1.35
Infection	24.36±1.56	45.67±3.29

Fig 2 *E. multiloculari* s infection cause liver damage. A.The HE staining of mouse liver. B.The Masson staining of mouse liver. C.The level of AST and ALT in serum.*P <0.05, **P <0.01.

miRNAs differential expression

E. multiloculari s infection can cause liver damage in mice, but the mechanism of damage is still unclear. This study mainly explores the mechanism of liver injury from the regulation of miRNAs. The mouse liver tissue close to the lesion site was isolated for transcriptome sequencing. A total of 71 differentially expressed miRNAs were screened out(Fig3B-D, Table S1), and deleted of non-mouse miRNAs, there are 36 miRNAs among them with a large difference of |FC|>0.585(Table3), and these miRNAs may be related to liver damage caused by *E. multiloculari* s.

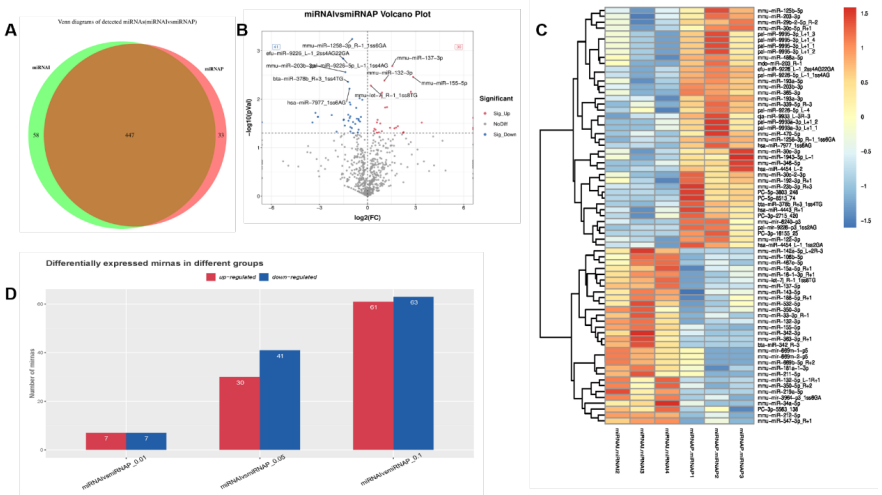

Fig 3. Differential expression of miRNAs. A. Venn diagrams of detected miRNAs. B. Volcano plot of miRNAs. C. heat map of differential expression miRNAs. D. Differential expressed miRNAs in different groups.

miRNAs target gene prediction and functional enrichment

In order to elucidate the mechanism of differentially expressed miRNAs, we predicted the target genes of miRNAs using Targetscan (V5.0) and miRanda (v3.3a). In the prediction results, the mRNAs with the highest targeting score was selected as the target gene of miRNAs(Table 3). To find the potential biological associations of differential miRNAs, we performed GO and KEGG pathway enrichment on these 36 mRNAs. The GO term is shown in Fig4A, GO enrichment results showed that differentially expressed miRNAs target genes were mainly involved in "inflammatory response", "immune response", "signal transduction", "cytokine-mediumed signaling pathway", "negative regulation of apoptotic process" and "aging" biologocal process. And these mRNAs may participate in the formation of membrance, cytoplasma, plasma membrance, cytosol and nucleus. KEGG enrichment analysis showed that pathways mainly included "cysteine and methionine metablism", "Notch signaling pathway", "ferroptosis", and "antigen processing and presentation" in the Echinococcus multilocularis infection(Fig 4B). These results showed that *E. multilocularis* infection can affect liver metabolic function and changes in signaling pathways.

Table 3 miRNA target gene prediction

NO	miRNA	mRNA	NO	miRNA	mRNA	NO	miRNA
1	mmu-miR-467e-5p	Ciita	13	mmu-miR-547-3p_R+1	Arhgef2	25	mmu-miR-350-3p
2	mmu-miR-155-5p	IL15RA	14	mmu-miR-212-5p	Ms4a2	26	mmu-let-7j_R-1_1ss8TG
3	mmu-mir-3964-p3_1ss6GA	Ccr1	15	mmu-miR-132-3p	Ucp2	27	mmu-miR-339-5p_R-3
4	mmu-miR-211-5p	Iigp1	16	mmu-miR-33-3p_R-1	Plekha1	28	mmu-miR-23b-3p_R+3
5	mmu-mir-669m-2-p5	Mal	17	mmu-miR-188-5p_R+1	Ubash3a	29	mmu-miR-30e-3p
6	mmu-miR-669b-5p_R+2	Mal	18	mmu-miR-16-1-3p_R+1	Adam17	30	mmu-miR-122-3p
7	mmu-mir-669m-1-p5	Mal	19	mmu-miR-181a-1-3p	Tnfsf13b	31	mmu-miR-29b-2-5p_R-2
8	mmu-miR-34a-5p	Rhoh	20	mmu-miR-106b-5p	Zfyve9	32	mmu-miR-192-3p_R+1
9	mmu-miR-142a-5p_L+2R-3	Ifngr1	21	mmu-miR-15a-5p_R+1	Ccr2	33	mmu-miR-30c-5p_R+1
10	mmu-miR-342-3p	Mefv	22	mmu-miR-350-5p_R+2	Fmr1	34	mmu-miR-125b-5p
11	mmu-miR-137-3p	Rassf4	23	mmu-miR-143-5p	Stmn1	35	mmu-miR-30c-2-3p
12	mmu-miR-132-5p_L-1R+1	Acs16	24	mmu-miR-532-5p	Armc8	36	mmu-miR-203-3p

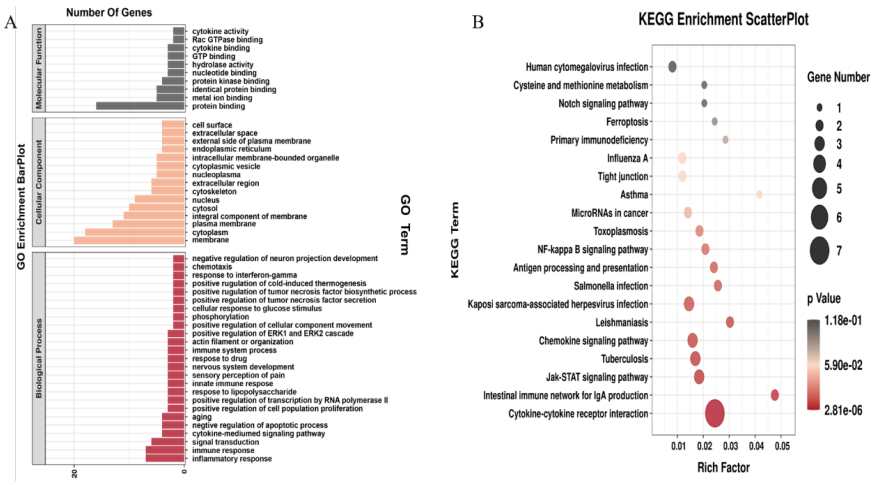

Fig 4 Functional enrichment of miRNA target genes. A. GO enrichment barplot. B. KEGG enrichment scatterplot.

Screening of miRNAs related to liver injury

E. multiloculari s enters the liver tissue along with the blood circulation, which induces an immune response in the liver and recruits a large number of inflammatory cells that release pro-inflammatory cytokines, and promotes the proliferation of fibroblasts. With the expansion of the lesion, immune tolerance appears in the liver, the expression of anti-inflammatory cytokines increases, the imbalance between fibrosis formation and degradation aggravates the degree of liver fibrosis, and the expression of collagen increases. Therefore, We screened 4 miRNAs related to liver injury(inflammatory response, immune response, fibrosis process)(Table 4). mmu-miR-30e-3p may target IL-1rn mRNA to participate in inflammatory response. mmu-miR-125b-5p may target was mRNA to regulate the immune response. mmu-miR-30c-2-3p may target prr5l gene to regulate the fibroblast migration. And mmu-miR-203-3p may target ctss mRNA to regulate the immune response.

Table 4 Liver injury-related miRNA expression

Number	miRNA name	miRNA sequence	mRNA	Target score
1	mmu-miR-30e-3p	CTTTCAGTCGGATGTTTACAC	IL-1rn	96
2	mmu-miR-203-3p	GTGAAATGTTTAGGACCACTAG	ctss	99
3	mmu-miR-125b-5p	TCCCTGAGACCCTAACTTGTA	Was	99
4	mmu-miR-30c-2-3p	CTGGGAGAAGGCTGTTTACTCT	Prr5l	97

Verification differentially expressed miRNAs

Sequencing results show the expression of mmu-miR-30e-3p, mmu-miR-203-3p, mmu-miR-125b-5p and mmu-miR-30c-2-3p were downregulation and the expression of IL-1rn, Ctss, Was and Prr5l were obviously up-regulation(Fig 5A). In order to evaluate the reliability of the sequencing results, the screened miRNAs and mRNAs were verified by RT-qPCR. The results showed that compared with the control group, the expression of mmu-miR-30e-3p, mmu-miR-203-3p, mmu-miR-125b-5p and mmu-miR-30c-2-3p in the liver of infected mice were significantly reduced, and the expression levels of IL-1rn, Ctss, Was and Prr5l were significantly increased(Fig 5B-C). The RT-qPCR results is consistent with the sequencing results.

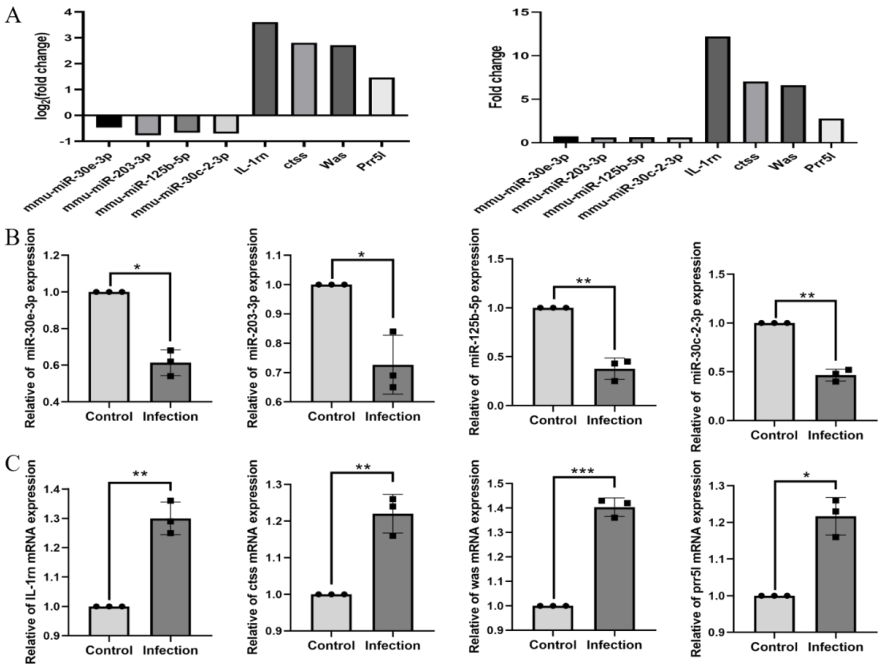

Fig 5 Validation of liver injury-related miRNAs and target genes by RT-qPCR. A.Change of log2(fold change) and fold change of differential expression gene. B.The level of liver injury-related miRNAs expression. C.The level of mRNA expression. $^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001.

Discussion

AE is a zoonotic parasitic disease that seriously endangers human health[20]. The study of *E. multilocularis* has always lacked stable experimental animal models. The traditional infection models are secondary infection mouse models constructed by intraperitoneal injection and liver puncture [21, 22]. Although it can reflect that the body is infected by *E. multilocularis* to a certain extent, the infection mode is far from the natural infection. During natural infection, humans become infected when they inadvertently ingest soil, water, or food contaminated with Echinococcus eggs excreted in dog feces. The eggs germinate in the small intestine to form hexacarcinoma, which can adhere and penetrate the intestinal wall, enter blood vessels, and reach and parasitize in the liver tissue along with the blood circulation[23, 24]. In recent years, Abuduaini Abulizi and his colleagues established a novel mouse model of infection by injecting *E. multilocularis* through the hepatic portal vein[25]. This model can more accurately simulate the natural infection process.

In this study, the infection mouse model was constructed by injecting*E. multiloculari* s through the hepatic portal vein. After 3 months of infection, B-ultrasound results found that the liver of the mice in the infection group had obvious lesions and *E. multiloculari* s antigen-specific antibodies in serum significantly increased, which indicates that the infection mouse model was successfully constructed. The results of HE and Masson staining showed that the infection of*E. multiloculari* s could cause liver damage, and a large number of collagen fibers were formed. In addition, the level of ALT and AST in serum were increased. These results suggested that *E. multiloculari* s infection can cause liver tissue damage, however, the mechanism of liver injury is still unclear. Thus, the present study explored the mechanism of liver injury from the perspective of miRNA by using RNA seq technique. Total 71 differential expression miRNAs were obtained and a total of 36 mouse miRNAs with |FC|>0.585 were screened out. Target gene prediction and functional enrichment of target genes were carried out on the 36 miRNAs screened out, the results showed that target gene were involved in many biologocal process, such as “inflammatory response”, “immune response” and “signal transduction”. KEGG analysis showed that a series of pathways(“Notch signaling pathway”, “ferroptosis”, “cysteine and methionine metablism”) were involved in liver disease, indicating that *E. multiloculari* s

induced the liver immunity reaction of hosts and activated signaling pathways related to immune response, such as Notch signaling pathway.

E. multiloculari s enters the liver tissue along with the blood circulation, the liver tissue will also generate immune response to resist infection and recruit a large number of inflammatory cells at the same time to release inflammatory cytokines, and finally play an anti-infection immune process. With the expansion of the parasite infection focus, the unbalanced between formation and degradation of fibrosis will aggravate the degree of liver fibrosis. Therefore, this study focuses on three aspects(inflammatory response, immune response and fibrosis formation) to search for miRNAs related to liver injury. In this study, we screened a total of four miRNAs and four miRNA target genes, which were mmu-miR-30e-3p and IL-1rn, mmu-miR-203-3p and ctss, mmu-miR-125b-5p and was, mmu-miR-30c-2-3p and prr5l. Wei Cong and colleagues suggested that mmu-miR-30e-3p were also differentially expressed in liver tissue from rats with acute *Toxoplasma gondii*infection[26], however, mmu-miR-30e-3p targeting IL-1rn regulates the inflammatory response caused by parasitic infections and no studies have been reported. Hu Y and his colleagues found that miR-203-3p may bind to vascular endothelial growth factor A (Vegfa)and participate in snhg8-mediated apoptosis and angiogenesis in AML cells[27]. Moreover, mmu-miR-203-3p identified as potential diagnostic biomarkers and indicators of Sjögren's Syndrome[28], however, there are no studies have been reported on mmu-miR-203-3p in liver injury caused by parasitic infections. mmu-miR-125b-5p is extensively involved in biological processes such as tua protein phosphorylation, cerebral ischemic tolerance and neuroregeneration[29]. In recent years, it has also been reported that mmu-miR-125b-5p was found to be downregulated in the peripheral blood of Trichinella-infected mice[30]. None of the four liver injury-associated miRNAs screened in this study have been studied in models of liver injury, and these miRNAs may serve as potential diagnostic targets for AE.

Materials and methods

Ethics

This study was conducted under the rules of the Guide for the Care and Use of Laboratory Animals and the Institutional Animal Use and Care Committee of Ningxia Medical University (approval number: KYLL-2021-765).

Animal

Female C57BL mice aged 6-8 weeks were purchased from the Experimental Animal Center of Ningxia Medical University. All mice were kept in a temperature-controlled, light-cycle room in animal facilities under specific pathogen-free conditions according to the national guidelines for animal care and use, with food and water ad libitum.

Isolation of protozoan and vitality identification

Protoscocras(PSCs) were obtained from E. multilocularis-infected gerbils. In briefly, E.multilocularis infected cyst tissue were isolated from gerbils and stripped components of mouse. PSCs were isolated immediately by pushing the cyst through a 300-mesh strainer in PBS buffer, and collected filtrate. The filtrate was naturaly settlemented for 5 minutes, discard the supernatant and wash 2 times with PBS. PSCs were stained with 1% eosin (Beyotime, China) to test vitality. If the vitality was over 95%, the PSCs could inject in mice.

Construction of infection mouse model

The infection mouse model was established by injecting PSCs via the hepatic portal vein. In briefly, The mice in infection group were injected with PSCs via portal vein and portal saline injection was defined as control group. A middle abdominal incision was made in mice under anaesthetic and 2000 PSCs/200μl sediment or saline was injected via portal vein by using a 0.45 × 15RWLB venous infusion needle. After injection, a cotton bud was pressed on the puncture site for 5 min to provide haemostasis and to prevent intraperitoneal spillage of the PSCs. The abdominal cavity was then closed and then the mice were put on the warm stage to promote waking.

RNA extraction

Liver tissue RNA was extracted by Trizol method. In brief, Approximately 50 mg of liver tissue was firstly placed in a mortar(Thermofisher, USA), ground into a powder with liquid nitrogen and transferred to an enzyme-free tube and 1ml TRIZOL(Invitrogen, USA) reagent was added to the homogenization to lyse sample for 5 minutes at room temperature in the dark. Then, the 200μl of chloroform per was added and shake tubes vigorously by hand for 15s. The sample was allowed to stand at room temperature for 3 minutes, and centrifuged at 12, 000 g for 10 min at 4°C. Pipette the supernatant into another new enzyme-free tube, 400 μl Isopropanol was added and shake tubes slightly by hands for 15s. The sample was allowed to stand at room temperature for 3 minutes, and centrifuged at 12, 000 g for 10 min at 4°C. Discard the supernatant, added 1ml 75% ethanol into tube and shake tube for 30s. centrifuged at 7, 500 g for 5 min at 4°C. Then, discard the supernatant and let the alcohol evaporate naturally. Finally, RNA pellet was redissolved with the 20μl Enzyme-free water. The RNA degradation and contamination were detected with 1% agarose gel test and RNA concentration was detected by NanoDrop Microvolume Spectrophotometers(Thermofisher, USA).

Small RNA library construction, sequence analysis, and identification of miRNAs

The experimental procedure mainly includes two parts: preparation of Library and sequencing experiment. Small RNA sequencing library preparation using TruSeq Small RNA Sample Prep Kits (Illumina, San Diego, USA). After library preparation, the libraries were sequenced using Illumina Hiseq2000/2500 with a single-end 1X50bp read length.

The miRNAs data analysis software is the self-developed ACGT101-miR (LC Sciences, Houston, Texas, USA). The analysis flow of the software is shown in Figure 1B. In brief, Remove 3' connectors and rubbish sequences to get clean data and screening of sequences with base length between 18-26nt. Then, the remaining sequences were compared to (without miRNA) mRNA, RFam and Repbase databases and filtered and obtaining valid data and comparing precursors and genomes for miRNAs identification. The miRNA identification base is linked to the miRBase and the genome of the species, and the degree of accuracy is highly correlated with the completeness of the database. Clean Data was used to identify small RNAs and calculate the miRNAs expression levels identified in each sample using ACGT101-miR. Expression was counted and used to assess the correlation of gene expression characteristics and differentially expressed miRNAs within and between groups of samples.

Target prediction of the differential miRNAs

Differential miRNAs target gene prediction was performed using both software A and B and target genes predicted by both software were screened according to scoring criteria. target genes with context score percentage less than 50 are removed in targetscan algorithm. Target genes with a maximum free energy (Max Energy) greater than -10 are removed in miRanda algorithm (i.e. the threshold is TargetScan score [?] 50, miranda_Energy $<$ - 10). Finally the intersection of these two software was taken as the final target gene of the differential miRNAs.

Functional annotation of the differential miRNAs

In order to analyze the biological function of differential miRNAs, the predicted miRNAs target genes were annotated using gene ontology enrichment (ftp://ftp.ncbi.nih.gov/gene/DATA/gene2go.gz) and Kyoto Encyclopedia of genes and genomes pathway analysis (http://www.genome.jp/kegg). The KEGG analysis provides information on the signal transduction and disease pathway of target genes. This information provides a basis for studying the function and involvement of differential miRNAs in pathways. GO is an internationally standardized gene function classification system. It includes three ontologies that describe the molecular functions of genes, cellular components and the biological processes involved.

HE

Hematoxylin and eosin (H&E) staining(Servicebio, China) was performed on thin slices that were embedded in a wax block of liver tissue. The whole dyeing process includes five contents: dewaxing, dyeing, dehydration, transparent and mounting. The overall characteristics of the tissue were observed under a low-power microscope, and then the images of representative areas were observed and collected.

Detection of ALT and AST in serum

The contents of AST and ALT in mouse serum were detected by an automatic biochemical analyzer(Thermofisher, USA).

Quantitative real-time PCR analysis

Following the manufacturer's instructions, total RNA was isolated from mouse liver using Trizol reagent (Invitrogen, USA). And then reverse-transcribed into cDNA using the qPCR RT kit(GeneCopoeia, USA),The cDNA was analyzed using real-time qPCR with a StepOnePlusTM Real-Time PCR System(Thermofisher, USA). Each reaction was performed in triplicate in a 96-well plate. The expression of each gene was normalized by the expression of GAPDH. miRNA and mRNA primers are listed in Table 1.

Table 1. The sequences of miRNA and mRNA primers

No	miRNAs/mRNAs	Forward primer	Reverse primer
1	mmu-miR-30e-3p	GGCTTTCAGTCGGATGTTTACAGC	GCGGTCGGACTACATCAT
2	mmu-miR-203-3p	CGCGTGAAATGTTTAGGACCACTAG	GCGGTCGGACTACATCAT
3	mmu-miR-125b-5p	TCCCTGAGACCCTAACTTGTGA	GCGGTCGGACTACATCAT
4	mmu-miR-30c-2-3p	CTGGGAGAAGGCTGTTTACTCT	GCGGTCGGACTACATCAT
5	IL-1Rn	CCCACCACCAGCTTTGAGTCAG	GGACGGTCAGCCTCTAGTGTTG
6	Was	CGAGGATGAAGAGGATGATGAATGG	ATGGCAGAGTGAGTGTGGAGAG
7	Prr5l	TGGTGAAGCAAGTGGTGTCTCC	CCGTGGTCATCAGAGAGGCATAG
8	Ctss	TTGGGGCCTTAACTTTGGTGATC	GCAATAACTAGCAATTCCGCAGTG
9	U6	CTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT
10	GAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC

Antibody testing in serum

According to the requirements of the reagent instructions, the antibodies in the serum were detected by Enzyme-linked immunosorbent assay(ELISA). In brief, 10 μg/ml Echinococcus multilocularis crude antigen was coated on the ELISA plate and placed at 4°C overnight. The plates were washed five times with PBST (0.05% Tween 20 PBS) and blocked with 5% skim milk powder in PBST at 37°C for 1 h. After washing five times with PBST, the plates were incubated with mouse serum (1:500) in 5% skim milk powder in PBST for 2 h and washed five times with PBST for 3 min. And one hundred microliters each of horseradish peroxidase (HRP)-conjugated anti-mouse IgM, IgG, IgA(abcam, USA) and IgE(Invitrogen, USA) were added enzyme plates and incubated at 37degC for 1 h. After washing, 100μl TMB Single-Component Substrate solution(Solarbio, China) was added for 8–10 min, and the reaction was stopped by 2 M H2SO4. The absorbance was measured at 450 nm using an ELISA reader (Thermo Fisher, USA).

Statistical analysis

Differential miRNAs expression levels were analyzed by One-way ANOVA test. For the sample data analysis of this experiment, the differential expression genes were screened by $P < 0.05$ as the threshold. The differentially expressed genes are defined as those with fold change[?]1 (upregulated) or < 1 (downregulated) between the groups.

Conclusion

miRNA is involved in the regulation of liver injury caused by Echinococcus multilocularis infection, and mmu-miR-30e-3p, mmu-miR-203-3p, mmu-miR-125b-5p and mmu-miR-30c-2-3p may serve as potential diagnostic targets for liver injury.

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Conflicts of Interest

The author declares no conflict of interest.

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