Original Paper

The Serum microRNA Profile of Intrahepatic Cholestasis of Pregnancy: Identification of Novel Noninvasive Biomarkers

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Key Words

Intrahepatic cholestasis of pregnancy • MicroRNA • Noninvasive biomarker • Microarray

Abstract

Background/Aims: Intrahepatic cholestasis of pregnancy (ICP) is a pregnancy-specific disease that significantly increases the risk of fetal complications. Here, we measured serum miRNA levels in ICP patients to identify candidate biomarkers for ICP. **Methods:** We used the Agilent miRNA array followed by reverse transcription-polymerase chain reaction assays to identify and validate the serum miRNA profiles of 40 pregnant women with ICP and 40 healthy pregnant controls. We used bioinformatics to identify metabolic processes related to differentially expressed miRNAs. **Results:** The expression levels of three miRNAs (miR-371a-5p, miR-6865-5p, and miR-1182) were significantly increased in ICP patients compared to controls; the areas under the receiver operating characteristic (ROC) curves (AUCs) were 0.771, 0.811, and 0.798, respectively. Multiple logistic regression analysis showed that a combination of the levels of the three miRNAs afforded a greater AUC (0.845), thus more reliably diagnosing ICP. The levels of all three miRNAs were positively associated with that of total bile acids. Furthermore, bioinformatics analysis indicated that the three miRNAs principally affected lipid phosphorylation, apoptosis, and the MAPK signaling pathway. **Conclusion:** This preliminary work improves our understanding of serum miRNA changes in pregnant women with ICP. The three miRNAs may serve as novel noninvasive biomarkers of ICP.

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Introduction

Intrahepatic cholestasis of pregnancy (ICP) is a pregnancy-related syndrome characterized by pruritus and elevated serum levels of liver enzymes and/or bile acids; the incidence varies geographically from 0.1 to 15.6% [1]. ICP is associated with increased risks of perinatal complications, including spontaneous preterm labor, fetal distress, and sudden intrauterine death [2]. Both genetic and environmental factors contribute to ICP pathogenesis [3]. Several studies have revealed ICP-associated changes in the expression levels of various genes and proteins in many functional categories, including apoptosis, oxidative stress, lipid metabolism, cell growth, and immune response [4-6]. However, the etiology of ICP remains poorly understood. A diagnosis of ICP is confirmed by an elevated serum level of total bile acids (TBA) and symptoms including pruritus and jaundice in the late second or third trimester of pregnancy without any sign of chronic liver disease. No specific diagnostic biomarker, other than the TBA marker, is currently available. However, serum TBA could not be used to distinguish the ICP patients with low pruritus from normal pregnant women [7], and even more, normal serum TBA concentrations have been observed in some cases with ICP [8]. So, new diagnostic and prognostic ICP biomarkers are urgently required.

MicroRNAs (miRNAs) are small, single-stranded non-coding RNAs (18–24 nt in length) that bind to upstream regions of mRNAs, negatively regulating mRNA function via either translational repression or degradation [9]. miRNAs affect various biological processes including cell proliferation, apoptosis, metabolism, inflammation, and tissue patterning during development [10, 11], highlighting the breadth of their functions. Recently, the roles of miRNAs in the pathogenesis of several human diseases have been studied [12]. MiRNAs are abundant in human serum, and are stable, easily assayed, and potentially disease specific [13]. Serum miRNA levels have been reported to serve as diagnostic and prognostic biomarkers of complications of pregnancy, including gestational diabetes mellitus (GDM) and pre-eclampsia [14, 15].

However, the serum miRNA profile of ICP patients has not been reported. To address this deficit, we used the Agilent miRNA array followed by quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) assays to identify correlations between levels of differentially expressed miRNAs and ICP clinical and biochemical parameters. We explored the diagnostic utilities of such miRNAs; further studies will increase our understanding of the complex molecular background of ICP.

Materials and Methods

Patient recruitment and blood sample collection

We performed a case-control study to identify maternal serum miRNAs that might be used to diagnose ICP. We enrolled 40 pregnant women with ICP and 40 healthy pregnant controls at the Affiliated Wuxi Maternity and Child Health Care Hospital of Nanjing Medical University from October 2016 to March 2017. All subjects were primiparous Chinese women with singleton pregnancies. The ICP diagnostic criteria and the inclusion and exclusion criteria used in the present study were described previously [5]. No patient underwent ursodeoxycholic acid treatment prior to blood sample collection. Ethical approval was granted by the Institutional Review Board of Nanjing Medical University, and

Table 1. The principal clinical characteristics of pregnant women with ICP and healthy pregnant controls. TBA, total bile acids; ALT, alanine transaminase; AST, aspartate transaminase. Statistical analysis was performed with the aid of the t-test; *P < 0.05 was considered to reflect significance

Variable	Screening samples			Validation samples		
	ICP (n=4)	Control (n=4)	P value	ICP (n=40)	Control (n=40)	P value
Maternal Age (y)	26.50±3.87	27.2±1.50	0.231	28.50±5.16	25.2±4.50	0.182
Gestational weeks	37.85±0.77	38.72±0.63	0.076	38.85±0.89	39.31±0.75	0.072
TBA (μmol/L)	58.33±12.4*	3.51±1.72	0.003	79.33±52.01*	4.45±2.20	0.015
ALT (IU/L)	68.25±24.76*	17.01±8.37	0.016	112.58±111.13*	15.0±7.57	0.023
AST (IU/L)	82.71±29.53*	19.36±5.42	0.028	113.32±94.29*	17.75±7.56	0.031

all participants gave written informed consent. Five-milliliter amounts of venous blood were collected from all participants, and sera were isolated within 4 h via centrifugation at 4,000 rpm for 10 min, followed by 12,000 rpm for 15 min to completely remove cell debris. Serum samples were stored at -80°C prior to analysis. The characteristics of all patients and their liver function test data are summarized in Table 1.

The miRNA array

Total RNAs (including small RNAs) were extracted from serum samples with the aid of the Trizol reagent (Invitrogen, Carlsbad, CA) and purified using a mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. miRNA profiling was performed using the Agilent miRNA array, which features eight identical subarrays per slide (in 8 x 60K format); each subarray contained probes interrogating 2,549 human mature miRNAs derived from miRBase ver. R21.0. Each miRNA was probed 30 times. The array also contains 2,164 Agilent control probes. All microarray experiments were performed as instructed by the manufacturer. Briefly, miRNAs were labeled using the Agilent reagent. Total RNAs (100-ng amounts) were dephosphorylated and ligated with pCp-Cy3, and the labeled RNAs were purified and hybridized to the array. Images were scanned using the Agilent device and gridded, and the data were analyzed using Agilent software ver. 10.10. The data were summarized, normalized, and quality controlled using GeneSpring software ver. 12 (Agilent). The default 90th percentile normalization method was employed for data preprocessing. When selecting differentially expressed miRNAs, we used threshold values of ≥ 2 and ≤ 2 -fold and a Benjamini-Hochberg corrected *P*-value of 0.05.

RT-qPCR

RT-qPCR was performed as previously described [16]. Complementary DNAs (cDNAs) were synthesized using 1- μg amounts of total RNA and a PrimeScript^{TM} RT Reagent Kit (Takara, China). The TaqMan microRNA assay (Applied Biosystems, Inc.) was employed to quantify relative miRNA expression levels using miR-16 as the endogenous control. All real-time amplifications were performed in triplicate using an ABI 7900 real-time PCR system (Applied Biosystems). The fold changes in miRNA levels were calculated using the $2^{-\Delta\Delta\text{CT}}$ method.

Functional classification of differentially expressed miRNAs

The gene ontology (GO) biological processes and the functional molecular annotations of genes targeted by the differentially expressed miRNAs ($P < 0.05$) were analyzed. The key GO biological processes are described for each miRNA, and include the numbers of genes targeted within each classification and their associated Korea Encyclopedia of Genes and Genomes (KEGG) pathways.

Statistical analysis

All statistical analyses were performed with the aid of SPSS ver. 16 software (SPSS, Inc., Chicago, IL, USA). Student's *t*-test was used to compare subjects' demographic and clinical characteristics and the expression levels of serum miRNAs between the ICP and control groups. We drew receiver operator characteristic (ROC) curves for all miRNAs and derived the associated sensitivities and specificities of discrimination (with 95% confidence intervals [CIs]) by reference to the areas under the curves (AUCs).

Table 2. Differentially expressed miRNAs in the sera of pregnant women with ICP and healthy pregnant controls as revealed by the Agilent miRNA array. miRNAs exhibiting changes ≥ 2 - or ≤ 2 -fold in pregnant women with ICP (P) compared to healthy pregnant controls (C), as defined in Experimental Procedures. The between-group average ratios (P:C) are shown

miRNA	Fold change (P : C ratio)	P value	Expression change
hsa-miR-514b-5p	8.39	1.49×10^{-2}	up
hsa-miR-4656	5.59	2.96×10^{-2}	up
hsa-miR-3614-5p	21.37	4.11×10^{-4}	up
hsa-miR-6829-5p	9.61	3.39×10^{-2}	up
hsa-miR-6861-5p	6.53	2.19×10^{-2}	up
hsa-miR-6806-5p	9.13	1.13×10^{-2}	up
hsa-miR-371a-5p	8.97	8.71×10^{-4}	Up
hsa-miR-3202	7.09	3.98×10^{-2}	up
hsa-miR-3156-5p	10.32	3.92×10^{-2}	up
hsa-miR-7109-5p	6.33	4.11×10^{-2}	up
hsa-miR-6865-5p	12.51	9.57×10^{-3}	Up
hsa-miR-8060	9.10	8.59×10^{-3}	up
hsa-miR-3138	12.86	3.84×10^{-2}	up
hsa-miR-1182	12.82	4.89×10^{-4}	Up
hsa-miR-4695-5p	5.90	1.98×10^{-2}	up
hsa-miR-1185-2-3p	6.30	1.82×10^{-2}	up
hsa-miR-6803-5p	2.14	1.88×10^{-3}	down
hsa-miR-6068	2.70	6.60×10^{-3}	down
hsa-miR-518e-5p	2.69	4.92×10^{-2}	down

Pearson correlations between the levels of differentially expressed miRNAs and serum TBA levels were also calculated. All results are expressed as means $\pm$ standard errors (SEs). A P -value <0.05 was considered to reflect statistical significance.

Results

Clinical characteristics of pregnant women with ICP and healthy pregnant controls

The clinical characteristics of 40 pregnant women with ICP and 40 healthy pregnant controls are summarized in Table 1. We found no significant between-group difference in either maternal age or body weight (both $P > 0.05$). Although pregnant women with ICP delivered slightly earlier than controls did, the difference was not significant ($P > 0.05$). However, the levels of TBA, alanine transaminase (ALT), and aspartate transaminase (AST) in pregnant women with ICP were significantly higher than those in healthy pregnant controls (Table 1).

Serum miRNA profiling of pregnant women with ICP and healthy pregnant controls

The Agilent miRNA array was used to identify differentially expressed miRNAs in serum samples from four pregnant women with ICP and four healthy pregnant controls. We calculated miRNA mean expression levels and fold changes compared to controls. We selected miRNAs that were ≥ 2 -fold upregulated or ≤ 2 -fold downregulated in the ICP group relative to controls for further validation. Preliminary screening revealed that 16 miRNAs were upregulated and 3 were downregulated (Table 2).

Validation of differential miRNA expression

Four differentially expressed miRNAs (three upregulated: miR-371a-5p, miR-6865-5p, and miR-1182; one downregulated: miR-6803-5p) were subjected to qRT-PCR in all 40 ICP patients and all 40 control subjects. Hsa-miR-16 served as the normalization control. The primer sequences used to amplify the miRNAs are summarized in Table 3. We found that miR-371a-5p, miR-6865-5p, and miR-1182 were significantly upregulated in pregnant women with ICP compared with healthy pregnant controls ($P = 0.006$, $P = 0.001$ and $P = 0.005$, respectively). The miR-6803-5p levels did not differ between the two groups ($P = 0.113$) (Table 4).

Table 3. Primers used in this study

Gene Name		Primer Sequence 5'→3'
hsa-miR-16	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCTAGGATACGACcgccaa
	AS	GCTTGTAGCAGCACGTAAATATTG
hsa-miR-371a-5p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCTAGGATACGACagtgcc
	AS	TGACTCAAACGTGGGCGGC
hsa-miR-6865-5p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCTAGGATACGACTgaagt
	AS	TAGGTGGCAGAGGAGGGACTT
hsa-miR-1182	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCTAGGATACGACgtcaca
	AS	GAGGGTCTTGGGAGGGATGT
hsa-miR-6803-5p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCTAGGATACGACacgcc
	AS	AAATCTGGGGGTGGGGGGCT
miRNA universal Sense primer		GTGCAGGGTCCGAGGT

Predictive utilities of the serum levels of miR-371a-5p, miR-6865-5p, and miR-1182

To evaluate the sensitivities and specificities of the serum miRNA signatures (both individually and in combination) in terms of ICP prediction, we drew ROC curves and calculated the AUCs for each miRNA (Fig. 1). The AUCs were 0.771, 0.811, and 0.798 for miR-371a-5p, miR-6865-5p, and miR-1182, respectively (Fig. 1A–C). We assessed the diagnostic utilities of miR-371a-5p, miR-6865-5p, and miR-1182 at cutoff values of 0.02, 0.0007, and 0.0018, which the Youden index method identified as the optimal diagnostic points. The sensitivities and specificities of miR-371a-5p, miR-6865-5p, and miR-1182 were, respectively, 72.5 and 75%, 72.5 and 81%, and 75 and 80%, respectively. Multiple logistic regression analysis of the combined miRNA data yielded an AUC of 0.845, and the sensitivity and specificity increased to 75 and 82.5%, respectively (Fig. 1D). Thus, a combination of the three miRNAs improved the reliability of the novel biomarker.

Fig. 1. The diagnostic utilities of maternal serum miRNAs in pregnant women with ICP. (A) miR-371a-5p; (B) miR-6865-5p; (C) miR-1182; (D) The ROC curve of a combination of the three miRNAs derived using multiple logistic regression analysis. The combination of the three miRNAs (miR-371a-5p, miR-6865-5p, and miR-1182) afforded the greatest area under the ROC curve (AUC).

Fig. 2. The correlations between serum miRNA and total bile acid levels in pregnant women with ICP. (A): miR-371a-5p ($R = 0.345$, $P = 0.029$); (B) miR-6865-5p ($R = 0.392$, $P = 0.012$); (C) miR-1182 ($R = 0.401$, $P = 0.010$).

Table 4. Validation of changed miRNA levels in pregnant women with ICP compared to healthy pregnant controls

miRNA	ICP (n=40)			Control (n=40)			2- $\Delta\Delta CT$	P value
	ΔCT	mean	SD	ΔCT	mean	SD		
miR-371a-5p	5.008	3.35*10 ⁻²	3.34*10 ⁻²	6.348	1.74*10 ⁻²	1.15*10 ⁻²	2.82	0.006
miR-6865-5p	9.893	1.11*10 ⁻³	9.67*10 ⁻⁴	10.841	5.85*10 ⁻⁴	2.12*10 ⁻⁴	2.36	0.001
miR-1182	8.727	2.42*10 ⁻³	1.84*10 ⁻³	9.506	1.52*10 ⁻³	6.37*10 ⁻⁴	1.93	0.005
miR-6803-5p	0.512	6.21*10 ⁻¹	1.45*10 ⁻¹	1.008	5.59*10 ⁻¹	1.92*10 ⁻¹	1.47	0.113

Table 5. Gene ontology (GO) biological functions of genes targeted by the differentially expressed miRNAs. The key GO biological processes are shown for each miRNA, and include the number of gene targets within each classification and their associated KEGG pathways

MicroRNA	No. of target genes	GO:biological function	No. of genes	P value	KEGG pathway	No. of genes	P value
miR-371a-5p	76	lipid phosphorylation	2	4.41*10 ⁻²	Protein processing in endoplasmic reticulum	2	4.21*10 ⁻³
		nucleoplasm	21	2.71*10 ⁻³	MAPK signaling pathway	2	4.33*10 ⁻²
		intracellular membrane-bounded organelle	8	5.09*10 ⁻³	Metabolic pathways	3	2.61*10 ⁻³
		nuclear speck	5	7.10*10 ⁻³			
		protein binding	44	1.00*10 ⁻³			
		zinc ion binding	11	1.21*10 ⁻²			
miR-6865-5p	339	intracellular signal transduction	18	7.82*10 ⁻⁴	Signaling pathways regulating pluripotency of stem cells	8	1.07*10 ⁻²
		cell adhesion	18	3.12*10 ⁻³	Insulin resistance	7	1.09*10 ⁻²
		peptidyl-threonine phosphorylation	5	4.25*10 ⁻³			
		regulation of cell aging	3	4.37*10 ⁻³	Prostate cancer	6	1.81*10 ⁻²
		protein kinase binding	17	8.95*10 ⁻⁴	MAPK signaling pathway	10	3.28*10 ⁻²
		mitogen-activated protein kinase kinase binding	4	2.43*10 ⁻³	Pathways in cancer	13	3.95*10 ⁻²
		MAP-kinase scaffold activity	3	5.91*10 ⁻²	Thyroid hormone signaling pathway	6	4.78*10 ⁻²
		transcription factor binding	12	1.08*10 ⁻²			
		mRNA 3'-UTR AU-rich region binding	3	1.48*10 ⁻²			
		peptidase activity	6	1.99*10 ⁻²			
		cellular lipid metabolic process	4	4.33*10 ⁻³	Adipocytokine signaling pathway	4	1.54*10 ⁻²
miR-1182	159	transport	9	7.14*10 ⁻³	GABAergic synapse	4	2.58*10 ⁻²
		regulation of transcription	23	1.33*10 ⁻²	FoxO signaling pathway	4	7.93*10 ⁻²
		RNA splicing, via transesterification reactions	3	1.71*10 ⁻²	Wnt signaling pathway	4	8.49*10 ⁻²
		protein binding	93	4.87*10 ⁻⁴			
		ligand-dependent nuclear receptor binding	4	6.52*10 ⁻⁴			
		thiol-dependent ubiquitin-specific protease activity	4	2.83*10 ⁻²			

Correlations between the serum levels of miR-371a-5p, miR-6865-5p, and miR-1182, and the TBA level in ICP patients

We calculated Pearson correlations of the levels of miR-371a-5p, miR-6865-5p, and miR-1182 with TBA. The levels of all three miRNAs were positively associated with the TBA level ($R = 0.345$, $P = 0.029$; $R = 0.392$, $P = 0.012$; and $R = 0.401$, $P = 0.010$ for miR-371a-5p, miR-6865-5p, and miR-1182, respectively) (Fig. 2).

Functions affected by the miRNAs

We determined the GO functional enrichments associated with the three miRNAs. As shown in Table 5, the targeted genes are involved in many biological processes including lipid phosphorylation, cell adhesion, regulation of cell aging, mitogen-activated protein kinase kinase binding, and the activity of the MAP-kinase scaffold. KEGG pathway analysis showed that the genes targeted by the three miRNAs were involved in protein processing in the endoplasmic reticulum (ER), the MAPK signaling pathway, various metabolic pathways, and the Wnt signaling pathway.

Discussion

It is well known that ICP can cause spontaneous preterm labor, fetal distress, and sudden intrauterine death. The fetal death rate can attain 2% [17]. Therefore, early and accurate ICP diagnosis is essential. Currently, this is based principally on the serum TBA level, which remains normal in some patients [18]. The etiology of ICP remains unclear. Therefore, it is essential to identify more sensitive and specific diagnostic indicators to improve the prognosis of ICP patients.

MiRNAs play important roles in both normal and pathological processes [19]. Many studies have found that plasma/serum miRNAs are promising noninvasive biomarkers aiding diagnosis; circulating miRNAs are very stable and are easily assayed [16],[20]. Wei et al. found that miR-222, miR-146a, and miR-30c might be useful diagnostic markers of polycystic ovary syndrome [12]. A multistage, retrospective, nested case-control study by Zhao et al. revealed that miR-29a, miR-222, and miR-132 were differentially expressed in women with GDM and controls, and served as biomarkers predicting GDM [15].

The circulating miRNA profile of ICP patients has not previously been investigated. Here, we used a miRNA array to screen the profiles of pregnant women with ICP and normal pregnant subjects, and validated our findings via qRT-PCR, identifying potential miRNA biomarkers of ICP. Sixteen miRNAs were upregulated and three were downregulated in ICP patients compared to normal controls (Table 2). Furthermore, qRT-PCR showed that the expression levels of three miRNAs (miR-371a-5p, miR-6865-5p, and miR-1182) were significantly increased in ICP patients. We drew ROC curves and calculated the AUCs, which were 0.771, 0.811, and 0.798 for miR-371a-5p, miR-6865-5p, and miR-1182, respectively (Fig. 1A–1C). When the levels of the three miRNAs were combined, multiple logistic regression analysis yielded a greater AUC (0.845), and the sensitivity and specificity increased to 75% and 82.5%, indicating that the combined miRNA level afforded more reliable ICP diagnosis than did the individual levels.

To date, miR-371-5p has been mentioned in works screening for or targeting prostate, colon, and gastric cancer, but few functional studies have appeared [21, 22]. miR-1182 regulates the expression of human telomerase reverse transcriptase (hTERT) in gastric cancer cells, affecting cell migration [23]. Associations of miR-371a-5p, miR-6865-5p, and miR-1182 with ICP have not been previously reported. Bioinformatics analysis showed that the three miRNAs principally regulated lipid phosphorylation, cell adhesion, cell aging, binding of the kinase that phosphorylates MAP kinase, and MAP-kinase scaffold activity. These results were consistent with our previous proteomic findings that defective lipid metabolism, apoptosis, growth dysfunction, and immune maladaptation are all important features of ICP pathophysiology [5]. KEGG pathway analysis showed that the three miRNAs may be principally involved in protein processing in the endoplasmic reticulum (ER), the MAPK signaling pathway, various metabolic pathways, and the Wnt signaling pathway. In particular, miR-371a-5p and miR-6865-5p may both affect the MAPK signaling pathway, which is, in turn, closely associated with apoptosis and lipid metabolism. Our earlier *in vitro* study showed that both the P38 MAPK pathway and ER stress played key roles in taurocholic acid (TCA)-induced apoptosis of human placental trophoblast HTR-8/SVneo cells, mirroring the pathogenesis of ICP *in vitro* [24]. Thus, in ICP patients, miR-371a-5p and miR-6865-5p may regulate the MAPK pathway, affording new insights into ICP pathophysiology and potential new targets of treatment.

Conclusion

In conclusion, we identified three differentially expressed miRNAs in the sera of ICP patients and found that the combination of miR-371a-5p, miR-6865-5p, and miR-1182 levels may serve as a new biomarker for ICP diagnosis. However, this is a preliminary work; the possible clinical applications require further investigation and optimization. Furthermore, the functional roles played by the three miRNAs remain unknown; the meaningful basic studies are required in our future work.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (grant no. 81671489 and 81401222), the Major Research Foundation of Jiangsu Science and Technology Department (grant no. BE2017628), the Foundation of Six Talent Peaks Project of Jiangsu Province (grant no. 2014-WSW-059), and the Foundation of Wuxi City Health and Family Planning (grants nos. MS201501 and Q201505). The authors thank all women who gave samples for the study.

Disclosure Statement

The authors declare that they have no competing interests.

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