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RESEARCH ARTICLE

Placental exosomal level of alanine aminotransferase is more accurate predictive biomarker of intrahepatic cholestasis of pregnancy than total bile acid

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Abstract

***Aims*:** As a serious liver abnormality, intrahepatic cholestasis of pregnancy (ICP) uniquely occurs in pregnant women. In the present study, we examined whether placental exosomes and their protein contents could serve as novel indicators to help early diagnosis of ICP.

***Methods*:** The current retrospective stratified study design included a 100 healthy pregnant females and a 100 patients with ICP. Plancental exosomes were isolated to determine the exosomal levels of alanine aminotransferase (ALT) and total bile acid (TBA). The predictive values of placental exosomal TBA and ALT were determined using receiver operating characteristic (ROC) curve analysis.

***Results*:** The placental exosomal TBA level did not exhibit any obvious differences between healthy and ICP pregnancies. Moreover, placental exosomal ALT level was significantly higher in ICP-affected pregnancy than healthy control. Analysis of the ROC curve further demonstrated that placental exosomal level of ALT yields better sensitivity and specificity to distinguish ICP patients from healthy individuals, than that of TBA.

***Conclusions*:** Placental exosomal ALT is a more accurate predictive biomarker of ICP than TBA and, therefore, can be employed with reliability as a characteristic abnormality in diagnosis of ICP.

Keywords: *ICP*; *exosome*; *TBA*; *ALT*; *biomarker*; *receiver operating characteristic curve analysis*

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Intrahepatic cholestasis of pregnancy (ICP) is a serious liver illness uniquely affecting pregnant women (1). The primary characteristics of ICP is pruritus starting around the second or third trimester of pregnancy that withdraws after labour (2). About 10% of the patients developed mild jaundice within 4 weeks following the onset of itching (3, 4). Other atypical symptoms include encephalopathy (5), abdominal pain and subclinical steatorrhea, which is associated with vitamin K deficiency (6, 7). Prevalence of ICP varies around the world and is also dependent on ethnicity, which could be up to 4% in Chile (8). Since 1883 when it was first described, ICP has been generally considered clinical minor disorder clinically (9).

The exact mechanisms causing ICP are still poorly understood to date, other than the belief that the pathological progress of ICP is multi-factorial, influenced by environmental factors, deficiencies of nutrients, genetic variations and predisposition, and fluctuations in hormone levels (10). Such nature greatly hindered the diagnosis as well as treatment of ICP. Prior studies demonstrated that the flux of total bile acids (TBA) from the mother to the foetus increased during ICP (11–13), and high level of maternal TBA may disturb the production and transport of hormones in the placenta, and chorionic constriction of vessels (14). The elevation in the serum level of TBA was thought to be the sign of ICP. TBA level in the serum higher than 11.0 μM was, therefore, used as the most accurate marker for early diagnostic of ICP (15), whereas the diagnostic end point for ICP that is widely accepted is 10–14 μM TBA in the serum. Based on serum levels of TBA, ICP is categorized into mild ICP (10–40 μM TBA) and severe ICP (above 40 μM TBA) (16). However, serum level of TBA varies depending on the ethnic population

studied (10, 16), arguing that the use of TBA clinically is insufficient as a biomarker for diagnosis (17).

Exosomes refer to stable and small lipid bilayer vesicles and have capacity to pack specific molecules (18). Studies have highlighted the involvement of exosomes in various diseases (19, 20). In particular, placental exosomes have been reported to serve as biomarkers in pregnancy-related conditions such as preeclampsia (21, 22). However, to the best of our knowledge, the predictive and diagnostic value of placental exosome as well as its cargoes has never been reported in clinical ICP patients.

Methods

Subjects

This is a retrospective study. During 2018 and 2021, 100 ICP patients and 100 healthy pregnant females, all in the last trimester of pregnancy, were selected to be enrolled into the study. All participants provided a written consent. Procedures in the current study are in conformity with the ethical guidelines of the Declaration of Helsinki of 1975 and approved by the Ethic Committee of Kuvempu University.

Inclusion criteria

The criteria for including mild ICP patients were as follows: 1) jaundice and/or pruritus; 2) serum level of TBA between 15 and 40 μM; 3) absence of dermatological illness with the exclusion of lesions caused by excessive scratching; 4) absence of biliary tract dilatation, current viral hepatitis, symptomatic cholelithiasis or chronic liver disorder; 5) no sign of endocervical or urinary infection, pre-eclampsia and fever; 6) recovery of routine liver function after delivery.

Isolation of placental exosomes

Isolation of exosomes was performed as previously described (22). Briefly, plasma (1 mL) was obtained from all participants during the second trimester and subsequently diluted in half with phosphate buffered saline (PBS). Centrifugation was then carried out at 2,000 g for 30–35 min at 4°C, followed by 12,000 g for 45 min. The supernatant was further centrifuged at 110,000 g for 2–3 h at 4°C, then the pellets were resuspended in PBS and filtered with a 0.22 μm filter. The filtrate was centrifuged at 110,000 g for 1 h, and the pellets were again re-suspended in PBS and centrifuged at 110,000 g for 1 h. The exosome pellets were then suspended in 1 mL PBS followed by purification with a 30% sucrose cushion. The final pellet was resuspended in 100 mL PBS and stored at -80°C for future use. Exosomal TBA level was determined by an enzymatic-fluorimetric assay (23) after solid-phase extraction with Sep-Pak C_{18} cartridges (24). The intra-assay coefficient of variation (CV) and inter-assay CV of TBA assay are <7 and <9%, respectively. Exosomal alanine aminotransferase (ALT) level was examined with the use of an ELISA kit (Sigma-Aldrich, MO, USA) as previously reported (15). The CV and inter-assay CV of ALT ELISA kit are <8 and <10%, respectively.

Serum aspartate aminotransferase level

Serum aspartate aminotransferase (AST) level was determined using commercially available ELISA kit (Sigma-Aldrich).

Statistical analysis

Results were presented as mean ± SD. Comparison were conducted using ANOVA analysis followed by Tukey's post hoc test, unpaired two-tailed Student's *t*-test. A receiver operating characteristic (ROC) curve was measured.

Results

Clinical characteristics

Among 136 patients with ICP, 100 patients with mild ICP were enrolled in the current study according to the inclusion criteria. Thirty-six patients were excluded due to severe ICP (TBA > 40 μM). Clinical characteristics in patients with ICP as well as healthy controls were presented in Table 1. Significant statistical differences were found between healthy controls and ICP patients with respect to new-born birth weight and gestational age at full term ($P < 0.05$). Labour was induced in 61 ICP patients, significantly higher than that in healthy pregnant women with merely four incidences of induced labour.

Placental exosomal characteristics

To establish an accurate biomarker for ICP diagnosis, we isolated placental exosomes and examined levels of exosomal ALT and TBA, both of which have been shown to be involved in ICP (15, 25). First, levels of placental exosomal TBA level were compared between the healthy participants and patients with ICP (Table 1). It was observed that TBA level in ICP patients had a trend of elevation compared to healthy controls, but it was not statistically significant. On the other hand, level of placental exosomal ALT in ICP patients was markedly higher than that in healthy controls (Table 1). In addition, serum AST levels were also measured at both symptom onset and confirmation of ICP diagnosis for all ICP patients (Table 2), which showed a marked increase.

ROC curve analysis

As shown in Fig. 1, in the analysis for TBA, we calculated the area under the ROC curve for diagnosing ICP to be merely 0.561, with 95% confidence interval (CI) 0.481 to 0.641 ($P > 0.05$). While in the analysis for placental exosomal ALT (Fig. 2), the area under the ROC curve

Table 1. Clinical and placental exosomal characteristics in healthy pregnant women and patients with ICP

Characteristics	Healthy (*n* = 100)	Mild ICP (*n* = 100)	*P*
Age at pregnancy (years)	28.3 ± 2.7	30.4 ± 3.0	0.4241
Onset of pruritus (weeks)	N.A.	28.3 ± 2.2	
Jaundice			
Incidence	0	56	
Appearance (weeks)	N.A.	30.9 ± 1.7	
Time of ICP diagnosis (weeks)	N.A.	34.1 ± 0.3	
Gestational age at term (weeks)	39.2 ± 0.7	36.9 ± 0.6	0.0376
Newborn birth weight (kg)	2.92 ± 0.41	2.61 ± 0.24	0.0212
Delivery method			
Spontaneous	79	26	
Induced	4	61	
Caesarean section	17	13	
Placental exosomal factors			
TBA (ng/mL)	27.0 ± 16.0	31.1 ± 18.9	0.1001
ALT (IU/L)	90.6 ± 39.1	172.8 ± 78.1	0.0007

Results were compared using unpaired two-tailed Student's *t*-test, performed on the basis of equal or unequal variance as appropriate. Values are mean ± SD. ICP, intrahepatic cholestasis of pregnancy; N.A., not applicable; TBA, total bile acid; ALT, alanine aminotransferase.

Table 2. Serum AST level at different time points in patients with ICP

Index	At symptom onset	At ICP diagnosis
Serum AST (U/L)	72.6 ± 7.1	112.5 ± 9.6

Values are mean ± SD. AST, aspartate aminotransferase; ICP, intrahepatic cholestasis of pregnancy.

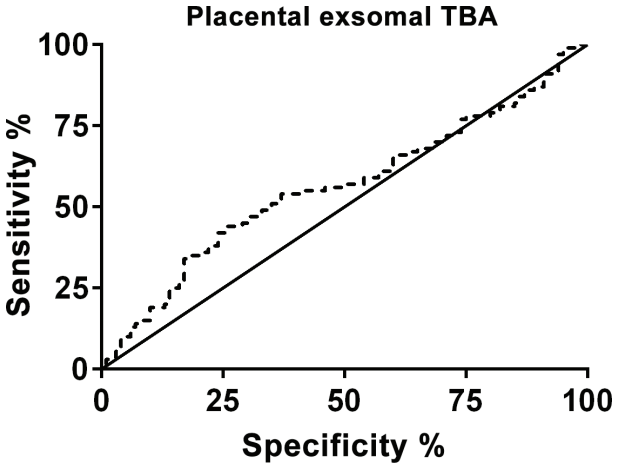

Fig. 1. Diagnostic values of placental exosomal levels of TBA in healthy pregnant participants and ICP patients were analysed by receiver operating characteristic curves. TBA, total bile acid; ICP, intrahepatic cholestasis of pregnancy.

for diagnosing ICP was calculated to be 0.813, with 95% CI 0.751 to 0.875, which was statistically significant ($P < 0.001$). Hence, we further determined the cut-off value of TBA to distinguish ICP from healthy pregnancy to be

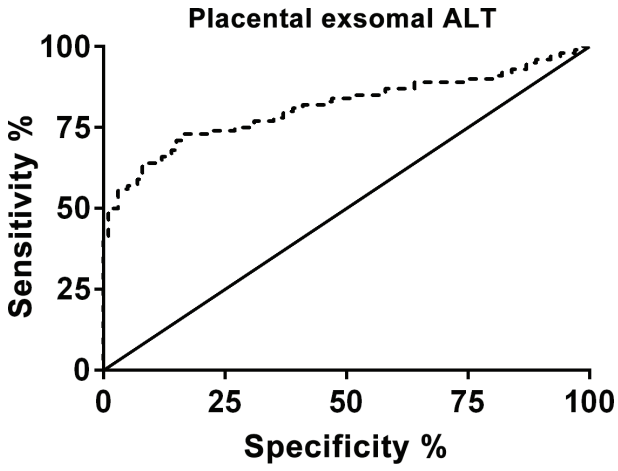

Fig. 2. Diagnostic values of placental exosomal levels of ALT in healthy pregnant participants and ICP patients were analysed by receiver operating characteristic curves. ALT, alanine aminotransferase; ICP, intrahepatic cholestasis of pregnancy.

114.3 IU/L, with 74.0% sensitivity (95% CI 64.3 to 82.3%) and 74.0% specificity (95% CI 64.3 to 82.3%).

Discussion

Elevation of the serum level of TBA is the most frequently observed laboratory sign of abnormality in ICP, along with elevations in ALT (26). ALT was initially characterized by Arthur Karmen and colleagues in the mid-1950s (27). ALT is most concentrated in the liver, catalysing the alanine cycle. Clinically, ALT is often measured for diagnostic assessment of hepatocellular injury or used as biomarkers for liver health together with serum level of

AST and AST/ALT ratio. With relation to ICP, ALT was also recently reported as a predictor of adverse perinatal outcomes in ICP women (25). However, its value as a biomarker to predict the onset of ICP has never been studied. In this context, our current study provides the first instance of data to support the role of ALT, in particular its level in placental exosomes during the second trimester of pregnancy, as an accurate biomarker to predict ICP.

In this study, we have also examined TBA in both healthy participants and ICP patients. Although it has been suggested that elevation of serum levels of TBA could be employed as a sign of ICP by several prior studies conducted on female patients with ICP from other ethnic groups (11–13), it is not the case in the present study, likely because the subjects in our current study were entirely of Chinese ethnics. In fact, the aetiology behind ICP appears heterogeneous and is still not completely clear, and a number of factors such as genetic predispositions have been suggested in the pathology of ICP (10). In line with this, our data demonstrated a trend of up-regulation in placental exosomal TBA levels among ICP patients compared to healthy participants. However, such increase was not statistically significant, therefore arguing against the use of TBA levels as a laboratory sign to predict ICP among Chinese patients. Hence, other biomarkers with sensitivity and accuracy, while being easily measurable, are urgently needed.

Exosomes function primarily through communication with both proximal and distal cells to modulate their phenotype as well as function (28). Investigations implicated that placental-derived exosomes are crucial in immune tolerance during either healthy or complicated pregnancies (29). It is suggested that exosomes exert immune regulatory roles in a successful pregnancy, based on the variable contribution of placental-derived exosomes and bioactivity in healthy pregnancies. The most likely function of placental-derived exosomes is to modify the bioactivity of target cells that are either adjacent or distal (30, 31). Therefore, perturbations in the secretion of exosomes and their cargo contents may result in profound physiological and/or pathological effects, through circulation, on various organs and tissues of human body. In this context, discovery in our study that placental exosomes and especially their cargo ALT levels are correlated with incidence of ICP provides yet another example of the usefulness of exosomes in predicting and diagnosing pregnancy-related diseases. In this context, drugs that inhibit exosome secretion may serve as potential treatment against ICP, which calls for further investigations. Nevertheless, there are certain limitations in our current study: 1) full liver function tests were not included in the analysis; 2) parameters were analysed using a multivariate analysis. Future study is to address these issues in a more detailed investigation.

In summary, in our retrospective clinical study, predictive values of placental exosomal TBA and ALT were compared between healthy pregnancies and ICP patients. To the best of our knowledge, our study is the first of its kind in investigating the potential utility of placental exosome in the diagnosis of ICP. More importantly, our data suggest that, compared to widely known biomarker TBA, ALT in the placental exosomes is a more accurate biomarker for distinguishing ICP from healthy controls with a better compromise between specificity and sensitivity. Our study, therefore, demonstrates the predictive value of placental exosomal ALT levels in women with ICP.

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Conflicts of interest and funding

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