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Hypomethylation along with increased *H19* expression in placentas from pregnancies complicated with fetal growth restriction

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ABSTRACT

The expression of imprinted genes is regulated by epigenetic modifications, such as DNA methylation. Many imprinted genes are expressed in the placenta and affect nutrient transfer capacity of the placental exchange barrier. The *H19* gene is abundantly expressed by the human placenta and is implicated in the pathogenesis of congenital growth disorders such as Beckwith-Wiedemann (BWS) and Silver-Russell (SRS) syndromes. The aim of this study was to investigate the role of DNA methylation on *H19* transcription and imprinting, in the pathophysiology of fetal growth restriction (FGR). Thirty one and 17 placentas from FGR-complicated and normal pregnancies were collected, respectively. We studied gene transcription, genotyping and methylation analysis of the AluI *H19* on exon 5 polymorphism. Placental expression levels of *H19* were significantly increased in the FGR group. The *H19* mRNA levels were similar between normal placental samples that demonstrated loss and maintenance of imprinting. Placentas from growth-restricted pregnancies had lower methylation levels compared to normals, in the H19 promoter region. We have demonstrated an increased H19 transcription in the FGR group of placentas. The hypomethylation of the H19 promoters is compatible with the aberrant expression. The association of these two findings is reported for the first time in placental tissues, however, its significance remains unknown. Whether the results of this study represent an adaptation of the placenta to hypoperfusion, or they are part of FGR pathophysiology has to be further investigated.

1. Introduction

Fetal Growth Restriction (FGR) comprises one of the leading obstetric complications. FGR indicates the presence of a pathophysiologic process occurring in utero that inhibits fetal growth [1]. It is associated with a spectrum of perinatal complications, including fetal morbidity and mortality, iatrogenic prematurity, fetal compromise in labor and cesarean delivery [2]. Placental insufficiency often represents the underlying cause of FGR although current classification systems propose three causative categories: fetal, maternal and uterine-placental factors [3].

Fetal growth is a complex process associated with multiple molecular factors and influenced by several nutritional and endocrine stimuli. A lot of research has been focused on genetic causes which regulate placental and fetal growth [4]. Imprinted genes are recognized to be involved in placental growth, development and function and are therefore considered suitable candidates for a role in FGR [5]. Genomic imprinting is a mechanism by which there is preferential expression of a gene based on the parental origin of the allele [6]. The genetic conflict hypothesis suggests that paternally derived genes influence nutrient acquisition in a way in which more resources are extracted from the mother whereas maternally derived genes balance the nutrient provision to the existing fetus with that of future offspring [7]. Hence maternally expressed genes are more conservative with regard to resource provision to the fetus [7]. The transcription of imprinted genes represents a phenomenon in which epigenetic mechanisms restrict gene expression through heritable DNA modifications such as cytosine methylation [8].

The *H19* imprinted gene, which is expressed exclusively from the maternal chromosome, produces an untranslated RNA which may have growth suppressing functions [9,10]. *H19* is clustered with the *IGF2* imprinted gene on human chromosome 11p15.5 and share common tissue specific expression patterns. One key factor of imprinting in 11p15 imprinted loci is the genomic methylation

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within regulatory differentially methylated regions (DMRs), which leads to transcriptional activation or silencing of gene expression. *IGF2* and *H19* transcription is controlled by a specific DMR located upstream of *H19* promoters which is an imprinting control region (ICR). Promoters from both genes compete for the same enhancers which are located downstream of *H19*. Access of the maternal *IGF2* allele to the enhancer, and subsequently transcription, is controlled by the imprinting control region (ICR) [11]. ICR when unmethylated binds to a CCCTC factor (CTCF) which possesses insulator activity and preferentially silences maternal *IGF2* allele while maternal *H19* transcription is allowed by promoter–enhancer interactions [11].

The implication of *H19* with congenital growth syndromes [12] motivated us to examine the *H19* transcription imprinting and epigenetic patterns in placentas deriving from pregnancies complicated with impaired fetal growth in order to investigate a possible role of *H19* in the pathophysiology of FGR. Herein we report the methylation analysis of two different regions which are involved in *IGF2/H19* imprinting aiming to investigate a possible relationship between *H19* function in placentas of growth restricted and normal pregnancies.

2. Materials and methods

2.1. 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 cesarean sections from 31 women with singleton pregnancies that where complicated with FGR and 17 uncomplicated pregnancies. FGR pregnancies were recruited in the immediate intrapartum period. Birth-weight (BW) was below the 5th percentile (www.gestation.net) 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). Gestational age at delivery was determined by the patient's last menstrual period or/and sonographic measurements at 11–14 weeks of gestation. Exclusion criteria were chorioamnionitis as well as chromosomal abnormalities and fetal anatomical defects by the use of antenatal sonogram, fetal karyotyping (in some cases) and clinical evaluation after delivery. Control placentas were obtained from pregnancies with healthy appropriate-for-gestational-age term neonates with (BW) $>$ 10th percentile and no other pregnancy complications. Biopsy specimens were collected from 6 different locations between the decidual and chorionic plates in order to limit tissue heterogeneity [13]. Each sample contained the deciduas basalis and villous placenta. Areas involving gross calcifications or infarcts were avoided. Care was taken by visual examination and dissection to minimize contamination from fetal membranes or maternal deciduas. Three fragments from each placenta were thoroughly washed in phosphate-buffered 0.9% saline to minimize blood contamination and then 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.

2.2. DNA and RNA extraction

Genomic DNA was extracted from FGR and normal placentas using 0.1 mg/ml proteinase K (Promega, Madison, WI) and subsequently, the phenol/chloroform protocol, as previously described [14]. For total RNA, tissue specimens were homogenized in TRIzol® reagent (Invitrogen, Carlsbad, CA) using a power homogenizer, followed by the addition of chloroform and centrifugation. Total RNA was precipitated from the supernatant with isopropanol, washed with 75% ethanol and resuspended in 50 μl DEPC-treated water. Total RNA was treated with DNase I before the reverse transcription reaction to avoid genomic DNA contamination. RNA and DNA concentration was calculated after the 260-nm absorbance and 260/280-nm absorbance ratio was measured on a UV spectrophotometer (Hitachi Instruments Inc., USA).

2.3. 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" according to the manufacturer's protocol (Promega, Madison, WI). Real-time PCR reactions were carried out on a Mx3000P thermal cycler (Stratagene, La Jolla, CA) using SYBR® Green I Master Mix (Stratagene, Greece) according to the manufacturer's instructions. The primer pairs were designed to span at least one intron in order to avoid amplification of contaminating genomic DNA along with the cDNA (Table 1). After initial denaturation at 95 °C for 10 min, samples were subjected to 40 amplification cycles comprised of denaturation at 95 °C for 30 s, annealing at 65°C–67 °C for 30 s and elongation at 72 °C for 30 s, 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/s. Negative controls were included in each PCR reaction, one with no cDNA template and one with no reverse transcription treatment. Normalized transcription levels were calculated for each pathological or normal sample using the following formula: normalized sample or Control $= (1 + E_{GOI})^{-\Delta Ct}/(1 + E_{GAPDH})^{-\Delta Ct}$.

2.4. Genotyping of H19 polymorphism

Genotypes of FGR and control tissues for one potential *H19* polymorphism were determined by PCR of genomic DNA as previously described [15]. 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 units of GoTaq Flexi DNA polymerase (Promega, Madison) and 200 ng of genomic DNA or cDNA. PCR conditions were 95 °C for 90 s, followed by 29 cycles of 95 °C for 25 s and optimal annealing temperature for 1 min, and finally 72 °C for 10 min. Optimal annealing temperatures for the primer pair were: H19-10248/10423, 67 °C. The PCR products were examined by electrophoresis on a 3% agarose gel and photographed on a UV transilluminator. Determination of the polymorphism required restriction enzyme digestion (Alu I). Four-five μg of PCR products was mixed with 20–30 units 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 an ultraviolet light transilluminator. The threshold for scoring a sample as LOI was a ratio of less than 5:1 between the more abundant and less abundant alleles. In general, samples with LOI demonstrated a ratio of 1:1 to 2:1 between expressed alleles.

2.5. Analysis of DNA methylation

Sodium bisulfite conversion (Zymo Research, Berlin, Germany) and MethyLight analysis (Applied Biosystems, Warrington, United Kingdom; Metabion, Munich Germany) were performed as previously described [16,17]. Briefly two sets of primers and probes, designed specifically for bisulfite-converted DNA, have been 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 SssI (New England Biolabs, Ipswich, MA) treated human white blood cell DNA (heavily methylated). The percentage of fully methylated molecules at a specific locus was calculated by dividing the GENE:COL2A1 ratio of a sample by the GENE:COL2A1 ratio of the SssI-treated human white blood cell DNA and multiplied by 100. The abbreviation PMR (Percentage of Methylated Reference) indicates this measurement. The analysis was performed blinded 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 FGR cases and controls.

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 1. In order to validate the MethyLight measurements we used four serial 4-fold dilutions in triplicate, ranging from 250 to 3.9, in order to construct standard curves.

Table 1
Primers and probe sequences for PCR and MethyLight qPCR, used in the study.

PCR Primer			
Primer	Sequence (5′ to 3′)		Optimal annealing temperature
H19-10248	CTTTACAACCACTGCACTACCTGAC		67 °C
H19-10423	GATGGTGTCTTTGATGTTGGGCTGA		
MethyLight Primers			
HUGO Gene Nomenclature	Forward Primer Sequence	Reverse Primer Sequence	Probe Oligo Sequence
H19 (AF087017) upstream region 5962-6413	5′-CGGAATTGGTTGTAGTTGTGGAAT-3′	5′-ACCCCAATTAAAACGAACTCGAA-3′	6FAM-CGCGGCGGTAGTGTAGGTTTATATA-BHQ-1
H19 (AF087017) promoter region 7574-7959	5′-AGATAGGAAAGTGGTTGCGAATG-3′	5′-TAAGTTCGGTTAATTGGATGGGAA-3′	6FAM-TTGCGGAAATCGCGGTGACGAG-BHQ-1

Table 2
Baseline characteristics[a].

	FGR $N = 31$	CONTROL $N = 17$	*P*-value
Maternal age (y)	29 ± 6 6.074449	27.8 ± 4.9	0.6657[c]
Weight (kg)	62.9 ± 10.4	61.1 ± 10.3	0.3906[c]
BMI (kg/m^2)	22.6 ± 3.5	23.5 ± 3.7	0.5092[c]
Smoking	21.4%	28.6%	0.608[b]
Parity Nulliparous	61.3%	52.9%	0.338[b]
Gestational age at delivery (wk)	35.4 ± 4.5	39.3 ± 0.9	0.0001[c]
Birth Weight kg	1775.2 ± 710.9	3389.4 ± 473.4	0.0000[c]
Fetal Gender Males	40%	35.3%	0.750[b]
Centiles	0.8 ± 1.5	49.8 ± 9.6	0.0000[c]

[a] Data presented as mean ± SD or percentage.
[b] Chi-square test for categorical data.
[c] Two-sample Wilcoxon rank-sum test.

Standard deviation ranged from 0.07 to 0.1. The slopes of the standard curve lines ranged from −3.29 to −3.33, whereas linearity was almost 1 (0.9997–1.000). All samples were treated in duplicate and the mean PMR value between the 2 measurements was chosen. The standard deviation of the H19 PMR values was 0.122, making the assay markedly sensitive in detecting small differences in methylation between the groups.

2.6. 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 chi-square test. The Spearman's rank correlation coefficient was estimated to assess the potential monotonic relationship between various continuous variables. Moreover, the association between mRNA or Percentage of Methylated Reference (PMR) values (independent variables) and characteristics of the study participants (for instance, BW or gestational age) was modelled using linear regression analysis. All tests of significance were two-sided and a *P*-value less than 0.05 declares significance. Statistical analysis was performed using SPSS 17 statistical package (*SPSS*, Inc., Chicago, IL[fx1]).

3. Results

3.1. 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 FGR pregnancies, with 90% of them being severely growth restricted, below the 3rd percentile. BW was between the 10th and 90th percentile in the control group. The percentiles were calculated based on individually customized fetal and BW percentile method (www.gestation.net). FGR cases and controls were compared in 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. As expected, there were significant differences between gestational age, BW and BW percentiles in FGR subjects versus controls. Baseline characteristics of the study are presented in Table 2.

3.2. Clinical and genetic follow up evaluation

In order to investigate the presence of Silver-Russell Syndrome (SRS) [18] or a late manifestation of other chromosomal disorders or genetic syndromes in the children who were born as growth restricted by the women recruited in this study, a follow up evaluation was carried out. In particular, all the children were examined by a team of a Paediatrician and a Clinical Geneticist 43–65 months after birth. ThePrice et al. [19] diagnostic criteria for SRS were used: birth weight below or equal to −2 SD from the mean; poor postnatal growth below or equal to −2 SD from the mean at diagnosis; preservation of occipitofrontal head circumference; classic facial phenotype; and asymmetry. None of the children demonstrated phenotypic characteristics of SRS nor presented with a phenotype indicative of an obvious chromosomal disorder or genetic syndrome, at the age of 3.6–5.2 years (median 4.3 years).

3.3. Imprinting analysis of H19 in placental tissues

One *H19* exon 5 polymorphism was evaluated in this study which involved the creation of a restriction enzyme site. PCR of genomic DNA with specific primers (Table 1), followed by restriction digestion, allowed the determination of informative samples (Fig. 1). RT-PCR of total RNA allowed for analysis of expressed alleles. Fourteen out of 31 (45%) FGR and 8 out of 17 control samples (47%) were informative for *H19*. Imprinting analysis of *H19* heterozygote is presented in Table 3. Of the 21 informative cases, LOI was shown in 5 specimens, 4 of which were control samples and 1 FGR (Fig. 1). Maintenance of imprinting (MOI) of *H19* was demonstrated in 13 FGR and 4 control specimens.

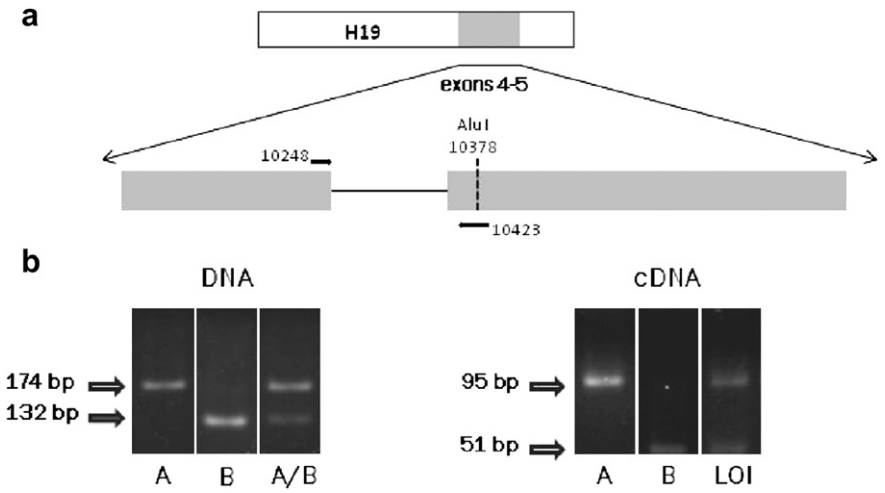

Fig. 1. a. H19 polymorphism studied (Alu I at bp 10378). Schematic of exons 4 and 5. Locations of PCR primers are indicated by numbered arrows. b. Genotyping analysis is presented in the gDNA figures. Representative samples of A homozygotes, B homozygotes, and A/B heterozygotes are shown. cDNA figures show the results of allelic expression analysis. Representative samples of H19 LOI. The PCR primers for the H19 Alu I polymorphism cross an intron, and thus products from cDNA are smaller than those from genomic DNA.

Table 3
Allelic expression of H19 gene.

Placental	Case Numbers		
	Loss of imprinting of H19	gDNA	cDNA
FGR	72	A/B	A/B
Control	102	A/B	A/B
Control	82	A/B	A/B
Control	85	A/B	A/B
Control	122	A/B	A/B
	Maintenance of imprinting of H19	gDNA	cDNA
FGR	7	A/B	A
Control	66	A/B	B
Control	87	A/B	A
FGR	96	A/B	A
FGR	97	A/B	A
Control	118	A/B	A
FGR	1	A/B	A
FGR	19	A/B	A
FGR	25	A/B	B
FGR	31	A/B	B
FGR	37	A/B	A
FGR	40	A/B	A
FGR	64	A/B	A
FGR	78	A/B	A
FGR	95	A/B	A
FGR	100	A/B	B
Control	113	A/B	B

3.4. Increased H19 expression in placentas complicated with FGR

We examined transcription of *H19* in FGR and control placentas. The mRNA levels of the *H19* transcript, were obtained after standardisation with quantitative data from the *GAPDH* "housekeeping" gene. Placental expression levels of *H19* were significantly increased in the FGR group ($P = 0.04$) (Fig. 2a). When a linear regression analysis was employed that included covariates such as BW, centiles and gestational age, there was no evidence of a relationship between *H19* expression with any of the parameters such as fetal growth, maternal characteristics or severity of FGR. H19 expression remained unaffected by gestational age, as shown in Fig. 2b. Interestingly *H19* mRNA levels were similar between normal placental samples that demonstrated biallelic and monoallelic expression of the *H19* gene ($P = 0.7$).

3.5. DNA methylation patterns in FGR placentas

A MethyLight qPCR assay was developed to determine the methylation status of regions that control *H19* transcription. We measured the Percentage of Methylated Reference (PMR) of two different potential DMR's. Methylation status of a region from 6156 to 6245 bp upstream of the *H19* transcription start site was examined (Fig. 3a). The particular site contains the sixth of seven of CTCF-binding sites and coordinates *IGF2* and *H19* reciprocal expression [11]. Compared with methylation values of normal

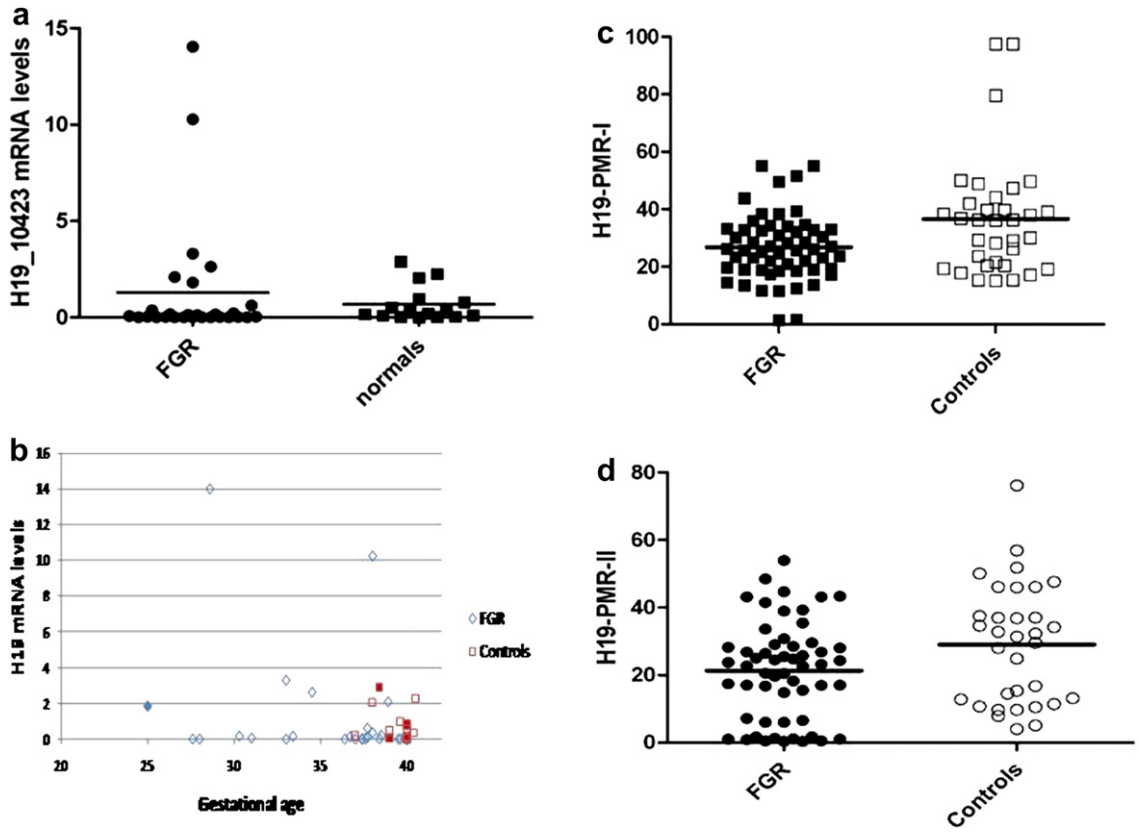

Fig. 2. a. Scatter plot depicting the increased *H19* mRNA levels in FGR placentas vs. controls ($p = 0.04$). b. Relative *H19* mRNA levels in placentas vs. gestational age. *H19* mRNA levels in placentas are demonstrated for each individual case. Control samples are shown as squares and FGR cases are shown as diamonds. There was no significant correlation between *H19* expression and gestational age. Solid diamonds and rectangles represent samples with LOI. c. PMR values of the DMR region ranging from 6156 to 6245 bp upstream of the H19 transcription start site. d. PMR values of the DMR region ranging from 7705 to 7809 bp upstream of the H19 transcription start site. FGR samples displayed significantly lower PMR levels vs. the normal placentas ($p = 0.02$).

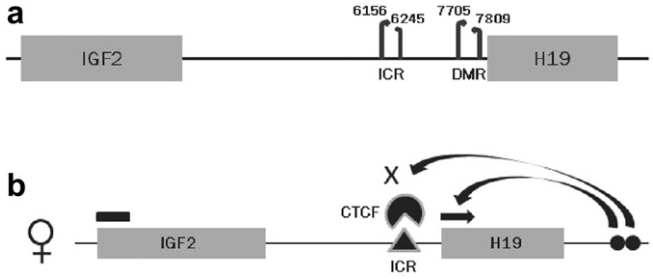

Fig. 3. a. Location of PCR primers used are indicated by numbered arrows. Two different DMRs were examined.H19 upstream region from base 6156 to 6245 which represent the ICR of the IGF2/H19 imprinting cluster and the H19 promoter region from base 7705 to 7809. b. Schematic of reciprocal imprinting of the IGF2/H19 imprinting cluster. On the maternal chromosome, the non-methylated ICR (solid triangle) bounds to the CTCF insulating the *IGF2* promoter from the enhancers located downstream of *H19* (solid circles) and allowing the H19 promoter unimpeded access to the enhancers. Maternal *H19* is subsequently transcribed. Black arrow indicates transcription.

placentas, placentas from growth restricted pregnancies had lower PMR, with mean values of 36.2% and 27.4% ($P = 0.15$) (Fig. 2c). There was no correlation between *H19* mRNA levels and methylation values in all of the placentas included in the study. *H19* PMR measurements of the specific region were also not associated with BW centiles ($P = 0.52$), BW ($P = 0.73$) nor gestational age ($P = 0.8$) in linear regression analysis.

The region from 7705 to 7809 bp close to the *H19* promoter was also assessed with respect to the methylation status (Fig. 3a). Samples from growth restricted pregnancies displayed significantly lower methylation levels compared to normal placentas with mean values of 22.3% and 32.4% respectively ($P = 0.02$) (Fig. 2d). There was no correlation between *H19* expression and methylation profile of this region. Linear regression analysis did not reveal any association between methylation levels and BW centiles ($P = 0.08$), BW ($P = 0.33$) and gestational age ($p = 0.52$).

We compared mean methylation values between four normal placental samples with retained imprinting and four samples with relaxed imprtinting. Our analysis showed that samples with normal imprinting of *H19* had higher but not significant methylation values of the CTCF binding region, than those with LOI (71% vs 32% respectively, $P = 0.43$). However when compared, methylation values of the H19 promoter, there was no difference between the two groups (46.3% samples with LOI, 44.2% samples with MOI, $P = 0.93$).

4. Discussion

The *H19* gene is one of the first imprinted genes studied [20]. Although *H19* codes an untranslated RNA of yet unknown biological significance it is abundantly expressed by the human intermediate trophoblasts and cytotrophoblasts [21]. Deletion of *H19* in mice resulted in increased placental size [22]. *H19* expression is modified during the different stages of placental growth based on a strict program which favours biallelic expression [23]. However there is hardly any data regarding *H19* expression and imprinting in late pregnancy. The epigenetic mechanisms which are involved in *H19* function in trophoblastic tissues are also obscure. The aim of this study was to investigate the role of DNA methylation of two separate regions which control *H19* expression and imprinting, and to evaluate a possible involvement in the pathophysiology of FGR.

Placentas obtained from FGR-affected pregnancies demonstrated increased *H19* expression in our study. To the best of our knowledge this is a novel finding, with yet undetermined importance. Although Guo et al. reported an increased trend of *H19* mRNA levels in the small for gestational age placentas that they have studied, this difference was not significant [24]. Experimental data in mice have linked increased *H19* expression with post-natal growth reduction [10]. It is difficult however to correlate *H19* expression with intrauterine growth restriction due to the established high variety of *H19* transcription patterns among different tissues [25]. Even if evidence exists that associates *H19* transcription with other placental abnormalities such as gestational trophoblastic disease, no definite conclusions can be drawn for the exact role of *H19* expression on placental function and fetal growth. In addition, it should be noted that the increased expression of *H19* in the FGR group was due to the abnormally high levels in some particular cases (Fig. 2a). This may have weakened the significance of the obtained results, and further investigation is required to confirm the findings of our study and to elucidate their implication in the pathogenesis of FGR. Hypomethylation at distal chromosome 11p15 represents a major cause of SRS, which is characterized by severe intrauterine growth retardation. The possibility, however, that the cases with increased H19 expression were associated with this condition was excluded after a clinical follow up 3.6–5.2 years (median 4.3 years) after birth.

Compared to previous studies, a significant amount of samples demonstrated LOI in our study. However, only one out of the 14 FGR informative samples revealed relaxed imprinting while 4 out of 8 normal informative samples presented with such an event. The preservation of the monoallelic gene expression in normal placentas [23,24] along with the report of a high prevalence of LOI in preeclampsia affected placentas [23], are two observations that are not compatible with our findings. However Guo et al reported only one case of growth affected placenta with LOI, among five informative samples that they have studied [24]. Since imprinting is a dynamic process that evolves during placental development, further investigation is clearly needed in order to elucidate the role of H19 imprinting on placental abnormalities.

Levels of H19 mRNA were similar, between normal placentas with relaxed or retained imprinting. This finding is inconsistent with several studies on cancer which advocate an increase in imprinted gene transcription whenever there is biallelic expression [26,27]. However, others postulate that in many cases, imprinting and gene transcription are not directly associated and that probably different mechanisms exist that mediate the two [28]. The same conclusion has been drawn from studies that focused their analysis on imprinted gene expression in placental tissues [29]. Our results are in agreement to those by Yu et al who found no correlation between imprinting defects and H19 expression in placentas from preeclamptic patients [23]. The authors of that study propose a possible association between LOI of H19 and abnormal trophoblast invasion that predisposes to preeclampsia. Their conclusion represents an attractive hypothesis that correlates H19 function with several pregnancy complications related to placental pathology, including FGR.

Several studies have suggested that the regulation of gene imprinting in the placenta is not subject to epigenetic mechanisms as in other tissues [30,31]. They concluded that histone modifications rather than DNA methylation are essential for gene imprinting in the placenta. In our study we have evaluated the methylation patterns of the ICR domain and at the *H19* promoter region. ICR hypomethylation is thought to be responsible for the low birth weight and poor post-natal growth observed in SRS patients [11]. Although PMR values were lower in the FGR group, in both DMRs studied statistical difference was shown only in the H19 promoter region. Methylation levels in the FGR placentas were lower in the CTCF binding site associated with SRS; however, the difference was not significant. Mean PMR values in the FGR group are similar to those reported by Bourque et al in the same region, 27.4% and 30.8%, respectively [32].

Hypomethylation of the H19 promoter region has been shown in the FGR group. Hypomethylation of the H19 promoter has been detected in all stages of normal placental development [21]. Reduced methylation levels at the same region were also reported in small for gestational age placentas. Hypomethylation in the promoter region of a gene is generally associated with increased gene expression. Hence the reduced PMR values in the FGR group that we have found is in consistency with the increased *H19* mRNA levels. There was no correlation, however, between PMR and mRNA levels.

We have detected lower but no statistically significant methylation values in the ICR domain in the placentas from the FGR group. Hypomethylation of that site is present in approximately one third of patients with SRS which is associated with post-natal growth restriction [12]. None of the children, who were born as growth restricted by the women recruited in this study, demonstrated phenotypic characteristics of SRS. The theory on *IGF2-H19* imprinting postulates that non-methylated ICR binds to CTCF, insulating the *IGF2* promoter from the enhancers and allowing the *H19* promoter to access them. *H19* is then transcribed while *IGF2* is not (Fig. 3b). Based on that theory, the reduced trend in methylation values of the ICR that we have found in the FGR samples matches the increased *H19* transcription in the same samples. Bourquet et al. established a significant reduction in methylation levels of the ICR in intrauterine growth restricted placentas [32]. Based on those results and since ICR is controlling the expression of both *H19* and *IGF2* genes which are known growth modulators, we conclude that aberrant methylation in the ICR represents a potential link between epigenetic modifications and abnormal fetal and placental growth.

Aberrant methylation patterns have been traditionally associated with imprinting defects. Based on that observation we have investigated whether samples that demonstrated relaxed imprinting, had also altered methylation patterns. There was a reduced trend in PMR values of the ICR domain in the group of normal placentas with LOI but with no statistical significance. PMR levels were similar in the DMR of the H19 promoter. Considering the limited number of samples from the same group of placentas that demonstrated imprinting defects, no speculation can be extracted from our results.

A broad range of PMR values were shown in the placentas of both groups examined in this study (Fig. 2c and d). This large variation has also been found by other investigators [33], probably suggesting that DNA methylation differences can be established during development [34]. An interindividual variation in DNA-methylation level is widespread in the human genome. In particular, the placenta has been reported to present high variability in overall DNA methylation compared to other tissues [35], probably in response to its role in mediating the conflicting demands of mother and fetus [36]. Moreover, DNA methylation variation at the imprinted genes IGF2/H19 has been shown [37,38], but also a high degree of interindividual differences in DNA methylation for other genes in the human placenta has been reported [39]. The nature of this variation as well as the biological significance it may have in human phenotypic variation, placental development and fetal growth requires further elucidation.

In this study, placentas from the third trimester FGR and normal pregnancies were examined. However, the absence of gestational age-match of cases and controls (Table 2) that would offer more appropriate groups for comparison should be noted. There are no data from the literature to show that placental H19 remains constant during the late gestational period. Despite this drawback, several conclusions can be drawn and also several questions can be raised from this study. The increased H19 transcription in the FGR placentas is a finding that is compatible with its known implication in post-natal growth restricted syndromes. The hypomethylation of the H19 promoters is in accordance with the increased expression in the FGR placentas. However, whether these two events represent the component of a molecular mechanism which leads to FGR or merely the consequence of placental dysfunction is unknown. Since DNA methylation and imprinting are particularly susceptible to environmental changes, imprinted genes may respond to placental hypoperfusion, by activating altered imprinting and methylation programs. Evidence provided from the present study may serve as a stimulus for larger studies that will elucidate the role of H19 in intrauterine growth restriction.

Conflict of interest

The authors declare no conflict of interest.

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