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Original Article

Serum microRNAs as non-invasive diagnostic biomarkers for intrahepatic cholestasis of pregnancy

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Abstract: Objectives: Intrahepatic cholestasis of pregnancy (IHCP) causes itching, preterm birth, and stillbirth. However, there is no accurate diagnostic method for IHCP. Currently, circulating microRNAs (miRNAs) have become candidate biomarkers for the diagnosis of multiple diseases. Here, we investigated the diagnostic value of miRNAs in IHCP and aimed to predict the molecular mechanism of IHCP pathogenesis. Methods: We analyzed differentially expressed miRNAs in both women with IHCP and normal pregnant women. The selected candidate miRNAs were validated in 46 IHCP cases and 46 normal pregnant subjects, and we constructed receiver operator characteristic curves of miRNAs. Pearson correlations between levels of total bile acid (TBA) and differentially expressed miRNAs were also calculated. In addition, we clustered functionally significant biological pathways using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. Results: The expression levels of 13 miRNAs were remarkably upregulated while the other 35 miRNAs were significantly downregulated, in women with IHCP ($P \leq 0.05$) when compared with healthy pregnant women. The areas under the curves of miRNA-7706, miRNA-877-3p, and miRNA-128-3p were higher than 0.90, indicating more reliable diagnosis of IHCP. The Pearson analysis showed that the levels of these miRNAs were positively correlated to TBA level. Additionally, the results of bioinformatics analysis revealed that the differentially expressed miRNAs mainly influenced fatty acid biosynthesis, the endoplasmic reticulum ubiquitin ligase complex, and the p53, and mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK) signaling pathways. Conclusion: The panel of three-miRNAs (miRNA-7706, miRNA-877-3p, and miRNA-128-3p) may be a useful noninvasive diagnostic biomarker of IHCP.

Keywords: miRNAs, microarray analysis, diagnostic biomarkers, bioinformatics analysis, intrahepatic cholestasis of pregnancy

Introduction

Intrahepatic cholestasis of pregnancy (IHCP), a pregnancy-specific liver abnormality that occurs most often during the third trimester, is characterized by pruritus and high level of liver total bile acid (TBA) [1]. The incidence of IHCP ranges from 1%-15% globally [2]. The maternal prognosis is usually benign, but there is a clear association between IHCP and the elevated risk of adverse perinatal outcomes such as preterm birth, fetal distress, and stillbirth [3, 4]. Although genetic, endocrinological, nutritional, and environmental factors have been implicated in IHCP, the etiology and diagnostic criteria of this condition are yet to be fully understood [5].

An accurate diagnosis plays an essential role in the treatment of IHCP. Currently, the diagnosis of IHCP is based on elevated serum TBA levels and symptoms of pruritus and jaundice [6]. Therefore, TBA is often used as a diagnostic marker for IHCP. However, its levels can fluctuate with fasting state or gestational age [7, 8], new prognostic and diagnostic biomarkers for IHCP are still urgently needed.

MicroRNAs (miRNAs) are a large family of small, noncoding RNAs of about 21-22 nucleotides and are regulators of essential biological processes [9], including cell apoptosis, proliferation, inflammation, and metabolism [10-12]. miRNAs are known to control approximately 30% of human genes that encode proteins [13] and inhibit target gene expression mainly

through translational inhibition or degradation of messenger RNAs (mRNAs) [14]. Previous studies have shown that changes in the expression of circulating miRNAs are associated with numerous diseases, such as hepatic carcinoma [15], stomach cancer [16], lung cancer [17], cholestasis [18], and alcoholic liver disease [19]. Currently, miRNAs in peripheral blood have been utilized as biomarkers for the diagnoses of acute and chronic liver disorders [20-22]. miRNAs have also been shown to reflect the physiological state of pregnancy and can be used as prognostic and diagnostic biomarkers to indicate complications in pregnancy such as preeclampsia and gestational diabetes mellitus [23, 24]. Thus, miRNAs may be further developed as important diagnostic indicators of IHCP.

In this study, we measured the expression levels of various miRNAs in patients with IHCP and healthy pregnant women and found miRNAs that were either remarkably upregulated or significantly downregulated. We have identified a panel of miRNAs that may be a useful noninvasive diagnostic biomarker for IHCP. The differentially expressed miRNAs identified by bioinformatics analysis have been found to be associated with fatty acid biosynthesis, the endoplasmic reticulum ubiquitin ligase complex, p53, mammalian target of rapamycin (mTOR), and the AMP-activated protein kinase (AMPK) signaling pathways, which will further our understanding and thus, increase the diagnostic and therapeutic value of such miRNAs for IHCP.

Materials and methods

Sample collection: patients and healthy controls

We conducted a case-control study to measure maternal peripheral blood miRNA levels indicative of an IHCP diagnosis. Peripheral blood specimens were drawn from women with IHCP (n=46) and control subjects (n=46) by researchers in the Department of Obstetrics and Gynecology, Tongji Hospital, Huazhong University of Science and Technology. All participants were Chinese women with singleton pregnancies. This research was approved by the Human Ethics Committee of the same institute (No. TJ-IRB20210520). Informed consent was obtained from all participants.

The inclusion criteria for IHCP were [25, 26]: (1) pruritus and jaundice; (2) elevated levels of aminotransferases, bilirubin, and TBA; and (3) normalization of cholestasis after delivery. The exclusion criteria were [27]: (1) presence of skin diseases or chronic liver and gall bladder diseases; (2) abnormal liver and kidney function, gestational diabetes, pregnancy-induced hypertension syndrome, or hematological diseases; and (3) pregnancy with preeclampsia, uterine fibroids, abnormal uterine development, placental adhesion or placenta accreta, placenta previa, or placental abruption. Only healthy pregnant women with normal coagulation and cognitive function were enrolled in the control group [26]. Peripheral blood specimens were collected in PAXgene tubes (PreAnalytiX GmbH, Feldbachstrasse, Hombrechtikon, Switzerland) for testing. Blood specimens were kept overnight at 4°C and stored until further analysis at -80°C.

RNA extraction

Peripheral blood specimens were thawed at 25°C for 2 h. Then, total RNA and miRNAs were isolated and purified using the PAXgene Blood miRNA kit (PreAnalytiX GmbH), following the kit's protocols. Quantification of purified miRNAs was conducted using the miScript® PCR system (Takara, Dalian, China). The yields and integrity of total RNA were examined using the Agilent High Sensitivity Reagents kit (Agilent Technologies, Santa Clara, CA, USA), employing the Agilent 2100 Bioanalyzer.

MiRNA expression profiling

The Qubit™ dsDNA HS Assay kit (Invitrogen, Carlsbad, California, USA, Q32854) was used for cDNA synthesis. MiRNA expression profiling was conducted using the Agilent miRNA array, designed to detect 2,705 miRNA sequences. Data normalization and quality control were performed using data collection software (Illumina). Data preprocessing was based on the default 90th percentile-normalization method. Benjamini-Hochberg-adjusted *P* values of 0.05 and threshold values of less than or equal to 2 and greater than 2-fold were used to identify differentially expressed miRNAs.

Quantitative, real-time PCR (qRT-PCR) assays

Nine candidate miRNAs (miRNA-7706, miRNA-877-3p, miRNA-128-3p, miRNA-1306-5p, miR-

Table 1. Sequences of miRNAs based on the miRNA sequences obtained from the miRBase database

miRNA	Species	miRBase ID	Sequence (5' to 3')
miR-7706	Human	MI0025242	UGAAGCGCCUGUGCUCUGCCGAGA
miR-877-3p	Human	MI0005561	UCCUCUUCUCCCUCCUCCCAG
miR-1306-5p	Human	MI0006443	CCACCUCCCCUGCAAACGUCCA
miR-128-3p	Human	MI0000447	UCACAGUGAACCGGUCUCUUU
miR-30c-5p	Human	MI0000736	UGUAAACAUCCUACACUCUCAGC
miR-3613-5p	Human	MI0016003	UGUUGUACUUUUUUUUUUGUUC
miR-379-5p	Human	MI0000787	UGGUAGACUAUGGAACGUAGG
miR-4772-5p	Human	MI0017414	UGAUCAGGCAAAAUUGCAGACU
miR-204-5p	Human	MI0000284	UUCCCUUUGUCAUCCUAUGCCU

Table 2. Main clinical characteristics of patients with ICP and healthy pregnant women

Variable	Control (N=46)	ICP (N=46)	*P* value
Maternal age, years	31.91±3.21	29.83±3.41	0.254
Gestational week	38.09±2.17	37.98±2.87	0.213
TBA, μmol/L	3.02±2.19	38.34±34.20*	0.014
TBIL, μmol/L	4.68±2.03	10.72±8.58*	0.061
DBIL, μmol/L	2.17±0.83	8.22±8.16*	0.031
AST, IU/L	16.73±2.69	151.08±166.20*	0.024
ALT, IU/L	9.34±1.86	225.04±196.28*	0.006
ALP, IU/L	93.45±6.55	203.58±85.83*	0.003
GGT, IU/L	11.82±3.43	49.00±29.99*	0.003

TBA: total bile acid; TBIL: total bilirubin; DBIL: direct bilirubin; AST: aspartate aminotransferase; ALT: alanine transaminase; ALP: alkaline phosphatase; GGT: glutamyltransferase. *P<0.05 vs. control.

NA-30c-5p, miRNA-3613-5p, miRNA-379-5p, miRNA-4772-5p, and miRNA-204-5p) were selected for miRNA expression profiling, and U6 was used as an internal control. The sequences of these miRNAs were chosen according to the miRNA sequences retrieved from the miRbase database (**Table 1**).

To confirm the candidate miRNAs detected by the microarrays, SYBR Green-based qRT-PCR was employed to analyze the relative miRNA expression. The RNA template was reverse transcribed, and the cDNA product was used for qRT-PCR. Using an miRNA-X-miRNA-qRT-PCR-TB-Green kit (Takara, Dalian, China), miRNA was polyadenylated using poly(A) polymerase and subsequently reverse transcribed into cDNA. Next, qRT-PCR analysis of the target genes was conducted using SYBR Green (Takara), following the kit protocol. PCR was initiated using a denaturing step at 37°C for 1 h and 87°C for 5 min, followed by 40 cycles of annealing at 95°C for 5 s and at 60°C for 20 s.

Functional and pathway analyses

The Gene Ontology (GO) database (http://www.geneontology.org/) was used to analyze the molecular functions of the differentially expressed miRNA genes ($P \leq 0.05$). Meanwhile, the relevant signaling pathway of each target gene was analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.kegg.jp/). The Fisher's exact test was applied to determine the *P* value for each pathway. A *P* value of less than or equal to 0.05 was deemed statistically significant.

Statistical analysis

Statistical tests were conducted with a one-way analysis of variance followed by Tukey's test using GraphPad Prism 8. An independent sample *t*-test was employed to compare the characteristics of healthy women and those with IHCP in this study. We constructed receiver operator characteristic (ROC) curves of miRNAs from peripheral blood (with 95% confidence intervals) to assess the diagnostic value of biomarkers of IHCP. Pearson correlations between serum TBA levels and differentially expressed miRNA levels were also calculated. All values are shown as the mean ± standard deviation. A *P* value of less than or equal to 0.05 was deemed statistically significant.

Results

Clinical features of women with IHCP and normal pregnant women

Table 2 summarizes the clinical features of women with IHCP and normal pregnant women. Maternal age and gestational week did not remarkably differ between the groups ($P > 0.05$). Serum levels of TBA, direct bilirubin (DBIL), total bilirubin (TBIL), gamma-glutamyltransferase (GGT), alkaline phosphatase (ALP), alanine transaminase (ALT), and aspartate ami-

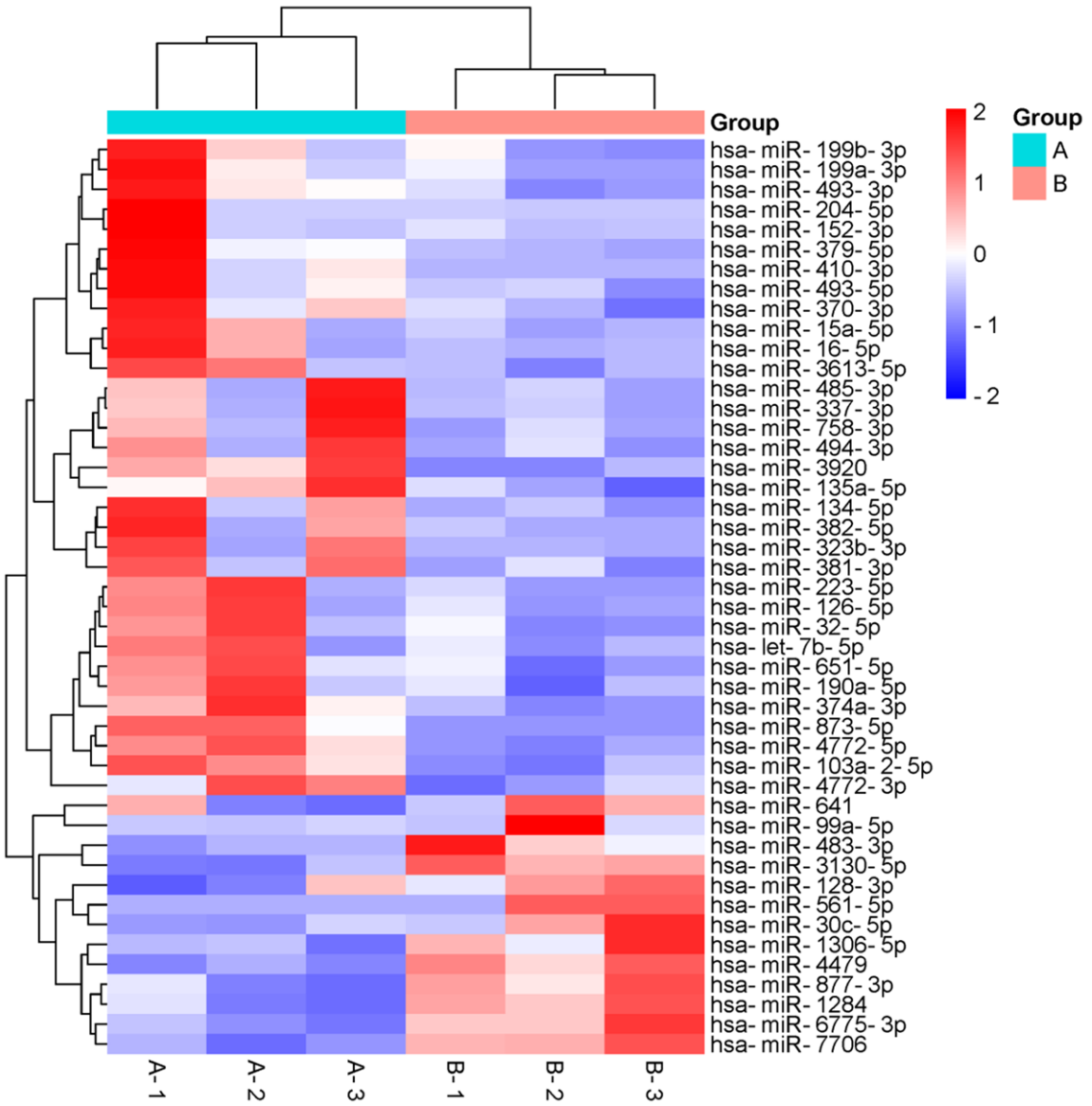

Figure 1. Heatmap of miRNA expression in patients with IHCP compared to controls. The heatmap shows the expression of 48 miRNAs that demonstrated significant differences between patients with IHCP and the control group. Compared to the control group, patients with IHCP were clustered based on the upregulation and downregulation of 13 and 35 miRNAs, respectively ($P \leq 0.05$). Each column represents an individual sample; columns A1-A3 represent the controls, and columns B1-B3 represent patients with IHCP. Each row corresponds to different miRNAs. The color scale illustrates the miRNA levels: blue, low expression; red, high expression.

notransferase (AST) were significantly elevated in women with IHCP compared to normal controls ($P<0.05$).

Differentially expressed miRNAs between women with IHCP and normal pregnant women

To determine differential miRNA expression in peripheral blood samples of women with IHCP and normal pregnant women, we employed miRNA array technology (Agilent) and determined the expression profiles of 2,705 mature human miRNAs. Of these, 13 miRNAs were remarkably upregulated ($P \leq 0.05$), including miRNA-7706, miRNA-877-3p, miRNA-128-3p, miRNA-1284, miRNA-1306-5p, miRNA-4479, miRNA-483-3p, miRNA-99a-5p, miRNA-561-5p, and miRNA-30c-5p, and 35 were downregulated ($P \leq 0.05$), including miRNA-3613-5p, miRNA-379-5p, miRNA-4772-5p, and miRNA-204-5p (**Figure 1**).

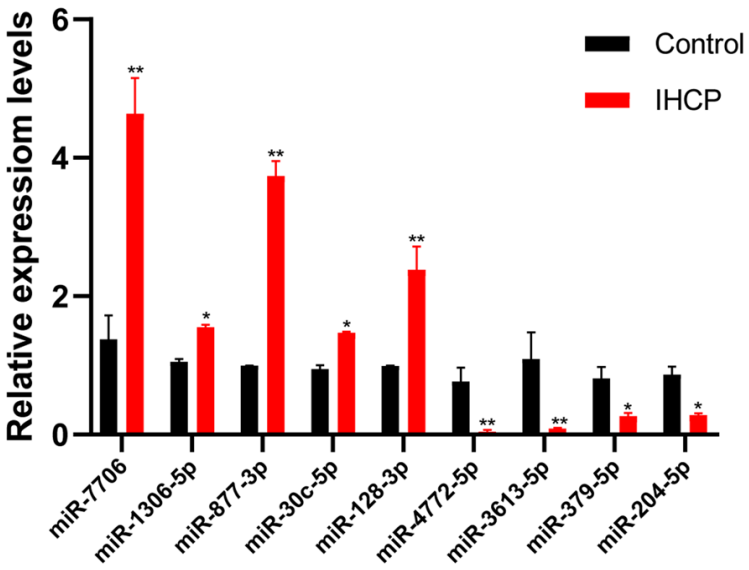

Figure 2. Differential expression of selected miRNAs in women with IHCP and healthy pregnant women. Expression of each miRNA was verified using qRT-PCR in 92 (46 control and 46 IHCP) peripheral blood specimens. $^{*}P<0.05$ vs. control, $^{**}P<0.01$ vs. control.

Verification of differential miRNA expression profiles for an IHCP diagnosis

To determine their potential as biomarkers in patients with IHCP, nine candidate miRNAs (five upregulated: miRNA-7706, miRNA-877-3p, miRNA-128-3p, miRNA-1306-5p, and miRNA-30c-5p; four downregulated: miRNA-3613-5p, miRNA-379-5p, miRNA-4772-5p, and miRNA-204-5p) were selected for further validation by qRT-PCR. U6 served as the normalization control. We found that all selected miRNAs could be detected in the collected samples, and the differentially expressed miRNAs between the IHCP and control groups were significantly different ($P\leq0.05$). The nine differentially expressed miRNAs between the IHCP and control groups were shown in **Figure 2**. The results showed that miRNA-7706, miRNA-877-3p, miRNA-128-3p, miRNA-1306-5p, and miRNA-30c-5p were markedly upregulated ($P\leq0.05$), whereas miRNA-3613-5p, miRNA-379-5p, miRNA-4772-5p, and miRNA-204-5p were significantly downregulated ($P\leq0.05$) in women with IHCP compared to normal pregnant women.

MiRNA-7706, miRNA-877-3p, and miRNA-128-3p could be used as biomarkers to predict the risk of IHCP

To assess the diagnostic values of differentially expressed miRNAs in patients with IHCP, we constructed ROC curves and analyzed the areas under the curves (AUCs) of miRNA-7706, miRNA-877-3p, miRNA-128-3p, miRNA-1306-5p, and miRNA-30c-5p. As shown in **Figure 3**, the AUCs were 1.00, 0.99, 0.97, 0.86, and 0.85 for miRNA-7706 (**Figure 3A**), miRNA-877-3p (**Figure 3B**), miRNA-128-3p (**Figure 3C**), miRNA-1306-5p (**Figure 3D**), and miRNA-30c-5p (**Figure 3E**), respectively. The AUCs of miRNA-7706, miRNA-877-3p, and miRNA-128-3p were higher than 0.90, indicating that these three miRNAs had sufficiently diagnostic value in predicting the risk of IHCP. The diagnostic values of miRNA-7706, miRNA-877-3p, and miRNA-128-3p at cut-off points of 1.00, 0.98, and 0.85, respectively, were assessed. The specificities of miRNA-7706, miRNA-877-3p, and miRNA-128-3p were 0.74, 0.77, and 0.76, respectively, while the sensitivities of miRNA-7706, miRNA-877-3p, and miRNA-128-3p were 0.74, 0.74, and 0.72, respectively. These results indicate that these miRNAs had sufficiently diagnostic value in predicting the risk of IHCP. Therefore, we explored the correlation of miRNA-7706, miRNA-877-3p, and miRNA-128-3p with clinical parameters and further investigated the potential value of these miRNAs in the pathogenesis of IHCP.

Correlation between miRNA-7706, miRNA-877-3p, and miRNA-128-3p and clinical parameters in women with IHCP

To examine the correlation between miRNAs and clinical parameters, we performed a correlation analysis between miRNA-7706, miRNA-877-3p, and miRNA-128-3p expression and TBA levels. The expression of these miRNAs positively correlated with TBA levels (r=0.20, P=0.17; r=0.83, P<0.001; and r=0.43, P=0.003 for miRNA-7706, miRNA-877-3p, and miRNA-128-3p, respectively) (**Figure 4**). Our findings suggest that these miRNAs may be potential biomarkers of an IHCP diagnosis.

Functional and pathway analyses

To further evaluate the functions of the identified miRNAs, we clustered functionally signifi-

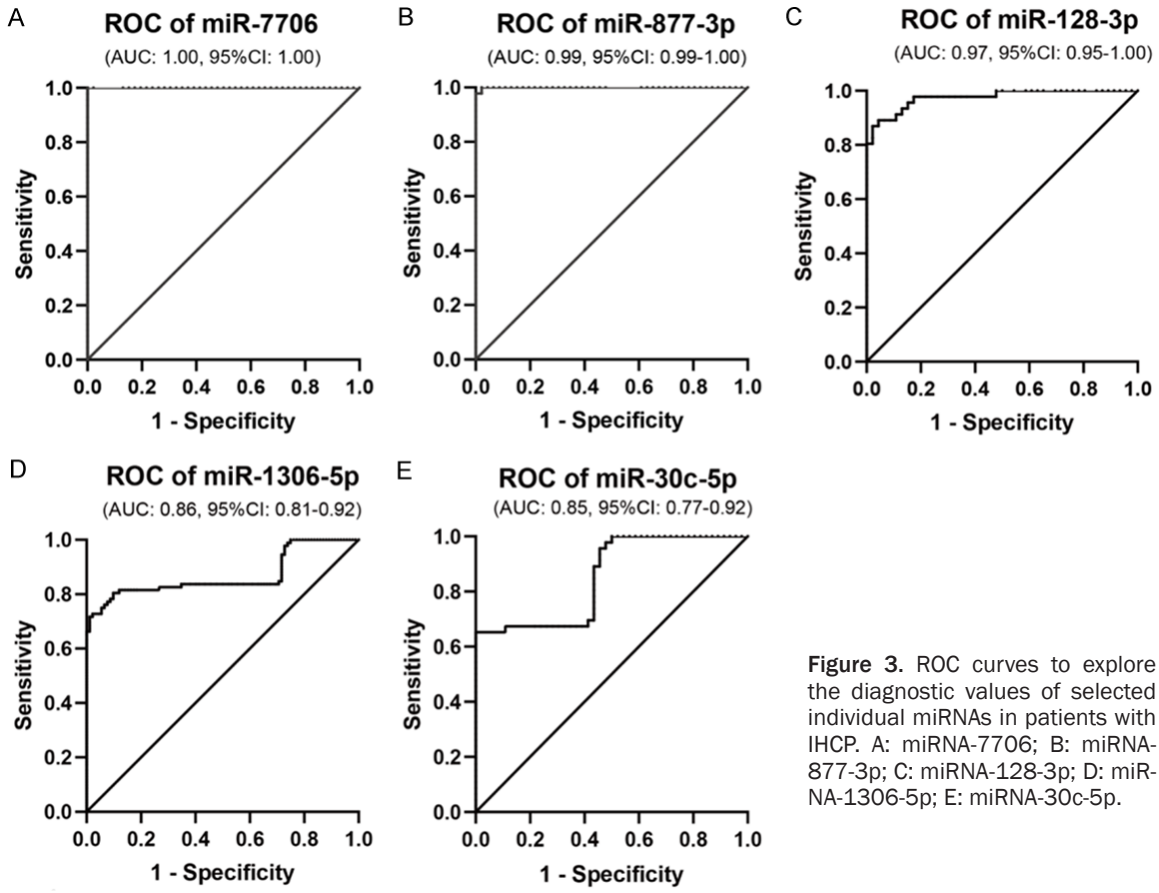

Figure 3. ROC curves to explore the diagnostic values of selected individual miRNAs in patients with IHCP. A: miRNA-7706; B: miRNA-877-3p; C: miRNA-128-3p; D: miRNA-1306-5p; E: miRNA-30c-5p.

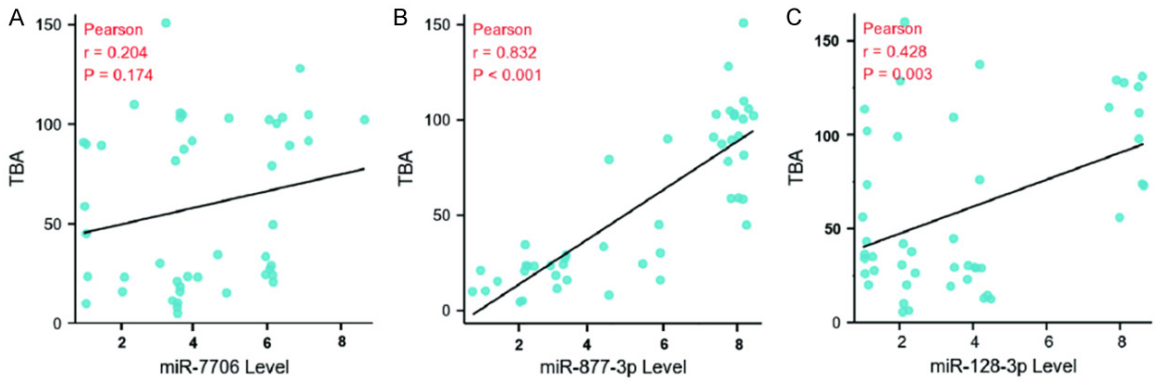

Figure 4. Correlations between peripheral blood miRNA levels and TBA levels in patients with IHCP. A: miRNA-7706; B: miRNA-877-3p; C: miRNA-128-3p.

cant biological pathways using GO and KEGG pathway analyses. The GO enrichment terms associated with miRNAs in women with IHCP were determined. As shown in **Figure 5A** and **5B**, the target genes were enriched in various biological processes such as "biological regulation", "cellular component organization or biogenesis", "cellular process", "immune system process", "localization", "metabolic process", and "positive regulation of gene silencing by miRNA". The target genes also appeared responsible for the formation of cellular components, such as "ER ubiquitin ligase complex", "cell junction", "membrane", "membrane-enclosed lumen", and "X chromosome". Several molecular functions such as "antioxidant activ-

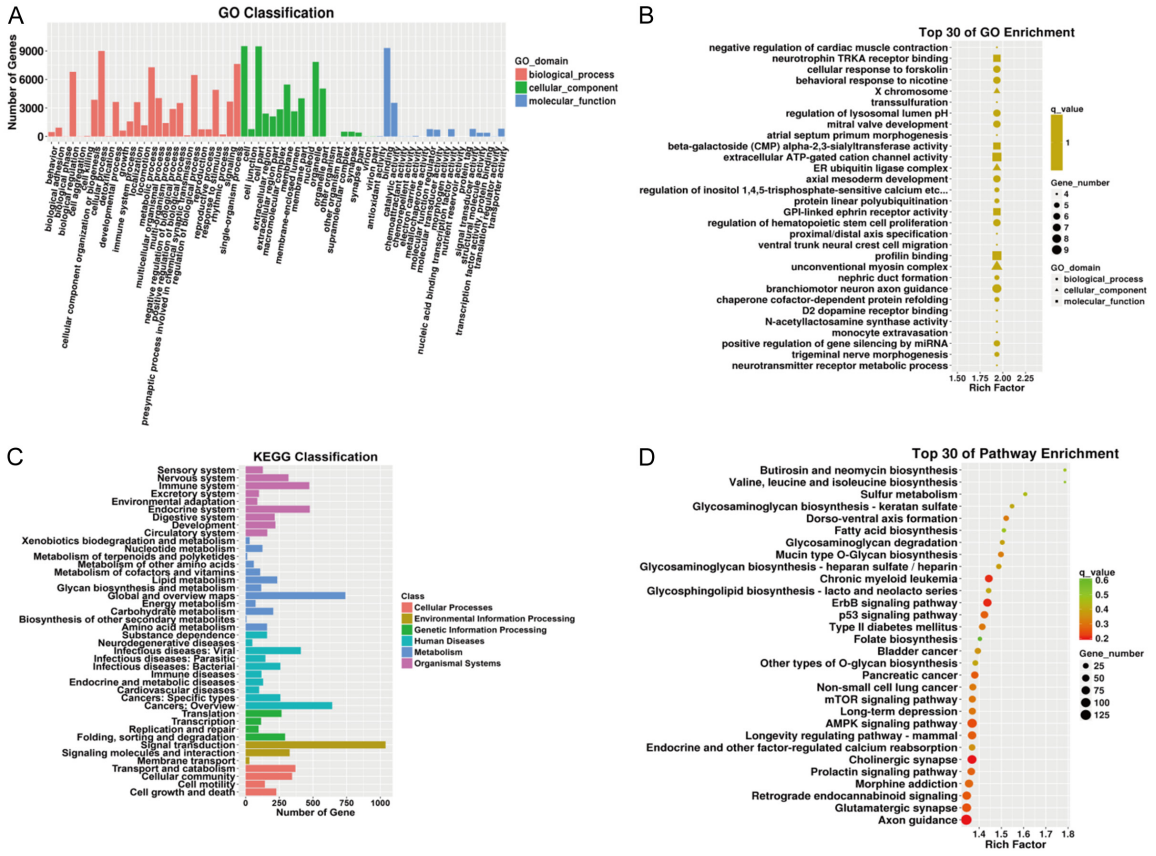

Figure 5. Functional and pathway analyses of differentially expressed miRNAs in women with IHCP. A: GO enrichment classification of differentially expressed miRNAs in women with IHCP, including cellular components (green), molecular functions (blue), and biological processes (red); B: KEGG pathways of differentially expressed miRNAs in patients with IHCP; C and D: Top 30 significantly enriched GO terms and KEGG pathways, respectively.

ity", "transporter activity", "protein tag", "structural molecule activity", "extracellular ATP-gated cation channel activity", "beta-galactoside alpha-2,3-sialyltransferase activity", "GPI-linked ephrin receptor activity", and "profilin binding" were also identified as possibly regulated by these miRNAs.

The signaling pathways modulated by these miRNAs were also evaluated using the KEGG database. The differentially expressed miRNAs participated in fatty acid biosynthesis, endoplasmic reticulum ubiquitin ligase complex, p53 signaling pathway, and mTOR and AMPK signaling pathways (**Figure 5C** and **5D**).

Discussion

In our study, we demonstrated that the expression levels of miRNA-7706, miRNA-877-3p, miRNA-128-3p, miRNA-1306-5p, and miRNA-30c-5p were increased in women with IHCP. Among them, strong correlations were found between levels of miRNA-7706, miRNA-877-3p, miRNA-128-3p, and TBA in women with IHCP, suggesting that these three miRNAs may be of certain clinical relevance as noninvasive biomarkers of IHCP.

IHCP is a pregnancy-specific disease with an incidence of 0.2%-5.6%, affecting 0.1%-2% of pregnant women [28, 29]. IHCP may cause adverse perinatal outcomes, including fetal asphyxia and preterm labor [30, 31]. Thus, an early and accurate diagnosis of IHCP is essential. However, the pathogenesis of IHCP and its relationship with the concentrations of biochemical markers remain unknown [32]. Currently, diagnosis of IHCP is based on symptoms of pruritus and elevated levels of TBA, ALT, ASL, and ALP levels. TBA is the most frequently used diagnostic marker for IHCP. However, TBA levels can fluctuate, depending on

fasting state or gestational age [33]. Thus, it is important to identify more specific and sensitive diagnostic markers to enhance the diagnosis for women with IHCP.

MiRNAs play a vital role in post-transcriptional target gene regulation by targeting the 3' untranslated region of mRNAs to reduce protein translation and/or increase mRNA degradation s [34, 35]. Many studies have reported miRNAs as novel molecules that could be used as diagnostic, prognostic, and therapeutic biomarkers [36]. Currently, miRNA profiling studies for liver disease are gaining considerable attention [37-39]. A study of patients with primary biliary cholangitis (PBC) showed that miRNA-505-3p and miRNA-197-3p were differentially expressed compared to healthy controls [40]. Sakamoto et al. reported that the elevation of let-7 miRNA and miRNA-520a-5p can be used as biomarkers in patients with refractory PBC [38]. Fernandez-Ramos et al. showed that miRNA-873-5p expression significantly increases in patients with primary sclerosing cholangitis [39]. Zhao and co-workers found that miRNA-132, miRNA-222, and miRNA-29a expression was significantly decreased in women with gestational diabetes mellitus [41]. Therefore, after using miRNA arrays to obtain miRNA profiles of healthy women and those with IHCP, validation of selected miRNAs using qRT-PCR to identify miRNA biomarkers could potentially be used to diagnose IHCP.

In this study, we observed that 48 miRNAs were differentially expressed in women with IHCP compared with the control group (**Figure 1**). Among candidate miRNAs, miRNA-7706, miRNA-877-3p, miRNA-128-3p, miRNA-1306-5p, and miRNA-30c-5p were significantly increased in women with IHCP. Moreover, the AUCs of miRNA-7706, miRNA-877-3p, and miRNA-128-3p were 1.00, 0.99, and 0.97, respectively (**Figure 3**), and Pearson correlations revealed that these three miRNAs were strongly correlated with TBA level (**Figure 4**), indicating that the expression levels of these miRNAs might provide reliable diagnostic value for IHCP. However, the molecular mechanisms by which these miRNAs are differentially regulated during IHCP pathogenesis have not been fully elucidated. The possible contributing factors may include environmental, nutritional, endocrinological, and genetic factors. Most studies have found that estrogen levels in women with IHCP are significantly increased, which may be the main reason for its pathogenesis. Besides, estrogen plays a critical role in regulating (either suppressing or stimulating) miRNA expression [42]. There are reports that the estrogen receptor regulates miRNAs via binding its promoter in an estrogen-dependent manner, which include upregulation and downregulation of miRNAs, such as miRNA-17-92, miRNA-106a-363, and miRNA-125b [42, 43]. Therefore, it is necessary to conduct further studies on the regulatory mechanism of differentially expressed miRNAs.

Results of a bioinformatics analysis revealed that differentially expressed miRNAs may be involved in localization, metabolism, the endoplasmic reticulum ubiquitin ligase complex, and the p53, mTOR, and AMPK signaling pathways in women with IHCP (**Figure 5**). Studies have shown that the p53, mTOR, and AMPK signaling pathways are involved in the pathogenesis of cholestasis [44-46], including bile acid homeostasis and the regulation of hepatic polarity, inflammation, oxidative stress, and fibrosis. Previous studies have reported that the activation of AMPK signaling disrupts bile acid homeostasis and promotes cholestasis [47]. Further, AMPK is responsible for the modulation of liver polarity, inflammation, and fibrosis in cholestasis and can affect the pathogenesis of cholestatic liver injury. Chen et al. revealed the promising role of p53 in modulating bile acid disposition-related genes via the enzymes and transporters responsible for bile acid metabolism [48]. Other studies reported that p53 can promote apoptosis during cholestatic liver injury [49, 50]. These differentially expressed miRNAs may regulate the aforementioned signaling pathways and participate in the pathogenetic process of IHCP. Thus, our study provides novel insights into the pathophysiology of IHCP and potential targets for its treatment.

To summarize, we identified significantly altered and specifically regulated miRNAs in women with IHCP compared with healthy controls. Our results suggest that miRNA-7706, miRNA-877-3p, and miRNA-128-3p may serve as a panel of potential biomarkers for the diagnosis of IHCP. The functional roles of these three miRNAs remain unclear. Due to the limitation of in

the present study (suah as small sample size), further studies are needed to corroborate our findings.

Appendix A. supplementary data

The original datas of miRNA expression profiling on the Supplementary File.

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Disclosure of conflict of interest

None.

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References

[1] Walker KF, Chappell LC, Hague WM, Middleton P and Thornton JG. Pharmacological interventions for treating intrahepatic cholestasis of pregnancy. Cochrane Database Syst Rev 2020; 7: CD000493.

[2] Lee RH, Goodwin TM, Greenspoon J and Incerpi M. The prevalence of intrahepatic cholestasis of pregnancy in a primarily Latina Los Angeles population. J Perinatol 2006; 26: 527-32.

[3] Aydin GA, Ozgen G and Gorukmez O. The role of genetic mutations in intrahepatic cholestasis of pregnancy. Taiwan J Obstet Gynecol 2020; 59: 706-710.

[4] Jurate K, Rimantas Z, Jolanta S, Vladas G and Limas K. Sensitivity and specificity of biochemical tests for diagnosis of intrahepatic cholestasis of pregnancy. Ann Hepatol 2017; 16: 569-573.

[5] Alemdaroglu S, Yilmaz BS, Durdag GD, Yuksel SS, Yetkinel S, Alkas YD, Kalayci H and Simsek E. Intrahepatic cholestasis of pregnancy: are in vitro fertilization pregnancies at risk? J Matern Fetal Neonatal Med 2021; 34: 2548-2553.

[6] Wood AM, Livingston EG, Hughes BL and Kuller JA. Intrahepatic cholestasis of pregnancy: a review of diagnosis and management. Obstet Gynecol Surv 2018; 73: 103-109.

[7] Chen J, Deng W, Wang J, Shao Y, Ou M and Ding M. Primary bile acids as potential biomarkers for the clinical grading of intrahepatic cholestasis of pregnancy. Int J Gynaecol Obstet 2013; 122: 5-8.

[8] Yule CS, Holcomb DS, Kraus AC, Brown CEL, McIntire DD and Nelson DB. Cholestasis: a prospective study of perinatal outcomes and time to symptom improvement. Am J Perinatol 2021; 38: 414-420.

[9] Chen L, Heikkinen L, Wang C, Yang Y, Sun H and Wong G. Trends in the development of miRNA bioinformatics tools. Brief Bioinform 2019; 20: 1836-1852.

[10] Zhang T, Zou P, Wang T, Xiang J, Cheng J, Chen D and Zhou J. Down-regulation of miR-320 associated with cancer progression and cell apoptosis via targeting Mcl-1 in cervical cancer. Tumour Biol 2016; 37: 8931-40.

[11] Fu X, Dong B, Tian Y, Lefebvre P, Meng Z, Wang X, Pattou FO, Han W, Wang X, Lou F, Jove R, Staels B, Moore DD and Huang W. MicroRNA-26a regulates insulin sensitivity and metabolism of glucose and lipids. J Clin Invest 2015; 125: 2497-509.

[12] Chen R, Li H, Cai J, Wang C, Lin Z, Liu C, Niu Y, Zhao Z, Li W and Kan H. Fine particulate air pollution and the expression of microRNAs and circulating cytokines relevant to inflammation, coagulation, and vasoconstriction. Environ Health Perspect 2018; 126: 017007.

[13] Wu ZB, Li WQ, Lin SJ, Wang CD, Cai L, Lu JL, Chen YX, Su ZP, Shang HB, Yang WL and Zhao WG. MicroRNA expression profile of bromocriptine-resistant prolactinomas. Mol Cell Endocrinol 2014; 395: 10-18.

[14] Correia de Sousa M, Gjorgjieva M, Dolicka D, Sobolewski C and Foti M. Deciphering miRNAs' action through miRNA editing. Int J Mol Sci 2019; 20: 6249.

[15] Li LM, Hu ZB, Zhou ZX, Chen X, Liu FY, Zhang JF, Shen HB, Zhang CY and Zen K. Serum microRNA profiles serve as novel biomarkers for HBV infection and diagnosis of HBV-positive hepatocarcinoma. Cancer Res 2010; 70: 9798-9807.

[16] Zhu C, Ren C, Han J, Ding Y, Du J, Dai N, Dai J, Ma H, Hu Z, Shen H, Xu Y and Jin G. A five-microRNA panel in plasma was identified as potential biomarker for early detection of gastric cancer. Br J Cancer 2014; 110: 2291-2299.

[17] Hu Z, Chen X, Zhao Y, Tian T, Jin G, Shu Y, Chen Y, Xu L, Zen K, Zhang C and Shen H. Serum microRNA signatures identified in a genome-wide serum microRNA expression profiling predict survival of non-small-cell lung cancer. J Clin Oncol 2010; 28: 1721-6.

[18] Kagawa T, Shirai Y, Oda S and Yokoi T. Identification of specific MicroRNA biomarkers in early stages of hepatocellular injury, cholestasis, and steatosis in rats. Toxicol Sci 2018; 166: 228-239.

[19] Beyoglu D and Idle JR. Metabolomic and lipidomic biomarkers for premalignant liver disease diagnosis and therapy. Metabolites 2020; 10: 50.
[20] Cai C, Lin Y and Yu C. Circulating miRNAs as novel diagnostic biomarkers in nonalcoholic fatty liver disease: a systematic review and meta-analysis. Can J Gastroenterol Hepatol 2019; 2019: 2096161.
[21] Krauskopf J, Caiment F, Claessen SM, Johnson KJ, Warner RL, Schomaker SJ, Burt DA, Aubrecht J and Kleinjans JC. Application of high-throughput sequencing to circulating microRNAs reveals novel biomarkers for drug-induced liver injury. Toxicol Sci 2015; 143: 268-276.
[22] Dubin PH, Yuan H, Devine RK, Hynan LS, Jain MK and Lee WM. Micro-RNA-122 levels in acute liver failure and chronic hepatitis C. J Med Virol 2014; 86: 1507-1514.
[23] Zou P, Luo L, Zhao C, Chen Z, Dong R, Li N, Wang Y, Wang J, Wang T, Chen M, Zhang T and Chen D. The serum microRNA profile of intrahepatic cholestasis of pregnancy: identification of novel noninvasive biomarkers. Cell Physiol Biochem 2018; 51: 1480-1488.
[24] Jiang PY, Zhu XJ, Jiang RA, Zhang YN, Liu L and Yang XF. MicroRNAs derived from urinary exosomes act as novel biomarkers in the diagnosis of intrahepatic cholestasis of pregnancy. Am J Transl Res 2019; 11: 6249-6261.
[25] Brown MA, Magee LA, Kenny LC, Karumanchi SA, Mccarthy FP, Saito S, Hall DR, Warren CE, Adoyi G and Ishaku S. The hypertensive disorders of pregnancy: ISSHP classification, diagnosis & management recommendations for international practice. Pregnancy Hypertens 2018; 13: 291-310.
[26] Joutsiniemi T, Timonen S, Leino R, Palo P and Ekblad U. Ursodeoxycholic acid in the treatment of intrahepatic cholestasis of pregnancy: a randomized controlled trial. Arch Gynecol Obstet 2014; 289: 541-547.
[27] Hague WM, Callaway L, Chambers J, Chappell L, Coat S, de Haan-Jebbink J, Dekker M, Dixon P, Dodd J, Fuller M, Gordijn S, Graham D, Heikinheimo O, Hennessy A, Kaaja R, Khong TY, Lampio L, Louise J, Makris A, Markus C, Marschall HU, Middleton P, Mol BW, Morris J, Newnham JP, Ovadia C, Peek M, Shand A, Stark M, Thornton J, Timonen S, Walker S, Warrilow D and Williamson C. A multi-centre, open label, randomised, parallel-group, superiority trial to compare the efficacy of URsodeoxycholic acid with RIFampicin in the management of women with severe early onset intrahepatic cholestasis of pregnancy: the TURRIFIC randomised trial. BMC Pregnancy Childbirth 2021; 21: 51.
[28] Williamson C and Geenes V. Intrahepatic cholestasis of pregnancy. Obstet Gynecol 2014; 124: 120-133.
[29] Fleminger J, Seed PT, Smith A, Juszczak E, Dixon PH, Chambers J, Dorling J, Williamson C, Thornton JG and Chappell LC. Ursodeoxycholic acid in intrahepatic cholestasis of pregnancy: a secondary analysis of the PITCHES trial. BJOG 2021; 128: 1066-1075.
[30] Bicocca MJ, Sperling JD and Chauhan SP. Intrahepatic cholestasis of pregnancy: review of six national and regional guidelines. Eur J Obstet Gynecol Reprod Biol 2018; 231: 180-187.
[31] Mor M, Shmueli A, Krispin E, Bardin R, Sneh-Arbib O, Braun M, Arbib N and Hadar E. Intrahepatic cholestasis of pregnancy as a risk factor for preeclampsia. Arch Gynecol Obstet 2020; 301: 655-664.
[32] Ovadia C, Seed PT, Sklavounos A, Geenes V, Di Ilio C, Chambers J, Kohari K, Bacq Y, Bozkurt N, Brun-Furrer R, Bull L, Estiu MC, Grymowicz M, Gunaydin B, Hague WM, Haslinger C, Hu Y, Kawakita T, Kebapcilar AG, Kebapcilar L, Kondrackiene J, Koster M, Kowalska-Kanka A, Kupcinskas L, Lee RH, Locatelli A, Macias R, Marschall HU, Oudijk MA, Raz Y, Rimon E, Shan D, Shao Y, Tribe R, Tripodi V, Yayla Abide C, Yenidede I, Thornton JG, Chappell LC and Williamson C. Association of adverse perinatal outcomes of intrahepatic cholestasis of pregnancy with biochemical markers: results of aggregate and individual patient data meta-analyses. Lancet 2019; 393: 899-909.
[33] Huang WM, Gowda M and Donnelly JG. Bile acid ratio in diagnosis of intrahepatic cholestasis of pregnancy. Am J Perinatol 2009; 26: 291-294.
[34] Fu X and Dong D. Bioinformatic analysis of MicroRNA sequencing data. Methods Mol Biol 2018; 1751: 109-125.
[35] Bushati N and Cohen SM. MicroRNA functions. Annu Rev Cell Dev Biol 2007; 23: 175-205.
[36] Mirzaei H, Momeni F, Saadatpour L, Sahebkar A, Goodarzi M, Masoudifar A, Kouhpayeh S, Salehi H, Mirzaei HR and Jaafari MR. MicroRNA: relevance to stroke diagnosis, prognosis, and therapy. J Cell Physiol 2018; 233: 856-865.
[37] Tadokoro T, Morishita A and Masaki T. Diagnosis and therapeutic management of liver fibrosis by MicroRNA. Int J Mol Sci 2021; 22: 8139.
[38] Sakamoto T, Morishita A, Nomura T, Tani J, Miyoshi H, Yoneyama H, Iwama H, Himoto T and Masaki T. Identification of microRNA profiles associated with refractory primary biliary cirrhosis. Mol Med Rep 2016; 14: 3350-3356.
[39] Fernandez-Ramos D, Fernandez-Tussy P, Lopitz-Otsoa F, Gutierrez-De-Juan V, Navasa N, Barbier-Torres L, Zubiete-Franco I, Simon J,

Fernandez AF, Arbelaiz A, Aransay AM, Lavin JL, Beraza N, Perugorria MJ, Banales JM, Villa E, Fraga MF, Anguita J, Avila MA, Berasain C, Iruzibieta P, Crespo J, Lu SC, Varela-Rey M, Mato JM, Delgado TC and Martinez-Chantar ML. MiR-873-5p acts as an epigenetic regulator in early stages of liver fibrosis and cirrhosis. Cell Death Dis 2018; 9: 958.

[40] Ninomiya M, Kondo Y, Funayama R, Nagashima T, Kogure T, Kakazu E, Kimura O, Ueno Y, Nakayama K and Shimosegawa T. Distinct microRNAs expression profile in primary biliary cirrhosis and evaluation of miR 505-3p and miR197-3p as novel biomarkers. PLoS One 2013; 8: e66086.

[41] Zhao C, Dong J, Jiang T, Shi Z, Yu B, Zhu Y, Chen D, Xu J, Huo R, Dai J, Xia Y, Pan S, Hu Z and Sha J. Early second-trimester serum miRNA profiling predicts gestational diabetes mellitus. PLoS One 2011; 6: e23925.

[42] Castellano L, Giamas G, Jacob J, Coombes RC, Lucchesi W, Thiruchelvam P, Barton G, Jiao LR, Wait R, Waxman J, Hannon GJ and Stebbing J. The estrogen receptor-alpha-induced microRNA signature regulates itself and its transcriptional response. Proc Natl Acad Sci U S A 2009; 106: 15732-15737.

[43] Zhang ZC, Liu Y, Xiao LL, Li SF, Jiang JH, Zhao Y, Qian SW, Tang QQ and Li X. Upregulation of miR-125b by estrogen protects against non-alcoholic fatty liver in female mice. J Hepatol 2015; 63: 1466-1475.

[44] Chao S, Xiaojun L, Haizhen W, Ludi F, Shaozhen L, Zhiwen S, Weiliang H, Chunhong J, Ying W, Fan W and Yunfei G. Lithocholic acid activates mTOR signaling inducing endoplasmic reticulum stress in placenta during intrahepatic cholestasis of pregnancy. Life Sci 2019; 218: 300-307.

[45] Bridle KR, Sobbe AL, De Guzman CE, Santrampurwala N, Jaskowski LA, Clouston AD, Campbell CM, Nathan Subramaniam V and Crawford DH. Lack of efficacy of mTOR inhibitors and ACE pathway inhibitors as antifibrotic agents in evolving and established fibrosis in $Mdr2^{-/-}$ mice. Liver Int 2015; 35: 1451-1463.

[46] Li X, Yuan Z, Liu R, Hassan HM, Yang H, Sun R, Zhang L and Jiang Z. UDCA and CDCA alleviate 17alpha-ethinylestradiol-induced cholestasis through PKA-AMPK pathways in rats. Toxicol Appl Pharmacol 2016; 311: 12-25.

[47] Xiang D, Xu Y, He W, Yang J, Zhang C and Liu D. Bioinformaticsbased identification of key pathways and candidate genes for estrogeninduced intrahepatic cholestasis using DNA microarray analysis. Mol Med Rep 2019; 20: 303-311.

[48] Chen P, Li D, Chen Y, Sun J, Fu K, Guan L, Zhang H, Jiang Y, Li X, Zeng X, Chen X, Huang M and Bi H. P53-mediated regulation of bile acid disposition attenuates cholic acid-induced cholestasis in mice. Br J Pharmacol 2017; 174: 4345-4361.

[49] Yang H, Li TW, Ko KS, Xia M and Lu SC. Switch from Mnt-Max to Myc-Max induces p53 and cyclin D1 expression and apoptosis during cholestasis in mouse and human hepatocytes. Hepatology 2009; 49: 860-870.

[50] Hu YY, Wang XD and Liu SY. The relationship between P53 and hypoxia-inducible transcription factor-1alpha in the placenta of patient with intrahepatic cholestasis of pregnancy under acute hypoxic condition. Sichuan Da Xue Xue Bao Yi Xue Ban 2006; 37: 901-903, 942.