RESEARCH NOTE

Genomic imprinting status of *IGF-II* and *H19* in placentas of fetal growth restriction patients

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Introduction

According to the intrauterine fetal programming theory (Ong and Dunger 2002), several authors have noted that fetal growth restriction (FGR) have a greater predisposition to develop metabolic syndrome later in life, manifesting as obesity, hypertension, hypercholesterolemia, cardiovascular disease, and type 2 diabetes mellitus.

In recent years, the morbidity of FGR is low. However, pregnancy-induced hypertension still may lead to fetal growth restriction, severe intrauterine hypoxia, fetal distress, and even intrauterine fetal death. The growth and development of the placenta is critical to fetal growth and development. Recently, some evidence showed that placental function is controlled by both fetal and maternal genes, therefore, imprinted genes may play an essential role in placental development and fetal growth (Zhang and Tycko 1992; Constância *et al*. 2004).

The gene for insulin-like growth factor-II (*IGF-II*) and its reciprocal imprinted gene *H19* have been reported to have an important regulatory function during the development of placenta and embryo (Constância *et al*. 2002). The aim of our study was to determine whether loss of imprinting (LOI) of *IGF-II* and *H19* existed in the placentas of FGR, and to explore the relationship between the high morbidity of FGR with hypertentive disorder complicating pregnancy and the LOI of *IGF-II* and *H19*.

Materials and methods

Subjects and groups

Our study is consistent with the World Medical Association Declaration of Helsinki and approved by the Experimental Committee and Ethics Committee at China Medical University. Placentas were obtained from women after delivery ($n = 40$) with a normal singleton pregnancy at term. Venous blood samples were collected from both the parents and three-day-old child. Informed consent was obtained from each patient before entering the study.

A piece of trophoblast (half way between the cord insertion and the edge of the placenta) was excised and put in 'RNA later' (Qiagen, Courtaboeuf, France) and stored at −80°C until total RNA preparation. 5 mL of blood sample was collected separately from the father, mother and three-day-old child and were kept at room temperature for 1 h, centrifuged, and serum was stored at −80°C for testing.

These samples were divided into four groups based on birth weight and pregnancy-related complications, and the standard criteria are defined according to *Williams Obstetrics* (Cunningham *et al*. 2002). We defined four groups, with 10 cases in each group: A1, FGR without pregnancy-related complications; A2, FGR with hypertentive disorder complicating pregnancy; B1, normal birth weight without pregnancy-related complications; B2, normal birth weight with hypertentive disorder complicating pregnancy.

DNA and RNA extraction and purification

Genomic DNA was extracted using the Genomic DNA isolation kit (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's protocol and stored at −20°C. Total RNA

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was extracted from placenta tissue (TaKaRa Biotechnology, Dalian, China).

IGF-II and H19 PCR

The primers used for *IGF-II* PCR are as follows: P1: 5′-TTGGACTTTGAGTCAAATTG-3′ and P2: 5′-GGTCGTGCCAATTACATTTCA-3′ (DQ104203). The PCR product was 292 bp. The primers used for *H19* PCR were as follows: H1: 5′-TACAACCACCTGCACTACCTG-3′ and H2: 5′-TGGAATGCTTGAAGGCTGCT-3′ (M32053). The PCR product was 655 bp.

Identification of genomic polymorphisms of IGF-II and H19

The amplified product of *IGF-II* was digested with the restriction endonuclease *Apa*I (polymorphic site GGGCC/C, TaKaRa Biotechnology, Dalian, China). The fragment of 292 bp was named fragment 'a' (lack of polymorphic sites). The fragments of 231 bp and 61 bp were named fragment 'b' (with polymorphic sites). The presence of both fragment 'a' and 'b' were identified as heterozygous status, while the presence of only fragment 'a' or 'b' was identified as homozygous status or loss of heterozygosity (LOH).

The amplified product of *H19* was digested with the restriction endonuclease *Rsa*I (polymorphic site GT/AC, TaKaRa Biotechnology, Dalian, China). The fragment of 655 bp was named fragment 'a' (lack of polymorphic sites). The fragments of 485 bp and 170 bp were named fragment 'b' (with polymorphic site). The presence of both fragments 'a' and 'b' was identified as heterozygous status, while the presence of only fragment 'a' or 'b' was identified as homozygous status or LOH.

Allele-specific gene expressions of IGF-II and H19

RT-PCR was performed using the RNA reverse transcription kit (including the reverse transcription to cDNA and PCR amplification for *IGF-II* and *H19*). The PCR products were digested with restriction endonucleases to determine the allele expressions of *IGF-II* and *H19*.

cDNA PCR for IGF-II: Primer sequences, conditions and reaction system as above. The PCR product was 292 bp, following extraction and purification, was then digested with *Apa*I. Fragment 'a' was the undigested fragment (292 bp, lack of polymorphic sites), and fragments 'b' were digested fragments (231 bp and 61 bp, with polymorphic sites). The presence of both fragment 'a' and 'b' were identified as biallelic expression or LOI; while the presence of only fragment 'a' or only fragments 'b' was identified as monoallelic expression or imprinting.

cDNA PCR for H19: Primer sequences, conditions and reaction system as above. The cDNA was amplified using primers H1 and H2 located at exons 4 and 5. The PCR product was 575 bp (unlike the gDNA PCR which amplifies a product of 655 bp due to an intron sequence within gDNA), following extraction and purification, was then digested with *Rsa*I. Fragment 'a' was an undigested fragment (575 bp, lack of polymorphic sites), and fragments 'b' were digested fragments (405 bp and 170 bp with polymorphic sites). The presence of both fragments 'a' and 'b' was identified as biallelic expression or LOI; while the presence of only fragment 'a' or only fragments 'b' was identified as monoallelic expression or imprinting.

Statistical analysis

SPSS 13.0 software was used for data processing, a difference was considered significant if the P value was below 0.05.

Results

Analysis of genomic polymorphisms of IGF-II and H19

Abnormal parental imprinting can only be studied when the transcripts from parental homologues are distinguishable in placental tissue, hence when the child is heterozygous for IGF-II and/or H19 genes polymorphisms and if one of the two parents is homozygous for the polymorphic site. So, as a first step, placenta samples informative for the *Apa*I polymorphism in IGF-II and for the *Rsa*I polymorphisms in H19 were searched.

The genomic polymorphisms of IGF-II and H19 of the four groups (six families of group A1, four families of group A2, five families of group B1 and four families of group B2) were informative for *IGF-II*; eight families of the group A1, eight families of the group A2, four families of the group B1 and five families of the group B2 were informative for *H19*.

Imprinting status of the IGF-II gene

Using the *Apa*I restriction site polymorphism in the human *IGF-II* gene, we examined the representation of the corresponding alleles in cDNAs from placentas. Expression of *IGF-II* was largely or exclusively from a single allele, the paternal allele and the maternal IGF-II allele was not express in placental tissues of all these four groups, either with or without FGR (figure 1).

Imprinting status of the H19 gene

Using the *Rsa*I restriction site polymorphism in the human *H19* gene, we examined the representation of the corresponding alleles in cDNAs from placentas. For the groups with normal birth weight (B1 and B2), the expressions of *H19* were largely or exclusively from a single allele, the maternal allele. (figure 2a). But for the groups with FGR (A1 and A2), bi-allelic expressions were detected, there were LOI of the *H19*, but there were no statistical difference between the LOI rates of group A1(1/7) and A2(2/6) (figure2 a and b)

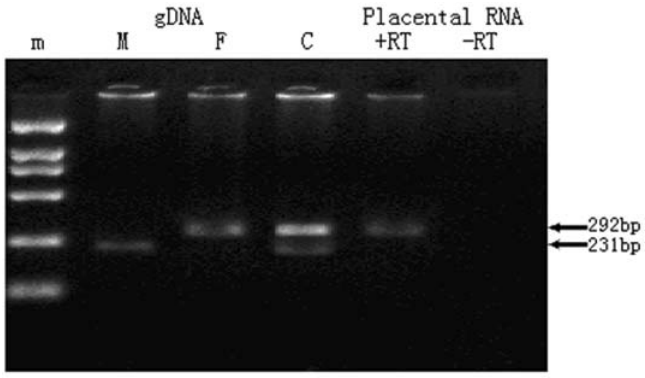

Figure 1. Genomic imprinting of *IGF-II* gene using *Apa*I polymorphism. After digestion with *Apa*I restriction enzyme, and electrophoresis on a 6% polyacrylamide gel, amplified cDNA obtained from placental RNA was compared to corresponding amplified genomic DNA (gDNA) from the mother (M), father (F) and child (C). The size of the DNA fragments (in bp) are indicated on the right side of the gel. RNA with reverse transcriptase (+RT), RNA without reverse transcriptase (−RT), DNA size markers (m).

Discussion

The current research on FGR mainly focusses on gestational nutrition, various growth factors, hormones and the regulating function of the placenta (Miles *et al*. 2005). We realized that the regulation of fetal growth and development involves multiple factors, steps and aspects with individualized and complex mechanisms. In order to find the underlying mechanism, we studied the role of imprinted genes as they are closely linked to fetal growth and development. Many studies have shown that the hypertensive disorder complicating pregnancy is associated with a higher complication rate of fetal growth restriction (Miles *et al*. 2005). The pathogenesis of the hypertensive disorder complicating pregnancy is well known, but the correlation between hypertensive disorder complicating pregnancy and genomic imprinting is not well-studied. Based on the above considerations, we explored the pathogenesis of FGR at the level of imprinted genes.

Imprinted genes are genes whose expression is determined by only one allele inherited from one of the parents while the other allele is inactivated (Miozzo and Simoni 2002). In mammals, many imprinted genes are expressed in placenta and fetus controlling resource usage (Constância *et al*. 2004), so they play an important role in fetal growth and placental function (Isles and Holland 2005).

As an imprinted gene, the paternal *IGF-II* allele is normally expressed. Its deletion or gene knockout is manifested as intrauterine fetal growth restriction (Angiolini *et al*. 2006). The main function of *IGF-II* is to promote cell proliferation, differentiation and metabolism, and so is involved in placental and embryonic growth (Constância *et al*. 2002). Sibley *et al*. (2004) demonstrated mouse growth restriction and reduced placental weight by destroying mouse *IGF-II* gene. *H19* is closely linked to *IGF-II* but only its maternal allele is expressed. It expresses only at the mRNA level and plays a role in gene expression regulation. Its deletion or gene knockout manifested as excessive *IGF-II* expression which resulted in fetal macrosomia (Murrell *et al*. 2004; Angiolini *et al*. 2006).

Our data clearly demonstrate that in the placentas of normal birth weight (groups B1 and B2), steady-state mRNA from only one *IGF-II* and *H19* gene copy is detected: the paternal copy for *IGF-II* and the maternal copy for *H19*. No loss of imprinting either of *IGF-II* or of *H19* genes was found in the groups with normal birth weight; in the placentas of FGR

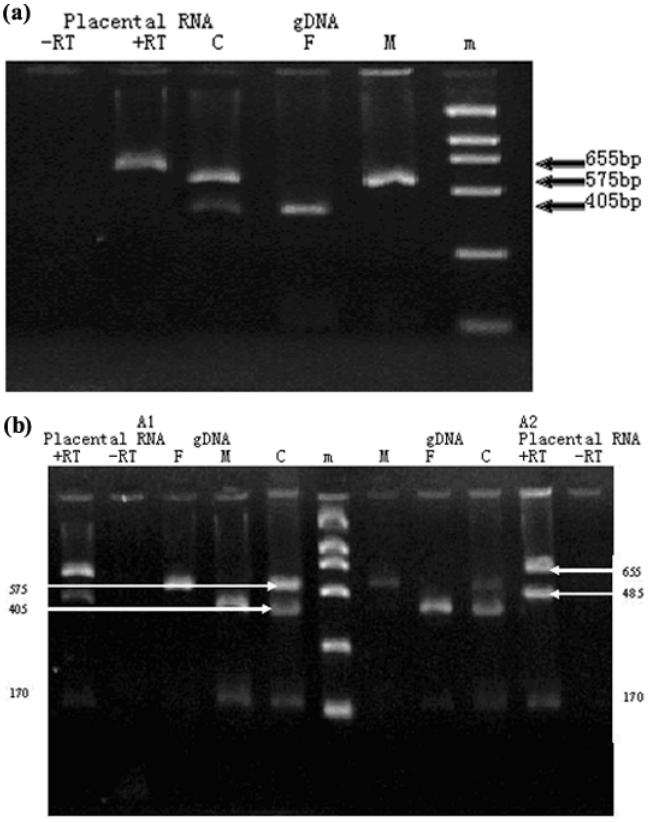

Figure 2. (a) The genomic imprinting status of *H19* gene of the groups of normal birth weight (B1, B2) using *Rsa*I polymorphism. After digestion with *Rsa*I restriction enzyme, and electrophoresis on a 6% polyacrylamide gel, amplified cDNA obtained from placental RNA was compared to corresponding amplified genomic DNA (gDNA) from the mother (M), father (F) and child (C).The size of the DNA fragments (in bp) are indicated on the right side of the gel. RNA plus reverse transcriptase (+RT), RNA without reverse transcriptase (−RT), DNA size markers (m). (b) The genomic imprinting status of *H19* gene of the groups of FGR (A1, A2) using *Rsa*I polymorphism. After digestion with *Rsa*I restriction enzyme, and electrophoresis on a 6% polyacrylamide gel, amplified cDNA obtained from placental RNA was compared to corresponding amplified genomic DNA (gDNA) from the mother (M), the father (F) and the child (C). The size of the DNA fragments (in bp) are indicated on the right side of the gel. RNA with reverse transcriptase (+RT), RNA without reverse transcriptase (−RT), DNA size markers (m).

(A1 and A2), steady-state mRNA from only one IGF-II gene copy is detected, the paternal copy; the maternal allele was not expressed (imprinted). No loss of imprinting of IGF-II however, bi-allelic H19 expressions were observed in FGR groups, there was LOI of the H19, indicating that LOI of H19 may play a part in the pathogenesis of FGR. Meanwhile, there was no statistical difference between the LOI rates of groups of FGR without pregnancy-related complications and FGR with hypertentive disorder complicating pregnancy, indicating that FGR frequently developed in hypertentive disorder complicating pregnancy cannot be explained by genetic factors, instead, is more likely related to vasospasm, placental hypoperfusion and hypoxia, as many studies had proved.

In a word, The pathogenesis of the FGR may be related with LOI of H19, no change of the imprinting status of the IGF-II gene. The high morbility of the FGR with hypertentive disorder complicating pregnancy has no relationships with the imprinting status of *IGF-II* and *H19* genes.

It is conceivable that only a proportion of the genes would be actually involved in, and essential for placental function, although all of the imprinted genes would show placental expression. Thus, important genes of this type could affect embryonal and postnatal growth, for example *IGF2R* (Monk *et al*. 2006), *PEG3* (Feng *et al*. 2008), *SLc38a4* (Smith *et al*. 2003), *PHLDA2* (Apostolidou *et al*. 2007; McMinn *et al*. 2006; Onyango *et al*. 2000), *MEST* (Lefebvre *et al*. 1998; Kosaki *et al*. 2000), are studied more and more. The mechanisms of these genes on the fetal growth and development will be the focus of our continual study. In view of the factors that may have impacts on the imprinting status of imprinted genes, the methylation of differentially methylated regions (DMRs) of imprinted genes is most critical. The focus of our next study project will be to determine the methylation status of certain CpG islands in the control region on the genomic DNA. By exploring the pathogenesis of adulthood metabolic diseases at the gene level, we hope to promote active prevention and intervention during gestation for future practice.

Acknowledgements

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