Study On Plasma Exosome Biomarkers Of Pregnant Women With Intrahepatic Cholestasis of Pregnancy

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Research

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Abstract

Background: Elevated serum total bile acid level is currently the main index for clinical diagnosis of intrahepatic cholestasis of pregnancy, but the use of TBA as a detection index has certain limitations. The early diagnosis of ICP and new treatment options still need to be further strengthened.

Methods: Plasma samples were collected, and exosomes were isolated. Key differential proteins were screened by bioinformatics methods. ELISA method was used to detect the concentration of the key differential protein cluster in plasma samples, and the ROC curve was drawn to find out the best critical value.

Results: There were 138 differentially expressed proteins between the ICP group and the normal group by quantitative analysis. Cluster protein was screened as a clinical validation index. The cluster protein concentration of plasma exosomes in the ICP group was significantly higher than that in the normal group (P<0.0001). ROC curve analysis showed that the best critical point for diagnosing ICP according to the plasma exosome cluster protein concentration of pregnant women was 255.28 ng/ml. In the ICP group, the best crucial point for predicting ICP with premature delivery is 286.72 ng/ml.

Conclusion: the plasma exosome cluster protein concentration of ICP pregnant women is significantly higher than that of normal pregnant women. When the plasma cluster protein concentration of pregnant women is more remarkable than 255.28ng/ml, ICP can be diagnosed. When the plasma cluster protein concentration of pregnant women is higher than 286.72ng/ml, ICP pregnant women are more likely to have a premature birth.

Background

Intrahepatic cholestasis of pregnancy (ICP) is a particular complication during the second and third trimester of pregnancy, which is mainly characterized by skin itching, abnormal liver function, and elevated serum total bile acid level (TBA). The pathogenesis of ICP is still unclear. ICP mainly affects the growth and development of the fetus, including premature birth, fetal distress, neonatal asphyxia, and even neonatal death. Ursodeoxycholic acid (UDCA) and S-adenosylmethionine are the main therapeutic drugs at present. Although the serum total bile acid level of pregnant women with ICP was decreased significantly after treatment, premature birth and perinatal death are still critical problems for both obstetricians and pregnant women.

The maternal serum total bile acid (TBA) level is the most commonly used laboratory parameter for the clinical diagnosis of ICP. However, many studies have found that the accuracy of TBA in diagnosing ICP may be overestimated. The use of TBA as the main indicator for the clinical diagnosis and prognosis of ICP has certain limitations. Therefore, it is very important to explore the pathogenesis of ICP and find more valuable biomarkers besides TBA for clinical diagnosis and prognosis judgment of pregnant women with ICP.
Exosomes were first found in sheep reticulocytes and were named “Exosome” in 1987. Abels et al. found that cells can exchange genetic materials through RNA in exosomes, which are widely distributed in biological fluids such as plasma, serum, saliva, urine, amniotic fluid, etc., and can maintain a stable state and biological characteristics at a long-term extremely low temperature, and are used to study many diseases. Therefore, exosomes analysis has broad application prospects in the pathogenesis, diagnosis, and treatment of diseases.

In this study, the plasma exosomes of pregnant women with intrahepatic cholestasis of pregnancy were isolated and analyzed by bioinformatics. The selected exosome cluster (CLU) protein was analyzed by ROC curve to preliminarily explore the significance of exosomes CLU as a biomarker for clinical diagnosis of pregnant women with ICP.

Results

2.1 Extraction and Identification of exosomes

To ensure the successful extraction of exosomes, we identified exosomes extracted from ICP and normal maternal plasma by TEM, NTA, and Western Blot. The purified exosomes have a typical structure, and a clear vesicle structure can be seen under the electron microscope (Fig. 1A). Conventional exosome markers CD63 and Hsp70 were detected in exosome samples (Fig. 1B), Hsp70 and CD63 were positively expressed, and CD63 was relatively high, indicating that exosome extraction was successful. NTA shows that the particle size of the measured sample is about 100nm, and the overall size of the sample meets the standard (Fig. 1C).

2.2 Data analysis of exosome proteins by mass spectrometry

According to MaxQuant search results, all samples' digestion efficiency is > 90%, which shows that the quality control of enzymatic hydrolysis is qualified. According to the mass spectrometry database, 591 non-redundant proteins, 5925 peptides (including redundant peptides), and 102575 general spectrograms were detected. According to the relative molecular weight distribution of all the identified proteins (Fig. 2A), Generally, it is considered that the protein with high credibility is a protein containing two unique peptide segments, and the distribution of the number of unique peptide segments is as follows (Fig. 2B). The results show that the number of proteins containing two unique peptide segments in this study is 463, accounting for 78.34% of the total protein. The distribution of peptide length (Fig. 2C) shows that the maximum peptide length is 11, and the average length is 15.30. The protein coverage distribution (Fig. 2D) shows that 31.13% of identified proteins are in the range of 0–10% coverage proteins, 48.9% identified proteins are in the range of ≥20% coverage proteins and the average identification coverage proteins is 24.93%.

2.3 Differential protein analysis of exosomes
A total of 591 proteins were detected in this study. The results of the qualitative analysis showed that 34 specific proteins were detected in the ICP group, and 39 specific proteins were detected in the normal group. The protein quantitative analysis results showed that 51 proteins were up-regulated and 71 proteins were down-regulated. By combining qualitative analysis and quantitative analysis, 138 kinds of differential proteins were obtained in this study.

2.4 Bioinformatics analysis of Mass Spectrometry data

The screening results of exosome differentially expressed proteins were drawn into a Volcano plot (Fig. 3A), and cluster analysis was carried out (Fig. 3B).

GO enrichment analysis: ICP-related proteins are mainly enriched in biological functions such as platelet degranulation, cell adhesion, etc. (Fig. 4A), especially related to exosomes, extracellular regions. (Fig. 4B), primarily involved in protein binding, calcium ion binding, and other molecular functions (Fig. 4C).

KEGG pathway enrichment analysis: KEGG analysis found that differentially expressed proteins were mainly related to the PI3K-Akt signaling pathway, Rap1 signaling pathway, etc. The first 15 pathways are selected and plotted as follows (Fig. 4D).

Protein interaction analysis: 139 different proteins were analyzed by the protein interaction network in STRING online database. The results showed that (Fig. 5A) most of the proteins had an interaction relationship, and many proteins were located in the center of the network and were closely related to the surrounding proteins. The results are imported into Cytoscape (Fig. 5B). The size of the node represents the number of interacting proteins, the color represents FC value, the red represents up-regulation, the green represents down-regulation, and the thickness of the connecting line represents the combine_score. The centrality Degree is calculated in Cytoscape to evaluate the importance of GeneCards. The proteins are sorted according to the Degree value, and the Top15 proteins are selected for analysis (Fig. 6A). The functions of them through GeneCards are shown in Table 2.

Table 2 Biological function of top 15 protein
<table>
<thead>
<tr>
<th>NO</th>
<th>Gene</th>
<th>Biological function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ACTB</td>
<td>Compose cell structure</td>
</tr>
<tr>
<td>2</td>
<td>ACTG1</td>
<td>Compose the cell structure/cell internal movement medium</td>
</tr>
<tr>
<td>3</td>
<td>ITGB3</td>
<td>Cell attachment/cell matrix attachment/integrin-mediated signal transduction pathway/development/hemagglutination</td>
</tr>
<tr>
<td>4</td>
<td>VCL</td>
<td>Cell-to-cell connection/cell attachment/participation in cell signal transduction</td>
</tr>
<tr>
<td>5</td>
<td>ITGA2B</td>
<td>Mediates the connection of signal molecules of fibronectin, fibrinogen, plasminogen, thrombospondin and vitronectin</td>
</tr>
<tr>
<td>6</td>
<td>CLU</td>
<td>Markers of various tumor cells</td>
</tr>
<tr>
<td>7</td>
<td>HP</td>
<td>Combined with free hemoglobin/low concentration of HP stimulates B cell proliferation, high concentration of HP inhibits B cell proliferation</td>
</tr>
<tr>
<td>8</td>
<td>CFL1</td>
<td>Actin binding protein to promote cell migration and movement</td>
</tr>
<tr>
<td>9</td>
<td>ACTA2</td>
<td>Movement of eukaryotic cells/response to enterovirus infection up-regulated</td>
</tr>
<tr>
<td>10</td>
<td>PTPRC</td>
<td>Interaction with human cytomegalovirus protein UL11/T cell activation</td>
</tr>
<tr>
<td>11</td>
<td>ACTA1</td>
<td>Participate in skeletal muscle contraction</td>
</tr>
<tr>
<td>12</td>
<td>ALDOA</td>
<td>Play a key role in glycolysis and gluconeogenesis/scaffold protein</td>
</tr>
<tr>
<td>13</td>
<td>FLNA</td>
<td>Promote the connection of actin filaments and membrane glycoproteins/act as a scaffold/cell adhesion for a variety of cytoplasmic signaling proteins on actin</td>
</tr>
<tr>
<td>14</td>
<td>APOA2</td>
<td>Maintain HDL structure/activate liver lipase</td>
</tr>
<tr>
<td>15</td>
<td>APOA4</td>
<td>Activate lecithin cholesterol acyltransferase (LCAT)/mediate cholesterol reverse transport</td>
</tr>
</tbody>
</table>

2.5 **Maternal plasma exosome CLU concentration of ICP pregnant women**

The CLU concentration of plasma exosomes in the ICP group was significantly higher than that in the normal group (P < 0.0001) (Fig. 6B). ROC curve analysis showed that the best critical point for diagnosing ICP according to the plasma exosome CLU concentration of pregnant women was 255.28 ng/ml, and its AUC was 0.995, sensitivity was 95%, and specificity was 100% (Table 3, Fig. 6C). The best critical point of predicting ICP with premature delivery by plasma exosome CLU concentration in pregnant women with ICP is 286.72 ng/ml, with an AUC of 0.854, a sensitivity of 91.7%, and specificity of 72.2% (Table 3 and Fig. 6D).

**Table 3  ROC curve analysis of plasma exosome CLU concentration in ICP pregnant women**
<table>
<thead>
<tr>
<th></th>
<th>ICP</th>
<th>ICP with premature birth</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC</td>
<td>0.995</td>
<td>0.854</td>
</tr>
<tr>
<td>standard error</td>
<td>0.008</td>
<td>0.069</td>
</tr>
<tr>
<td>Optimal critical point (ng/ml)</td>
<td>255.28</td>
<td>286.72</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>95</td>
<td>91.7</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>100</td>
<td>72.2</td>
</tr>
<tr>
<td>Youden index</td>
<td>0.95</td>
<td>0.639</td>
</tr>
</tbody>
</table>

Note: AUC, area under ROC curve; Youden index = sensitivity + specificity -1.

Discussion

3.1 The importance of exploring new biomarkers for clinical diagnosis of ICP

ICP is a special complication during the second and third trimester of pregnancy, which has a significant influence on perinatal outcomes. Early diagnosis and treatment of ICP may improve perinatal outcomes and reduce the risk of premature delivery and fetal death. At present, the commonly used indicator for clinical diagnosis of ICP is the serum total bile acid (TBA) level of pregnant women. However, many studies have found that the accuracy of TBA in diagnosing ICP may be overestimated. Taking TBA as the primary indicator of clinical diagnosis and prognosis of ICP has certain limitations. Many pieces of evidence showed that some pregnant women with ICP still suffered from fetal death, although maternal serum TBA level decreased to very low concentration or even normal after UDCA treatment. Therefore, it is very important to find more valuable biomarkers besides TBA for clinical diagnosis and prognosis judgment of pregnant women with ICP. One of the characteristics of exosomes is that they are enriched with cell-specific protein or miRNA, which can be used as a molecular marker for disease diagnosis and prognosis. For example, Sardar et al. found that neuron exosomes can be easily separated in blood and other body fluids and contain specific marker molecules, which can be used as diagnostic markers for Alzheimer's disease. Multiple studies showed that exosome miRNA might be used as a marker for the diagnosis and prognosis of liver cancer. With the rapid development of gene detection technology and protein omics, it is possible to use exosomes in disease diagnosis. Therefore, this study explored the feasibility of exosomes as a diagnostic biomarker of ICP.

3.2 The signal pathway mediated by plasma exosome protein in ICP pregnant women may be involved in the pathophysiological process of ICP

In this study, the exosome protein of ICP pregnant women was analyzed by KEGG (Fig. 8). The results showed that the exosome protein of ICP pregnant women was closely related to the P13K-Akt pathway and RaP1 signaling pathway. PI3k/Akt signaling pathway belongs to the casein kinase receptor transduction pathway. Many growth factors can activate PI3k to
produce lipid products and then activate downstream signal protein Akt. Akt regulates various cell functions through different downstream targets, including metabolism, protein synthesis, cell cycle progression, anti-apoptosis, tumor growth, and angiogenesis, and finally regulates cell proliferation, differentiation, migration, and invasion. Yin\textsuperscript{16} found that the effect of EGF and U0126 on the ERK signaling pathway affected the apoptosis rate of ICP placental trophoblasts, suggesting that ERK signaling pathway may be involved in the pathogenesis of ICP, and the activation of ERK is closely related to the RaP1-MEK pathway. All of the above suggests that the PI3K-Akt pathway and RaP1 signal pathway may be involved in the pathophysiological process of ICP.

3.3 Maternal plasma exosome cluster protein can be used as a biomarker of clinical diagnosis and premature birth prediction of ICP pregnant women

Cluster (CLU) protein is a multifunctional secretory glycoprotein, which exists in many different biological fluids, including urine, plasma, serum, semen, breast milk, and cerebrospinal fluid. It plays an essential role in lipid metabolism regulation, cell proliferation, complement activity regulation, and cell apoptosis. Scaltriti et al.\textsuperscript{17, 18} found that CLU can promote cell apoptosis by preventing Bcl-xL from binding to Bax. In some cells, such as cardiomyocytes and neurons, CLU activates PI3K/AKT signaling pathway, which leads to cell survival or proliferation\textsuperscript{19}. Oztas et al.\textsuperscript{20} found that the increased expression of CLU in the placenta was related to IUGR. Shin et al.\textsuperscript{21, 22} found that the expression of CLU in the placenta and serum of pregnant women with hypertensive disorder complicating pregnancy were significantly higher than those of normal pregnant women. Vacínová\textsuperscript{23} found that there was no significant difference in CLU level between gestational diabetes mellitus and normal pregnant women. Ivana et al.\textsuperscript{24} found that the concentration of CLU in the amniotic fluid of pregnant women with preterm premature rupture of membranes during pregnancy was lower than that of normal pregnant women. However, there is no previous report on the CLU level in ICP.

In this study, the ROC curve was used to evaluate the value of plasma exosome CLU in diagnosing ICP and predicting ICP with premature delivery. ROC curve analysis\textsuperscript{25} showed that the larger the Youden index or AUCindicated greater the diagnostic value. If AUC is greater than 0.9, it means a great diagnostic value. AUC 0.7–0.9 means medium diagnostic value; AUC 0.5–0.7 means poor diagnostic value. AUC Less than 0.5 means no diagnostic value. Table 5 shows that the ROC curve AUC of plasma exosome CLU concentration in diagnosis of ICP is greater than 0.9, which means good diagnostic value and high sensitivity and specificity. ROC curve AUC of maternal plasma exosome CLU concentration in diagnosis of ICP with premature delivery is between 0.7 and 0.9, which means medium diagnostic value, high sensitivity, and low specificity. The results of this study showed that the best critical point for diagnosing ICP according to the plasma exosome CLU concentration of pregnant women was 255.28 ng/ml, and the best critical point for predicting ICP with premature delivery was 286.72 ng/ml.

Conclusions
Under plasma exosome analysis of ICP pregnant women, it demonstrated that the PI3K-Akt signal pathway and RaP1 signal pathway might be involved in the pathophysiological process of ICP. The plasma exosome cluster protein (CLU) in pregnant women with ICP is an important biomarker for clinical diagnosis and prediction of premature delivery of ICP.

**Methods**

**5.1 Sample collection**

ICP pregnant women who delivered in the First Affiliated Hospital of Chongqing Medical University from January 2020 to December 2020 were involved in this study. Before the termination of pregnancy (natural delivery or cesarean section), 5ml of blood from the elbow vein was collected in an anticoagulant tube containing EDTA. After centrifugation (5000r, 5min), the plasma was stored in a −80°C refrigerator. This study was approved by the ethical committee of the First Affiliated Hospital of Chongqing Medical University, within which the work was undertaken and that it conforms to the provisions of the Declaration of Helsinki (as revised in Tokyo 2004). Written informed consent was obtained from all participants.

Inclusion criteria of clinical blood sample collection: ICP group pregnant women (10 cases): Maternal serum TBA level ≥ 10 µmol/L. There were no other complications, such as gestational diabetes mellitus, gestational hypertension, hepatitis, pregnancy complicated with hyperthyroidism, etc. The clinical symptoms and liver function of pregnant women returned to normal after delivery within 42 days. All pregnant women had no abnormal liver function before pregnancy and no history of liver-related operation, hepatitis B and hepatitis C. Normal group (10 cases): no other complications, and the liver function was normal. The comparison of clinical data and laboratory examination is shown in Table 1.

**Table 1 Clinical data analysis of ICP and normal pregnant women**

<table>
<thead>
<tr>
<th>Group</th>
<th>NO</th>
<th>age</th>
<th>Delivery gestational age</th>
<th>TBA (mmol/L)</th>
<th>ALT (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>29.6±4.48</td>
<td>39.4±1.12</td>
<td>3.10±1.51</td>
<td>12.58±5.06</td>
</tr>
<tr>
<td>ICP</td>
<td>10</td>
<td>29.8±4.85</td>
<td>35.4±1.88*</td>
<td>39.10±36.86*</td>
<td>242.40±134.26*</td>
</tr>
</tbody>
</table>

**5.2 Extraction and enrichment of exosomes**

**5.2.1 Extraction and ultrafiltration of exosomes**

The plasma samples were taken out of the refrigerator at -80°C, thawed in the water bath at 25°C. According to the manufacturer's instructions, the qEV original kit (SP5, Izon Science, New Zealand) was used to enrich and purify plasma exosomes in the normal group and ICP group. According to the manufacturer's instructions, The 10K ultrafiltration tube (amicon ultra-0.5, millipore, American) was used for ultrafiltration of exosomes.
5.3 Identification of exosomes

5.3.1 Nanoparticle tracking detection (NTA)

Exosome samples were diluted, and the concentration and size distribution of exosomes were determined by nanoparticle tracking analysis (Zeta) (analysis software version Zeta View 8.04.02) on ZetaView S/N 17–310 (Particle Metrix, Germany).

5.3.2 Transmission electron microscope (TEM)

The sample was tested by nano transmission electron microscope (TEM) at 80kV of Tecnai G2 Spirit BioTwin (FEI, USA).

5.3.3 Western Blot

M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, USA) was added for lysis and protein extraction. The dilution ratio of cd63 (SBI, exoab-cd63a-1) and Hsp70 (Abcam, ab2787) primary antibodies was 1/1000 and incubated overnight at 4°C. Secondary antibodies were incubated with CD63 (SBI, 180202-001) and Hsp70 (Abcam, ab131368), respectively.

5.4 Liquid chromatography-mass spectrometry (LC-MS) analysis

The polypeptide products were analyzed by QE-HF-X mass spectrometer and EASY-nLC 1200 nano-upgraded liquid chromatography. Tandem mass spectrometry detection adopts Data Dependent Acquisition (DDA) mode. The total scan resolution is 60,000 (FWHM), the range of mass-to-charge ratio is set to m/z 350–2000, and the collision energy is set to 28% in HCD fragmentation mode.

5.5 Data processing of proteome

5.5.1 MaxQuant

The MaxQuant database was used to search for mass spectrometry data, and each original data and corresponding database were uploaded to max quant 1.5.8.3 institute for biochemistry, Germany. After searching the database, remove the Reverse and Potential Contaminants proteins, and make statistics on the search results.

5.5.2 Screening of differential proteins

According to the MaxLFQ algorithm embedded in MaxQuant software, this quantitative value (Intensity) is calculated to obtain normalized quantitative value LFQ. Quantitative analysis of proteins in all samples was carried out according to LFQ values. In pairwise comparison between groups, the normalized signal mean of all samples in each group was calculated to calculate the Ratio between groups, and the P values of the two groups were calculated by Mann-Whitney rank-sum test. Screening proteins that meet
the following two conditions as difference proteins between groups: ratio between groups $\geq 1.5$ or $\leq 0.67$ (i.e., $1/1.5$); $P < 0.05$.

5.6 Bioinformatics analysis

5.6.1 Screening of differentially expressed proteins

For the experimental design with biological repetition ($n \geq 3$), the data of samples detected by mass spectrometry were tested by T-test between groups, and two-parameter values, P-value and Fold change, were obtained. The obtained differential proteins were processed by data to draw a Volcano plot.

5.6.2 Cluster analysis

Unsupervised hierarchical cluster analysis was performed for the differential proteins modulated between groups. Through protein expression data, the expression of differential proteins among multiple samples is calculated, and the direct correlation between samples is calculated by the expression of selected differential proteins.

5.6.3 GO enrichment analysis

The function of differentially expressed proteins between normal pregnant women and ICP pregnant women were annotated, and the primary biological process (BP), cellular component (CC), and molecular function (MF) of these differentially expressed proteins were analyzed by GO enrichment.

5.6.4 KEGG Pathway enrichment analysis

Kegg (Kyoto encyclopedia of genes and genomes) is the central database for systematic analysis of gene function, genome and proteome information. Different proteins perform their biological behaviors in coordination with each other, and protein and expression information are studied as a whole network.

5.6.5 Protein interaction network interaction analysis

Protein-protein interaction is the most critical way of signal transduction and protein function in cells. Protein-protein interaction (PPI) analysis of protein interaction network helps discover the protein which is in the core position of regulation among a large number of different proteins.

5.7 Clinical specimen verification experiment

5.7.1 Screening and verification indicators

We consulted literature and Gene Cards, screened 15 differential proteins, and selected CLU, which was up-regulated as an index, to verify the experiment.

5.7.2 Maternal plasma sample collection of ICP
Inclusion criteria were the same as before (2.1 sample collection). 35 pregnant women (aged 21–34 years) who visited the First Affiliated Hospital of Chongqing Medical University from March 2020 to December 2020 were randomly selected as research objects, including 11 cases of normal pregnant women and 24 cases of ICP pregnant women.

5.7.3 Extraction, Identification, and concentration of exosomes

The exosome was extracted, identified, and concentrated by the same experimental method as before, and finally, 35 concentrated exosome samples of 200ul were obtained.

5.7.4 Detection of plasma exosome cluster protein

CLU was detected by ELISA (R&D, USA), reagents, samples, and standards were prepared in turn according to the instructions, and read the OD value immediately at 450nm (corrected at 570nm).

5.7.5 Statistical analysis

GraphPad Prism6 was used to calculate the t-test, and a histogram was drawn to compare the level of plasma exosome CLU between the normal and ICP groups. P < 0.05 indicates statistical significance. The results were statistically analyzed with IBM SPSS statistics 23 software, and the ROC curve was drawn with 1-specificity as abscissa and sensitivity as ordinate, and the area under the curve (AUC) was calculated.

Abbreviations

ICP Intrahepatic cholestasis of pregnancy

Declarations

Authors’ contributions

YS initiated and concepted the study. FF did all the experiments and wrote the first draft of the article. Other authors collected samples. All authors have agreed on the final version of the article.

Funding

This work is funded by the National Natural Sciences Foundation of China (No.81471473) and the Chongqing Health Planning Commission project (No. 2019ZDXM055).

Availability of data and materials

The datasets analysed during the current study available from the corresponding author on reasonable request.
Ethics approval and consent to participate

This study was approved by the ethical committee of the First Affiliated Hospital of Chongqing Medical
University within which the work was undertaken and that it conforms to the provisions of the Declaration
of Helsinki (as revised in Tokyo 2004).

Consent for publication

Not applicable.

Competing interests

No Conflict of interest can be declared.

Acknowledgments

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Chongqing Health Planning Commission project (No. 2019ZDXM055)

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perinatal outcomes of intrahepatic cholestasis of pregnancy with biochemical markers: results of


diagnosis of intrahepatic cholestasis of pregnancy in an high incidence area: Portugal.Eur J Obstet


Table 5

Table 5 is not available in this version.

Figures
Figure 1

The purified exosomes have a typical structure, and a clear vesicle structure can be seen under the electron microscope (Figure A). Conventional exosome markers CD63 and Hsp70 were detected in exosome samples (Figure B), Hsp70 and CD63 were positively expressed, and CD63 was relatively high, indicating that exosome extraction was successful. NTA shows that the particle size of the measured sample is about 100nm, and the overall size of the sample meets the standard (Figure C).
Figure 2

According to the relative molecular weight distribution of all the identified proteins (Figure A), Generally, it is considered that the protein with high credibility is a protein containing two unique peptide segments, and the distribution of the number of unique peptide segments is as follows (Figure B). The results show that the number of proteins containing two unique peptide segments in this study is 463, accounting for 78.34% of the total protein. The distribution of peptide length (Figure C) shows that the maximum peptide length is 11, and the average length is 15.30. The protein coverage distribution (Figure D) shows that 31.13% of identified proteins are in the range of 0-10% coverage proteins, 48.9% identified proteins are in the range of \( \geq 20\% \) coverage proteins and the average identification coverage proteins is 24.93%.
Figure 3

The screening results of exosome differentially expressed proteins were drawn into a Volcano plot (Figure A), and cluster analysis was carried out (Figure B).
Figure 4

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KEGG pathway enrichment analysis: KEGG analysis found that differentially expressed proteins were mainly related to the PI3K-Akt signaling pathway, Rap1 signaling pathway, etc. The first 15 pathways are selected and plotted as follows (Figure D).
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The proteins are sorted according to the Degree value, and the Top15 proteins are selected for analysis (Figure A). The CLU concentration of plasma exosomes in the ICP group was significantly higher than that in the normal group (P<0.0001) (Figure B). ROC curve analysis showed that the best critical point for diagnosing ICP according to the plasma exosome CLU concentration of pregnant women was 255.28 ng/ml, and its AUC was 0.995, sensitivity was 95%, and specificity was 100% (Table 3, Figure C). The best critical point of predicting ICP with premature delivery by plasma exosome CLU concentration in pregnant women with ICP is 286.72 ng/ml, with an AUC of 0.854, a sensitivity of 91.7%, and specificity of 72.2% (Table 3 and Figure D).