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Research article

Elevated GABRP expression is correlated to the excessive autophagy in intrahepatic cholestasis of pregnancy

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ABSTRACT

In intrahepatic cholestasis of pregnancy (ICP) patients, high concentrations of bile acids altered the normal maternal-fetal-unit physiological condition and could bring negative influence on placenta functionality. GABRP is the pi subunit of the gamma-aminobutyric acid type A receptor ($GABA_A$) and plays pivotal role in regulating $GABA_A$ receptor's physiological function. Here we presented evidence that increased expression of GABRP in parallel with autophagic biomarkers, LC3 and ATG14, in patients with ICP. Methods: A total of 27 participants, including 18 ICP patients and 9 healthy pregnancies were recruited according to strict inclusion criteria. Placentas of ICP patients and controls were collected immediately after cesarean section before labor onset. GABRP and autophagic markers expression in placenta were investigated by immunohistochemistry (IHC), RT-qPCR, and Western blot. Results: The neonatal birthweight and gestational weeks were significantly lower in severe ICP group, while the hepatic enzymes were elevated in ICP group. Semiquantitative analysis of IHC revealed the AOD of GABRP in severe ICP patients was higher than that in mild ICP patients and control pregnancies. Western blot and RT-qPCR analysis both indicated that the expression of GABRP and ATG14 were significantly elevated in severe ICP patients. Moreover, GABRP was correlated with TBA ($r = 0.64$, $p < 0.05$), ATG14 ($r = 0.87$, $p < 0.05$), direct bilirubin ($r = 0.54$, $p < 0.05$), ALT ($r = 0.72$, $p < 0.05$), and AST ($r = 0.67$, $p < 0.05$). Conclusion: There were elevated expression of GABRP, ATG14 and LC3 in ICP placentas compared with uncomplicated pregnancies. The expression of GABRP was associated with autophagy and was correlated with the TBA levels.

1. Introduction

Intrahepatic cholestasis of pregnancy (ICP) is a pregnancy related disorder of unknown etiology. Maternal risk of ICP is ignorable compared with the fetal risk as ICP significantly elevated the risk of perinatal mortality [1,2]. The incidence of ICP varies widely from 0.1 to 15.6% due to geographical and demographic differences [2,3]. In ICP placentas, high concentrations of bile acids can bring biochemical and even morphological changes in placenta, the physiological functionality of placenta in ICP pregnancy is interrupted

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[4,5]. As an important factor for embryogenesis, trophoblast implantation, and pregnancy maintenance, disrupted autophagy in trophoblasts is one of the possible underlying mechanisms for adverse pregnancy outcomes in ICP patients. Autophagy is an evolutionarily conserved process which allows eukaryotic organisms to survive in harsh conditions. Autophagy plays an irreplaceable role in embryogenesis, implantation, and pregnancy maintenance [6,7,8]. During autophagy, unique double-membraned autophagosomes are formed to engulf excessive, dysfunctional or toxic intracellular components [9]. As a dynamic process, the beginning of autophagy is marked by the formation of double membrane autophagosomes, which then fused with lysosomes for degradation. The core machinery of autophagy consists of over 30 autophagy-related proteins, including autophagy related 14 homolog (ATG14) and microtubule-associated protein 1A/1B-light chain 3 (LC3). LC3 is a soluble protein, which functioned as an important structural component of the autophagosome [10]. ATG14 is an essential autophagy-specific regulator of the class III phosphatidylinositol 3-kinase complex [11]. ATG14 played an important role in promoting autophagosome fusion with lysosome by directly binding to STX17 autophagosomes with LAMP2-labeled lysosomes. In ICP pregnancies, impairment in trophoblast autophagy has been investigated by previous studies with controversial results [12,13,14].

As the major inhibitory neurotransmitter in the central nervous system, gamma-aminobutyric acid (GABA) is also widely distributed in the peripheral systems including endocrine organs, gastrointestinal tract and reproductive organs [15,16]. GABRP is the pi subunit of the $GABA_A$ receptor. As a recombinant receptor composed of different subunits, GABRP has important role in regulating for $GABA_A$ receptor's function and the downstream signal transduction [15,16]. GABRP has been found in several reproductive organs, including uterus, prostate, ovaries and placenta [17,18,19]. The GABAergic activation could enhance antimicrobial response by promoting autophagy activation via macrophage $GABA_A$ receptor [20]. The correlation of GABRP with dysfunction in trophoblast apoptosis has been identified in the pathogenesis of preeclampsia by previous studies [17]. Expression of GABRP in human and mouse trophoblastic cells is also associated with pathological implantations [17,21]. However, the underlying mechanisms of GABRP in pregnancy complications remains largely unknown.

In the present study, we aimed to delineate the potential role of GABRP in the process of trophoblast autophagy, especially the impact on LC3 and ATG14 expression in ICP pregnancies.

2. Materials and methods

2.1. Study subjects

From March 2021 to September 2021, a total of 27 participants, including 18 ICP patients and 9 healthy pregnancies were recruited at West China Second University Hospital in Chengdu, China following approval of the Ethics Committee of West China Second University Hospital.

ICP cases were identified if the patient was presented with serum total bile acids above 10 mmol/L and raised liver transaminase enzymes with no additional identifiable causes for their liver dysfunction [1,22]. Severe ICP was defined according to TBA levels as >40 mmol/L [1,22–25]. Pregnancies without maternal and fetal complications were included in the control group. Most of participants in the control group chose selective cesarean section due to scarred uterus. The exclusion criteria were patients diagnosed with hypertensive disorders, twin pregnancies, diabetes mellitus, cancer, chronic kidney disease, chronic heart disease and chronic liver disease. Patients who had vaginal delivery or had emergency CS during labor were excluded. In ICP group, patients were excluded if their elevated liver enzyme or pruritus could be attributed to causes other than ICP (e.g. hepatitis, gallstones, cholecystitis or liver cirrhosis). Suspicion of hepatitis virus, Epstein Barr virus and cytomegalovirus were screened. Consent form for their blood sample and placenta were obtained from all participants at admission.

3. Samples

Placentas of ICP patients and controls were collected immediately after cesarean section before labor onset. Villous samples were taken from each lobules of each placenta (approximately 0.5 g each). The tissue was washed with 0.9% saline solution repetitively and were briefly rinsed in ice-cold phosphate-buffered saline (PBS) and transferred from hospital to the laboratory within 30 min. Snap-frozen of these samples were performed immediately in liquid nitrogen and stored at −80 °C until use for RNA extraction and protein analysis. Two cylindrical samples of placental tissue were obtained near the insertion of umbilical cord and near the margin of placenta. The cylindrical samples with a diameter of 2.5–3 cm were obtained by cutting through the placenta from the maternal surface down to the fetal surface. After repetitive wash with 0.9% saline solution, these tissue samples were sectioned into blocks of 10-mm

Table 1
Specific primers for the four genes.

GABRP	Sense	*5′-CGACCGTGTTATCAATGACC-3′*
	Antisense	*5′-CCCCAAACACAAAGCTAAAGCA-3′*
LC3B	Sense	*5′-GTTACGGAAAGCAGCAGTGTA-3′*
	Antisense	*5′-CAGAAGGGAGTGTGTCTGAATG-3′*
ATG14	Sense	*5′-CATTATGAGCGTCTGGCAAATC-3′*
	Antisense	*5′-GTCTCTCACACCCGTCTTTAC-3′*
ACTB	Sense	*5′-GGATCAGCAAGCAGGAGTATG-3′*
	Antisense	*5′-AGAAAGGGTGTAACGCAACTAA-3′*

thick, and then were immersed in formalin at room temperature for further analysis of morphology.

3.1. Real-time PCR analysis

GABRP, LC3, and ATG14 mRNA expressions in placentas were analyzed by real-time PCR method. Total RNA (about 5 μg) was extracted from around 5 mg sample using the RNeasy Kit (Foregene, Chengdu, China) and dissolved in 20 μl of RNase-free water. The cDNAs were reverse transcribed from 1 μg of total RNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (RR047A; Takara Bio, Inc., Kusatsu, Japan). Specific primers were then used amplify the four genes (Table 1).

RT-PCR assays were performed using SYBR Premix Ex *Taq*II (RR820A; Takara Bio, Inc., Kusatsu, Japan) in a CFX96™ Real-Time PCR detection system (Bio-Rad, Berkeley, CA, USA) 2-ΔΔCt method was used to calculate the relative quantitative value. This assay was repeated three times. Transcript levels for the genes of interest were normalized to those of the ACTB housekeeping gene assayed in parallel.

4. Western blotting

Placental samples (100 mg) were homogenized in ice-cold lysis buffer containing protease inhibitors. Protein concentrations were determined using BCA Protein Kit (Biotech Corporation, Shanghai, China). Homogenates were incubated for 30 min on ice and then subjected to centrifugation at 12,000 g for 20 min at 4 °C. The supernatants were collected, and total protein concentration in the supernatants was quantitated using the Bio-Rad assay (BioRad, Hercules, CA, USA). Equal amounts of protein were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Subsequently, the proteins were transferred onto polyvinylidene difluoride membranes. Membranes were blocked for 2 h at room temperature in Tris-buffered saline containing 5% skim milk and 0.1% Tween 20, followed by incubation with different primary antibodies for 4 h with slow shaking and additional incubation at 4 °C overnight. The primary antibodies included the following: anti-GABRP (1:1000, ab26055; Abcam, Cambridge, UK), anti-LC3 (1:1000, ab48394; Abcam, Cambridge, UK), anti- ATG14 (1:1000, 28021-1-AP; ProteinTech Group, Wuhan, China). β-Actin (1:1000, ab8227; Abcam, Cambridge, UK) was used as an internal control. The membrane was washed 3 times with washing buffer for 10 min and then were incubated with secondary antibody (1:3000, ab6721; Abcam, Cambridge, UK) at room temperature for 1 h. Relative band densities were quantitated by densitometry using Quantity One software (Bio-Rad). All experiments were repeated at least three times.

4.1. Immunohistochemical analyses

The paraffin-embedded placentas were sectioned at a thickness of 4–5 μm. Then, 5% hydrogen peroxide was incubated, following pre-treatment with 5% bovine serum albumin (BSA) for 30 min, the sections were performed with anti-GABRP (1:200, ab26055; Abcam, Cambridge, UK), anti-LC3 (1:200, ab48394; Abcam, Cambridge, UK), anti- ATG14 (1:200, 28021-1-AP; ProteinTech Group, Wuhan, China). Phosphate-buffered saline as a substitute for the primary antibody at 4 °C overnight. The Histofine Simple Stain PO (M) kit (Nichirei, Tokyo, Japan) was used to detect the antibody binding. Incubation the sections with DAB substrate was applied to detect peroxidase activity. Brown deposits indicated positive staining. Sections were counterstained with hematoxylin. All slides were examined, and representative photographs were taken using an Olympus BX41 microscope (Olympus, America, Melville, NY). The staining results were analyzed by Image-J software (NIH, United States). Semiquantitative analyses were applied by calculating the average optical density (AOD) of positive staining for GABRP, LC3, and ATG14. The AOD was calculated by integral optical density (IOD) compared with area.

4.2. Statistical analysis

Analysis of the data were conducted using IBM SPSS 23 software and R version 4.0.4 (IBM corp., Armonk, NY, USA). For the quantitative variables, data are presented as means ± standard deviation. Independent sample T test or one-way analysis of variance

Table 2
Clinical and obstetric characteristics of participants.

Characteristics	Control (9)	Mild ICP (9)	p^a	Severe ICP (9)	p^a
Age (years)	31.67 ± 4.30	28.67 ± 5.22	0.202	30.67 ± 4.53	0.637
Body mass index (cm/kg2)	20.69 ± 2.27	21.75 ± 2.57	0.367	21.20 ± 2.70	0.666
Gravidity	2.00 ± 1.41	1.56 ± 0.53	0.390	1.89 ± 1.17	0.858
Parity	1.11 ± 0.33	1.22 ± 0.44	0.555	1.33 ± 0.50	0.284
Gestational weeks (week)	38.93 ± 0.64	37.96 ± 1.30	0.052	35.05 ± 1.15	<0.001[b]
Neonatal weight	3091.11 ± 197.32	3011.78 ± 190.25	0.404	2116.67 ± 210.67	<0.001[b]
10 min Apgar score	10 ± 0	9.89 ± 0.33	0.332	9.67 ± 0.50	0.063
Preterm birth	0/9	2/9	0.134	9/9	<0.001[b]
Meconium-stained amniotic fluid	0/9	2/9	0.134	4/9	0.023[b]
History of ICP	0/9	0/9	0.134	2/9	0.134

[a] : Compared with control group.
[b] Significant statistical difference.

(ANOVA) test was applied in statistical analysis. Chi-square tests was used to compare the data for categorical variables. For the correlation matrix, Pearson's parametric correlation test was utilized. A two-sided $P < 0.05$ was considered statistically significant.

5. Results

5.1. Clinical characteristics of participants

Tables 1 and 2 showed the clinical characteristics and biochemical parameters of these three groups. The maternal age, pre-pregnancy BMI, gravidity and parity were comparable between ICP groups and control group. The neonatal birthweight and gestational weeks were significantly lower in severe ICP group. Only 2 participants in the severe ICP group were diagnosed with ICP in previous pregnancy. The alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bile acid (TBA) were significantly higher in the mild ICP group and severe ICP group (Tables 2 and 3).

5.2. Immunohistochemical analysis of GABRP and autophagic markers expression in placental tissue between ICP patients and normal pregnancy

Immunohistochemical (IHC) analysis was utilized on placental tissues of ICP patients and control participants. GABRP was mainly located in the cell membrane of trophoblasts (Fig. 1(A-C)). Semiquantitative analysis revealed the AOD of GABRP in severe ICP patients was higher than that in mild ICP patients and control pregnancies. The AOD of GABRP in mild ICP group was higher than that of control group. LC3 Fig. 1D-F) and ATG14 Fig. 1G-H) expression were mainly expressed in cytoplasm of trophoblasts. There was significantly higher expression of LC3 and ATG14 in severe ICP group compared with that of control pregnancies.

5.3. Comparison of protein and mRNA expressions of GABRP and autophagic markers in placental tissue between ICP patients and normal pregnancy

Western blot analysis showed that GABRP expression was significantly higher in severe ICP group in comparison to mild ICP group and normal controls ($P < 0.05$) (Fig. 2A and B). There were no statistical differences between mild and severe ICP group compared with control group in expression of LC3. The expression of ATG14 was significantly higher in severe ICP group and mild ICP group compared with control group.

Real-time quantitative PCR analysis showed that expression of GABRP mRNA was significantly higher in ICP patients. The expression of GABRP was higher in severe ICP group than in mild ICP group (Fig. 2C). The LC3 mRNA expression was higher in severe ICP group compared with control. And analysis of ATG14 mRNA expression also revealed elevated expression in severe ICP group compared with controls.

5.4. Correlation between protein expression of GABRP and autophagic markers in placenta of patients with ICP and their relationships with other biochemical parameters

The correlation matrix was constructed by testing the Pearson parametric correlation coefficients among maternal serum biochemical indicators related to hepatic and biliary function with GABRP and autophagic markers expressed in the placenta. There are significant and positive correlations between GABRP with ALT ($r = 0.72$, $p < 0.001$), AST ($r = 0.67$, $p = 0.001$), TBA ($r = 0.64$, $p = 0.001$), direct bilirubin ($r = 0.54$, $p = 0.004$), and ATG14 ($r = 0.87$, $p < 0.001$). Positive correlations between ATG14 with ALT ($r = 0.75$, $p < 0.001$), AST ($r = 0.67$, $p = 0.001$) and TBA ($r = 0.53$, $p = 0.005$) were found (Fig. 3).

6. Discussion

Placental dysfunction was the underlying reason for adverse pregnancy outcomes during the late trimester of pregnancy in ICP

Table 3
Biochemical parameters of participants.

	Control (9)	Mild ICP (9)	p^a	Severe ICP (9)	p^a
Alanine aminotransferase (U/L)	19.11 ± 8.28	201.33 ± 78.63	<0.001[b]	376.78 ± 132.13	<0.001[b]
Aspartate aminotransferase (U/L)	21.44 ± 6.44	153.33 ± 77.57	0.001[b]	241.78 ± 60.52	<0.001[b]
Total bile acid (μmol/L)	3.31 ± 1.63	19.47 ± 7.31	<0.001[b]	74.84 ± 24.61	<0.001[b]
Total bilirubin (μmol/L)	8.14 ± 2.62	9.46 ± 3.61	0.391	11.37 ± 3.86	0.055
Direct bilirubin (μmol/L)	2.07 ± 1.01	3.74 ± 2.74	0.104	6.88 ± 2.93	0.001[b]
Indirect bilirubin (μmol/L)	6.07 ± 2.10	5.71 ± 2.37	0.732	4.49 ± 1.76	0.101
Lactic dehydrogenase (μmol/L)	367.10 ± 97.84	270.84 ± 94.77	0.369	551.00 ± 276.77	0.193
γ-glutamyl transferase (U/L)	18.56 ± 9.74	47.44 ± 31.45	0.018[b]	141.56 ± 61.90	<0.001[b]
Alkaline phosphatase (U/L)	168.67 ± 59.79	333.44 ± 130.12	0.003[b]	352.67 ± 161.45	0.006[b]

[a] : Compared with control group.
[b] Significant statistical difference.

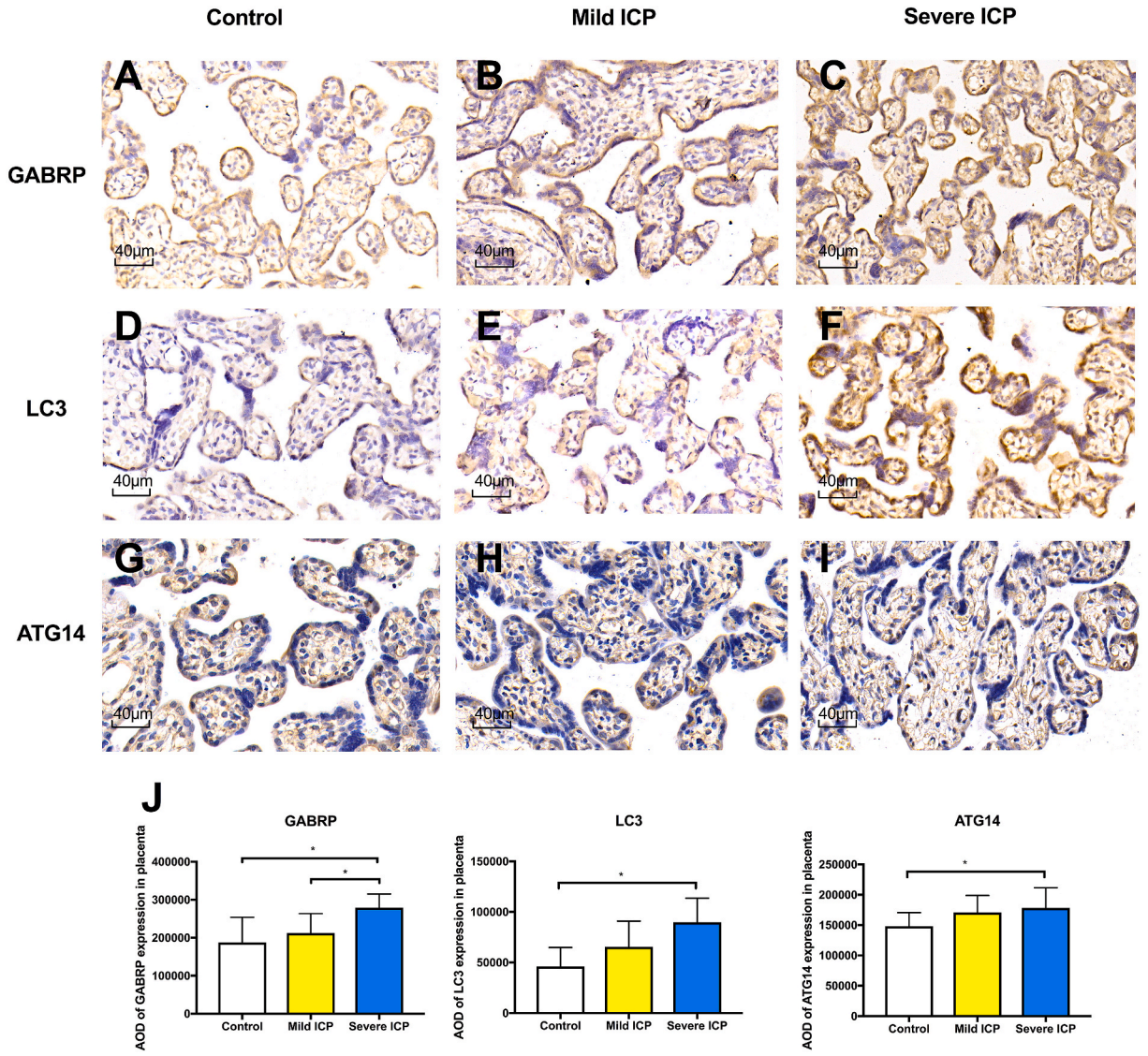

Fig. 1. (A) (B) (C) Expressions of GABRP in placental tissue by immunochemistry in normal pregnancy, mild ICP patients and severe ICP patients. (D) (E) (F) Expressions of LC3 in placental tissue by immunochemistry in normal pregnancy, mild ICP patients and severe ICP patients. (G) (H) (I) Expressions of ATG14 in placental tissue by immunochemistry in normal pregnancy, mild ICP patients and severe ICP patients. (J) The AOD of GABRP was significantly increased in severe ICP group compared with control ($P < 0.05$), there was significant difference between mild ICP group and severe ICP group. In the comparison of AOD of LC3 and ATG14 expression, severe ICP group had increased expressions of these autophagic markers compared with control group.

patients [2,26]. High concentrations of TBA could damage maternal vital organs and put great threats to the fetus through uterine-placental-fetal unit. As the major components of placenta, trophoblasts were the main target of circulating bile acids [27,28]. Here we demonstrated that the expressions of autophagic biomarkers LC3 and ATG14 were elevated in trophoblasts of ICP placentas. Our data revealed the important role of GABRP as a novel potential influencing factor in regulation of LC3 and ATG14 of ICP pregnancies. We also found positive correlations between GABRP with the autophagic biomarkers in placenta and serum circulatory TBA and biochemical parameters.

Dysfunction of autophagy in trophoblasts were recognized as a contributing factor for disrupted placental function in many pregnancy complications [29]. However, limited researches with controversial results investigated autophagy in ICP pregnancies. Autophagy is activated under starvation and hypoxic conditions. Autophagic cell death is another way of programmed cell death. Hu et al. found the increased expression of LC3 in ICP placentas were caused by Linc02527 [12]. By sponging miR-3185, Linc02527 could promote autophagy in HTR8/SVneo cells. Proteomic analysis of TCA treated term placental villous fragments revealed that chronic exposures to toxic BAs altered the expression of autophagy related proteins [14]. Of note, the hydrophobic BAs are potent regulating factors for endoplasmic reticulum stress, reactive oxygen species (ROS) system, immunology and inflammatory reactions in

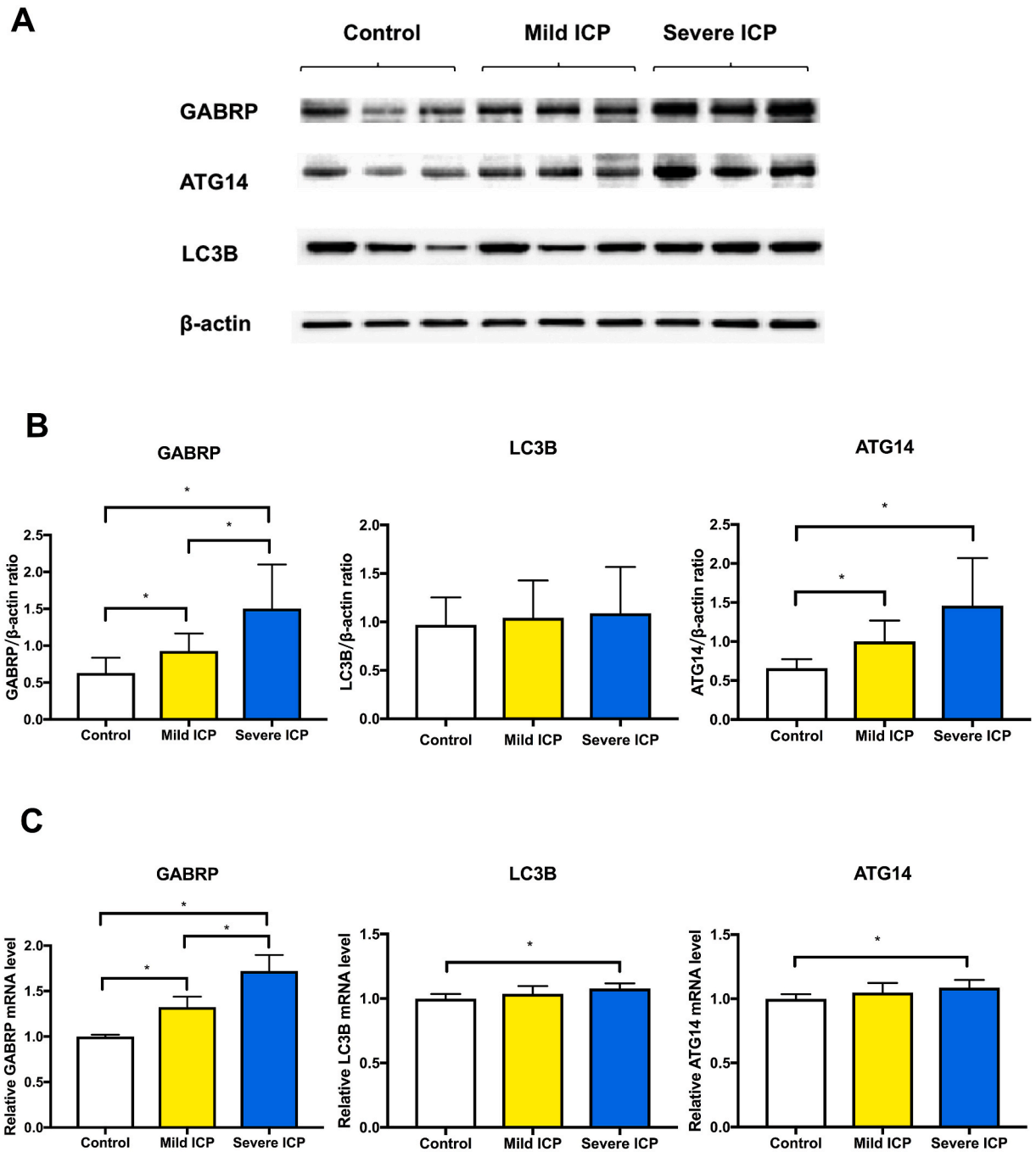

Fig. 2. (A) Protein expressions of GABRP and autophagic parameters in ICP placentas and normal control. (B) There were significantly higher protein expressions of GABRP in placentas with ICP compared with control group ($P < 0.05$), and there was significant difference between mild ICP group and severe ICP group ($P < 0.05$). No difference of LC3 expression was found in the comparison between ICP group and control group. There were significantly higher protein expressions of ATG 14 expression in placentas of ICP patients compared with control group ($P < 0.05$). (C) The expression of GABRP mRNA were significantly increased in ICP groups compared with control ($P < 0.05$), there was significant difference between mild ICP group and severe ICP group. In the comparison of LC3 and ATG14 mRNA expression, severe ICP group had increased expressions of these autophagic markers compared with control group. The uncropped Western blot picture are shown in Supplementary Fig. S1.

trophoblasts [28], which, of no doubt, will lead to impairment in autophagy and even trigger autophagic cell death. Our results provided the evidence in excessive expressions of autophagy related biomarkers in ICP placentas. These findings indicated that the underlying reason for the impaired functionality of ICP placenta might be caused by excessive autophagy.

Our study first demonstrated GABRP expression was significantly elevated in ICP patients and were correlated with the two

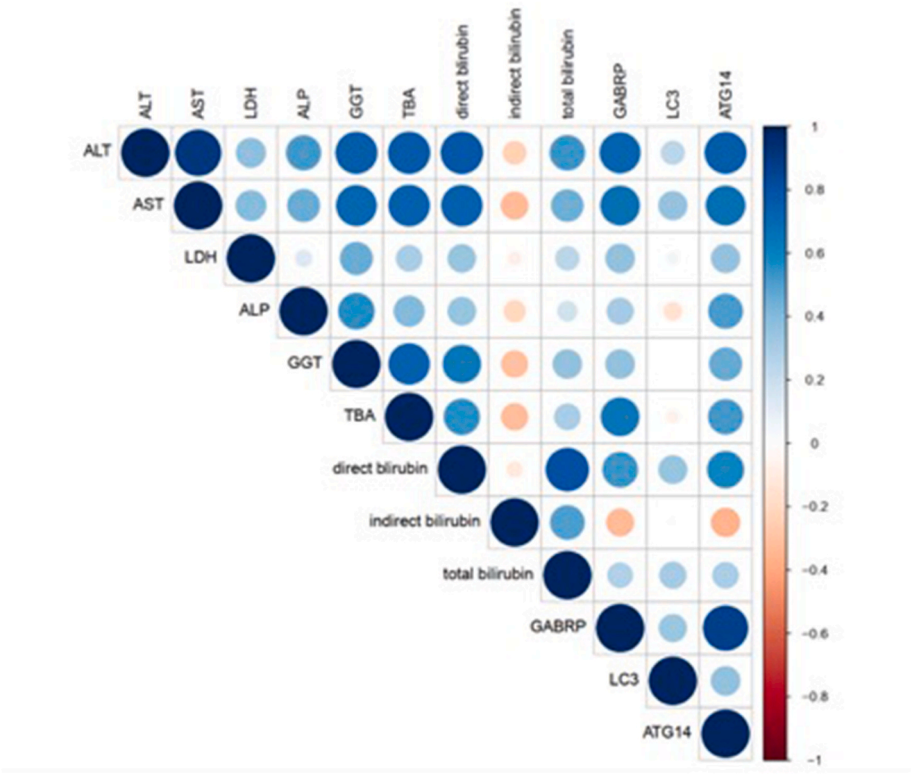

Fig. 3. Pearson correlation matrix of GABRP, autophagic markers of placenta and serum biochemical parameters. The color-coded map displays the value of the Pearson correlation coefficient. Positive correlation was indicated as blue and negative correlation was indicated as red. The size and color intensity of the circle are in proportion with the correlation coefficients. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

autophagic biomarkers. Results from IHC analysis, real-time quantitative PCR and Western blot analysis demonstrated there was significantly higher expressions of ATG14, LC3 and GABRP in placentas of severe ICP patients compared with that of normal control group. These data indicated that GABRP might be a potential sensitive target of circulating bile acids and involved in the regulation of autophagy in trophoblasts. As the modulating subunit of $GABA_A$ receptor, GABRP was reported to play an important role in female reproduction and gestational disorders. In mouse oocytes and cumulus cells, GABRP was responsible for oocyte maturation [30]. In early pregnancy, GABRP was also involved in the suppression of remodeling of decidua [21]. In patients with preeclampsia, Lu found the elevated expression of GABRP was associated with the increased apoptosis in trophoblasts of placentas [17]. In patients with pancreatic ductal adenocarcinoma, Jiang found increased expression of GABRP could interact with potassium calcium-activated channel subfamily N member 4 (KCNN4) to induce ca^{2+} entry and led to activation of several downstream signaling pathways and finally cause macrophage infiltration [15]. Although the possible underlying mechanism of GABRP in ICP placentas remains to be elucidated. Our findings revealed a new role of GABRP in ICP pregnancies. The correlations between GABRP, ATG14 and LC3 with TBA and hepatic enzymes in patients with ICP also needed concern. These findings reflected the consistency of the toxic effects of bile acids in both placenta and liver, and provided another reason for the strict monitoring of TBA level in the third trimester in ICP pregnancies.

However, our findings should be interpreted with caution due to the following limitations. First, our findings are quite superficial. The underlying mechanism of GABRP's potential role in regulation of autophagy in ICP pregnancy still needs to be explored. The mechanism of how TBA influence GABRP expression remains unsolved. Second, the methods used in this research were primitive. The authenticate reverse verification of GABRP's function was not tested. Detection of autophagy were only by measuring the two classic autophagy biomarkers. Data on cell experiment and animal experiment were lacking. Third, limited number of participants was included and all ICP patients in our study were treated with UDCA. Although no previous studies reported the influence of UDCA on the expression of GABRP. The possibility of influencing effects could not be ruled out. Fourth, some of the clinical confounding factors, including gestational age and status of amniotic fluid could have potential influence on trophoblasts. Due to the limitation in the number of participants, these confounding factors could not be counterbalanced. Larger number of participants will be included in the future studies, more detailed clinical and experimental petameters will be collected. Moreover, verification of results will be carried out in in-vitro experiments in the future.

7. Conclusion

In summary, our data indicated the elevated expression of GABRP, ATG14 and LC3 in ICP placentas compared with uncomplicated pregnancies. GABRP and ATG14 were positively correlated with the biochemical indicators and TBA level. GABRP might be associated with the elevated TBA level in ICP pregnancies and were also correlated with the increased expression of autophagy biomarkers. However, the underlying mechanism of GABRP and excessive autophagy in ICP still needs to be elucidated.

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Author contribution statement

Ruihong Dong: Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Yayi Hu: Conceived and designed the experiments. Qian Chen: Contributed reagents, materials, analysis tools or data. Dan Shan: Conceived and designed the experiments; Wrote the paper. Yuxia Wu: Analyzed and interpreted the data.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heliyon.2023.e13221.

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