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Placental expression of the insulin receptor binding protein *GRB10*: Relation to human fetoplacental growth and fetal gender

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ABSTRACT

Introduction: Imprinted genes play an important role in mammalian fetoplacental growth and development. We have evaluated whether the placental expression of two imprinted genes, growth factor receptor-binding protein 10 (*GRB10*) and pleckstrin homology-like domain, family A, member 2 (*PHLDA2*) correlate with human fetoplacental growth parameters.
Methods: Placentae (n = 77) were collected from small- (SGA) and appropriate- (AGA) for gestational age full-term singleton pregnancies (n = 36 SGA and 41 AGA). Placentae and neonates were weighed at birth. Realtime quantitative PCR was performed to assess placental transcript abundance of *GRB10* and *PHLDA2* normalized to a panel of reference genes.
Results: Placental *GRB10* transcript abundance associated positively with placental weight (r = 0.307, $P = 0.007$), birth weight (r = 0.267, $P = 0.019$) and neonatal head circumference (r = 0.280, $P = 0.014$). Placental *GRB10* transcript levels were significantly lower in male SGA placentae compared to the male AGA placentae. Placental *PHLDA2* transcript abundance did not show any associations with maternal, placental or neonatal parameters.
Discussion: Placental *GRB10* expression was found to be associated positively with placental weight, birth weight, and neonatal head circumference, especially in males. Hence, we speculate that placental *GRB10* plays a role in regulating fetoplacental growth and thereby in the pathophysiology of fetal growth restriction in the context of fetal gender.

1. Introduction

A large number of small for gestational age (SGA) babies are born in India [1]. Fetal growth is dependent on maternal, environmental, fetal and placental factors. Dysregulated expression of imprinted genes that are critical regulators of fetal and placental growth is a plausible mechanism for linking modifiable environmental factors, such as maternal nutrition, with intrauterine growth and thereby, fetal growth restriction. Imprinted genes in placentas from intrauterine growth restricted human pregnancies [2–4] have consistently been reported to be either upregulated [pleckstrin homology-like domain, family A, member 2 (*PHLDA2*), paternally-expressed gene 3 (*PEG3*), paternally-expressed gene 10 (*PEG10*)] or downregulated [pleiomorphic adenoma gene 1 (*PLAG1*)]. A two-fold increase in expression of *PHLDA2* leads to a significant reduction in placental weight, underlying its importance in placental development, even though the biochemical function of PHLDA2 has not yet been elucidated. On the other hand, knockout mice exhibit an increase in placental weight [5].

Growth factor receptor-binding protein 10 (*GRB10*) is an

Abbreviations: BMI, Body mass index; *GRB10*, growth factor receptor-bound protein 10; *PHLDA2*, pleckstrin homology-like domain, family A, member 2; *IGF1*, insulin-like growth factor 1; *INS*, insulin; SGA, small for gestational age; AGA, appropriate for gestational age; IUGR, intrauterine growth restriction; FFPE, Formalin-fixed paraffin embedded.

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imprinted gene that is expressed from the maternal allele in mouse [7]. Maternal uniparental disomy of the region containing *GRB10* leads to growth failure. Conversely, paternal uniparental disomy leads to prenatal overgrowth. *GRB10* plays a crucial role in regulating placental size as well as efficiency in mice [8]. Though *GRB10* is imprinted in humans as well, it is expressed biallelically in most tissues. However, *GRB10* is expressed from the maternal allele in both human placental villous trophoblasts and mouse labyrinthine trophoblast cells [9]. *GRB10* codes for growth factor receptor-binding protein 10, an adapter protein that can modulate multiple signaling pathways by interacting with multiple cell surface receptor tyrosine kinases and other signaling molecules. The human GRB10 had been identified as an insulin receptor binding protein [6], and a series of intriguing recent reports suggest that it acts as a regulator of glucose and lipid metabolism. *GRB10* knockdown in adult mice pancreas and in human pancreatic islet cells reduces insulin and glucagon secretion, an effect that has been shown in the mice pancreas to be mediated by induction of apoptosis of both exocrine (glucagon producing alpha cells) and endocrine cells (insulin producing beta cells) [10,11]. Adipocyte-specific *GRB10* knockout leads to increase in mTORC1 signaling in adipose tissues and reduction in lipolysis and thermogenic function [12].

A systematic evaluation of the association between *GRB10* and *PHLDA2* transcript abundance in human placenta and fetoplacental growth, especially in the setting of SGA, is lacking. The objective of this study was to examine whether placental expression of *GRB10* and *PHLDA2* are associated with fetoplacental growth in a set of placentae from SGA and AGA births.

2. Subjects and methods

2.1. Study population

The study population is a subset of a larger cohort that has been established at St. John's Research Institute and the departments of obstetrics and gynecology, and pathology of St. John's Medical-College Hospital (SJMCH) Bangalore, India; to study the effect of maternal determinants of fetal growth on placental morphology and histopathology. The 77 pregnant women had been recruited between 2009 and 2012, and the placentae had been collected at birth. The experimental protocols were approved by the Institutional Ethical Review Board. All study participants gave written informed consent at enrollment.

Pregnant women attending the routine antenatal care in the first trimester of gestation were invited to participate in the study. Exclusion criteria were: multiple pregnancies, a clinical diagnosis of chronic illness (e.g. diabetes mellitus, hypertension, heart disease, thyroid disease, and epilepsy), and a positive test for hepatitis B surface antigen, HIV, or syphilis. The routine antenatal tests were carried out at recruitment and as part of the routine antenatal care, folic acid, iron, and calcium supplements and tetanus toxoid were given.

The subjects' age and obstetric history were recorded at recruitment. Assessment of gestational age was done from the first day of the last menstrual period and it was confirmed by ultrasound within 2 weeks of the initial visit. Height was measured to the nearest 0.1 cm on a calibrated stadiometer. Body weight was recorded (Soehnle, Germany) to the nearest 0.1 kg during each monthly visit. Calculation of gestational weight gain per week (GWG) was done between measurements. During the course of pregnancy the morbidity and clinical outcomes were recorded. Baby parameters were measured and birth outcomes were recorded at delivery. Maternal complications at delivery such as pre-rupture of membrane (PROM) were also recorded. Infant birth weight was measured to the nearest 0.01 kg on a digital weighing scale (Phoenix, BWS 101) and birth length measured on a standard infantometer to the nearest 0.1 cm. Birth outcomes were categorized as preterm births (born < 37 weeks of gestation), low birth weight (LBW; birth weight < 2.5 kg), and small for gestational age baby (SGA; birth weight <10th percentile for gestational age) [13].

2.2. Placenta collection

Placentae were collected at birth and fixed in 10% neutral buffered formalin. Placental weights were recorded after trimming of the placental membranes. Formalin-fixed paraffin embedded (FFPE) placental tissues from fetal sites of the placenta were used for gene expression analysis.

2.3. RNA extraction, reverse transcription and quantitative PCR (qPCR)

Placental RNA was extracted from two 20 micron sections per specimen as described previously [14]. Total RNA was reverse transcribed and used for estimations of transcript abundance. qPCR reactions were performed in duplicates in 10 μl reaction volume with a SYBR Green master mix. Primer sequences are available in Supplemental Table 1. Specimens with poorly preserved RNA were excluded based on transcript abundance of the reference gene beta-actin (*ACTB*). Relative transcript abundance was computed based on a modification of the ΔC_t method using *ACTB*, beta-2-microglobulin (*B2M*) and Tyrosine 3-Monooxygenase/Tryptophan 5-Mono oxygenase Activation Protein, Zeta (*YWHAZ*) as reference genes [$\Delta C_t = C_{t\ (\text{target gene})} - C_{t\ (\text{RG mean})}$] [15]. The ΔC_t values were converted to relative normalization unit (RNU $= 15 - \Delta C_t$) according to Korlimarla et al. [14] where 15 represents the approximate dynamic range of the assay. This also helps in converting the C_t values from a scheme where a lower numerical value represents a higher abundance to a more intuitive representation of transcript abundance. The least RNU value across all samples was then subtracted from the RNU values and expressed as adjusted RNU (aRNU) which corresponds to target gene transcript abundance relative to the reference genes on a $\log_2$ scale.

2.4. Statistical analysis

All quantitative results are presented as mean ± s.d. or median (Interquartile range). All characteristics were compared between the groups by either Independent sample t test or Mann-Whitney U test based on the satisfaction of assumptions of normality. The normal distribution was examined using Q–Q plots. The associations of maternal, placental and newborn measurements (including birth weight and placental weight) with placental *GRB10* transcript abundance were examined using either Pearson's correlation coefficient or Spearman's rank correlation coefficient. Linear regression analyses were performed to examine the significant associations in correlation analysis after adjusting for probable confounders such as mother's height, first trimester weight, age, parity. Regression coefficient b and the corresponding *P* value are reported. $P < 0.05$ was considered statistically significant. Data analysis was conducted using SPSS software (version 13; SPSS Inc., Chicago, IL, USA).

3. Results

The anthropometric and clinical data of 77 mothers and the neonates are summarized in Table 1. Mean age of the subjects was 23 ± 3 years (range 17–31 years). 55% of the subjects were primiparous. The average body mass index (BMI) and height at