Journal of Assisted Reproduction and Genetics (2021) 38:791–801
https://doi.org/10.1007/s10815-020-02047-3

REPRODUCTIVE PHYSIOLOGY AND DISEASE

Deregulation of imprinted genes expression and epigenetic regulators in placental tissue from intrauterine growth restriction

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Received: 5 May 2020 / Accepted: 21 December 2020 / Published online: 3 January 2021

Abstract

Purpose Intrauterine growth restriction (IUGR) is a fetal growth complication that can be caused by ineffective nutrient transfer from the mother to the fetus via the placenta. Abnormal placental development and function have been correlated with abnormal expression of imprinted genes, which are regulated by epigenetic modifications at imprinting control regions (ICRs). In this study, we analyzed the expression of imprinted genes known to be involved in fetal growth and epigenetic regulators involved in DNA methylation, as well as DNA methylation at the KvDMR1 imprinting control region and global levels of DNA hydroxymethylation, in IUGR cases.

Methods Expression levels of imprinted genes and epigenetic regulators were analyzed in term placental samples from 21 IUGR cases and 9 non-IUGR (control) samples, by RT-qPCR. Additionally, KvDMR1 methylation was analyzed by bisulfite sequencing and combined bisulfite restriction analysis (COBRA) techniques. Moreover, global DNA methylation and hydroxymethylation levels were also measured.

Results We observed increased expression of *PHLDA2*, *CDKN1C*, and *PEG10* imprinted genes and of *DNMT1*, *DNMT3A*, *DNMT3B*, and *TET3* epigenetic regulators in IUGR placentas. No differences in methylation levels at the KvDMR1 were observed between the IUGR and control groups; similarly, no differences in global DNA methylation and hydromethylation were detected.

Conclusion Our study shows that deregulation of epigenetic mechanisms, namely increased expression of imprinted genes and epigenetic regulators, might be associated with IUGR etiology. Therefore, this study adds knowledge to the molecular mechanisms underlying IUGR, which may contribute to novel prediction tools and future therapeutic options for the management of IUGR pregnancies.

Keywords Genomic imprinting · DNA methylation · DNA hydroxymethylation · Intrauterine growth restriction · Placenta

Abbreviations

5-hmC	5-Hydroxymethylcytosine
5-mC	5-Methylcytosine
DNMT	DNA methyltransferase
ES cells	Embryonic stem cells
ICR	Imprinting control region
IUGR	Intrauterine growth restriction
TET	Ten eleven translocation

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Introduction

Intrauterine growth restriction (IUGR) is a clinical complication that occurs during pregnancy and is characterized by the inability of the fetus to achieve its genetic growth potential, being the second leading cause of perinatal mortality [1]. The placenta is a fetal-maternal endocrine organ that enables the exchange of nutrients, oxygen, and water [2], secrets hormones and growth factors, and safeguards the fetus from the mother's immune system [3]. Most IUGR cases are caused by placental insufficiency, which is characterized by alterations

in nutrient and oxygen delivery to the placenta and transference of those components across the placenta, influencing the regulation of growth processes [4].

In placental mammals, genomic imprinting plays an important role in fetal development and placentation [5]. Genomic imprinting is an epigenetic mechanism in which genes are mono-allelically expressed, from only one of the parental alleles. It is thought that it first occurred 210–310 million years ago after the divergence of monotremes from marsupials and placental mammals, being evolutionarily linked to placentation [6]. The placenta exhibits enriched expression of many imprinted genes, for example *PHLDA2*, *H19*, *IFG2*, *MEST*, *ZFAT*, *PLAGL1*, *AIM1*, *MEG3*, *RTL1*, *PEG10*, and *DLK1*, and the majority of these have an important role in placental function and fetal development [1, 7]. The importance of imprinted genes during placental and embryonic development is supported by the parental-conflict theory that postulates that expression of paternally expressed genes contributes to increasing the allocation of nutrients during gestation, while maternally expressed genes limit the allocation of nutrients to the fetus [8]. Therefore, genomic imprinting influences maternal resources allocation and, in turn, fetal development [6]. Additionally, imprinted genes are associated with placenta physiology, controlling early developmental processes that establish the organ (e.g., *Peg10*), modulate labyrinth size and the surface area for exchange (*Igf2* and *Grb10*), and vascular branching density (*Aquaporin*) in mice [9]. Deregulation of epigenetic mechanisms such as genomic imprinting may cause placental dysfunction, culminating in IUGR [6]. Imprinted genes expression is regulated by epigenetic modifications at imprinting control regions (ICR), which include, but are not restricted to, DNA methylation [10]. DNA methylation consists in the incorporation of a methyl group, originating from the S-adenosylmethionine donor (SAM), in cytosines located in CpG dinucleotides, resulting in a 5-methylcytosine (5-mC). This is achieved by DNA methyltransferases (DNMTs). DNA hydroxymethylation, in which pre-existing 5-mC is oxidized into 5-hydroxymethylcytosine (5-hmC) by ten eleven translocation (TETs) family of enzymes, is an epigenetic intermediate involved in the process of active DNA demethylation [11].

The human chromosome 11p15.5 contains an imprinted gene cluster with genes involved in fetal growth. The ICR1 at the 11p15.5 cluster is generally methylated on the paternal allele and regulates the paternally expressed *IGF2* (*insulin-like growth factor 2*) and the maternally expressed *H19* imprinted gene (Fig. S1). *IGF2* is a major fetal growth hormone in mammals, promoting cellular growth and proliferation. *H19* is a noncoding transcript and appears to play an important role in early development. The dysregulation of ICR1 is associated with abnormal fetal growth [12, 13]. *Pleckstrin homology like domain, family A, member 2* (*PHLDA2*), *cyclin-dependent kinase inhibitor 1C* (*CDKN1C*), and *potassium voltage-gated channel subfamily Q member 1* (*KCNQ1*) are imprinted genes regulated by a second ICR at 11p15.5 - ICR2 or KvDMR1. On the paternal unmethylated allele, transcription of the non-coding RNA *KCNQ1OT1* is one of the mechanisms that results in silencing of the flanking imprinted genes. On the maternal chromosome, the ICR2 is methylated and the anti-sense transcript *KCNQ1OT1* is not transcribed, resulting in the expression of the flanking genes [14] (Fig. S1). *PHLDA2* is a maternally expressed gene associated with intracellular trafficking, cell signaling, and membrane-cytoskeletal interactions [15]. Additionally, in mouse, *Phlda2* was associated with cell cycle inhibition resulting in suppression of trophoblast cell proliferation [16]. *CDKN1C* is a maternally expressed gene that negatively regulates cell proliferation, inhibiting G1 cyclin/cyclin-dependent kinase complexes [17]. *KCNQ1* is a maternally expressed gene that codes a protein with function of a voltage-gated potassium channel and functions as the promoter for the KCNQ1OT1, a long non-coding RNA (lncRNA). *KCNQ1* is associated with Silver-Russell syndrome, a restriction growth syndrome, and contains the KvDMR1 within intron 10 [18].

Imprinted genes located on human chromosome 7 are also associated with fetal growth, namely two paternally expressed genes, the *mesoderm-specific transcript (MEST/PEG1)* and the *paternally expressed gene 10* (*PEG10*) [19]. Maternal uniparental disomy (mUPD) of chromosome 7 in humans is associated with intrauterine and postnatal growth restriction [20]. *MEST* encodes an α/β hydrolase fold family enzyme that is thought to be involved in angiogenesis in human trophoblast and decidua [21]. *PEG10* is a retrotransposon-derived gene associated with trophoblast proliferation [22].

Given the importance of imprinted genes in placental function, we here hypothesized that deregulation of imprinted genes, as well as epigenetic regulators and epigenetic modifications (DNA methylation and hydroxymethylation), could be associated with IUGR. Hence, we evaluated the expression of seven imprinted genes, known to be involved in fetal growth (*PHLDA2*, *CDKN1C*, *H19*, *KCNQ1*, *IGF2, PEG10*, and *MEST/PEG1*) and six epigenetic regulators (*DNMT1, DNMT3A, DNMT3B, TET1, TET2*, and *TET3*), in term placentas from IUGR pregnancies. Additionally, epigenetic modifications were also evaluated, such as DNA methylation at the KvDMR1 and, global 5-mC and 5-hmC levels in both control and IUGR placentas.

Material and methods

Placental samples collection

Placental tissue samples were collected from 9 control and 21 IUGR pregnancies, with gestational ages between 35 and 41

weeks (Table 1), in collaboration with the Gynecology and Obstetric Department of Centro Hospitalar de São João (CHSJ), Porto. The IUGR was classified by an obstetrician and the criteria included biometrical parameters of the fetus below percentile 10 for gestational age, fetal anatomy, and placenta evaluation [23, 24]. Only cases with normal karyotype were used in the present study. This study was approved by the Health Ethics Committee of Hospital São João/Faculty of Medicine of Porto, and informed consent was signed and obtained from the patients. Placental samples were collected next to the insertion of the umbilical cord and by the same obstetricians to avoid placenta variability [25, 26].

After collection, the anonymized placental samples were processed in approximately 5-mm^3 segments and stored at − 80 °C in RNA later (Ambion), until RNA and DNA extraction.

RNA and DNA extraction from IUGR and controls placentas

For RNA and DNA extraction, placental tissue was homogenized in a Triple-Pure™ zirconium beads tube (Bertin Techonologies) by a Minilys homogenizer (Bertin Techonologies) with 1 mL of TRIzol reagent (Thermo Fisher Scientific), according to manufacturers' instructions. Finally, DNA and RNA purity and quantification were determined in a NanoDrop 2000 UV-vis Spectrophotometer (Nanodrop Technologies).

Expression analysis of imprinted genes and epigenetic regulators by quantitative real-time PCR

For cDNA synthesis, 1 μg of total RNA was treated with DNaseI (Thermo Fisher Scientific) and reverse transcription was performed using 1 μg of DNase-treated total RNA and 4 μL of qScript™ cDNA SuperMix (Quanta Biosciences). The samples were incubated at 25 °C for 5 min, followed by 42 °C for 30 min and 85 °C for 5 min.

Table 1 Samples characteristics according to presence or absence of IUGR

Sample characteristics	Normal (n = 9)	IUGR (n = 21)	p
Maternal age at birth (years)	33.78 (4.24)	31.30 (5.75)	0.258[a]
Maternal smoking (%)	11%	15%	0.779[b]
Gestational age (weeks)	38.54 (1.26)	37.12 (1.10)	0.005[a]
Birth weight (g)	3091 (323)	1997 (416)	< 0.001[a]
Fetal sex	Female: 67% Male: 33%	Female: 65% Male: 35%	0.445[b]

Data are expressed as mean (SD) and percentage

IUGR intrauterine grown restriction

[a] t test, [b] X^2 test

Transcript levels of seven imprinted genes (*PHLDA2*, *CDKN1C*, *H19*, *KCNQ1*, *IGF2, PEG10* and *MEST/PEG1*), DNA methyltransferases (*DNMT1*, *DNMT3A*, and *DNMT3B*) genes, and ten eleven translocation (*TET1*, *TET2*, and *TET3*) genes were measured by quantitative real-time PCR (RT-qPCR)on a StepOnePlus™ Real-Time PCR System (Life Technologies Corporation) using 5x HOT FIREPol® EvaGreen® qPCR Supermix (Solis Biodyne) and gene-specific primers, according to manufacturers' instructions. Gene expression was normalized using two reference genes, *RPLP0* (ribosomal protein, large, P0) and *TBP* (TATA box binding protein) [27]. Primers were selected and designed to be exon-spanning and amplify only the cDNA (primer sequences are available in Table S1). RT-qPCR was performed for each gene and sample, in duplicate, and a negative control for each gene was included to detect any contamination. For each gene, we concomitantly tested normal and IUGR placental samples on the same plate to exclude the effects of intra-plate variation.

The raw data obtained after RT-qPCR was introduced in qbasePlus (BioGazelle), which calculate the relative expression levels. This software converts the quantification cycle values (Cq) to calibrated normalized relative quantities (CNRQ) [28].

Sodium bisulfite conversion and KvDMR1 amplification

Isolated DNA (1 μg) was treated with EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's instructions. This procedure allows the conversion of unmethylated cytosines to uracil with 5-mC remaining unchanged.

Modified DNA was subjected to PCR amplification of the KvDMR1 with HotStarTaq enzyme (Qiagen) and specific primers (Supplementary table 1) [29]. KvDMR1 is an ICR located at 11p15.5 (NCBI Reference Sequence: NC_000011.10; nucleotides 2,608,328-2,699,994), within a CpG island in intron 10 of *KCNQ1* that overlaps with the promoter region of *KCNQ1* overlapping transcript 1 *(KCNQ1OT1)* ncRNA, which regulates some of the imprinted genes included in this study. After amplification, the resulting PCR fragment contains 359 base pairs (bp) and 24 CpGs (Genbank Accession Number U90095; nucleotides 66492-66850). The amplification was performed in a 50 μL reaction mix containing 1X buffer with 1.5 mM MgCl2 (Qiagen), 0.12 μM of each dNTP (Invitrogen), 0.5 μM of each KvDMR1 primer (Metabion) and 1.5 U HotStarTaq enzyme (5 U/μL; Qiagen), and 2 μL of the bisulfite modified DNA. The PCR conditions were as follows: initial activation of the HotStarTaq for 15 min at 95 °C, followed by 40 cycles of 1 min denaturation at 94 °C, annealing for 1 min at 60 °C and extension for 1 min at 72 °C, and a final 20 min extension step at 72 °C.

Combined bisulfite restriction analysis of a KvDMR1 CpG and global DNA methylation

Combined bisulfite restriction analysis (COBRA) is a quantitative technique to determine DNA methylation levels at specific gene loci [30]. Briefly, COBRA takes advantage of a restriction site for one restriction enzyme—we utilized HpyCH4IV enzyme—that is modified after bisulfite treatment and allows determination of the methylation level at this specific CpG. First, bisulfite-treated DNA was used for COBRA analysis of KvDMR1 methylation and global DNA methylation by LINE1 analysis [31]. After amplification, PCR products were used for restriction digestion for 15 min at 37 °C with the restriction enzyme HpyCH4IV (New England Biolabs), following an inactivation step at 65 °C for 20 min. This enzyme cleaves only originally methylated DNA as it recognizes and cleaves 5′-ACGT-3′ (5′-A↓CGT-3′; 3′-TGC↑A-5′). This enzyme site selection allows the study of the 13th CpG present in the KvDMR1 fragment, previously amplified. COBRA analysis was run in triplicate for KvDMR1 and duplicate for LINE1, in each sample.

The stained gel was visualized and scanned in a ChemiDoc™ XRS+ System (Bio-Rad Laboratories). Acquired images were processed using Image Lab software (Bio-Rad).

Methylation quantity was calculated using the uncleaved band (~ 359 bp) representing the unmethylated portion and the average intensity of the digested bands (~ 202 bp and ~ 157 bp) which represent the methylated portion. Firstly, the intensity of each band is calculated comparing to the uncut control (which is set to 1). Then, the relative quantity of each band is normalized, in which the relative value of each band is calculated considering the value previously calculated as 1 (band relative quantity normalized = band relative quantity/sum of the uncut band with the mean value of the two cut bands). Finally, the value obtained for the uncleaved fragments represents the value for unmethylated CpG and the mean value of the two cleaved fragments is the value for methylated CpG.

Bisulfite sequencing of KvDMR1

Bisulfite sequencing allows the analysis of methylation at each individual CpG site of the 24 CpGs included in the PCR fragment. The amplified KvDMR1 fragment was purified using Agencourt® AMPure XP (Beckman Coulter Krefeld), according to the manufacturer's instructions. For this analysis, 18 samples were selected, 9 control and 9 IUGR samples. The selection of IUGR samples considered the highest values of *CDKN1C* and *PHLDA2* expression and fetal sex. The samples were balanced for males and females to avoid differences in methylation due to fetal sex—3 males and 6 females in each group [32, 33].

Adenine residues were added to the PCR fragments after purification for a more efficient ligation to the TOPO TA vector (Invitrogen, Thermo Scientific). The next step was the cloning of purified PCR products with the TOPO TA cloning kit (Invitrogen), using pCR™II-TOPO® (Invitrogen) and 1 μL of the KvDMR1 fragment. After ligation, transformation and cloning were performed using NZY5α chemically competent Escherichia coli cells (NZYTech). Colony PCR was performed with 5 U/μL Taq DNA polymerase (Thermo Scientific). The conditions were as follows: 94° for 10 min, followed by 35 cycles at 94 °C for 45 s, 50 °C for 45 s and 72 °C for 1 min, finally 10 min at 72 °C. After purification with Agencourt® AMPure® XP (Beckman Coulter Krefeld), around 15 independent clones containing the fragment of interest of each sample were sequenced using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc) and were analyzed in an ABI PRISM® 3500 Genetic Analyzer.

The bisulfite sequencing data were analyzed in BiQAnalyzer software, which aligns sequencing results with a reference sequence and determines the methylation status and levels of each CpG [34]. Only clones with more than 90% of non-CpG cytosines converted were included.

Global DNA hydroxymethylation levels

The 5-hmC content of the DNA samples (100 ng) was measured, in duplicate for each sample, using QUEST 5-hmC^{TM} DNA ELISA kit (Zymo research), following manufacturer's instructions. This procedure allowed the detection and quantification of 5-hmC using anti-5hmC polyclonal antibody and anti-DNA HRP antibody. The color development was measured after 30–60 min (Abs 405), on a 10-min interval, on an ELISA plate reader (Sunrise). This procedure allowed the detection and quantification of 5-hmC (in percentage), comparing the absorbance of each sample to controls and using a standard curve. The standard curve was obtained using 5 controls, in duplicate (control A 0%, control B 0.03%, control C 0.12%, control D 0.23%, control E 0.55%).

Statistical analysis

The Mann-Whitney *U* test was used to compare the expression of genes between the two groups (IUGR vs control) and the gene expression analysis method was $2^{-\Delta\Delta Cq}$ by Livak and Schmittgen[35]. For gene expression analysis, the definitive outliers for each gene and group were identified in the Statistical Package for Social Sciences (SPSS, IBM) software v24.0 and excluded from the calculations (for *DNMT3A* analysis, two controls were excluded and for *TET1* and *TET3* one IUGR sample).

For KvDMR1 analysis, the normalized CpG methylation values, obtained by COBRA, from each group were compared

using the Mann-Whitney *U* test in SPSS. The methylated CpG values after bisulfite sequencing were calculated by dividing the number of observed methylated CpGs by the total number of CpGs analyzed. The mean values and the individual CpG values of each group were compared and analyzed using the *t* test in SPSS.

Additionally, the linear regressions based in ANOVA between gene expression and normalized KvDMR1 CpG methylation (obtained with COBRA), fetal percentile and normalized KvDMR1 CpG methylation (obtained with COBRA), genes expression and fetal percentile, and lastly, fetal percentile and normalized KvDMR1 CpG methylation (obtained with COBRA) were performed using SPSS. The same was also evaluated with the relative KvDMR1 values obtained with BS-sequencing.

For the global hydroxymethylation study data, the percentage of 5-hmC for each sample was calculated according to manufacturers' instructions, using the slope and y-intercept values obtained from the standard curve of the known controls. The comparison between groups was evaluated using *t* test in SPSS.

The results of all tests were considered significant when the *p* value was below 0.05.

Results

Increased expression of imprinted genes and epigenetic regulators in IUGR term placentas

We observed an increased expression in IUGR placentas of three imprinted genes, two maternally expressed -*PHLDA2, CDKN1C*-, and one paternally expressed- *PEG10* (Fig. 1). Additionally, four epigenetic regulators were also more highly transcribed, three involved in DNA methylation- *DNMT1, DNMT3A, DNMT3B* - and one in the removal of methylation marks -*TET3* (Fig. 1). Analysis of correlation, by linear regression, between the fetal percentile and imprinted genes expression, in IUGR, showed no significant correlation (Fig. 2d–f).

Methylation at KvDMR1 is unaltered in IUGR placentas

Since altered expression of imprinted genes controlled by KvDMR1 was observed, namely for *PHLDA2* and *CDKN1C* (Fig. 1a and S1), we analyzed methylation at this ICR using combined bisulfite restriction analysis (COBRA) and bisulfite sequencing. Our results, using both methods, showed no differences between control and IUGR groups, in KvDMR1 methylation levels (Figs. 3, 4, and 5). Also, analysis of each CpG individually again did not show differences between the groups (Fig. S2), and the same is true for the CpG13 which was the CpG analyzed by COBRA.

Comparing gene expression and KvDMR1 methylation levels (by COBRA analysis) in IUGR samples, we observed a moderate linear association between *CDKN1C* expression and KvDMR1 CpG methylation (Fig. 2a; $p = 0.017$; ANOVA) but not with *PHLDA2* expression (Fig. 2b) or with fetal percentile (Fig. 2c).

Global 5-mC and 5-hmC levels are similar in IUGR and control placentas

Due to the increased expression of *DNMT*s in IUGR placentas, we analyzed global DNA methylation levels by LINE1 retrotransposon methylation [31] in IUGR placentas and control placentas ($p = 0.082$; *t* test) (Fig. 6a). Since we also observed an increased expression of *TET3* in IUGR cases, we analyzed global DNA hydroxymethylation levels using an

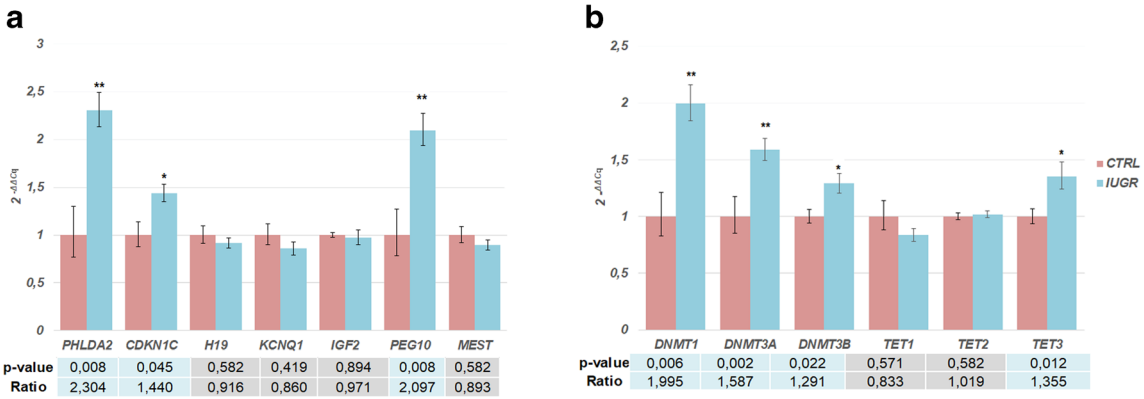

Fig. 1 Relative gene expression in placental samples with IUGR ($n = 21$) and control placental samples ($n = 9$). **a** Relative expression of imprinting genes (*PHLDA2, CDKN1C, KCNQ1, IGF2, H19, MEST*, and *PEG10*). The expression of *PHLDA2*, *CDKN1C*, and *PEG10* is increased in IUGR. **b** Relative expression of epigenetic regulators (*DNMT1, DNMT3A, DNMT3B, TET1, TET2*, and *TET3*). The expression of *DNMT1, DNMT3A, DNMT3B*, and *TET3* is increased in IUGR. The bars represent the mean expression for each gene with respectively error bar ($2^{-\Delta\Delta Cq} \pm$ SEM) * represent the *p* value < 0.05; ** represent the *p* value < 0.01. Mann-Whitney *U* test. CTRL, control samples; IUGR, intrauterine growth restriction samples

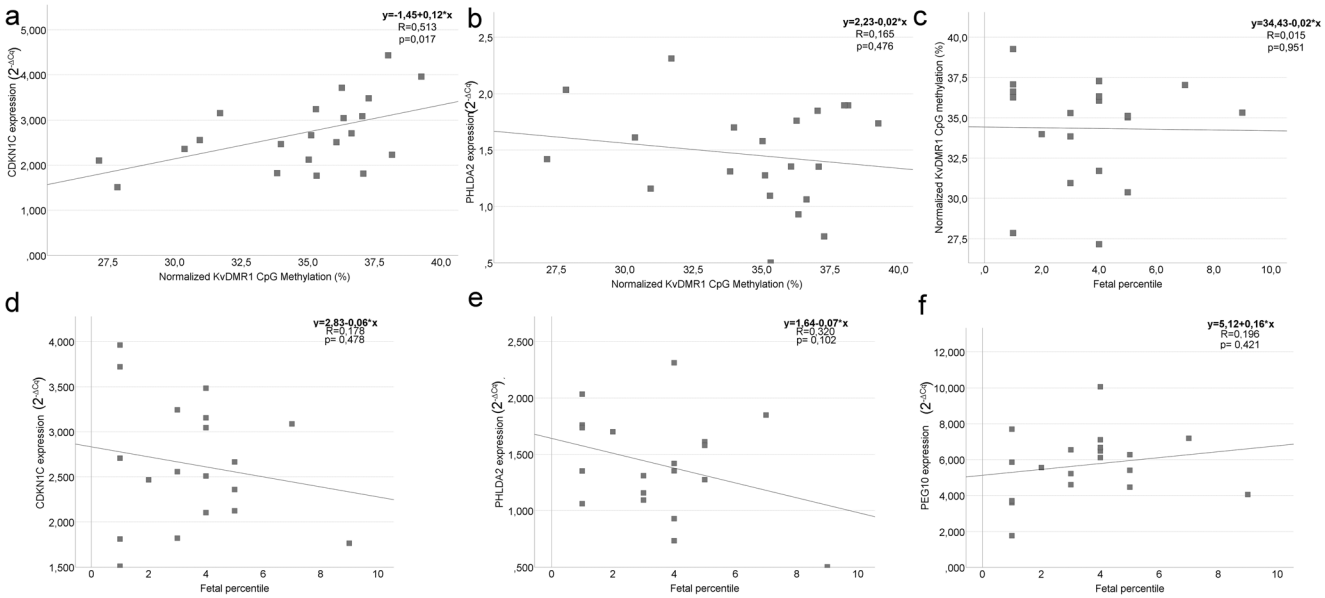

Fig. 2 Linear regression between gene expression and relative KvDMR1 CpG methylation, fetal percentile and relative KvDMR1 CpG methylation, genes expression and fetal percentile or fetal percentile, and relative KvDMR1 CpG methylation. **a** *CDKN1C* expression ($2^{-\Delta Cq}$) values and KvDMR1 CpG methylation of placental samples with IUGR, observing a moderate linear correlation. **b** *PHLDA2* expression ($2^{-\Delta Cq}$) values and KvDMR1 CpG methylation of placental samples with IUGR. **c** Fetal percentile and KvDMR1 CpG methylation of placental samples with IUGR. **d** *CDKN1C* expression ($2^{-\Delta Cq}$) values and fetal percentile of placental samples with IUGR. **e** *PHLDA2* expression ($2^{-\Delta Cq}$) values and fetal percentile of placental samples with IUGR. f-*PEG10* expression ($2^{-\Delta Cq}$) values and fetal percentile of placental samples with IUGR. The dots represent each IUGR sample of this study

ELISA-based technique. The IUGR samples exhibited a mean global DNA hydroxymethylation level of 0.058% and the control samples of 0.069% (p = 0,451; t test) (Fig. 6b).

Discussion

Placental imprinted genes may be involved in IUGR etiology, as it is well documented that they play an important role in fetal and placental development [36]. Piedrahita proposed that, in IUGR, imprinted genes may be grouped into two categories: genes that participate in enhancing IUGR or reducing fetal growth (negative effectors) and genes that, through a compensatory mechanism, sense that the fetus is at risk and act to increase fetal growth (positive effectors) [37]. We here observed increased expression of two genes that restrict placental and fetal growth (*PHLDA2* and *CDKN1C*) and one gene (*PEG10*) that enhances fetal growth, therefore

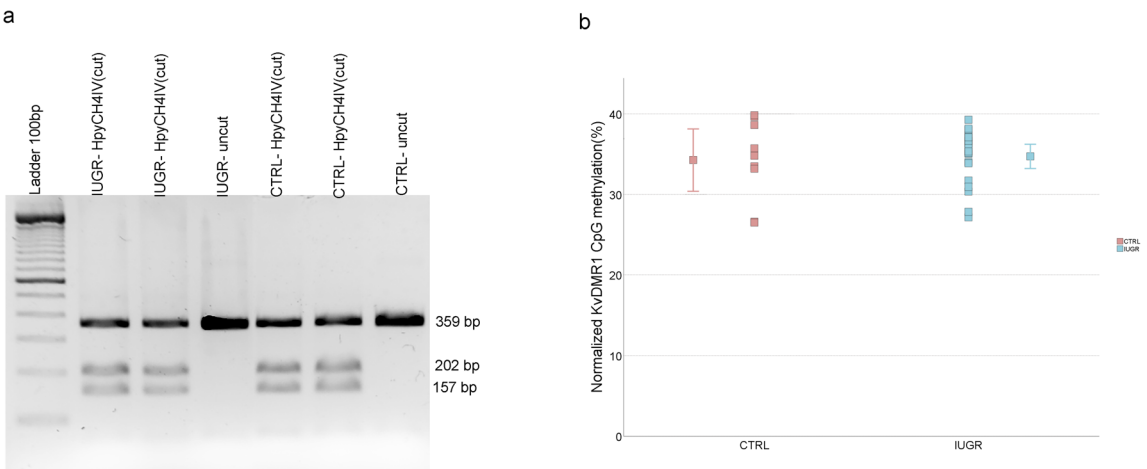

Fig. 3 KvDMR1 methylation analysis in IUGR placental samples comparing with control samples, using COBRA. **a** Example of COBRA analysis of KvDMR1 in IUGR placenta and in normal placenta (CTRL). **b** Comparison of normalized KvDMR1 methylation, using COBRA. No significant difference was observed between the IUGR cases and non-IUGR (CTRL) cases using Mann-Whitney U test. The dots represent the methylation for each sample and the mean methylation for each group. CTRL, control samples; IUGR, intrauterine growth restriction samples

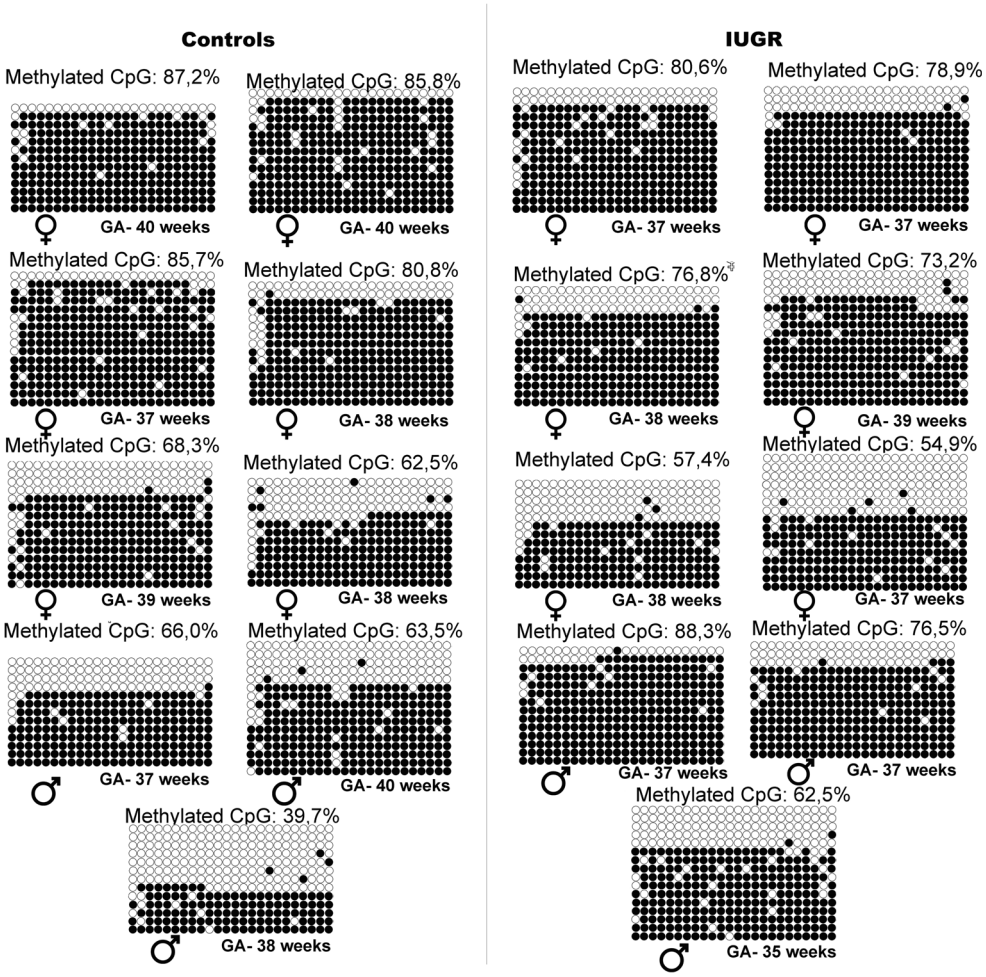

Fig. 4 Methylation profiles of 24 CpGs KvDMR1 of IUGR cases and non-IUGR (CTRL) cases. The circles represent the CpGs: methylated (black) and non-methylated (non-colored). Each sample has the KvDMR1 methylation percentage, the corresponding fetal sex and each line that represents one clone. CTRL, control samples; IUGR, intrauterine growth restriction samples

potentially acting in a compensatory manner. Overexpression of *PHLDA2* and *CDKN1C* in the human placenta and the association with IUGR was previously described, both in single pregnancies [38–44] and in the placental shares of the smaller fetus in cases of selective IUGR [45, 46]. *PHLDA2* is highly expressed in the placenta and encodes a protein with a Pleckstrin-homology domain, where phosphatidylinositol lipids can bind, and it is associated with the inhibition of cell

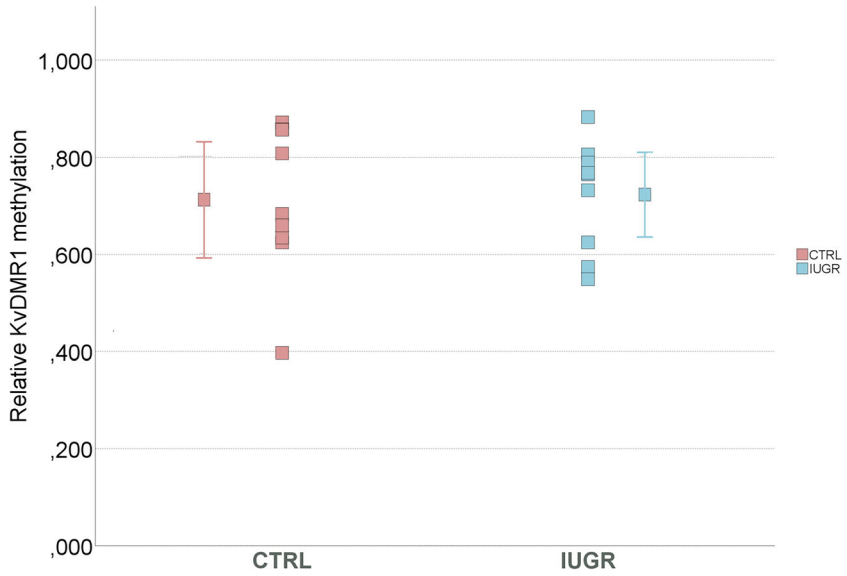

Fig. 5 KvDMR1 methylation analysis in 9 IUGR placental samples comparing with 9 control samples, using BS-sequencing. The mean methylation for each group with respectively error bar. No significant difference was observed between the IUGR cases and non-IUGR (CTRL) cases, using *t* test. CTRL, control samples; IUGR, intrauterine growth restriction samples

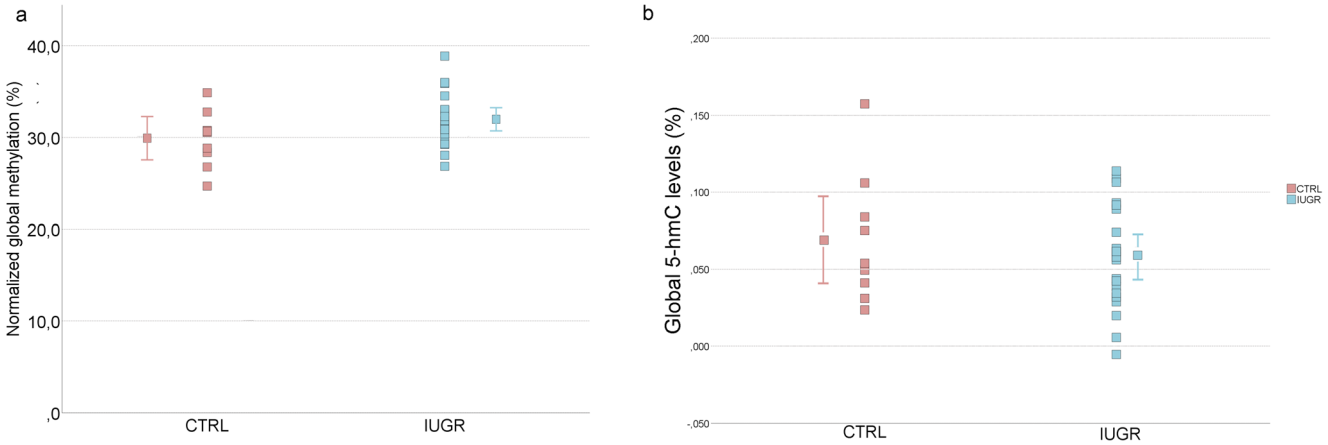

Fig. 6 **a** Global DNA methylation by LINE1 analysis in IUGR and control samples. The mean global methylation for each group with respectively error bar. No significant difference was observed between the IUGR cases and non-IUGR (CTRL) cases, using *t* test. **b** Comparison of the mean percentage of global 5-hmC between the two groups (IUGR vs. controls). The mean global hydroxymethylation for each group with respectively error bar. No significant difference was observed between the IUGR cases and non-IUGR (CTRL) cases, using *t* test. CTRL, control samples; IUGR, intrauterine growth restriction samples

proliferation, migration, and invasion, leading to growth restriction [47]. *CDKN1C* is a gene that encodes the p57Kip2 protein- CIP/Kip family- which bind and inhibit cyclin/cyclin-dependent kinase complexes, inhibiting the cell cycle [17]. Taking this into account, it is suggested that these two genes may have an important role in IUGR.

The conflict theory for explaining the occurrence of genomic imprinting advocates that paternally expressed genes promote fetal growth [8]. However, we observed overexpression of *PEG10*, a paternally expressed gene, in placentas from pregnancies with IUGR. Other studies also observed a *PEG10* overexpression in IUGR, suggesting that this gene is acting in a compensatory manner to increase the growth of IUGR fetus [37, 40]. *PEG10* and *MEST* paternally expressed genes are located on human chromosome 7. *PEG10* and the contiguous gene *SGCE* are regulated by an ICR located on 7q21.3 that is methylated in the maternal allele. The *MEST* ICR is also maternally methylated and is located on human chromosome 7q32 [48].

Several studies evaluated methylation at the KvDMR1 or ICR2 that regulate *PHLDA2* and *CDKN1C* imprinted genes in placental samples from IUGR [12, 13, 38, 39, 49, 50]. However, as in our study, others studies did not find changes in methylation at the KvDRM1, suggesting that other epigenetic modifications or methylation at other regions might be contributing to the observed changes in imprinted gene expression [12, 13, 38, 39, 49, 50]. Indeed, Ishida and collaborators observed that the promoter dysregulation altered the expression of *PHLDA2* gene, altering human fetal growth [51]. In other types of models like human osteosarcomas, the *PHLDA2* expression is regulated by promoter methylation [52] and in goat, Wang and collaborators observed that methylation of *PHLDA2* promoter leads to *PHLDA2* inhibition in the placenta [53]. Nevertheless, the moderate linear correlation that we observed between *CDKN1C* imprinted gene and KvDMR1 CpG methylation supports the association between gene expression and KvDMR1 methylation. In humans, it has also been shown that ICR2 deletion in the paternal allele leads to the silencing of KCNQ1OT1 and activation of *CDKN1C* and *PHLDA2*, causing IUGR [54].

On the other hand, DNA hydroxymethylation in human placentas from IUGR cases is less explored [55–57]. In our study, we observed increased expression of *TET3*, one of the dioxygenases involved in oxidation of 5-mC into 5-hmC, in IUGR cases; however, no significant differences in global DNA hydroxymethylation levels were observed. In the placenta, it was shown that the enzymes TET have a role in the specialized trophoblast cells differentiation and regulation [58]. Although increased expression of *TET3* and *DNMT*s was observed in IUGR samples, no significant changes in global levels of methylation and hydroxymethylation occurred, suggesting that regional or gene-specific changes might be the target of this increased expression. It is also plausible that increased transcript levels might be not be reflected into higher protein levels or enzyme activity.

Interestingly, it was shown that TET1 is the principal enzyme responsible for 5-mC oxidation into 5-hmC, while TET2 and TET3 enzymes promote the removal of 5-hmC in the oxidative cascade [59]. In a previous study by our group, we also observed a higher expression of *TET2* and *TET3* with no significant changes in DNA hydroxymethylation global levels in placental samples from idiopathic spontaneous abortion [49]. The higher expression of *TET3* may lead to a faster 5-hmC turnover, by conversion into 5-formylcytosine (5-fC) and 5-carboxycytosine (5-caC); however, we need to take in account that placenta has different cell types and that those cells may have different expression levels that we do not detect, supporting the need to do single-cell analysis. In fact, a

study has shown that cell columns, extravillous trophoblast, and syncytiotrophoblast expressed TET1-3 whereas only TET3 was expressed in villus cytotrophoblast cells in first trimester and term human placentas [60].

Nevertheless, analysis of 5-hmC levels specifically at the affected imprinted genes would be of importance to assess whether 5-hmC levels at imprinted genes are altered in IUGR placentas. Indeed, it has been shown that 5-hmC is present in many imprinted genes, both in the human brain and placenta, and it is enriched in the transcribed allele, suggesting 5-hmC is positively associated with transcription at imprinted loci [61]. Furthermore, it has been shown that 5-hmC levels are lower in placenta than brain [62, 63], which positively correlates with transcription in actively transcribed genes [64]. Moreover, the levels of 5-mC at KvDMR and *IGF2* DMR0 and 5-hmC at *H19* gene body were shown to positively correlate with size at birth [56]. We here confirmed the presence of 5-hmC in placental samples with similar levels to the ones reported by other studies (0.06%) [62, 63].

As IUGR is a multifactorial pathology [4], the dysregulation of growth-related genes, such as *PHLDA2* and *CDKN1C*, may be only one of the factors involved in this pathology. Several other genes involved in different pathways—inflammatory, cardiovascular and metabolic genes—and their altered methylation status in the placenta may be associated with uterine growth [65, 66]. In this study, we showed that *PHLDA2* and *CDKN1C* genes could serve as potential biomarkers for human IUGR. In future, studies will be interesting to investigate which cell types mostly contribute to the observed changes in genes expression and analyze methylation and hydroxymethylation in a genome-wide manner to explore gene-specific epigenetic changes.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10815-020-02047-3.

Acknowledgments We thank the obstetricians at the Department of Gynecology/Obstetrics of Hospital São João, Porto, Portugal, for collaboration with sample and patients' data collection. We are also thankful to members of the Genetics Department of FMUP for technical help and insightful discussions.

Author contributions C.C. performed the experiments, analyzed the results, and wrote the first draft of the manuscript; S.V. performed experimental work and revised the manuscript; C.R. (MD, Obstetrician) collected placental samples and patients' data; C.J.M supervised experimental work, analyzed the data, and wrote the final manuscript; S.D. designed the study, analyzed the data, and wrote the final manuscript. All authors revised and approved the final version of the manuscript.

Funding This work was partly funded by the Portuguese Foundation for Science and Technology (FCT) – UID/BIM/04293/2013 and FCT Investigator award to CJM (IF/00047/2012; CEECIND/00371/2017). C.C. is a recipient of a PhD studentship by FCT (SFRH/BD/141855/2018). S.V. is a recipient of a PhD studentship by FCT (SFRH/BD/147440/2019).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Patient consent All patients provided a signed informed consent for this study.

Ethics approval This study was approved by the Health Ethics Committee of Hospital São João/Faculty of Medicine of Porto (N 70/17).

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