Loss of imprinting and aberrant methylation of *IGF2* in placentas from pregnancies complicated with fetal growth restriction

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Abstract. The objective of this study was to investigate the hypothesis that the altered epigenetic mechanisms that regulate *IGF2* imprinting in placentas from fetal growth restricted (FGR) pregnancies affect *IGF2* expression leading to impaired fetal growth. We investigated gene transcription, genotyping and the methylation patterns of *IGF2* from 31 and 17 placentas from FGR-complicated and normal pregnancies, respectively. A statistically significant decrease in *IGF2* mRNA levels was observed in the placentas from the FGR pregnancies. Loss of imprinting (LOI) was only detected in the abnormal placentas. The evaluation of the percentage of the methylated reference (PMR) of two different potentially differentially methylated regions (DMR) demonstrated significant PMR values in both sites for the normal and FGR pregnancies with no significant differences. Our results suggest the involvement of the *IGF2* imprinted gene in placental function and fetal growth and the possible association of epigenetic alterations with the pathophysiology of fetal growth restriction.

Introduction

It is well known that fetal growth restriction (FGR) is not a single disorder, but instead has various causes (1). Placental dysfunction ranks high among the most common causes of FGR. Although numerous placental histopathological changes have been described, little is known about the precise etiology and the contribution of placental genes in this disorder (2,3).

Imprinted genes are known to be involved in regulating placental growth and function and are therefore considered to be suitable candidates for a role in FGR development (4,5). Genomic imprinting is the preferential silencing of one copy of an autosomal gene while the other copy is expressed (6). This parent-of-origin-specific expression is regulated by epigenetic modifications, such as DNA methylation. Imprinted genes that are paternally expressed (maternally imprinted) promote growth of the fetus, while maternally expressed (paternally imprinted) genes act as growth suppressors to ensure the appropriate allocation of limited maternal resources to each offspring (7). Insulin-like growth factor 2 (*IGF2*) and *H19* represent two oppositely expressed genes located adjacent to each other at 11p15.5 that share the same transcription regulatory epigenetic mechanisms (8). *IGF2* is highly expressed in mouse and human placenta and affects both the functional capacity of the placenta to transfer nutrients to the fetus and placental size (9,10). It is expressed in most tissues only from the paternal allele, with the maternal allele being transcriptionally silent.

Imprinting occurs primarily by allelic-specific methylation of cytosines in areas of DNA that are rich in CpG dinucleotides (11). In most human tissues the imprinting of *IGF2* depends on a differentially methylated region (DMR) which is located upstream of *H19* promoters (12). This region functions as a methylation-sensitive insulator that binds to a CCCTC factor (CTCF) on the unmethylated maternal allele preventing the interaction of *IGF2* with the enhancer located downstream of *H19*, and partitioning the two alleles into transcriptionally 'silent' and 'active' regions (11,13). Two regions of allele-specific methylation also exist within the human *IGF2*. The region spanning exons 2 and 3 is homologous to the mouse dmr0 and another region within exon 9 is similar to mouse dmr2 (4).

IGF2 expression is characterized by a delicate epigenetic regulation through several promoters exhibiting developmental-dependent expression patterns (14). Aberrant DNA methylation which can modify imprinted gene expression may provide an attractive mechanism linking environmental causes to placental insufficiency and subsequently to the development of intrauterine growth restriction. To investigate the role of the imprinted *IGF2* in the pathogenesis of FGR, we aimed to determine whether altered *IGF2* expression correlates with relaxed imprinting in FGR placentas. We also examined the epigenetic profiles of the *IGF2/H19* domain in these tissues to determine whether deregulated methylation status correlates with *IGF2* transcription and potential imprinting deregulation. We postulated that altered epigenetic mechanisms affect *IGF2* imprinting and deregulate its expression leading to FGR.

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Materials and methods

Sample collection. This study was approved by the Research and Ethics Committee of the University Hospital of Heraklion, Crete, Greece. Informed consent was obtained from all patients. Placentas were obtained after vaginal deliveries or caesarean sections from 31 women with singleton pregnancies that where complicated by FGR, as well as from 17 normal pregnancies. The FGR pregnancies were recruited in the immediate intrapartum period. The estimated fetal weight was below the 5th percentile and all pregnancies demonstrated sonographic findings of FGR such as reduced fetal size, abnormal Doppler measurements of the umbilical artery, and/or oligohydramnios (data not shown). Exclusion criteria were multiple pregnancy, chorioamnionitis, chromosomal abnormalities and fetal anatomical defects. Control placentas were obtained from pregnancies with healthy appropriate-for-gestational-age term neonates with birth weight (BW) between the 10th and 90th percentile and no other pregnancy complications. Biopsy specimens were collected from six locations between the decidual and chorionic plates in order to limit tissue heterogeneity (15). Each sample contained the deciduas basalis and villous placenta. Areas involving calcification or infarcts were avoided. Three fragments from each placenta were thoroughly washed in phosphate-buffered 0.9% saline to minimize blood contamination and subsequently snap-frozen and stored at -80°C for further treatment. Baseline demographic characteristics and medical history information (maternal weight, height, age, parity, smoking, mode of delivery, pregnancy complications, fetal gender, and BW) were recorded. Gestational age at delivery was determined by the mother's last menstrual cycle and/or sonographic measurements at 11-14 weeks of gestation.

DNA and RNA extraction. Genomic DNA and total RNA were extracted from FGR and normal placentas as previously described (16). RNA and DNA concentration was measured on a UV spectrophotometer (Hitachi Instruments Inc., USA).

Reverse transcription and real-time PCR. Reverse transcription reactions for the preparation of first-strand cDNA from 2 μg of total RNA were performed using the reverse transcription system as previously described (17), followed by DNase treatment in order to ensure avoidance of genomic DNA contamination. Real-time PCR reactions were carried out on an Mx3000P thermal cycler (Stratagene, La Jolla, CA) using SYBR® Green I Master Mix (Stratagene, Greece) and the primers listed in Table I (18). After initial denaturation at 95°C for 10 min, samples were subjected to 40 amplification cycles comprising denaturation at 95°C for 30 sec, annealing at 65°C for 30 sec and elongation at 72°C for 30 sec, followed by a melt curve analysis in which the temperature was increased from 55 to 95°C at a linear rate of 0.2°C/sec. Negative controls were included in each PCR reaction. Normalized transcription levels were calculated for each pathological or normal sample using the formula: Normalized sample or Control = $(1+E_{GOI})^{-\Delta Ct}/(1+E_{GAPDH})^{-\Delta Ct}$.

Genotyping of IGF2 polymorphisms. Genotypes of FGR and control tissues were determined by PCR of genomic DNA as previously described (18). Briefly, PCR reactions were performed in a total volume of 25 μl containing 5 μM of 5X Green GoTaq®

Reaction Buffer, 1.5 mM MgCl$_2$, 0.2 mM of each deoxynucleotide triphosphate (dNTPs), 0.6 U of GoTaq Flexi DNA polymerase (Promega, Madison) and 200 ng of genomic DNA or cDNA. PCR conditions were 95°C for 90 sec, followed by 29 cycles of 95°C for 25 sec and optimal annealing temperature for 1 min, and finally 72°C for 10 min. The optimal annealing temperature was 65°C. The PCR products were examined by electrophoresis on a 3% agarose gel and photographed on a UV transilluminator. Determination of the IGF2 exon 9 bp 819 polymorphism required restriction enzyme digestion with *Apa*I. PCR products (4-5 μg) were mixed with 20-30 U of the appropriate enzyme (Promega, Madison) in a total volume of 20 μl, and digested at 37°C for ~6 h. Products were electrophoresed on 3% agarose and photographed on a UV transilluminator.

Analysis of DNA methylation. Sodium bisulfite conversion (Zymo Research, Berlin, Germany) and MethyLight analysis (Applied Biosystems, Warrington, UK; Metabion, Munich Germany) were performed as previously described (19). Each MethyLight reaction, at a specific locus, covered on average 5-7 CpG dinucleotides. A detailed list of primer and probes for all analyzed loci is provided in Table I. Briefly two sets of primers and probes, designed specifically for bisulfite-converted DNA, were used: a methylated set for the gene of interest and a reference set (COL2A1) to normalize for input DNA. Specificity of the reactions for methylated DNA was confirmed separately using *Sss*I- (New England Biolabs; www.newenglandbiolabs.co.uk) treated human white blood cell DNA (heavily methylated). The percentage of fully methylated molecules at a specific locus was calculated by dividing the IGF2:COL2A1 ratio of a sample by the IGF2:COL2A1 ratio of the *Sss*I-treated human white blood cell DNA and multiplied by 100. The abbreviation PMR (percentage of methylated reference) indicates this measurement. The analysis was performed in a blinded manner, and cases and controls were randomly mixed for bisulfite treatment and real-time PCR. The concentration of bisulfite-modified DNA (assessed by the level of the reference gene COL2A1) was the same between the cases and controls.

Statistical analysis. Mean values and standard deviations were calculated for continuous parameters. Data were presented as percentages in case of categorical variables. The distribution of continuous characteristics within two groups of individuals was compared using the non-parametric Wilcoxon rank-sum test. The potential association between categorical variables was examined by the χ^2 test. The Spearman's rank correlation coefficient was estimated to assess the relationship between various continuous variables. Moreover, the association between mRNA or PMR values and characteristics of the study participants (BW, gestational age, etc.) was modelled using linear regression analysis. All tests of significance were two-sided, and a P<0.05 indicated significance. Statistical analysis was performed using *SPSS* 17 statistical package (SPSS, Inc., Chicago, IL).

Results

Sample characteristics. We analyzed 31 placental samples from FGR-complicated pregnancies and 17 samples from normal pregnancies. BW was below the 5th percentile in all the FGR pregnancies, with almost 90% of them being below the 3rd

Table I. PCR primers used in the study.[a]

Primer	PCR primer	
	Sequence (5' to 3')	Optimal annealing temperature
IGF2-758 IGF2-857	CTT GGA CTT T(G/T)A GTC AAA TTG GC GCG GTA CGA GCG ACG TGC CCA C	65°C

MethyLight primers

HUGO Gene Nomenclature	Forward primer sequence	Reverse primer sequence	Probe oligo sequence
IGF2 (AC006408) 65759-64972	5'-TCGTAGTTTTTGGATAGTGGATAAAGAG-3'	5'-CCAAAAACACCCCCTACTTTAATAATAAC-3'	6FAM-TTTTTTCGAAATTGTTTGGACGATGATTTGTC-BHQ-1
H19 (AF087017)' upstream region 5962-6413	5'-CGGAATTGGTTGTGAGTTGTGGAAT-3'	5'-ACCCCAATTAAAACGAACTCGAA-3'	6FAM-CGCGGCGGGTAGTGTAGGTTTATATA-BHQ-1

[a]Primers and probe sequences for MethyLight used in the study.

Table II. Allelic expression of *IGF2*.

Placental samples	Case nos.	gDNA	cDNA
Loss of imprinting of IGF2			
FGR	10	A/B	A/B
FGR	13	A/B	A/B
FGR	16	A/B	A/B
FGR	22	A/B	A/B
FGR	25	A/B	A/B
FGR	37	A/B	A/B
FGR	43	A/B	A/B
FGR	48	A/B	A/B
FGR	98	A/B	A/B
Maintenance of imprinting of IGF2			
FGR	7	A/B	A
FGR	50	A/B	B
Control	58	B	
FGR	62	B	
Control	66	A/B	A
Control	75	A/B	B
FGR	76	B	
Control	79	A/B	A
Control	87	A/B	B
Control	88	A	
FGR	96	A/B	B
FGR	97	A/B	A
FGR	99	A/B	A
Control	102	A/B	A
Control	118	A	
Control	128	A/B	A

percentile. The percentiles were calculated based on an individually customized fetal and BW percentile method (www.gestation.net). FGR cases and controls were compared with respect to baseline characteristics and outcome. The distributions of maternal age, weight, BMI, fetal gender, parity and smoking were well balanced between the two groups (data not shown). As expected, there were significant differences between gestational age, BW and BW percentiles in the FGR subjects versus controls.

Imprinting analysis of IGF2 in placental tissues. Exon 9 *IGF2* polymorphism, which involved the creation of a restriction enzyme site, was evaluated in this study. PCR of genomic DNA with specific primers (Table I), followed by restriction digestion, determined the informative samples. RT-PCR of total RNA allowed for the analysis of the expressed alleles. In total, 16 out of 31 placental FGR samples and 9 out of 17 control placentas were informative (heterozygous) for *IGF2* (Table II). In a total of 25 informative cases, LOI was found in 9 specimens, all of which were FGR samples (Fig. 1). Normal placentas did not reveal a biallelic expression of *IGF2*. Therefore, a statistically
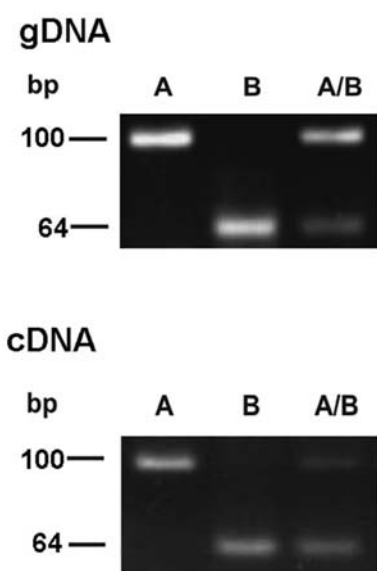
Figure 1. Genotyping and imprinting of the IGF2 gene in the placenta. Representative samples of *IGF2* LOI. Upper gel indicates representative samples of *Apa*I-digested PCR products for genotyping of IGF2. Heterozygous cases were labeled with A/B, which were considered as informative. Lower gel indicates representative samples of *Apa*I-digested RT-PCR products for imprinting status analysis. Loss of imprinting was demonstrated in sample labeled A/B.

significant difference in the incidence of LOI of *IGF2* was demonstrated between the FGR and control placentas (P=0.02). The remaining 16 informative samples of *IGF2* revealed maintenance of imprinting (MOI) in 7 FGR and 9 control samples.

Quantitative analysis of the IGF2 gene. We tested the expression levels of *IGF2* in the FGR and control placentas. *IGF2* transcripts derived from FGR placentas were down-regulated compared to controls (P=0.01) (Fig. 2A). A linear regression analysis did not reveal a relationship between *IGF2* expression and any of the covariates including BW percentile, gestational age at birth, and maternal characteristics.

In order to examine the effect of imprinting defects on expression, we also examined the relative expression of *IGF2* in samples that demonstrated aberrant imprinting patterns. Hence, we tested whether there were different mRNA levels in placentas with a biallelic expression of *IGF2*, compared to those that maintained the imprinting status. Nine FGR samples that demonstrated LOI for *IGF2* showed decreased mRNA expression compared to FGR samples that maintained the imprinting pattern; however, this decrease was not significant.

DNA methylation patterns in FGR placentas. We measured the percentage of methylated reference (PMR) of two different potential DMRs (Fig. 3). *IGF2* exon 2/3 region from base 65545
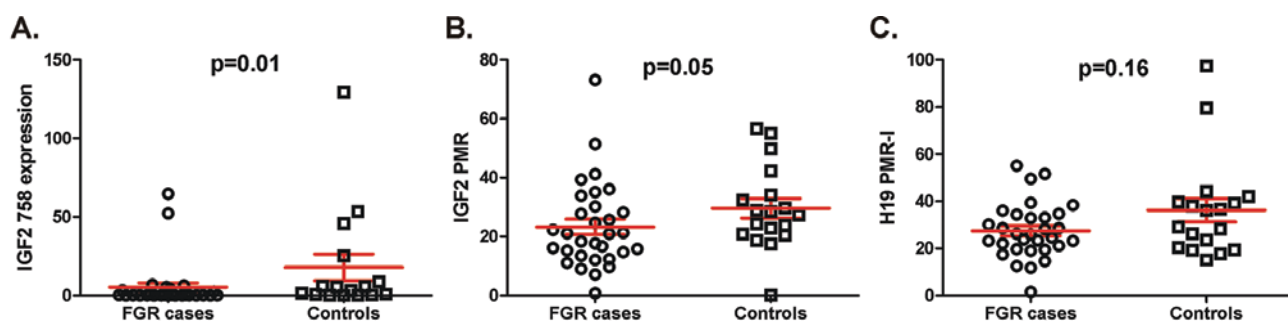

Figure 2. (A) *IGF2* transcripts derived from FGR placentas were significantly down-regulated compared to controls (P=0.01). Mean values with SEM are depicted. (B) Percentage of methylated reference (PMR) values in the *IGF2* exon 2/3 region. FGR samples demonstrated lower methylation values than controls (P=0.05). Bars indicate mean values and SEM. (C) Percentage of methylated reference (PMR) values in the *H19* transcription start site. Bars indicate mean values and SEM. P-values are indicated.
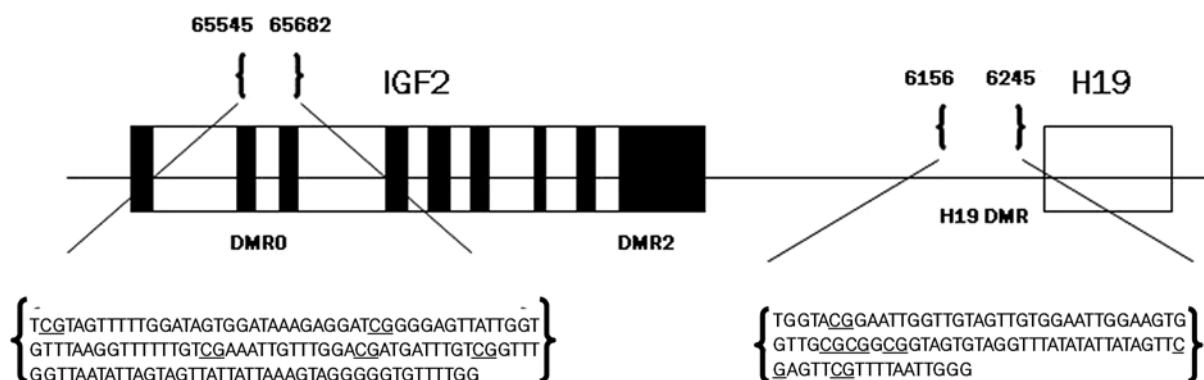

Figure 3. *IGF2/H19* imprinted cluster. Black vertical bars indicate *IGF2* exons. Location of the PCR primers used are indicated by numbered parentheses. Detailed view of the sequence is presented between brackets. CpG dinucleotide locations are underlined.
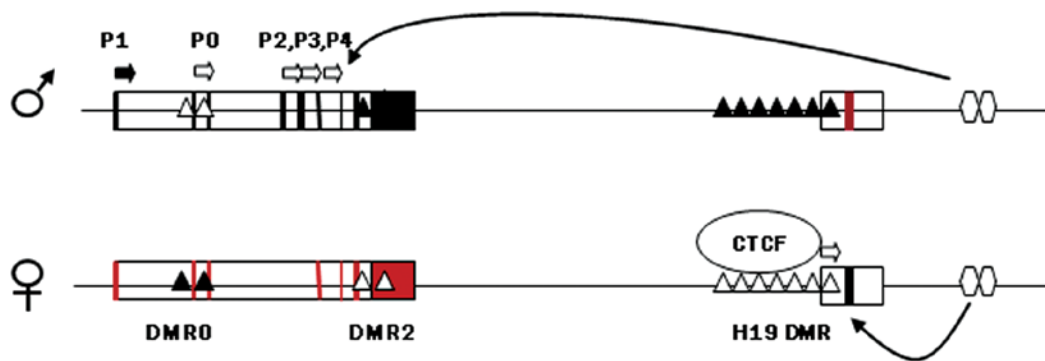
Figure 4. Schematic representation of the imprinted regulation of *IGF2/H19* cluster. Maternal and paternal chromosomes are indicated. Vertical black bars represent exons from the expressed allele; red vertical bars represent exons from the silenced allele. Black arrows show the direction of transcription (white arrows, active transcription from *IGF2* P0, P2, P3, P4 and *H19* promoter; black arrow, non-imprinted P1 *IGF2* promoter). Ovals represent enhancers. Arrowed lines represent enhancer activity. Three known differentially methylated regions (DMR) are presented as sets of triangles. Solid triangles represent methylated DMRs and clear triangles represent unmethylated regions. DMR upstream *H19* harbors the binding sites for CTCF. Methylation of this region on the paternal chromosome prevents binding with CTCF and allows the *IGF2* promoter to assess enhancers located downstream of *H19*. *IGF2* is subsequently transcribed. On the maternal chromosome the non-methylated *H19* DMR is bound to CTCF insulating the *IGF2* promoter from the 3' enhancers and allowing the H19 promoter unimpeded access to the enhancers. Maternal *H19* is subsequently transcribed. There are also differentially *IGF2*-methylated regions (DMR0, DMR2). DMR0 is methylated on the maternal allele. Although DMR at exon 9 is methylated on the paternal allele, this region most likely represents a site of tissue-specific methylation which is not involved in *IGF2* imprinting.

to 65682 was evaluated in order to determine the methylation patterns of our samples. FGR placentas demonstrated lower methylation references compared to controls, with mean PMR values of 23.3 and 29.6%, respectively, a difference that was marginally significant (P=0.05) (Fig. 2B). Methylation levels of the *IGF2* promoter did not correlate with gene expression. Additionally, as shown in linear regression analysis, *IGF2* PMR values were not associated with BW percentiles (P=0.17), BW (P=0.14) and gestational age (P=0.2). We extended our methylation analysis to the subset of samples that demonstrated LOI for *IGF2* compared to those that maintained monoallelic gene expression. The two groups showed similar methylation values (22.9 vs. 22.8%, respectively).

Methylation status of the region spanning from 6156 to 6245 bp upstream of the *H19* transcription start site, which contains CTCF-binding sites (20), was also assessed (Fig. 3). Compared with methylation values of the normal placentas, those from the growth-restricted pregnancies had lower PMR, (mean value 27.4 vs. 36.2%, P=0.15) (Fig. 2C). *H19* PMR measurements of the specific region were not associated with BW percentiles (P=0.52), BW (P=0.73) or gestational age (P=0.8) in linear regression analysis. Since methylation of this site controls *IGF2* transcription we investigated the impact of methylation on *IGF2* mRNA levels. No correlation was noted between the *H19* DMR methylation levels and *IGF2* expression. Samples with LOI for *IGF2* had higher PMR values compared to those with MOI with mean values of 31.3 and 26.1%, respectively. This difference did not reach statistical significance (P=0.84).

Discussion

In the present study we investigated the epigenetic characteristics of the *IGF2/H19* imprinted domain in FGR placentas in the context of altered *IGF2* expression and imprinting. Alterations in the methylation profile of specific regions within the *IGF2/H19* imprinted domain were detected. We verified the previously reported reduced *IGF2* expression noted in growth-restricted placentas. Interestingly a high prevalence

of LOI was only noted in the placentas associated with FGR. However, there was a lack of correlation between these two events. Our results highlight the importance of the human chromosome 11p15.5 imprinting cluster in fetal and placental growth and the possible association of epigenetic alterations with the pathophysiology of FGR.

Our results are consistent with previously reported human and animal studies demonstrating a reduced *IGF2* mRNA level in placentas from pregnancies with FGR (5,21). *IGF2* underexpression results in a reduction in all placental layers and affects the diffusional properties of the placenta (22). Guo *et al* suggested a correlation between the relative *IGF2* expression and BW percentile (21). We were not able to reproduce this observation since there was no association between *IGF2* expression and FGR severity in our study.

The biallelic expression of *IGF2* was established exclusively in the FGR samples. Similar conclusions were drawn in a study by Yu *et al* who evaluated the imprinting status of *H19* in normal and preeclamptic placentas (23). LOI of *H19* was observed only in preeclamptic placentas and was correlated with the severity of the disease (23). Similarly, relaxation of the *IGF2* imprinting was demonstrated in tissues with gestational trophoblastic disease unlike the normal placentas (24). Since LOI was also established in the affected placentas in our study, we hypothesize that relaxed *IGF2* imprinting might correlate with placental dysfunction which represents a component of the original pathogenetic process of FGR.

Fetal growth restricted samples that displayed LOI had similar *IGF2* mRNA levels compared to those with normal imprinting. This observation implies that there is probably no correlation between *IGF2* expression and the imprinting profile in the placenta. Diplas *et al* concluded that even when a correlation between imprinted genes and the pathophysiology of FGR is present, it appears that mechanisms other than imprinting contribute to altered gene transcription (25).

The model of reciprocal *IGF2* and *H19* expression predicts that the DMR upstream of *H19* if methylated in both alleles would result in biallelic expression of *IGF2* (20) (Fig. 4). Our

methylation analysis included the region located upstream of the *H19* promoters (Fig. 3). Control and FGR samples demonstrated similar methylation values (36.2 vs 27.4%) which corresponds to that previously reported in FGR as well as in control placentas in this region (21,26). There was no correlation between the PMR values of the specific DMR region that we detected and the *IGF2* mRNA values.

We compared 9 tissues from the FGR group with LOI and 8 with MOI based on their PMR values. Samples with relaxed imprinting had comparable PMR values compared to those with MOI (31.3 vs. 26.1%). Increased methylation levels in the placentas with imprinting defects would agree with the above-mentioned model of *H19* DMR methylation controlling *IGF2* imprinting (Fig. 4). Since we did not demonstrate such a difference, no definite conclusions can be drawn regarding the association of *IGF2* imprinting status and methylation patterns in placentas; thus, this event warrants further investigation.

Similarly the difference noted in the *IGF2* PMR values between FGR placentas and controls was marginally significant (23.3 vs. 29.6%, p=0.05). Aberrant methylation of this site has been linked to imprinting defects in cancer as well as in normal tissues (27). Other investigators have postulated that the human DMR0 is a methylation-dependent insulator that regulates *IGF2* expression independently of the *H19* DMR (28). Since LOI results from failure of the cell to maintain monoallelic expression and can be associated with hypomethylation, we speculated that the reduced trend of *IGF2* DMR PMR levels is attributed to the high prevalence of samples with imprinting defects in the FGR group. However, methylation levels of *IGF2* DMR in the FGR samples that demonstrated either LOI or MOI in *IGF2* were similar. Guo *et al* found hypomethylation of *IGF2* DMR2 in exon 9 in placentas from SGA neonates, but normal methylation values in the *H19* DMR for the same group (21). There is no obvious explanation as to why samples with LOI had similar methylation levels in the *IGF2* DMR to those with MOI. Since *IGF2* DMR methylation patterns are reported to be tissue-specific (4), more data are needed to elucidate these findings.

Several conclusions can be drawn and also several questions can be raised from this study. Whether the observed *IGF2* down-regulation is a causative factor of FGR or merely a consequence of placental dysfunction remains unexplained. An abnormal biallelic placental expression of imprinted genes has been reported in cases of abnormal placentation such as preeclampsia and gestational trophoblastic disease (23,24). We hypothesize that in FGR cases the placenta responds to chronic hypoperfusion by activating a program of gene expression via genomic imprinting. *IGF2* LOI may represent an effort made by the placenta to compensate its reduced functional capacity by leading to a biallelic production of a potent growth factor. The direct effect of *IGF2* imprinting in placental tissues is not yet clear.

Our results showed a possible relationship between aberrant DNA methylation and imprinting relaxation that might be associated with FGR. Although we did not include placental weights and histopathological findings, it would be interesting to examine the possible correlation of the above with imprinted gene expression in placental tissues and in umbilical cord blood. Given the complexity of imprinted gene regulation, more studies are needed on the role of imprinted genes in human placenta pathology and for a more effective compre-hension of the relationships between molecular mechanisms and phenotypic events in FGR pregnancies.

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