

Epigenetic modulation of the *IGF2/H19* imprinted domain in human embryonic and extra-embryonic compartments and its possible role in fetal growth restriction

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Abbreviations: AGA, appropriate for gestational age; BWS, Beckwith-Wiedemann syndrome; DMR, differentially methylated region; ICR, imprinting control region; IUGR, intrauterine growth restriction; PBL, peripheral blood lymphocytes; SGA, small for gestational age; SRS, Silver Russell syndrome; UCT, umbilical cord tissue; UCB, umbilical cord blood; UPD, uniparental disomy

Genomic imprinting, resulting in parent-of-origin-dependent gene expression, is mainly achieved by DNA methylation. *IGF2* and *H19*, belonging to the same cluster of imprinted genes and regulated by ICR1, DMR2 and *H19* promoter elements, play a major role in fetal/placental growth. Using quantitative approaches, we explored the epigenetic modulation of *IGF2/H19* during human development in 60 normal and 66 idiopathic IUGR (Intrauterine Growth Restriction) pregnancies, studying embryonic (cord blood) and extraembryonic (placenta and umbilical cord) tissues. We found ICR1 normal methylation levels (~50%) and *H19* promoter/DMR2 hypomethylation in extra-embryonic tissues. In contrast, in embryonic samples the three loci displayed normal methylation values comparable to those in postnatal blood. This feature is stably maintained throughout gestation and does not vary in IUGR cases. We reported asymmetric allelic expression of *H19* and *IGF2* as a common feature in pre- and post-natal tissues, independent of *H19* promoter and DMR2 methylation levels. In addition, we excluded in IUGR post-transcriptional *IGF2* interference possibly related to miRNA 483-3p (*IGF2*, intron 2) expression defects. Through LINE1 methylation analysis, we observed a methylation gradient with increasing methylation from pre- to post-natal life. The involvement of UPD (Uniparental Disomy) in IUGR aetiology was excluded. Our data indicate that: (1) ICR1 methylation status is a necessary and sufficient condition to drive the imprinting of *IGF2* and *H19* present in embryonic as well as in extra-embryonic tissues; (2) hypomethylation of *H19* promoter and DMR2 does not influence the expression pattern of *IGF2* and *H19*; (3) there is a gradient of global methylation, increasing from extra-embryonic to embryonic and adult tissues. Finally, because of placental hypomethylation, cautions should be exercised in diagnosis of imprinting diseases using chorionic villi.

Introduction

Genomic imprinting is a phenomenon peculiar to the mammalian genome, contributing to several aspects of pre- and post-natal life: embryo viability and growth, placental development, as well as brain functions and behavior.¹ This process results in a reversible parent-of-origin specific marking of the genome, leading to monoallelic expression of a subset of genes. Silencing of the imprinted allele mainly occurs through cytosine methylation of key regulatory elements termed differentially-methylated regions (DMRs), or imprinting control regions (ICRs). Most of the imprinted genes are arranged in clusters (imprinted domains) and

each cluster is under the control of an ICR² and often contains noncoding RNAs that are silenced according to their parental origin. These RNAs can be broadly classified into long noncoding RNAs and short regulatory RNAs (small nucleolar RNAs and microRNAs).^{3,4}

Human chromosome region 11p15.5 harbors two clusters of imprinted domains controlled by ICR1 and ICR2. ICR1 regulates *IGF2* and *H19* monoallelic expression,⁵ leading to *IGF2* transcription from the paternal and *H19* from the maternal allele. Expression of these two genes is controlled by differential and coordinated methylation of ICR1, DMR2 and the *H19* promoter,⁶ constituting a parentally-inherited epigenetic trait. ICR1

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is located 2 kb upstream of the *H19* promoter and contains seven boundary elements for the zinc-finger CTCF protein. Binding of ICR1 to CTCF mediates higher-order chromatin conformation, partitioning paternal and maternal *IGF2* alleles into active and inactive chromatin domains.^{6,7} CTCF binding to the maternal unmethylated ICR1 results in a specific change in chromatin loop structure and prevents the *IGF2* gene promoter from interacting with enhancers downstream of the *H19* gene, thus resulting in transcriptional silencing of the maternal *IGF2* allele.⁶ On the paternal allele, DNA methylation prevents CTCF binding and is permissive for *IGF2* gene activation. The methylation status of the sixth binding site within ICR1 was found to be most consistently associated with the transcriptional status of both *IGF2* and *H19*.⁸

Our understanding regarding this imprinted domain has recently been enhanced by the description of miRNA 483-3p located within intron 2 of *IGF2*.⁹ As the intronic miRNAs may share common promoters with their host genes,¹⁰ we evaluated the possibility that miRNA 483-3p expression could correlate with *IGF2* expression profile. We also speculated that miRNA 483-3p could have a role in post-transcriptional regulation of *IGF2*.

In humans, deregulation of the *IGF2/H19* imprinted region is associated with the overgrowth and tumor predisposition-related Beckwith-Wiedemann Syndrome (BWS, OMIM: #130650) and with the Silver Russell Syndrome (SRS; OMIM: #180860), mainly characterized by pre- and post-natal growth deficiency.

Sparago et al.¹¹ demonstrated aberrant ICR1 methylation in patients with BWS and SRS and Gicquel et al.¹² described that ICR1 hypomethylation is responsible for biallelic expression of *H19* in SRS patients.

In mice, it has been demonstrated that placental *Igf2* and *H19* play a major role in fetal growth, through their effects on nutrient transfer capability from the mother to the fetus.^{13,14} Using knockout experiments, a direct effect of *Igf2/H19* gene silencing on fetal and placental growth has been demonstrated. In particular, placental *Igf2* knockout leads to Intrauterine Growth Restriction (IUGR),¹⁵ whereas *H19* silencing results in fetal overgrowth.¹³ These phenotypes mirror human SRS and BWS, respectively.

IUGR is a complex disorder that can be due to fetal, maternal or environmental causes. It can be isolated or in association with other phenotypic defects. Chromosomal imbalances, monogenic disorders or uniparental disomies (UPDs) constitute the recognizable fetal causes of syndromic IUGR,^{14,16} whereas the etiology of the majority of isolated IUGRs remains unknown.

The importance of genomic imprinting in human fetal growth prompted investigation of its role in intrauterine growth. Regarding *IGF2* and *H19*, the majority of reports have focused on evaluation of their expression level changes in the fetal/placental unit, in association with intrauterine growth defects.¹⁷⁻²² Conflicting evidence regarding *IGF2* expression levels in the placenta has been reported, given that only some authors^{17,22} describe decreased expression in association with growth deficiency. *H19* placental expression has been poorly investigated, though it has been reported to be unchanged in SGA (Small for Gestational Age)¹⁷ and in IUGR,¹⁹ when compared to AGA (Appropriate for Gestational Age).

The allele-specific expression dosage of *IGF2* and *H19* has also been evaluated. *IGF2* biallelic expression, defined as loss of imprinting (LOI), was found in both normal and IUGR placentae, whereas LOI of *H19* was specifically observed in IUGR¹⁹ and SGA¹⁷ placentae.

The methylation status of the *IGF2/H19* imprinting domain in human placenta has been investigated by Guo et al.¹⁷ The authors reported *H19* promoter non-methylation and DMR2 hypomethylation in the placenta when compared to post-natal blood and suggested that the placenta does not maintain differential methylation of these regions. Conversely, ICR1 in both tissues exhibited normal methylation for an imprinted locus (~50%).

Imprinting regulation during development has been intensely investigated in the mouse, while knowledge in humans is less comprehensive. Reported data reveal differences between human and mouse in the assessment and maintenance of imprinting during development. Indeed, relaxation of imprinting was observed in human for a few loci, such as *IGF2R*²³ and *ESX1*,²⁴ and for X chromosome inactivation,²⁵ suggesting limited evolutionary conservation of imprinting between the two species.²⁶

To examine the process of methylation assessment during human development, we firstly investigated the *IGF2/H19* imprinting domain in embryonic and extra-embryonic tissues at different gestational ages. Secondly, the genomic methylation profile was delineated in the same samples using LINE1 methylation measurement as a marker for global methylation levels. Finally, we included in the study embryonic and extra-embryonic tissues from a wide cohort of isolated idiopathic IUGR pregnancies to assess the importance of epigenetic/genomic imprinting changes in fetal growth restriction. In these cases, changes in the expression levels of miRNA 483-3p and the presence of UPD for all chromosomes were also explored.

Results

Epigenetic evaluation of ICR1, DMR2 and the *H19* promoter in normal and IUGR pregnancies. Extraembryonic tissues. Pyrosequencing experiments were performed on the following imprinting regulatory regions of *IGF2/H19*: ICR1, DMR2 and the *H19* promoter (Fig. 1).

We studied a total of 79 third trimester placental samples from 33 AGA and 46 IUGR. We also analyzed umbilical cord tissue fragments (UCT) from a subset of AGA (19 cases) and IUGR (7 cases). In addition, placental specimens from two volunteered abortions at 11 wg (weeks of gestation) were included.

As reference for the methylation levels of *IGF2/H19*, peripheral blood lymphocytes (PBLs) from 17 healthy individuals, one case of SRS and one case of BWS were analyzed. As shown in Figure 2A, mean methylation levels of ICR1, DMR2 and the *H19* promoter in PBLs from healthy individuals were: 45.8%, 48.8% and 49.4%. These results are consistent with methylation levels expected for imprinted loci in physiological conditions. As expected, SRS case exhibited hypomethylation (ICR1: 11.8, DMR2: 31.2, *H19* promoter: 13.0, in mean), and BWS hypermethylation (ICR1: 83.5, DMR2: 68.0, *H19* promoter: 82.0, in mean). Figure 2B shows the pyrograms of PBLs from one healthy

individual, one SRS and one BWS case. Notably, each investigated region showed homogeneous methylation values in all the analyzed CpGs.

The methylation pattern of both AGA and IUGR third trimester placentae is shown in Figure 3A. Mean methylation values for ICR1, DMR2 and the *H19* promoter were: 46.1%, 26.3%, 12.3% in AGA and 45.2%, 21%, 11.9% in IUGR samples. Taken together, these results highlight a placental-specific methylation pattern for the *IGF2/H19* imprinted region, characterized by homogeneous and significant hypomethylation ($p < 0.001$) of DMR2 and the *H19* promoter and normal methylation values of ICR1 relative to levels in PBLs from healthy individuals. Methylation levels of the three loci did not show significant differences between AGA and IUGR placental samples.

In order to verify the stability of the methylation pattern during gestation, we analyzed chorionic villi samples from two volunteered abortions at 11 wg with normal karyotype and observed a methylation profile similar to that of term placenta (Fig. 3A). This finding suggests that the placental methylation profile does not vary from the first trimester to the end of pregnancy.

To assess whether the methylation pattern was homogeneous in different extra-embryonic tissues we analyzed 26 UCT samples. As shown in Figure 3B, methylation levels of ICR1 (46%) and the *H19* promoter (23.3%) in UCT were similar to those found in term placental samples. In addition, DMR2 values (44%) were significantly higher than those in placental samples ($p < 0.05$) and significantly lower ($p < 0.05$) than those in PBLs from healthy individuals. No significant differences were observed in UCTs from AGA and IUGR pregnancies (Fig. 3B).

We can conclude that hypomethylation of the *H19* promoter and DMR2 is a signature of the placenta, irrespective of the gestational age and the presence of IUGR.

The umbilical cord, although considered an extraembryonic tissue, displayed DMR2 hypermethylation with respect to the placenta. In all the analyzed tissues, ICR1 maintains methylation levels close to 50%.

Embryonic tissues. We completed epigenetic analysis of the fetal/placental unit by assessing the methylation pattern of ICR1, DMR2 and the *H19* promoter in neonatal umbilical cord blood (UCB) from AGA (5 cases) and IUGR (10 cases) pregnancies and in fetal fragments from the two volunteered abortions. As summarized in Figure 3C, we found that: (1) UCB methylation values were similar in AGA and IUGR cases (ICR1: 46.2% vs. 46.1%, DMR2: 48.2% vs. 49% and *H19* promoter: 48.4% vs. 48.2%); (2) the mean methylation levels in UCB resembled those in PBLs from normal individuals; (3) first trimester fetal fragments showed methylation levels similar to term UCB (ICR1: 43.6%, DMR2: 40.2% and *H19* promoter: 45.2%).

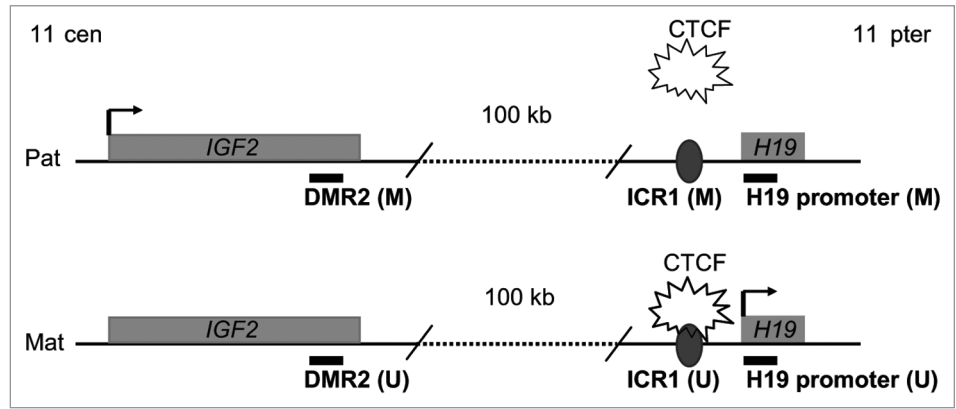


Figure 1. Schematic representation of the *IGF2/H19* imprinted region. The methylation status of DMR2 (Differentially Methylated Region 2), ICR1 (Imprinting Control Region 1) and *H19* promoter is specified (U = unmethylated; M = methylated). Arrows indicate the transcribed allele. Paternal chromosome (Pat): DMR2 (M) activates *IGF2* transcription, ICR1 (M) is unavailable for CTCF binding, thus promoting *IGF2* and blocking *H19* expression; *H19* promoter (M) blocks *H19* transcription. Maternal chromosome (Mat): DMR2 (U) prevents *IGF2* transcription, ICR1 (U) binds the insulator CTCF, enabling *H19* and repressing *IGF2* expression; *H19* promoter (U) facilitates *H19* transcription.

These results suggest that the methylation pattern of the *IGF2/H19* region in embryonic tissues remains stable during gestation and throughout life.

Relationship between *IGF2/H19* methylation and expression levels in normal and IUGR placentae. To establish whether the methylation profile of the *IGF2/H19* region correlated with *IGF2* and *H19* expression levels, we performed Real-Time Reverse Transcriptase (RT)-PCR of the two genes in 42 AGA and 50 IUGR placental RNA samples. The median expression values for either gene was not significantly different between the two groups. In details, *IGF2* median values (expressed as Log_{10}) were 2.8 (25° percentile: 2.4, 75° percentile: 3) in AGA and 2.8 (25° percentile: 2.5, 75° percentile: 3.1) in IUGR. *H19* median values were 3.7 (25° percentile: 1.9, 75° percentile: 4.1) in AGA and 3.9 (25° percentile: 1.8, 75° percentile: 4.1) in IUGR (Fig. 4A). Moreover, expression data were analyzed considering different clinical parameters (gestational age, fetal and placental weight and IUGR severity), but we failed to find any significant correlation (data not shown).

This confirmed the results for *IGF2* placental expression previously obtained by our group¹⁷ using a smaller cohort of IUGR cases and we also report here similar observations for *H19*. In addition, a wide range of values in both normal and pathological placental samples were observed, leading us to conclude that *IGF2* and *H19* expression levels do not represent a robust marker for evaluation of appropriate fetal growth. Moreover, we showed that the methylation profiles of the *IGF2/H19* domain do not correlate with their own expression levels, as depicted in Figure 4B for ICR1.

Allele-specific expression of *H19* and *IGF2*. Given that hypomethylation of an ICR often correlates with biallelic expression at imprinted loci,²⁷ we investigated allele-specific expression of *H19* and *IGF2* in placental tissues. We used a quantitative allelic discrimination approach on a subset of 35 placental cDNA (both from AGA and IUGR pregnancies) and in PBLs samples from four normal individuals.

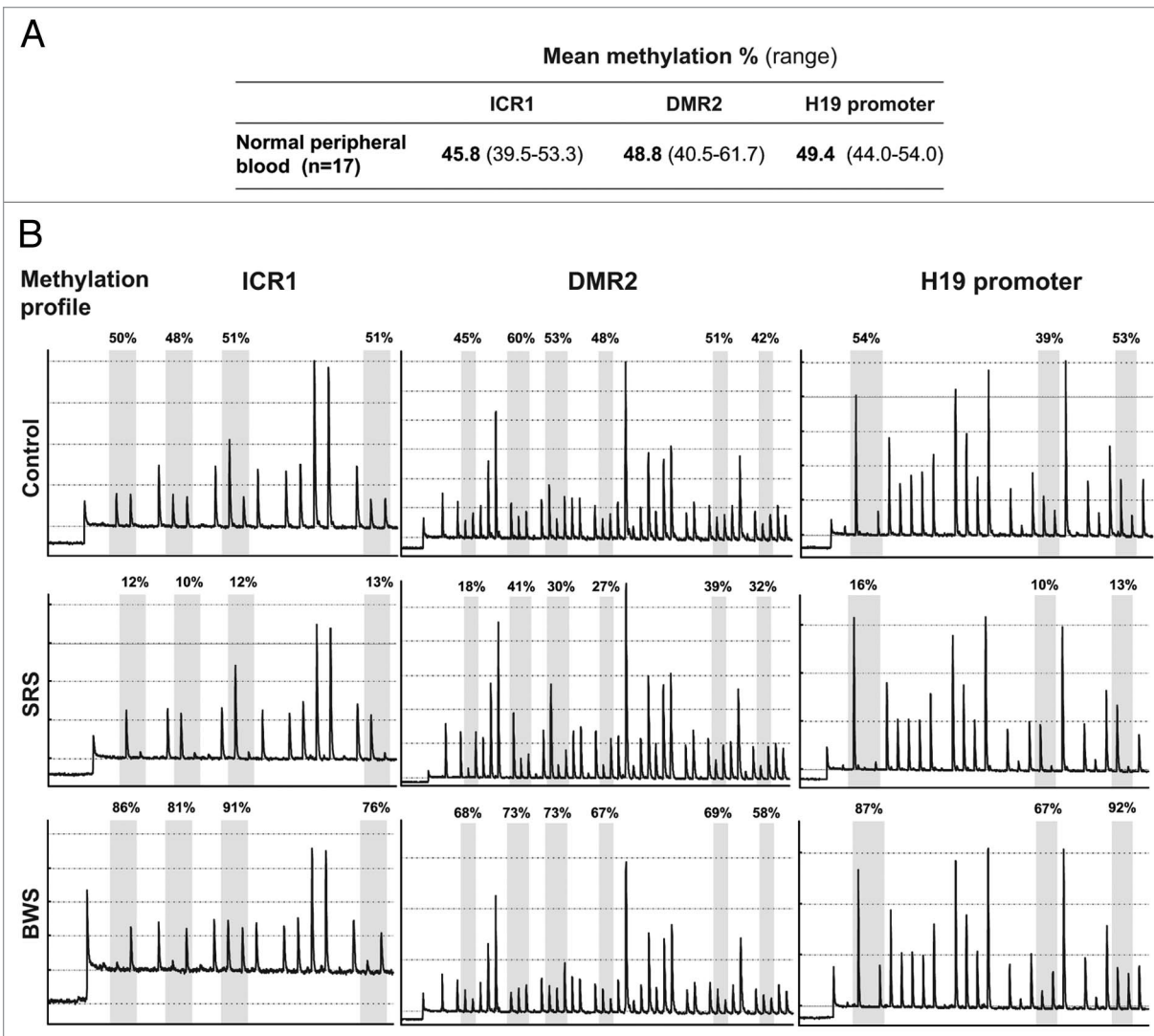


Figure 2. Quantitative CpG methylation analysis of ICR1, DMR2 and *H19* promoter. Pyrosequencing results of healthy individuals (controls), Beckwith-Wiedemann Syndrome (BWS) and Silver Russell Syndrome (SRS) peripheral blood samples. (A) Mean methylation levels and methylation ranges in 17 peripheral blood samples. (B) Representative pyrograms showing the methylation profile in one control, one SRS and one BWS case. The potentially-methylated cytosines (C or T after bisulphite treatment) are highlighted in grey. Methylation levels at individual CpG positions are shown as the percentage of methylation above the respective positions.

H19. Twenty-two placental samples were informative for the Single Nucleotide Polymorphism (SNP) rs2839702, located at *H19* exon 5. Eight of these showed expression from both alleles (Fig. 4C). Quantitative analysis revealed that the contribution of the two allelic products was unequal, with the levels of transcript from the predominantly-expressed allele ranging from 67–93%.

IGF2. Fourteen samples were informative for the SNP rs680 located at *IGF2* exon 9. Two of these displayed expression from both alleles as represented in Figure 4C. The allelic-expression levels were also unbalanced for *IGF2*, with the percentage of transcript from the predominantly-expressed allele ranging from 87–94%.

Although *IGF2* and *H19* “biallelic” expression was previously reported in a subset of placental samples from SGA pregnancies,¹⁷ we showed that imbalanced expression is a frequent

event in placenta and not restricted to fetal growth defects. Notably, we did not observe a correlation between methylation status and the type of expression (mono- or biallelic) and detected hypomethylation associated with mono- as well as biallelic expression.

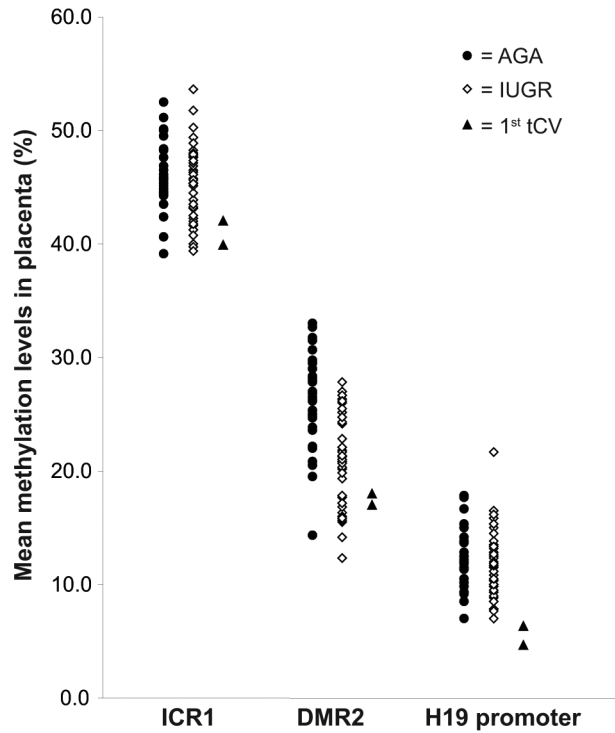
This skewed expression is due to low level activity of the “repressed allele.”

We also observed *IGF2* or *H19* imbalanced expression, with a preferentially-transcribed allele in two out of four PBLs samples from normal individuals.

Finally, one of the two volunteered abortions was informative for the *H19* SNP and showed monoallelic expression in cDNA from fetal fragments, whereas basal expression was revealed in chorionic villi (90.3% G vs. 9.7% T allele), probably from the “imprinted” allele.

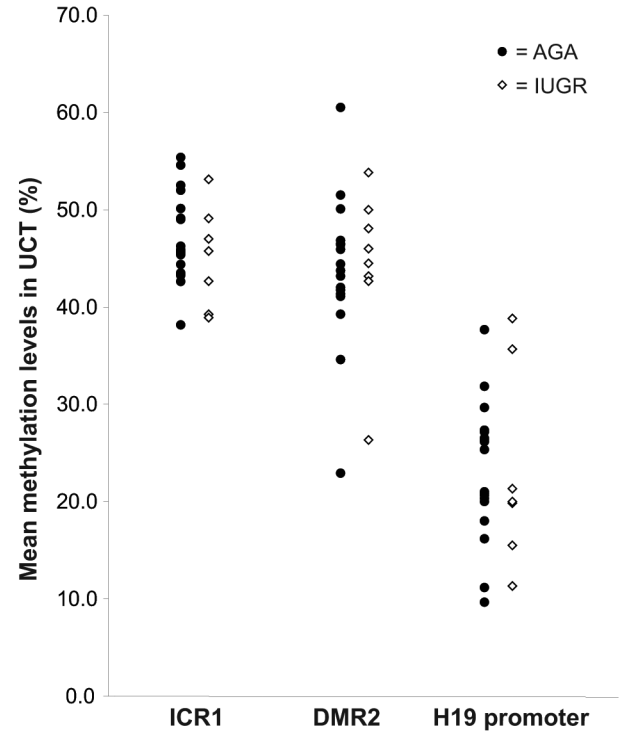
A

Placenta	Mean methylation % (range)		
	ICR1	DMR2	H19 promoter
AGA (n=33)	46.1 (39.1-52.5)	26.3 (14.3-33.0)	12.3 (7.0-17.8)
IUGR (n=46)	45.2 (39.4-53.6)	21.0 (12.3-27.8)	11.9 (2.7-21.7)
1st tCV (n=2)	41.5 (40.5-42.5)	17.5 (17.0-18.0)	5.5 (4.7-6.3)



B

UCT	Mean methylation % (range)		
	ICR1	DMR2	H19 promoter
AGA (n=19)	46.9 (38.2-55.4)	43.6 (22.9-60.5)	23.3 (9.7-37.7)
IUGR (n=7)	45.1 (38.9-53.1)	44.3 (26.3-53.8)	23.2 (11.3-38.8)



C

UCB	Mean methylation % (range)		
	ICR1	DMR2	H19 promoter
AGA (n=5)	46.1 (43.2-48.4)	49.0 (40.7-58.3)	48.2 (44.2-56.5)
IUGR (n=10)	46.2 (42.9-51.6)	48.2 (43.3-51.0)	48.4 (43.3-56.5)
1st tE (n=2)	43.6 (43.2-44.0)	40.2 (38.5-42.0)	45.2 (44.0-46.3)

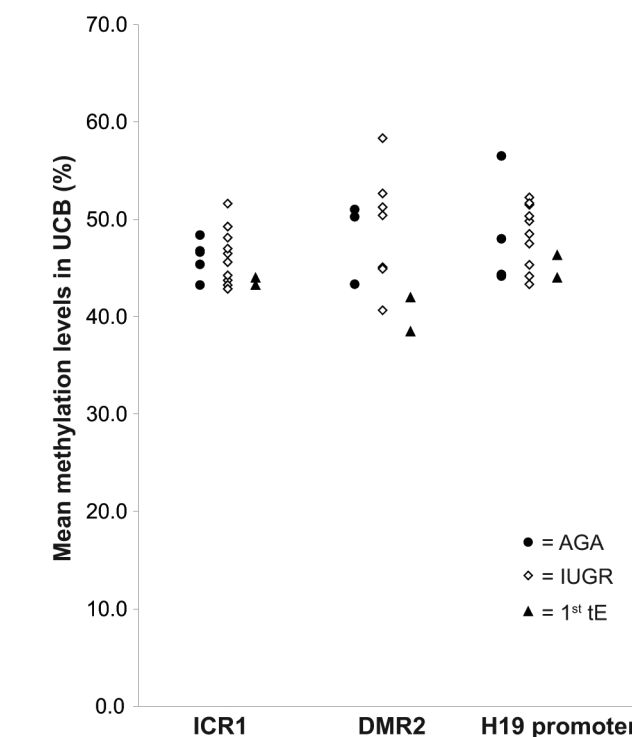


Figure 3 (See previous page). Quantitative CpG methylation analysis of ICR1, DMR2 and *H19* promoter. Pyrosequencing results of: (A) placental samples, (B) fragment of umbilical cord tissues (UCT) and (C) umbilical cord blood (UCB) samples, from normal (AGA = Appropriate for Gestational Age) and IUGR (Intrauterine Growth Restriction) third trimester pregnancies. Methylation levels of chorionic villi (1st tCV) and fetal fragments (1st tE) from first trimester volunteered abortions are also displayed in (A) and (C), respectively. Tables (A–C) summarize the mean methylation levels and methylation ranges. Graphs (A–C) represent single methylation values of each analyzed case.

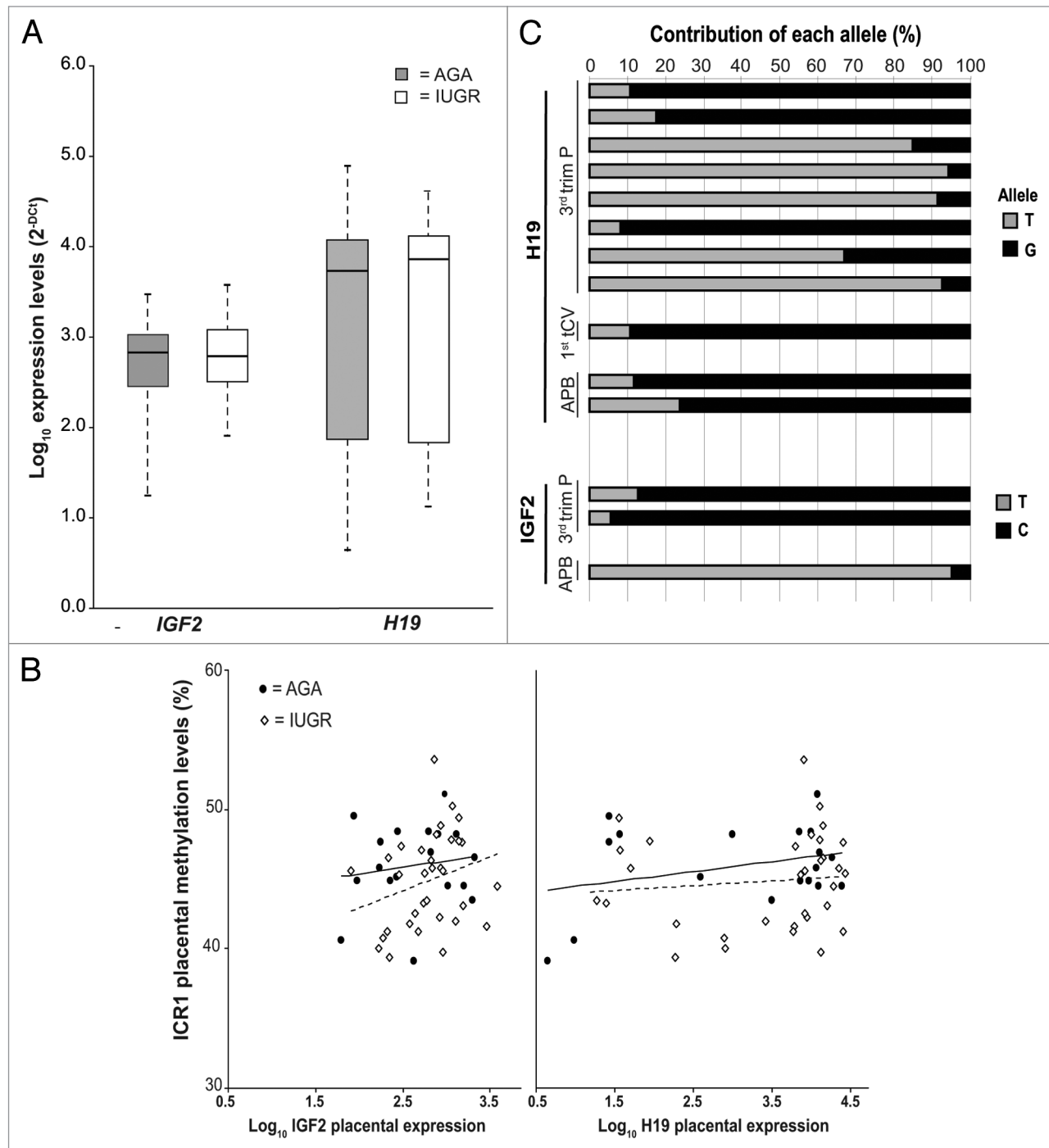


Figure 4. Placental expression pattern of *IGF2* and *H19*. (A) Results of expression level analysis for *IGF2* and *H19* in AGA (Appropriate for Gestational Age) (grey boxes) and IUGR (Intrauterine Growth Restriction) (white boxes) samples by Real-Time RT-PCR. Each box represents: 75^o percentile, median, 25^o percentile of expression levels; dotted lines show the interval values. (B) Correlation between ICR1 methylation values and *IGF2*/*H19* mRNA expression in AGA and IUGR placentae. Regression curves are represented as a continuous line for AGA and a dotted line for IUGR. (C) Quantitative allelic discrimination analysis for *H19* (top) and *IGF2* (bottom) by pyrosequencing. Bars represent the contribution of each allele (in percentage) in: third trimester placentae (3rd trim. P), first trimester chorionic villi (1st tCV) and adult peripheral blood (APB) samples.

Global methylation analysis in normal and IUGR pregnancies. The methylation pattern is flexible during prenatal life and, in the embryo, low methylation levels correlate with the earlier developmental stages, in which pluripotency-associated genes are expressed.²⁸ Since DNA methylation of repetitive elements is a marker of the global DNA methylation status of the genome,²⁹ we evaluated the LINE1 methylation profile in 46 placental specimens (21 AGA and 25 IUGR cases) and in 81 peripheral blood samples from normal individuals. In addition, UCB (from 3 AGA and 7 IUGR) and UCT (from 15 AGA and 5 IUGR) samples were included in the analysis.

We observed significantly lower methylation levels in placenta (mean value 41.8 ± 4.5) compared to peripheral blood (mean value 67.3 ± 5.3) ($p < 0.01$). Both UCB (mean methylation 60.1 ± 3) and UCT (57.7 ± 4.3) samples showed methylation levels that were intermediate between placenta and peripheral blood values (Fig. 4). Of note, no differences between AGA and IUGR samples were observed, either in the placenta or in neonatal UCB and UCT samples. Consequently, methylation levels were represented in Figure 5 without distinguishing between the two groups.

We also analyzed both chorionic villi and fetal tissues from the two first trimester volunteered abortions and observed mean methylation values of 39.2% and 57.1% for placental and fetal tissues respectively (Fig. 5). LINE1 methylation levels mirror a gradient of methylation that is low in extra-embryonic and increases in embryonic tissues, reaching the maximum value postnatally.

Expression levels of miRNA 483-3p in IUGR and normal placenta. Since miRNAs can have a role in genomic imprinting,⁹ we hypothesized a possible role of miRNA 483-3p in transcriptional and/or post-transcriptional regulation of *IGF2*, according to its position within intron 2 of *IGF2*.

We evaluated expression of miRNA 483-3p by Real-Time RT-PCR in 27 IUGR and 28 AGA placental RNA samples and did not detect a significant difference between the two groups. Specifically, miRNA 483-3p median values were 3.5 (25° percentile: 2.5, 75° percentile: 4.4) in AGA and 2.7 (25° percentile: 2.4, 75° percentile: 4.1) in IUGR samples (Fig. 6). These data suggest that miRNA 483-3p expression levels are similar in normal as well as in IUGR samples. In addition, we did not detect significant correlation between miRNA 483-3p and *IGF2* expression levels.

UPD testing in IUGR pregnancies. UPD exclusion was performed in 25 IUGR pregnancies by allelic segregation from parents to placental and UCB DNAs using a panel of STRs (Short Tandem Repeats) covering all autosomes and the X chromosome (Suppl. Table 1). UPD was not detected in any case.

Discussion

Genomic imprinting is a mammalian-specific phenomenon mainly achieved by DNA methylation, and marks the imprinted

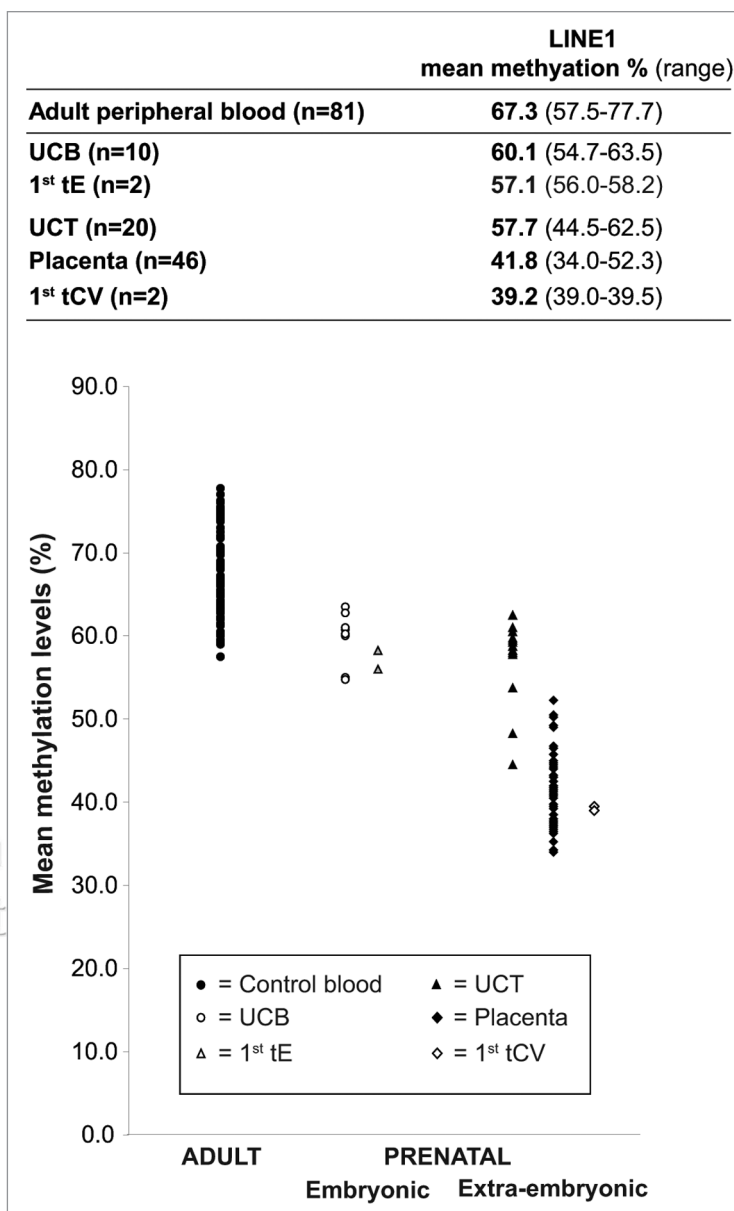


Figure 5. Quantitative CpG methylation analysis of LINE1. Pyrosequencing results of extra-embryonic and embryonic tissues from first and third trimester pregnancies and in peripheral blood of healthy adults (controls). (UCB = Umbilical Cord Blood; UCT = Umbilical Cord Tissue; 1st tE = fetal fragment; 1st tCV = first trimester chorionic villi). The table summarizes the mean methylation levels and methylation ranges. The graph represents single methylation values of each analyzed case.

allele in the gamete. This epigenetic phenomenon has been extensively studied in the mouse, and investigations in humans have revealed consistent differences with respect to the mouse, probably due to a relaxation of imprinting observed in humans.²⁶ Because of the difficulties in studying imprinting during human development, many gaps remain in our comprehension of this phenomenon. The *IGF2* and *H19* imprinted genes play a major role in fetal/placental growth, and are regulated by *H19* promoter, ICR1 and DMR2 elements.

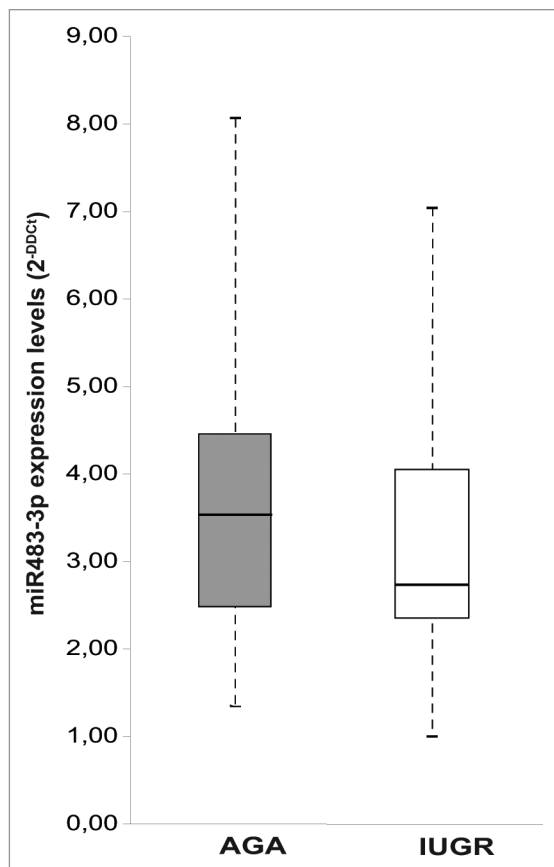


Figure 6. Analysis of miR483-3p expression levels in AGA and IUGR samples by Real-Time RT-PCR. Each box represents: 75° percentile, median, 25° percentile of expression levels; dotted lines show the interval values. AGA (Appropriate for Gestational Age) values are represented in grey, IUGR (Intrauterine Growth Restriction) in white boxes.

We investigated the methylation status of the *IGF2/H19* imprinted domain in embryonic and extra-embryonic tissues at different stages of human pregnancy.

We found that a peculiar methylation pattern is stably maintained throughout gestation in embryonic and extra-embryonic tissues. In embryonic tissues, ICR1, the *H19* promoter and DMR2 displayed a methylation status of about 50%, typical of imprinted genes and comparable to that observed in postnatal blood from healthy individuals. This methylation pattern was stable throughout gestation, since we found similar results in the first as well as in the third trimester pregnancy. In contrast, in the placenta, the *H19* promoter and DMR2 were hypomethylated (~9 and 22% respectively), while ICR1 displayed normal methylation levels (~50%). This observation is in keeping with those of Guo L, et al.¹⁷ In the umbilical cord we confirmed this picture, although DMR2 displayed methylation levels intermediate between the placental and adult blood values.

In the mouse, imprinted loci tend to maintain their particular methylation pattern established in the gamete, thus escaping the demethylation wave of early embryogenesis, during the morula stage.²⁸ In humans, we confirmed this feature for ICR1, demonstrating methylation stability in pre- and post-natal tissues.

Conversely, DMR2 and the *H19* promoter displayed variations of the methylation levels in extra-embryonic tissues relative to the embryonic levels.

The imprinting pattern of *IGF2/H19* in mouse and human spermatozoa consists of full methylation of ICR1, the *H19* promoter and DMR2.³⁰⁻³² Based on these data and our observations, we surmise that placental hypomethylation of the regulatory loci of *IGF2/H19*, excepting ICR1, could arise from the demethylation wave during the morula stage. Subsequently, these loci could regain DNA methylation in the embryo-derived cells. We believe that the imprinting signature of the *IGF2/H19* region in the early stages of embryogenesis is maintained by ICR1 methylation.

ICR1 methylation is sufficient for allele-specific silencing; indeed, we found that in pre- as well as in post-natal tissues one of the two alleles was preferentially expressed, irrespective of the DMR2 and *H19* promoter methylation levels. It is conceivable that there exist additional mechanisms for regulating parental-specific expression, such as chromatin modifications.

We reported in ante-natal tissues and in post-natal peripheral blood from healthy controls an imbalanced expression of *IGF2* and *H19* alleles, ranging from 67–93% for *H19* and from 87–94% for *IGF2* alleles. We observed homogeneity among pre- and post-natal samples and embryonic and extra-embryonic compartments. Although both alleles were expressed, it is important to demonstrate that the allele with the lower expression level most likely reflects basal expression of the imprinted allele. A similar pattern of imbalanced biallelic expression of imprinted genes was reported in placenta^{19,33} as well as in normal adult tissues.³⁴

Taken together, our results indicate that: (1) biallelic, but asymmetric, expression of *H19* and *IGF2* is a common feature in pre- and post-natal tissues; (2) given that similar results for the allelic expression pattern were found in all analyzed tissues, this supports the idea that in placenta, *IGF2* and *H19* are also imprinted; (3) the expression ratio between the two alleles in placenta is independent of the methylation level of the *H19* promoter and DMR2; (4) in placenta, the ICR1 methylation status is sufficient to guide the imprinting of *IGF2* and *H19*.

Although the hypomethylation observed in extra-embryonic tissues was specific for DMR2 and *H19* promoter, we observed lower global methylation in placenta, umbilical cord and cord blood relative to post-natal blood, using LINE1 quantitative methylation analysis. Among these tissues, the placenta showed the lowest methylation levels. This finding reinforces previous observations of low methylation in placenta relative to post-natal blood.³⁵ Our data suggest that methylation tends to increase from pre-natal to post-natal life, and seems to follow the stages of tissue differentiation. Given that the methylation profile in the placenta is different from that of the embryo, caution should be exercised in the case of diagnosis of imprinting diseases from chorionic villi. In particular, our data suggest that ICR1 but not *H19* promoter methylation is an appropriate target for SRS and BWS diagnosis using placental DNA.

The idea that imprinted genes can affect fetal growth is becoming increasingly intriguing, as it has been shown that most of the imprinting diseases are associated with pre- and post-natal growth defects.^{36,37} To examine whether *IGF2/H19* methylation

Table 1. Clinical features of the overall population analyzed

	Maternal age at delivery (mean value–range)	Fetal weight (mean value–range)	Placental weight (mean value–range)	Gestational age (mean value–range)
AGA (60 cases)	34 y (22–41)	3231.5 g (1900–3960)	519.5 g (156–850)	265.1 days (210–290)
IUGR (66 cases)	33.3 y (20–42)	1414.7 g* (451–262)	235.7 g* (96–600)	230.2 days* (147–271)

*p < 0.05 in IUGR vs. AGA cases.

and expression defects were associated with an IUGR phenotype, we analyzed 66 pregnancies complicated by idiopathic isolated (non syndromic) IUGR. Comparison of the results obtained with those of normal pregnancies did not reveal significant differences in epigenetic signatures of *IGF2/H19* between the two groups. In particular, regarding the allelic expression pattern of *IGF2* and *H19*, we found in IUGR similar results to those obtained in normal pregnancies. This is in contrast to the observations of Guo L, et al.¹⁷ who reported biallelic expression in one out of ten IUGR cases, and monoallelic expression in 14 placentae from normal pregnancies. This discrepancy may be due to the different methodological approaches and to the low number of pathological cases included in that study. Loss of imprinting of *IGF2* and *H19* in normal and IUGR placenta was recently reported by Diplas A et al.¹⁹ This observation is in apparent disagreement with our results, probably because Diplas et al. did not verify the imprinting status and allele-specific expression of the analyzed genes in embryonic as well as in post-natal tissues.

In addition, the methylation profile of LINE1 was similar between normal and IUGR pregnancies.

We investigated the expression levels of miRNA 483-3p in normal and IUGR placentae. According to its position within intron 2 of *IGF2*, we hypothesized a possible role for miRNA 483-3p in transcriptional and/or post-transcriptional regulation of *IGF2*. However, we excluded the involvement of miRNA 483-3p in IUGR and we did not evidence correlation between its and *IGF2* expression levels. Overall, these results support previous evidence that *IGF2* perturbancies are not associated with idiopathic IUGR,^{18,20} probably because *IGF2/H19* defects lead to complex phenotypes such as SRS.

Imprinting diseases can also originate from UPD and most of the UPDs reported in humans are characterized by pre-natal and/or post-natal growth defects.^{38,39} To evaluate the impact and relevance of UPD in IUGR we investigated placentae from 25 IUGR pregnancies. All autosomes and X chromosome were investigated in order to identify possible UPDs involving chromosomal regions so far unreported in the isolated IUGR phenotype. No UPD was found, thus suggesting that UPDs are not a main determinant of IUGR.

In summary, we presented here a study on 60 normal and 66 IUGR pregnancies. This represents the major cohort of in utero diagnosed IUGR in which was explored the epigenetic modulation of the *IGF2/H19* imprinted domain, global methylation and UPD testing. Our data indicate that: (1) both normal and IUGR extra-embryonic tissues are characterized by global hypomethylation relative to embryo and post-natal blood;

(2) extra-embryonic hypomethylation of *H19* and DMR2 does not influence the expression pattern of *IGF2* and *H19*; (3) *IGF2* and *H19* are imprinted in normal and IUGR placenta; (4) ICR1 methylation status is a necessary and sufficient condition to drive the imprinting of *IGF2* and *H19* loci during development; (v) idiopathic and isolated IUGR is not associated with *IGF2/H19* defects, nor with UPDs.

Materials and Methods

Population. Samples included in the study were third trimester placentae (in total 60 AGA and 66 IUGR), UCT (in total 19 AGA and 7 IUGR) and UCB (in total 5 AGA and 10 IUGR) recruited by the obstetrics units of San Paolo Hospital and Policlinico Mangiagalli Hospital (Milan, Italy). Two chorionic villi and fetal fragment samples from two volunteered abortions at 11th wg with normal karyotype were also analyzed. Cases were recruited from 2001 to 2005 and informed consent was obtained from all individuals involved in the study.

All pregnancies were singleton, and gestational age was calculated from the last menstrual period and confirmed by ultrasound performed at 11–13 weeks' gestation. Fetuses with major malformations and/or chromosomal abnormalities or an evident maternal cause for IUGR (smoke, metabolic disorders, infections, thrombophilia) were excluded from the study. IUGR was defined during pregnancy as: (1) a percentile reduction of the abdominal circumference greater than 40% compared with the previous measurements by ultrasound, and (2) a birth weight below the 10th percentile (according to Italian standards for sex and gestational age). IUGR severity was classified as previously reported.⁴⁰

AGA placentae were recruited among women who had healthy term neonates with birth weight between the 10th and 90th percentiles, according to Italian standards. All women delivered by elective caesarean section in the absence of labour. Indications for caesarean section were breech or repeat caesarean section in the control group; pregnancy was terminated by caesarean section in the interest of the fetus in the IUGR group. The characteristics of the population are summarized in Table 1. As expected, AGA and IUGR differences were statistically significant in fetal and placental weight as well as in gestational age.

We also collected peripheral blood lymphocytes (PBLs) from 81 healthy controls, and one BWS and one SRS patient. The DNA methylation defects of the *H19* DMR and promoter in these two cases were previously detected by Southern blotting (data not shown), following standard procedures.⁴¹

Table 2. Primers for *IGF2* and *H19* genotyping and specific allelic expression experiments

Method	SNP	Primer sequences	Hybridization temperature	Product length
Sequencing	H19 SNP rs2839702	F: 5'-GGCCTTCTGAACACCTTA-3' R: 5'-CTGGAGGAGCTCAGCTCTG-3'	62.2°C	536 bp
Pyrosequencing	H19 SNP rs2839702	F: 5' biotin—CGTGTGCTATCTCTAGG-3' R: 5'-CTGGAGGAGCTCAGCTCTG-3' Seq: 5'-GATGAGGTCTGGTTCC-3'	56°C	147 bp
Sequencing	IGF2 SNP rs680	F: 5'-CTTGACTTTGAGTCAAATTGG-3' R: 5'-GGTCGTGCCAATTACATTTCA-3'	58°C	292 bp
Pyrosequencing	IGF2 SNP rs680	F: 5' biotin—CTTGACTTTGAGTCAAATTGG-3' R: 5'-GGTCGTGCCAATTACATTTCA-3' Seq: 5'-TGCCACCTGTGATTCTGGG-3'	58°C	292 bp

DNA methylation analysis. Genomic DNA was isolated by Trizol reagent (Invitrogen, Life Technologies, Cergy, France) following standard procedures. Sodium bisulphite conversion of DNA (700 ng) was performed using the EZ DNA Methylation-Gold Kit (Zymo Research Corporation, Orange, CA, USA); bisulphite-converted DNA was eluted with 30 μ L of Elution Buffer and concentration was determined by the NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

ICR1, DMR2, *H19* promoter and LINE1 PCR were carried out using 20 ng of bisulphite-treated DNA and 10 pmol forward and reverse primers, one of them being biotinylated. For LINE1, we utilized PCR and pyrosequencing conditions as previously described²⁹ in order to quantify four CpG sites. Pyrosequencing assays for ICR1, DMR2 and *H19* promoter were carried out using a prototype kit (RUO) by Explera (Ancona, Italy), providing PCR and sequencing primers specific for quantifying four CpG sites for ICR1, six CpG sites for DMR2 and three CpG sites for *H19* promoter. Quantitative DNA methylation analyses were performed using the Pyro Mark ID instrument (Biotage AB, Uppsala, Sweden) in the PSQ HS 96 System (Biotage), with the PyroGold SQA reagent kit (Biotage AB, Uppsala, Sweden) according to the manufacturer's instructions. Raw data were analyzed using the Q-CpG software v1.0.9 (Biotage AB, Uppsala, Sweden), which calculates the ratio of converted C's (T's) to unconverted C's at each CpG, giving the percentage of methylation.

For each sample, the methylation value represents the mean between at least two independent PCR and pyrosequencing experiments.

Genotyping and allelic expression of *IGF2* and *H19* genes.

Assays were based on genotyping of two SNPs: rs2839702 (variation: T/G) located at exon 5 of *H19*, and rs680 (variation: A/G) at exon 9 of *IGF2*.

Firstly, genomic DNA was sequenced using the ABI PRISM 3130 (Applied Biosystems, Foster City, CA, USA) to identify heterozygous samples for one and/or both SNPs. cDNAs of informative cases, synthesized using the Super ScriptTM III Platinum (Two-Step qRT-PCR Kit, Invitrogen, Life Technologies, Cergy, France), were analyzed using the Pyro Mark ID instrument (Biotage AB, Uppsala, Sweden) to assess mono- or biallelic SNP expression. Data quantification was performed using the Pyro Mark ID software v1.0.9 (Biotage AB, Uppsala, Sweden) which

gives the percentage ratio of each allele. For both *IGF2* and *H19*, standard samples with a known proportion of the two alleles were used to generate a standard curve, allowing raw data normalization. For each sample, allelic expression values represent the mean of at least two independent PCR and pyrosequencing experiments.

Primers used for sequencing and allelic expression determination are listed in Table 2.

Real-time RT-PCR. Total RNA was isolated from tissues using Trizol reagent (Invitrogen, Life Technologies, Cergy, France) following the manufacturer's instructions, treated with a "DNA-free kit" (Ambion, Austin TX, USA) to remove potential contaminating DNA. RNA concentration was determined using the NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). For *IGF2* and *H19* expression studies, 500 ng of total RNA were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and the obtained cDNA served as template for quantitative Real-Time PCR, based on TaqMan methodology, using the ABI PRISM 7500 Fast Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

The amount of *IGF2* and *H19* RNA was calculated using the 2^{- Δ Ct} method⁴² relative to hypoxanthine-guanine phosphoribosyltransferase (HPRT) and succinate dehydrogenase complex-subunit A (SDHA) housekeeping genes, selected from a pool of tested housekeeping genes because they showed a similar amplification efficiency in a scale of RNA concentration. All the assays were provided by Applied Biosystems (TaqMan Gene Expression Assays: ID# Hs01005963_m1 *IGF2*; Hs00262142_g1 *H19*; Hs99999909_m1 HPRT; Hs00417200_m1 SDHA). All samples were reverse-transcribed in duplicate and cDNA was run in triplicate to allow assessment of sample homogeneity and technical variability. Log₁₀-transformed results were used to obtain normally-distributed values.

miRNA 483-3p expression was evaluated by the 2^{- Δ Ct} method⁴³ relative to RNU48 as the endogenous normalization control. Both assays were provided by Applied Biosystems (TaqMan MicroRNA Expression Assays, ID# 002339 miRNA 483-3p, 001006 RNU48). Equal amounts (5 ng) of small RNA (<200 nucleotides) were reverse transcribed with the specific primers for both miRNA 483-3p and RNU48, using the TaqMan MicroRNA

Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The qRT-PCR was performed with the TaqMan MicroRNA Assays Human Kit (Applied Biosystems, Foster City, CA, USA), using the Step-ONE Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each sample was reverse transcribed in duplicate and cDNA was run in triplicate for both miRNA 483-3p and RNU48, to allow assessment of sample homogeneity and technical variability.

Real-time data were analyzed using the Sequence Detector software, and statistical analyses were performed by the non-parametric Kruskal-Wallis test.

UPD testing. UPD screening was carried out by genotyping placental samples and UCB from 25 IUGR pregnancies and their parents. Segregation analysis was performed with STRs mapping to each of the 22 autosomes and to chromosome X, obtaining information on primers and the physical order by the UCSC browser (www.genome.ucsc.edu). **Supplementary Table 1** shows the STRs and their cytogenetic localization. Amplification of polymorphic DNA regions was performed by fluorescent PCR using one 6-FAM labelled primer, in reactions containing standard reagents (Go Taq polymerase, Promega Italia s.r.l., Milan, Italy). The amplification conditions were 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 50 to 62°C depending on the optimal annealing temperature, and 30 s at 72°C. The PCR products were run on the Fluorescent Capillary System ABI PRISM 310 (Applied Biosystems, Foster City, USA).

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