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Screening for microRNA-based diagnostic markers in hepatic alveolar echinococcosis

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Abstract

This study aims to screen differentially expressed host miRNAs that could be used as diagnostic markers for liver alveolar echinococcosis (LAE).

Differentially expressed miRNAs were first screened by miRNA microarray in liver tissues from2 LAE patients and normal liver tissues from 3 LAE patients, followed by qRT-PCR validation in 15 LAE tissues and 15 normal tissues. Target genes of differentially expressed miRNAs were predicted using Targetscan, PITA and microRNAorg database, and the overlapped predicted target genes were analyzed by GO and KEGG.

The hsa-miR-1237-3p, hsa-miR-33b-3p, and hsa-miR-483-3p were up-regulated whereas the hsa-miR-4306 was down-regulated in LAE tissues compared with normal controls ($P < .05$). The expression change of miR-483-3p was further confirmed in both liver tissues and plasma. Several predicted targets of miR-1237-3p, miR-4306, and miR-483-3p were related to DNA-dependent transcriptional regulation, developmental regulation of multicellular organisms, and biological functions such as cellular immune responses (T cell proliferation). The overlapped predicted target genes of the 4 differentially expressed miRNAs were enriched in mRNA surveillance, cancer signaling pathway, intestinal immune network, and other signal pathways.

Our results indicate that miR-483-3p is a potential marker for the diagnosis of LAE, and targets of this miRNA could be the focus of further studies.

Abbreviations: HNSCC = HPV-negative head and neck squamous cell carcinoma, HPV = human Papillomavirus, LAE = liver alveolar echinococcosis.

Keywords: *Echinococcus multilocularis*, GO and KEGG analysis, liver alveolar echinococcosis, miR-483-3p

1. Introduction

Liver alveolar echinococcosis (LAE) is a disease caused by larvae stage of *Echinococcus multilocularis (E multilocularis)* in humans. This disease is invasive and has tumor-like characteristics, which is called "bug cancer".[1,2] LAE has the characteristics of long incubation period, no obvious symptoms at the beginning of infection, and easy transfer in the later stage.[3] In China, it is mainly reported in Qinghai, Tibet, Xinjiang and other animal husbandry areas, of which Sanjiangyuan area in Qinghai Province is one of the most reported area.[2]

At present, the diagnosis of LAE mainly relies on traditional diagnostic methods such as clinical diagnosis, imaging diagnosis, and immunological diagnosis. However, these diagnostic methods have certain deficiencies, such as strong subjectivity and inconsistency among patients for clinical diagnosis,[4] and low sensitivity and specificity for imaging and immunological diagnosis.[5] The development of molecular technology in recent years greatly improved the early diagnosis of patients with LAE.[6]

MicroRNAs (miRNAs) are a class of endogenous non-coding RNA with the length of 22nt, which could regulate gene expression at post-transcriptional level by targeted cleavage of mRNA or repression of translation and participate in the regulation of several physiology and pathology processes.[7,8] For example, it is found that miR211 can promote cell proliferation, migration, and differentiation by targeting *ZFPM2* gene in human osteoblasts.[9] Several differentially expressed miRNAs are found during the growth of Hela cells.[10] Deep sequencing and miRNA microarray technology reveal that the expression of miRNAs in host cells or tissues are regulated during parasite infection and play an important role in host response to pathogens [Xu, 2013 #4118;Yadong, 2013 #4121]. Jin et al found that infection with *E multilocularis* disrupted the expression of 40% of genes involved in miRNA synthesis in

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mouse liver, including AGO1 and AGO2, and thus 46 differentially expressed miRNAs were found by deep sequencing analysis.[11] In the process of *E multilocularis* infection in humans, miRNAs also play important roles,[12] and some of these miRNAs participate in the immune and inflammatory responses to parasite infection.[13] Therefore, detection of miRNAs involved in pathogen-infection process could improve the sensitivity and specificity of early diagnosis for parasite.[14]

Circulating miRNAs are a class of molecule that are stably present in body fluids such as serum and plasma, and are stably detected in blood or body fluids of humans and animals infected with worms.[15–17] Circulating miRNAs have been used as potential biomarkers in various tumors, heart diseases, liver damage, and other diseases for early diagnosis, classification, and prognosis.[18–22] Circulating miRNAs have also been reported as biomarkers in parasitic infections.[23–25] For example, serum miR-146a and miR-223 were reported to be used as biomarkers for the diagnosis of sepsis, with high specificity and sensitivity.[26,27]

In this study, we used miRNA microarray to detect miRNA expression in patients with LAE. The significantly dysregulated miRNAs between LAE patients and normal controls were selected for further validation in the liver tissues and plasma of a larger cohort of patients. Our results will provide candidate for circulating miRNAs that could be used as biomarkers for LAE diagnosis.

2. Materials and methods

2.1. Patients

Two cases with LAE (1 male and 1 female with average age of 34.75) who were diagnosed between Jun, 2016 and May, 2018 at the Affiliated Hospital of Qinghai University, and 3 healthy controls (1 male and 2 female with average age of 41.5) were enrolled for microRNA microarray. A total of 15 cases of LAE patients (7 male and 8 female with average age of 46.1) who were diagnosed between, 2016 and May, 2018 at the Affiliated Hospital of Qinghai University, and 15 healthy controls (7 male and 8 female with average age of 44.6) were enrolled for validation. Prior written and informed consent were obtained from every patient and the study was approved by the ethics review board of the Hospital affiliated to Qinghai University.

2.2. Inclusion and exclusion criteria

All patients with LAE were first diagnosed and confirmed according to the diagnostic criteria by Expert consensus on diagnosis and treatment of liver echinococcosis (2015 edition). The patients were at early stage of LAE when the tissue samples were collected. Patients or normal controls with hepatitis B or tuberculosis or other liver diseases were excluded from this study.

2.3. Sample collection

A total of 0.5 to 250 mg normal or infected liver tissues were collected by resection, and stored in liquid nitrogen until analysis. EDTA-K_2 treated whole blood was collected from patients and healthy controls and plasma was isolated by centrifugation for 5 minutes at 1000 rpm at 4°C. The plasma was stored at −80°C until further analysis.

2.4. RNA extraction and microRNA microarray

miRNAs were isolated from liver tissues using mirVanaTM RNA Isolation Kit (AM1561, Thermo Fisher Scientific) according to the manufacturer's instructions. The miRNA was quantified using NanoDrop ND-2000 (Thermo Fisher Scientific) and the integrity of RNA was determined using Agilent 2100 Bioanalyzer (Agilent Technologies).

Agilent Human miRNA (8*60K, Design ID: 070156) microarrays were used. The RNA labeling and array hybridization were conducted using the miRNA Complete Labeling and Hyb Kit (Agilent technologies, 5190–0456). Slides were scanned by Agilent Scanner (#G2505C Agilent technologies).

Raw data were obtained by Agilent Feature Extraction software (version 10.7.1.1; Agilent Technologies), and the quantile algorithm was performed using Genespring software (version 12.5; Agilent Technologies) with default settings.[20] The total gene signal between groups was normalize to the 75th percentile signal intensity. After acquiring the raw data, the differently expressed miRNAs were calculated using the *t* test. Those with fold change ≥ 2 and P value $< .05$ were regarded as significantly different. Volcano plots and heat maps were generated in R software.

2.5. Target gene prediction and analysis

Targets of miRNAs were predicted using Targetscan (http://www.targetscan.org), miroRNAorg (http://www.microrna.org/) and PITA (https://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html). The function analysis was conducted using the GO analysis (http://www.geneontology.org). The pathway analysis was conducted using the KEGG (https://www.genome.jp/kegg/).

2.6. Quantitative real-time PCR

Total RNAs were isolated from liver tissues using Trizol Reagent (Thermo Fisher Scientific). Plasma miRNA was extracted using serum/plasma miRNA extraction and separation kit (#DP503, Tiangen Biotech, China). RNA was reverse transcribed using miRNA First Strand cDNA Synthesis (Tailing Reaction) into cDNA (Sangon Biotech Co. Ltd., China). Quantitative real-time PCR was performed using miRNA Fluorescence Quantitative PCR Kit (Dye Method) (Sangon Biotech Co. Ltd., China) on an ABI 7500 Real-time PCR system (Thermo Fisher Scientific). Primers used were as follows: hsa-miR-1237–3p forward 5'-agtccttctgctccgtc -3'; hsa-miR-483-3p forward 5'- gcagtcactcctctcct -3'; hsa-miR-4306 forward 5'- cgcagtggagagaaagg -3'. Reverse primer was universal primer from Sangon (Shanghai, China). Primers for cel-miR-39 were purchased from Tiangen Biotech (Beijing) Co. Ltd. The relative expression levels were evaluated using the $2^{-\Delta\Delta Ct}$ method. The expression of cel-miR-39 was used as internal control.

2.7. Statistical analysis

Data analysis was carried out using the SPSS 19.0 (IBM Corp, Chicago, IL, USA) and expressed as mean ± SD. Differences

Table 1

MiRNAs with significantly different expression ($P < .05$) in livers between 2 cases of LAE and 3 healthy controls.

miRNAs	*P* value	Fold change	Case/control	mirbase accession No
hsa-miR-1237-3p	.015545277	10.927992	up	MIMAT0005592
hsa-miR-1246	.00933136	1.3349183	up	MIMAT0005898
hsa-miR-33b-3p	.025067944	8.267294	up	MIMAT0004811
hsa-miR-3620-5p	.011374874	1.5686951	up	MIMAT0022967
hsa-miR-4306	.014589614	41.6154	down	MIMAT0016858
hsa-miR-483-3p	.044761777	8.867646	up	MIMAT0002173
hsa-miR-6739-5p	.043787543	1.6227034	down	MIMAT0027379
hsa-miR-8063	.021557998	1.3592225	up	MIMAT0030990
hsa-miR-940	.01566861	1.3666642	up	MIMAT0004983

LAE = liver alveolar echinococcosis.

between groups were evaluated for significance using independent sample T test. $P < .05$ was considered as statistically significant.

3. Results

3.1. Screening for differentially expressed miRNAs

To identify miRNAs that may play important roles in LAE, we investigated the differentially expressed miRNAs in LAE liver tissues of 2 patients with LAE and normal liver tissues from 3 controls. As shown in Table 1 and Figure 1A, there were 9 miRNAs that were differently expressed between the 2 groups (|Fold change|>1.3, $P < .05$ & coefficient of variation%<12%) among the 2549 investigated miRNAs. In these 9 miRNAs, we further focused on miRNAs with |log2(Fold change)|>1, which were deemed as differentially expressed miRNA. As a result, we detected the upregulation of hsa-miR-1237-3p (log2 (fold change) = 3.4), hsa-miR-33b-3p (log2 (fold change) = 3.03) and hsa-miR-483-3p (log2 (fold change) = 3.13), and the downregulation of hsa-miR-4306 (log2 (fold change) = −5.73) in LAE tissues compared with normal tissues (Fig. 1A). Unsupervised clustering of these 4 miRNAs showed the obvious differences of their expression between LAE tissues and controls (Fig. 1B). These results suggest that these 4 miRNAs of hsa-miR-1237-3p, hsa-miR-33b-3p, hsa-miR-483-3p, and hsa-miR-4306 were significantly differentially expressed between LAE and control tissues and that they may play a role in the development of LAE.

3.2. Validation of miRNA expression

To confirm the expression changes of these miRNAs, 3 miRNAs with higher fold changes were selected for validation by qRT-PCR in a larger set of samples. Compared with normal tissues, only miR-483-3p showed significant upregulation in LAE tissues ($P < .01$), whereas no significant changes were found for miR-1237-3p and miR-4306 (Fig. 2A). Expression of miR-483-3p was further investigated in plasma, in which similar results were obtained ($P < .01$; Fig. 2B). These data indicate that miR-483-3p may be a potential diagnostic marker for the early detection of LAE.

3.3. Target gene prediction for miRNAs

We first predicted the target genes of the differentially expressed miRNAs using 3 database of Targetscan (http://www.targetscan.

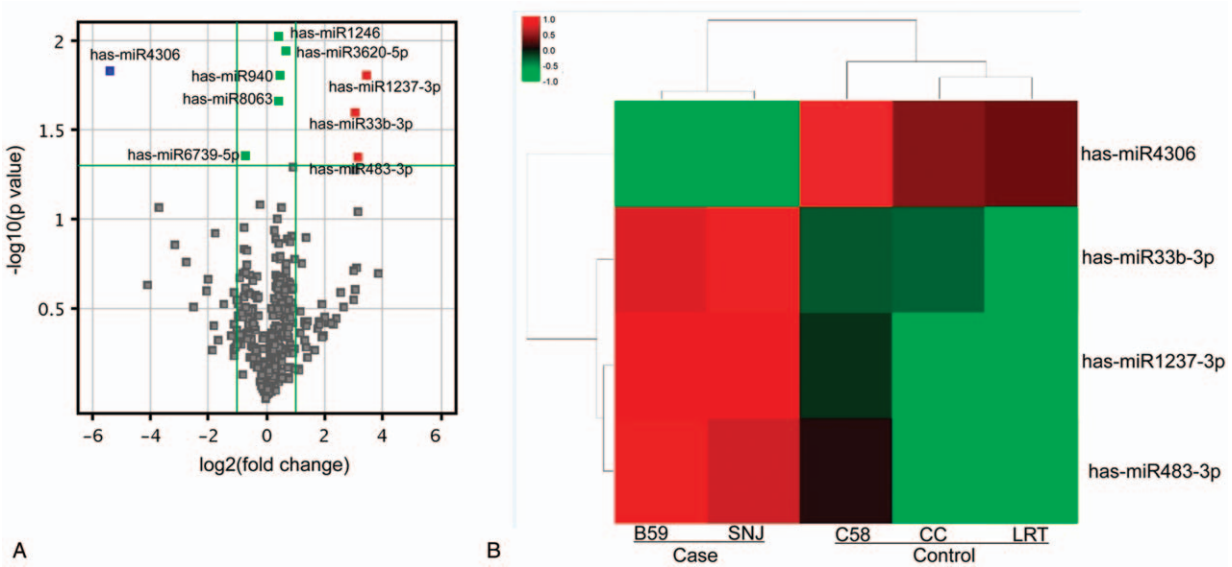

Figure 1. Differential expression of miRNAs in patients with LAE and healthy controls. Microarray of normal liver tissues from 3 healthy controls or *E. multilocularis* infected liver tissues from 2 cases of LAE. (A) Volcanic plot of the differentially expressed miRNAs. Gray dots, $P \geq .05$; green dots, |log2(FC)|<2 and $P < .05$; red dots, |log2(FC)| ≥2 and $P < .05$. (B) Unsupervised clustering of the 4 significantly differenced miRNAs between cases and controls.

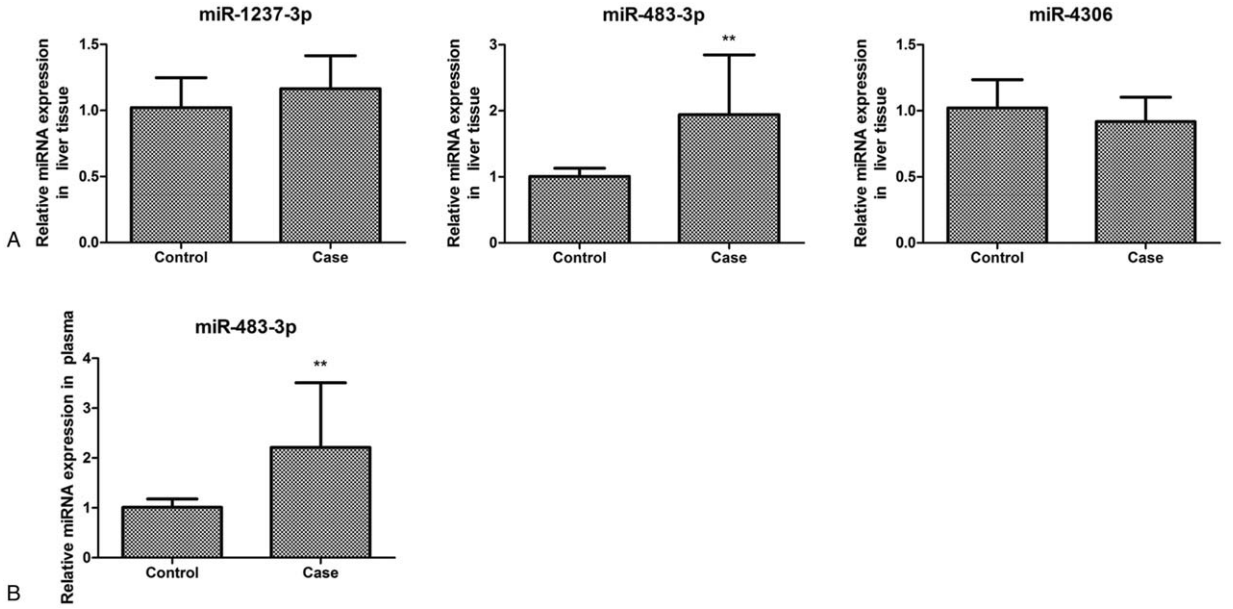

Figure 2. Real time PCR analysis of miRNAs. Real time PCR was used to validate miRNA expression in liver tissues and plasma. (A) Expression of miR-1237-3p, miR-483-3p, and miR-4306 in normal liver tissues from 60 healthy controls and from 47 cases of LAE (case). (B) Expression of miR-483-3p in plasma from 60 healthy controls (control) and 47 cases of LAE (case). $^{**}P < .01$, independent sample *t* test.

org), miroRNAorg (http://www.microrna.org/), and PITA (https://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html). Different numbers of predicted target genes were obtained (Table 2). A total of 137 common target genes were obtained by overlapping the results from the 3 databases (Fig. 3). The target genes of miR-1237-3p, miR-4306, and miR-483-3p included genes related to immune responses and liver lesion.[28–30]

3.4. GO analysis of predicted target genes

We next performed GO analysis to analyze the functions of predicted target genes. As shown in Figure 4, the target genes of differentially expressed miRNAs were mainly involved in biological processes such as DNA-dependent transcriptional regulation, developmental regulation of multicellular organisms, and cellular immune responses (T cell proliferation). Their molecular function was enriched in proteins such as sequence -specific DNA binding protein, ubiquitin-binding enzyme, and reverse transporters. They also enriched in cell components related to mRNA cleavage factor complex and vesicle membrane. The results of GO analysis provided a set of genes for subsequent studies on LAE.

3.5. KEGG analysis of predicted target genes

The pathway analysis of the predicted target gene was performed using the KEGG database. In the KEGG mapping of the differentially expressed miRNA target genes, the top 20 entries were displayed. These target genes were enriched in signaling pathways such as mRNA surveillance pathways, cancer signaling pathways, and intestinal immune networks (Fig. 5).

4. Discussion

In this study, we compared the host miRNA expression in the infected liver tissues and normal tissues from LAE patients using

Table 2

Target gene prediction for the 4 differentially expressed miRNAs.

	Number			
miRNAs	**PITA**	**Targetscan**	**microRNAorg**	**LAE related genes (gene ID)**
hsa-miR-1237-3p	462	3210	2962	CES1 (1066) IL5 (3567) IL17A (3605) CD40LG (959)
hsa-miR-33b-3p	N/A	1727	1910	N/A
hsa-miR-4306	163	N/A	2143	TAP2 (6891) TNF (7124) CD40LG (959)
hsa-miR-483-3p	N/A	4955	3292	LBR (3930)

Gene ID was accessible from www.ncbi.nlm.nih.gov. CD40LG = CD40 ligand, CES1 = Carboxylesterase 1, IL17 = Interleukin 17A, IL5 = Interleukin 5, LAE = liver alveolar echinococcosis, LBR = Lamin-B receptor, TAP2 = Antigen peptide transporter 2, TNF = tumor necrosis factor.

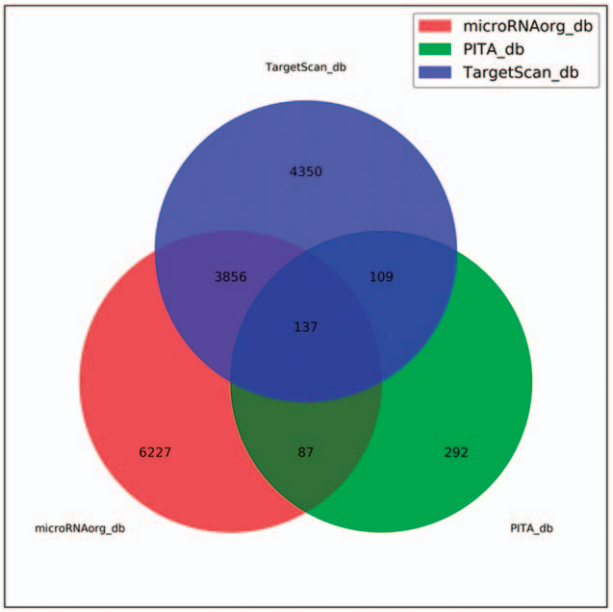

Figure 3. Venn graph showing the prediction results of miR-1237-3p, miR-483-3p, miR-4306, and miR-33b-3p targets in PITA, Targetscan, and microRNAorg softwares.

miRNA microarray. Among the 2549 detected miRNAs, a total of 4 miRNAs were differentially expressed, of which 3 (miR-1237-3p, miR-33b-3p, and miR-483-3p) were significantly upregulated and 1 (miR-4306) were significantly downregulated in LAE tissues. Consistent with the microarray data, qRT-PCR results showed that the expression level of miR-483-3p was significantly elevated in LAE tissues.

All of the 4 differentially expressed miRNAs were reported to be related with human diseases. For example, decreased expression of miR-1237-3p is associated with poor survival in patients with chordoma.[31] It is predicted that miR-33b-3p can regulate metabolic processes in arthritic patients and serum miR-33b-3p expression could be used as a potential biomarker for assessing the risk and progression of arthritis.[32] In hepatic carcinoma cells, miR-145-5p, and miR-483-3p have dual effect on cell proliferation dependent on the concentration of glucose.[33] Hsa-miR-4306 is an important marker of disease recurrence and survival in human Papillomavirus (HPV)-negative head and neck squamous cell carcinoma (HNSCC) patients.[34] Here, to the best of our knowledge, this is the first study on host miRNA expression changes upon *E multilocularis* infection in Qinghai, China. The differential expressions of these 4 miRNAs indicate that these miRNAs may play important roles in the process of *E multilocularis* infection.

Circulating miRNAs are a class of molecules that are stably present in body fluids such as serum and plasma, which are involved in the transmission of information among cells.[35,36] Cell-free circulating miRNAs are usually present in ribonucleoprotein complexes or high-density lipoproteins, or released from liposomes, exosomes, or apoptotic bodies.[36] Therefore, circulating miRNAs can reflect the body's homeostasis and disease progression. Since circulating miRNAs are stable and resistant to endogenous RNase digestion, they are appropriate biomarkers for the diagnosis and prognosis of diseases and clinical applications of circulating miRNAs are present in areas such

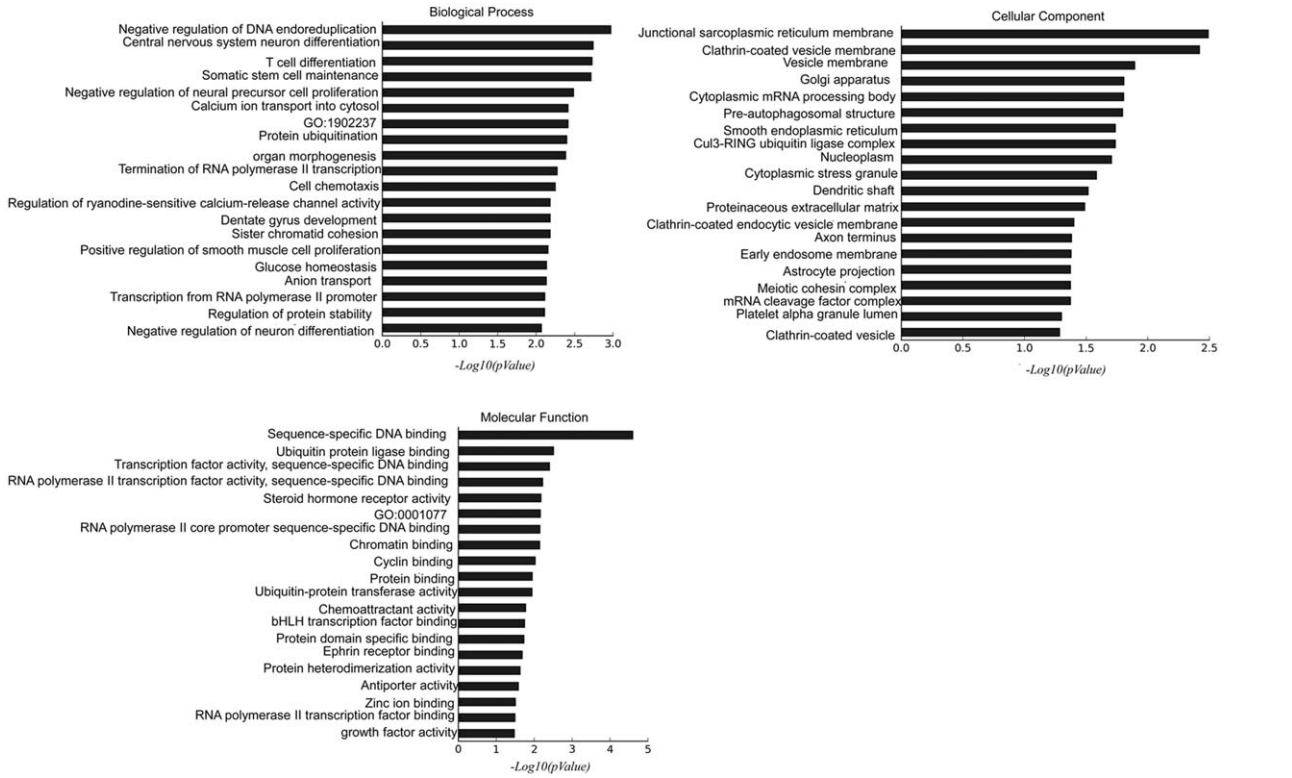

Figure 4. GO analysis of the predicted target genes for miR-1237-3p, miR-483-3p, miR-4306, and miR-33b-3p.

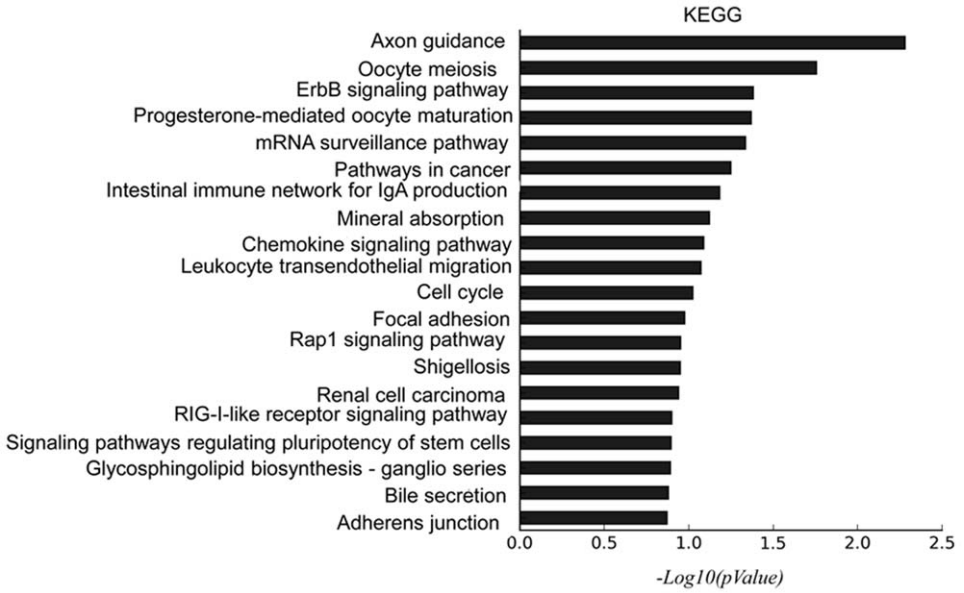

Figure 5. KEGG pathway analysis of the predicted target genes for miR-1237-3p, miR-483-3p, miR-4306, and miR-33b-3p. The top 20 terms were shown.

as cancer, diabetes, and neurological disorders.[27,37–39] However, studies on circulation miRNAs in LAE progression is just emerging, which prompts us to find a circulating miRNA as possible diagnostic marker for LAE. We found significantly increased expression of miR-483-3p in the plasma of LAE patients, indicating that miR-483-3p may be used as a diagnostic marker for echinococcosis.

Roles of hsa-miR-483-3p have been reported in wound healing and cancer progression.[40–42] It is an important regulator of endothelial integrity in patients with type 2 diabetes, and is a potential treatment target for rescue endothelial regeneration.[40–43] Hsa-miR-483-3p also plays a negative regulatory role in diabetes-related heart disease.[44] In summary, expression of miR-483-3p is dysregulated in several diseases and plays a role in regulating disease progression.

A previous study in *E multilocularis*-infected mice found that several targets of *E multilocularis*-responsive miRNAs were associated with the host immune responses.[45] In this study, 137 predicted target genes were found for the 4 differentially expressed miRNAs when predicted by TargetScan, PITA and microRNAorg database. The predicted targets of miR-1237-3p, miR-4306, and miR-483-3p are involved in host immune responses and hepatic echinococcosis. For instance, CD40LG (IgM) and antigen peptide transporter 2 (TAP2), the predicted targets of miR-4306, are correlated with hepatic echinococcosis infection.[46,47] The mean concentrations of IgG, IgA, and IgM in patients with alveolar hydatidosis and the mean levels of IgG and IgM in patients with cystic hydatid disease were significantly higher than those in the normal control group,[47] while the polymorphism of the *TAP2* gene is related to cystic hydatidosis.[46] IL17A and IL5 were predicted targets of miR-1237-3p, and these 2 genes were reported to be downregulated after cystic echinococcosis infection, leading to significantly reduced severity of OVA-induced airway inflammation.[48] As the predicted target of miR-483-3p, lamin B receptor is a multi-transmembrane protein of nuclear membrane that is often used as a "reporter" of nuclear membrane dynamics.[49] Lamin B receptor is associated with primary biliary cirrhosis.[50]

GO analysis of the overlapped target genes showed that these genes are related to biological processes such as cellular immune response, which in line with the finding that infection of *E multilocularis* can elicit immune responses in patients.[47] KEGG analysis indicated that these genes were enriched in signaling pathways such as mRNA surveillance pathways, cancer signaling pathways, and intestinal immune networks. These signaling pathways may have implications for the prevention and treatment of LAE in the future.

This study has some limitations. First, the sample size was relatively small. Second, only 1 miRNA was identified as the potential marker for the diagnosis of LAE and thus the number was limited. Further studies are warranted.

To sum up, based on the differentially expressed miRNAs identified by liver tissue miRNA microarray, we found that plasma miR-483-3p is significantly differentially expressed between normal and LAE liver tissues, which indicates that miR-483-3p is a potential circulating marker for LAE. Our study thus provides new approach for the clinical diagnosis of LAE.

Author contributions

Data curation: Haining Fan.
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Funding acquisition: Yi Lv.
Methodology: Ying Zhou, Haining Fan.
Project administration: Yi Lv.
Resources: Haijiu Wang, Cairang Yangdan.
Software: Ying Zhou.
Writing – original draft: Bin Ren, Haijiu Wang, Li Ren, Cairang Yangdan.
Writing – review & editing: Yi Lv.

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