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Original Research

Transthyretin and retinol-binding protein 4 in patients with fetal growth restriction

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Summary

The goal of this study was to analyze the relationship between maternal serum levels of transthyretin (TTR), retinol-binding protein 4 (RBP4), and the birth weight in fetal growth restriction (FGR). *Materials and Methods:* Retrospective case control study including 30 FGR patients and 30 normal healthy pregnancies with the same week of gestation. Serum concentrations of TTR and BRP4 were assessed by ELISA; placenta concentration of TTR and RBP4 by Immunohistochemical method. *Results:* FGR patients were characterized by reduced TTR compared to controls .The concentration of TTR in FGR was 4.05 ± 0.32 ng/ml and control was 5.20 ± 0.27 ng/ml ($p < 0.05$); however, RBP4 remained unchanged in two groups (FGR *vs*. controls RBP4 35.36 ± 2.15 ng/ml *vs*. 35.09 ± 1.58 ng/ml, $p > 0.05$). The expression of TTR had a positive correlation with the birth weight in FGR ($r = 0.620$, $p < 0.05$). Placenta of FGR showed a strong staining for TTR, while control was only weak by using immunohistochemistry. The expression of RBP4 was poor in FGR and control. *Conclusions:* The higher the maternal plasma in TTR, the better the fetal development. TTR may be the marker to diagnose FGR and forecast the sequelae of the fetus.

Key words: Fetal growth restriction; Transthyretin; Retinol blinding protein; Birth weight.

Introduction

Fetal growth restriction (FGR) is a serious pregnancy related the early impaired placentation and restricted growth of the fetus. The pathogenesis of idiopathic FGR is complex. However this theory accepted the characterized poor placentation, shallow trophoblast invasion, and impaired transformation of uterine spiral arteries. These pathological changes could reduce transfer of nutrients and oxygen from the placenta to the fetus followed and developed the fetal growth restriction [1, 2].

Human transthyretin (TTR) is a 55-kDa homotetrameric protein carrier for thyroxin (T4) which is mainly synthesized by the liver, meninges, choroid plexus, pancreas, intestine, and the syncytiotrophoblast of human placenta. Its function is to carry T4 by binding with thyroxine-binding protein (TBG) in plasma [3, 4] and play an important role in the transport of T4. Moreover, TTR is responsible for transporting retinol by forming a complex with retinol-binding protein 4 (RBP4). It has been known that retinol plays an important role of many physiological processes, including immune function, reproduction, and normal embryonic and fetal development [5]. RBP4 is a 21-kDa adipokine which highly expressed in liver, fat, and placenta during pregnancy. The complex of TTR-RBP4 (in a 1:1 molar ratio) is the specific receptor which can prevent retinol form excreting in the kidney, increase the stability and solubility, and reduce the toxicity of retinol [6, 7].

Recent studies showed that point mutations of the TTR gene was responsible for many diseases such as gestational diabetes mellitus and familial amyloidosis [8]. Although some studies showed TTR was dysregulated in FGR, but the relationship with complex of TTR-RBP4 and fetal birth weight has not been investigated in FGR. The aim of this study was to evaluate the concentration of TTR and RBP4 in maternal blood and placentas in FGR, and furthermore to study the relationship between TTR-RBP4 and birth weight.

Materials and Methods

This was a retrospective study on maternal serum and placentas which collected during the period between 2015 and 2016 at the Beijing Luhe Hospital, Capital Medical University, China. Medical and obstetric history was obtained for each woman using the institutional data base (Obstetrics and Gynecology, Beijing Luhe Hospital, Capital Medical University, China). Gestational age was determined by the last menstruation or ultrasonic gestational age by calculated embryonic crown-rump length (CRL). All the samples were collected and kept at 4°C for 24 hours until samples were stored at –80°C. Before screening program and collecting sample, an informed consent was signed. This retrospective study was conducted with the Declaration of Helsinki.

The study consisted of 60 pregnant women who were assessed between the 32rd and 36th gestational week, including 30 FGR and 30 normal pregnancies. The FGR patients had no other complication such as gestational diabetes mellitus, preeclampsia, and medical disease. All collected samples were matched with respect

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to gestational age of sampling. Maternal serum were selected before pregnant women accepted treatment in FGR. Placentas were selected among consecutive pregnancies that delivered during the period 2015–2016 at the Clinic for Obstetrics and Gynecology, Beijing Luhe Hospital, Capital Medical University, China. FGR was defined as the birth weight lower than the average weight of the fetus with two standard deviations or 10 percent, or having a birth weight less than 2,500 grams after 37 weeks. Doppler parameters showed absent/reversed end-diastolic umbilical artery flow (ARED) and increased resistance in uterine artery Doppler [9, 10]. Controls were selected normal women with singleton pregnancies and undergoing pregnancy legal termination.

Concentrations of serum TTR and RBP4 were measured by using enzyme-linked immunosorbent assay (ELISA) kit using polyclonal rabbit anti-human antibodies).

Tissue samples were fixed in 10% buffered formalin for 24 hours. Sections (2 μm thickness) of formalin-fixed paraffinembedded tissues were deparaffinized according to standard procedures. The sections were dehydrated with xylene and graded alcohol/water mixtures, deparaffinized, rehydrated, and treated with 3% H_2O_2 for ten minutes to inhibit endogenous peroxidase. Antigen retrieval was performed by incubating sections in citrate buffer at 95°C for 15 minutes. After blocking with normal goat serum for 30 minutes, sections were incubated with anti-TTR and anti-RBP4 (1:150) at 4°C overnight. Sections were then incubated with a sheep secondary antibody for one hour and stained using a diaminobenzidine kit. Visualization was performed by incubating the slides with AEC chromogen single solution for 10 minutes. Negative controls were performed by omitting the primary antibody. Sections were counterstained with hemalaun.

The statistical analysis was performed with R (version3.0.1). The normality of data distribution was checked using the Kolmogorov–Smirnov test. A p-value of < 0.05 was considered significant. Data that were normally distributed were presented as mean (± standard deviation). Correlation analyses were performed using Pearson's coefficient (parametric data) or Spearman's coefficient (non-parametric data). Then, the following statistical analysis was also using Wilcoxon test, t-tests, and Kruskall–Wallis test. In case of multivariate analysis was performed using logistic regression.

Results

The present study reports features of the study population as follows: maternal age in FGR (mean ± SD) of 28.70 ± 0.77 (28.87 ± 0.88 in controls, $p > 0.05$) years, gestational age at delivery (mean ± SD) of 34.99 ± 0.55 (34.96 ± 0.54 in controls, $p > 0.05$) weeks, gravidity of 1.67± 0.19 (1.73 ± 0.23 in controls, $p > 0.05$), and parity of 1.07 ± 0.05 (1.10 ± 0.07 in controls, $p > 0.05$). The authors found no significant differences in maternal age, gestational week, gravidity, and parity (Table 1).

FGR patients were characterized by reduced TTR in maternal serum compared to controls (mean ± SD: 4.05 ± 0.32 ng/ml *vs*. 5.20 ± 0.27 ng/ml $p < 0.05$). However RBP4 did not show changes in two groups (FGR *vs*. controls RBP4 35.36 ± 2.15 ng/ml *vs*. 35.09 ± 1.58 ng/ml, $p > 0.05$) (Figure 1). The authors also studied the relationship between fetal birth weight and the concentration of TTR and RBP4 in FGR patients. As Figure 2 shows, the birth weight had a positive relationship with TTR in FGR and it was significantly correlated ($r = 0.620$, $p < 0.05$). However, the concentration of RBP4 had not significant correlation ($r = 0.105$, $p < 0.05$).

Table 1. — Features of the study population.

Group	Age (years)	Gestational Week (weeks)	Gravidity	Parity
FGR	28.70±0.77	34.99±0.55	1.67±0.19	1.07±0.05
Control	28.87±0.88	34.96±0.54	1.73±0.23	1.10±0.07

There were no significant differences in maternal age, gestational week, gravidity, and parity in two groups ($p > 0.05$).

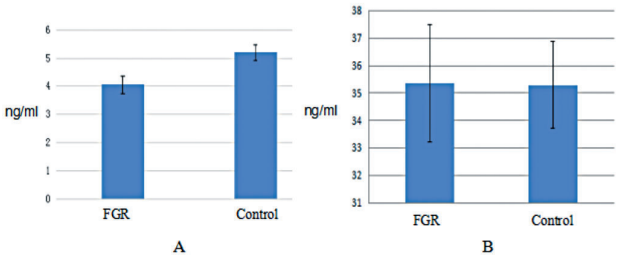

Figure 1. — Serum concentrations of TTR and BRP4 in FGR and control. (A) The concentration of TTR in FGR is reduced ($p < 0.05$). (B) RBP4 did not distinguish changes in two groups ($p > 0.05$).

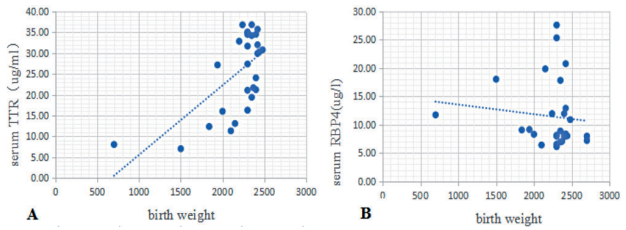

Figure 2. — Relationship between fetal birth weight and the concentration of TTR and RBP4 in FGR patients. (A) Plot of linear regression between fetal birth weight and TTR, showing no significant correlation ($r = 0.620$, $p < 0.05$). (B) Linear regression between fetal birth weight and RBP4 ($r = 0.105$, $p < 0.05$). The r-values in the plots refer to Pearson's correlation coefficient.

All placentas of the two groups was performed with sections by immunohistochemistry. Figure 3 shows characteristic staining patterns of TTR in FGR compared with control. It is obvious that FGR shows a strong staining for TTR, while control only a weak one. Staining is mostly localized on the villous trophoblast. The expression of RBP4 was poor in FGR and control. Semi-quantitative analysis of the staining pattern of TTR in villous trophoblast of preterm placentas was also performed. The authors invited two independent pathologists to analyze the semi-quantify TTR staining focussing on intensity and distribution within the villous trophoblast. The staining intensity was graded as follows: -, +, ++, +++, ++++. Quantification of staining reveals that there is a significant difference between the two

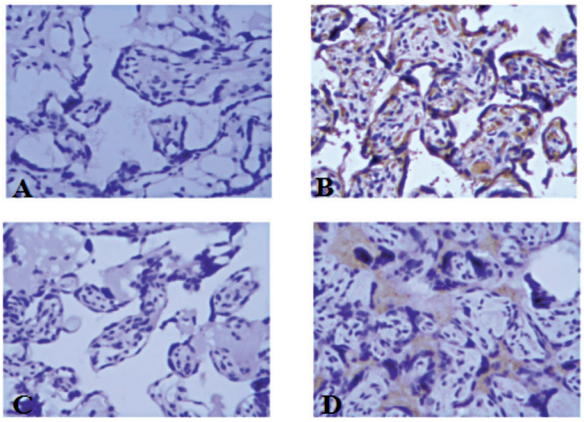

Figure 3. — Immunohistochemical staining for TTR and RBP4 in preterm placenta. The images (A) show representative staining patterns of TTR in preterm controls. (B) Staining patterns of TTR in FGR. (C) Representative staining patterns of RBP4 in preterm controls. (D) Staining patterns of TTR in FGR. FGR shows a stronger staining for TTR than the control, and the expression of RBP4 are poor in the two groups.

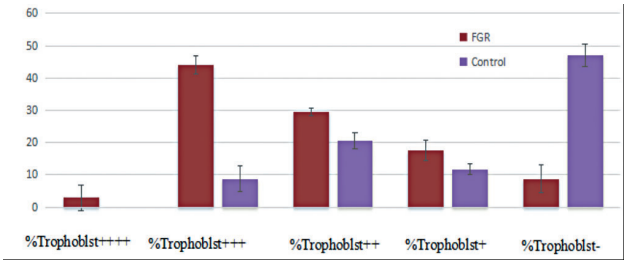

Figure 4. — Semi-quantitative analysis of the staining pattern of TTR in villous trophoblast of preterm placentas. Staining intensity of villous trophoblast is classified into five categories (++++, +++, ++, +, -) and staining is quantified using systematic random selection of images and point grid analysis. There is a significant difference between two groups with a significantly increased staining in the groups FGR ($p < 0.05$).

groups with a significantly increased staining in FGR in Figure 4 (red and blue bars).

Discussion

This is the first study to consider the relationship between TTR and fetal birth weight of FGR in maternal serum. The authors also researched the expression of RBP4, which forms a complex with TTR to transfer retinol from the placenta to the fetus [7, 8]. Retinol is important in fetal development [5]. Some studies have shown that the expression of retinol had decreased FGR in animal models, but few studies focused on the complex TTR-RBP4 metabolism in the placenta. Gharesi-Fard *et al.* reported TTR of placentas increased compared to normal pregnancies versus pregnancies complicated by using a proteomic analysis [11]. Patel *et al.* analyzed the expression of TTR in normal placenta at different gestational weeks and described a significant correlation between TTR levels and gestational age in the first trimester of pregnancy, but the expression of TTR did not increase in the second trimester [12, 13]. No previous study has investigated the relationship between TTR \ RBP4 and the fetal birth weight of FGR.

In the present study, the authors demonstrated that TTR of plasma concentrations decreased in FGR compared to normal pregnancies, while the RBP4 had no significant difference. TTR acts as carrier to transport the retinol-RBP4 complex and forms a ternary complex with retinol-RBP4 in the blood [14]. However, such ligands to the human fetus through the placenta are mostly not yet elucidated. Recently, other studies reported the deficiency of RBP4 may affect the metabolites of retinol following development in FGR [15, 16]. However, the present data show plasma RBP4 concentration is not significantly different between FGR and normal pregnancies ($p > 0.05$). Some studies indicated maternal serum concentration of RBP4 had no significant difference in FGR [17]; this result is the same as the present. Whether RBP4 plays a role in the pathogenesis of FGR still needs further study. The present study reports that the deficiency of TTR is associated with the onset of FGR, which is in agreement with other research [18-20]. Furthermore, the present data showed that there was a significant positive correlation between the birth weight and the concentration of TTR in FGR ($r = 0.620$, $p < 0.05$).

In this study, the authors describe an enhancement of TTR immunoreactivity in placentas of FGR. It is obvious that FGR shows a distinguished increased expression of TTR in the villous trophoblast compared to controls. It has been reported that the expression of TTR in placenta is increased because of the relative hypoxia [13]. Also, other studies detected that TTR is modified in several types of post-translational modifications especially under oxidative conditions [21-23]. Post-translational modifications of the cysteine residue in position 10 (Cys10) in TTR can affect the stability of the TTR tetramer [24]. Arrigo *et al.* found that relative deficiency of the amount of TTR modified by chemical modification of cysteine side chain to form glycine Cys10 in TTR is located away from the sites of interaction of TTR with RBP4, and it seems unlikely to have an effect on the interaction with such TTR ligands. Moreover, RBP4 can undergo post-translational modifications, resulting from the truncation of RBP4 at the C-terminus by one or two leucine residues. Thus, it is possible that augmented TTR expression in placentas of FGR in this study may be the result of placental hypoxia. Because the post-translational modifications in TTR under hypoxia conditions alter its stability, the placenta needs more TTR to transport T4 or the complex retinol-RBP4. The concentration of TTR in maternal serum was positively correlated

with birth weight in FGR ($r = 0.620$, $p < 0.05$). Possibly, the higher the maternal plasma in TTR, the better the fetal development. Moreover, TTR may be a marker to diagnose FGR and forecast the sequelae of the fetus.

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References

[1] Redman C.W., Sargent I.L.: "Latest advances in understanding preeclampsia". *Science*, 2005, *308*, 1592.
[2] Huppertz B.: "Placental origins of preeclampsia: challenging the current hypothesis". *Hypertension*, 2008, *51*, 970.
[3] McKinnon B., Li H., Richard K., Mortimer R.: "Synthesis of thyroid hormone binding proteins transthyretin and albumin by human trophoblast". *J. Clin. Endocrinol. Metab.*, 2005, *90*, 6714.
[4] Richardson S.J.: "Evolutionary changes to transthyretin: evolution of transthyretin biosynthesis". *FEBS J.*, 2009, *276*, 5342.
[5] Kalkunte S.S., Neubeck S., Norris W.E.: "Transthyretin is dysregulated in preeclampsia, and its native form prevents the onset of disease in a preclinical mouse model". *Am. J. Pathol.*, 2013, *183*, 1435.
[6] Christou G.A., Tselepis A.D., Kiortsis D.N.: "The metabolic role of retinol binding protein 4: an update". *Horm. Metab. Res.*, 2012, *44*, 6.
[7] Wiwanitkit V.: "Retinol-binding protein 4, insulin resistance and pregnancy". *Metabolism*, 2012, *61*, e9.
[8] Ando Y., Nakamura M., Araki S.: "Transthyretin-related familial amyloidotic polyneuropathy". A*rch. Neurol.*, 2005, *62*, 1057.
[9] Mitra S.C., Seshan S.V., Riachi L.E.: "Placental vessel morphometry in growth retardation and increased resistance of the umbilical artery Doppler flow". *J. Matern. Fetal Med.*, 2000, *9*, 282.
[10] Sankaran S., Kyle P.M.: "Aetiology and pathogenesis of IUGR". *Best Pract. Res. Clin. Obstet. Gynaecol.*, 2009, *23*, 765.
[11] Gharesi-Fard B., Zolghadri J., Kamali-Sarvestani E.: "Proteome differences of placenta between pre-eclampsia and normal pregnancy". *Placenta*, 2010, *31*, 121.
[12] Patel J., Landers K.A., Li H., Mortimer R.H., Richard K.: "Ontogenic changes in placental transthyretin". *Placenta*, 2011, *32*, 817.
[13] Patel J., Landers K.A., Li H., Mortimer R.H., Richard K.: "Expression and uptake of the hyroxine-binding protein transthyretin is regulated by oxygen in primary trophoblast placental cells". *J. Endocrinol.*, 2012, *212*, 159.
[14] Fruscalzo A., Londero A.P., Driul L., Henze A., Tonutti L., Ceraudo M,. *et al.*: "First trimester concentrations of the TTR-RBP4-retinol complex components as early markers of insulin-treated gestational diabetes mellitus". *Clin. Chem. Lab. Med.*, 2015, *53*, 1643.
[15] Zinkhan E.K., Chin J.R., Zalla J.M., Yu B., Numpang B., Yu X., et al.: "Combinafion of intrauterine growth restriction and a high fat diet impairs cholesterol elimination in rats". *Pediatr. Res.*, 2014, *76*, 432.
[16] Metzler-Zebefi B.U., Lang I.S., Gors S., Brussow K.P., Henniq U., Nurnberg G., *et al.*: "High-protein-low-carbohydrate diet during pregnancy alters maternal plasma amino acid concentration and placental amino acid extraction but not fetal plasma arslino acids in pigs". *Br. J. Nutr.*, 2012, *108*, 2176.
[17] Vaisbuch E., Romero R., Mazaki-Tovi S., Erez O., Kim S.K., Chaiworapongsa T., et al.: "Retinol binding protein 4—a novel association with early-onset preeclampsia". *J. Perinat. Med.*, 2010, *38*, 129.
[18] Vascotto C., Salzano A.M., D'Ambrosio C., Fruscalzo A., Marchesoni D., di Loreto C., *et al.*: "Oxidized transthyretin in amniotic fluid as an early marker of preeclampsia". *J. Proteome Res.*, 2007, *6*, 160.
[19] Fruscalzo A., Schmitz R., Klockenbusch W., Kohler G., Londero A.P., Siwetz M., *et al.*: "Human placental transthyretin in fetal growth restriction in combination with preeclampsia and the HELLP syndrome". *Histochem. Cell. Biol.*, 2012, *138*, 925.
[20] Pecks U., Seidenspinner F., Rower C., Reimer T., Rath W., Glocker M.O.: "Multifactorial analysis of affinity-mass spectrometry data from serum protein samples: a strategy to distinguish patients with preeclampsia from matching control individuals". *J. Am. Soc. Mass. Spectrom.*, 2010, *21*, 1699.
[21] Gericke B., Raila J., Sehouli J., Haebel S., Konsqen D., Mustea A., et al.: "Microheterogeneity of transthyretin in serum and ascitic fluid of ovarian cancer patients". *BMC Cancer*, 2005, *5*, 133.
[22] Gericke B., Raila J., Deja M., Rohn S., Donaubauer B., Naqi B., *et al.*: "Alteration of transthyretin microheterogeneity in serum of multiple trauma patients". *Biomark Insights*, 2007, *2*, 299.
[23] Henze A., Rohn S., Gericke B., Raila J., Schweiqert F.J.: "Structural modifications of serum transthyretin in rats during protein-energy malnutrition". *Rapid Comm. Mass Spectrom.*, 2008, *22*, 327.
[24] Zhang Q., Kelly J.W.: "Cys10 mixed disulfides make transthyretin more amyloidogenic under mildly acidic conditions". Biochemistry, 2003, *42*, 8756.

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