Pre-implantation and placental development in humans and mice

He, N.

CHAPTER 5

Spatial pattern of methylation in *IGF2/H19* ICR1 in human first-trimester placenta

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Abstract

Allele-specific DNA methylation is an important tool in studies of genomic imprinting. The *IGF2/H19* imprinting control region (ICR1) is essential for normal fetal and placental growth and development. Its pattern of methylation during human early pregnancy is poorly understood. We aimed to search for possible non-CpG single nucleotide polymorphisms (SNPs) on ICR1 and to investigate the pattern of methylation of in different placental villi of first trimester human placenta and embryonic tissue using bisulfite DNA sequencing. We were able to select two informative non-CpG SNPs, rs2071094:G>T and rs2107425:C>T in two first-trimester pregnancies, which allowed us to discriminate the paternal and maternal alleles. Our results indicate a normal imprint pattern in methylation of the *IGF2/H19* ICR1in first trimester embryos and placental villi.

Introduction

The imprinted *IGF2/H19* cluster domain, controlled by the *IGF2/H19* imprinting control region (ICR1), plays an essential role in growth and development of placental mammals [1, 2]. In humans, the *IGF2/H19* ICR1, located on the 11p15.5 chromosomal region, controls the paternally expressed *IGF2* and maternally expressed long non-coding RNA *H19*. One hypothesis for the regulation of this imprinting cluster is the insulator model for imprinting expression (reviewed in [3]), whereby the *IGF2/H19* ICR1 unmethylated maternal allele allows the CCCTC-binding factor (CTCF) to bind to its (seven) insulator consensus sequences, whereas CTCF cannot bind to CTCF-binding sites in the methylated paternal allele.

In humans, *H19* has been reported to be biallelically expressed in cytotrophoblasts until 10 weeks of gestation (W10), but to become monoallelically expressed in placenta at term, even though this could be due to the increased amount of mesodermal tissue in the placental villi [4-7]. In agreement, term placentas seem to show a normal imprinted or slightly hypomethylated *IGF2/H19* ICR1 [5, 8-11]. However, of those studies, only Turan and colleagues used informative single nucleotide polymorphisms (SNPs) (in that case rs10732516 present in the 6th CTCF binding site) to discriminate the parental origin of DNA methylation and reported not only variation between individual placentas but also pronounced spatial variation in the same term placenta [11].

Buckberry and colleagues compared the methylation status of the *IGF2/H19* ICR1 between term placentas and first-trimester placentas (W6-W12) and found no differences in average methylation levels in the 6th CTCF binding site (53.5%±7.2% versus 54.8%±6.9%, respectively) [5]. However, those authors used only one site of collection in the placentas and might have missed spatial variation in methylation. Moreover, although they discriminated allelic-specific expression, no discrimination was made between the parental alleles regarding the DNA methylation of the *IGF2/H19* ICR1 and only global DNA methylation levels were reported. Villous cytotrophoblast proliferate and generate trophoblastic columns, from the tips of which extravillous trophoblast cells (EVTs) detach and invade the maternal decidua [12]. Here, we have investigated for the first time the spatial pattern of methylation of the *IGF2/H19* ICR1 in first-trimester placental villi, using informative SNPs to discriminate between the parental alleles. We report a normal imprinted pattern for *IGF2/H19* ICR1 in all investigated placental villi samples, as well as in the respective embryos.

Materials and Methods

Tissue samples and sex genotyping

The collection and use of the material for this study was approved by the Human Ethics Committee of the Leiden University Medical Center, Netherlands (P08.087). Informed consent was obtained on the basis of Helsinki Declaration by the World Medical Association (WMA). Tissues were collected from elective abortion of uncomplicated pregnancies, without medical indication. The embryonic tissue was sex genotyped as previously described, using polymerase

chain reaction (PCR) for AMELX/AMELY [13]. Endometrium and embryonic tissue from first trimester was used for genotyping analysis to identify informative SNPs in *IGF2/H19* ICR1 region. In addition, four intact placental villi from different parts of the same placenta (V1 to V4) were cut at the basal connection with the placental plate and individually stored at -80°C for DNA methylation analysis.

Detection of genomic SNPs in the *IGF2/H19* ICR1 region

To identify genomic SNPs in the *IGF2/H19* ICR1 region flanking the 6th binding site for CTCF (Table 1), genomic DNA of decidua and embryo was purified [14] and used for PCR (FW 5'-GTA TTT CTG GAG GCT TCT CCT TC-3' and RV 5'-GAT CAT CAC ATA AGT AGG CGT GAC -3') with conditions 95°C 5 minutes, 35 cycles (95°C 30 seconds, 56°C 30 seconds, 72°C 30 seconds), and 72°C 5 minutes. The DNA fragments were sequenced by LGTC (www.lgtc.nl) and their sequence was aligned to identify SNPs (Table 1).

Bisulfite DNA sequencing

Genomic DNA from four different placental villi was modified and purified using the EpiTec Bisulfite Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. On bisulfite-treated DNA, PCR was performed using previously described bisulfite primers for the *IGF2/H19* ICR1 region flanking the 6th binding site for CTCF [15] (FW 5'-TATGGGTATTTTTGGAGGTTTTTT-3' and RV 5'-AATCCCAAACCATAACACTAAAAC-3'), using AmplitaqTaq Gold DNA Polymerase (Applied Biosystems, CA, USA). PCR conditions were 95°C 5 minutes, 50 cycles (94°C 45 seconds, 59°C 45 seconds, 72°C 45 seconds) and 72°C 10 minutes. The resulting amplicons were cloned using pENTR 5'-TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The bacterial transformation was carried out in *E. coli* TOP10 (Invitrogen, Carlsbad, CA). The obtained colonies were screened by PCR and positive clones were sequenced as above.

Results

We have investigated first the presence of informative genomic SNPs in the *IGF2/H19* ICR1 in six aborted pregnancies from first trimester (Table 1). Three SNPs (rs10732516:G>A, rs2071094:G>T, rs2107425:C>T) were heterozygous in Pregnancy 1 embryonic DNA and homozygous in corresponding maternal DNA (Table 1). Pregnancy 5 showed one SNP (rs2107425:C>T) to be heterozygous in embryonic DNA and homozygous in maternal DNA. However, the other four pregnancies (Pregnancies 2, 3, 4 and 6) showed same homozygous or heterozygous SNPs in both embryonic and maternal DNA. Therefore, only Pregnancies 1 and 5 were included in further analysis.

The *IGF2/H19* ICR1 region analyzed contains 23 CpG sites (Fig 1). Interestingly, one of three identified informative genomic SNPs, rs10732516:G>A, is located on a CpG (CpG7 in Fig 1). For CpG7 (rs10732516) in Pregnancy 1, maternal alleles were AA (or TT in the reverse

strand) in DNA while embryonic alleles were AG (or TC). After bisulfite treatment, maternal T and methylated paternal C stay as T and C respectively, whereas unmethylated paternal C will be converted into T. Consequently, in bisulfite sequence of embryo, we could not tell whether the "T" in CpG7 is from maternal allele "T" or is from conversion of the unmethylated paternal allele "C". Therefore, CpG7 was not taken into account in the analysis of methylation of *IGF2/H19* ICR1. We investigated the spatial pattern of methylation of the *IGF2/H19* ICR1 in 4 different placental villi (and embryo) from Pregnancy 1. The density of methylation in the placental villi (excluding CpG7) was 124/147 (84.4%) in V1, 107/142 (75.4%) in V2, 128/149 (85.9%) in V3, 108/112 (96.4%) in V4 and 68/86 (79.1%) in the embryo (Fig 1A). From the presence of two informative SNPs (rs2071094:G>T, rs2107425:C>T), the general hypermethylated pattern of DNA resulted from a technical bias and that in each of the placental villi (and embryo) analyzed

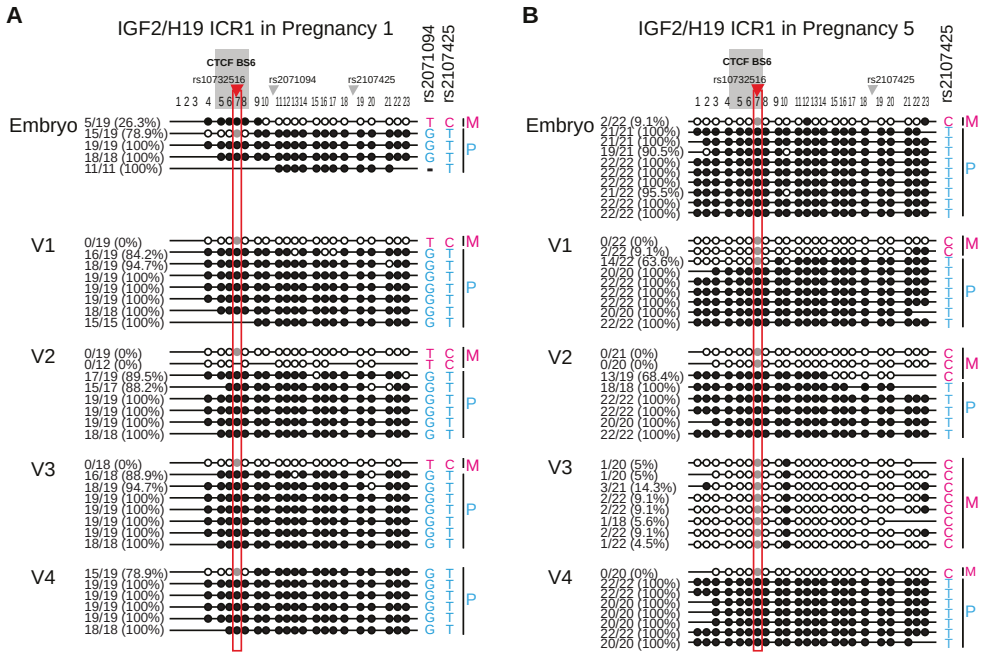

Fig 1. *IGF2/H19* ICR1 methylation profile in two pregnancies.

(**A**,**B**) The individual methylation sequence of the IGF2/H19 ICR1 as determined by bisulfite sequencing of the individual placenta villi V1, V2, V3 and V4 and the embryo tissue in Pregnancy 1 (**A**) and Pregnancy 5 (**B**). A black circle indicates a methylated CpG site and an open circle represents an unmethylated CpG site. A grey circle indicates the allele "T" in CpG7 of bisulfite sequence. The percentage of methylated CpG is given as well as the number of methylated CpG sites and total number of sequenced CpG sites. The nucleotides of the informative SNP sites rs2071094 and rs2107425 in *IGF2/H19* ICR1 are depicted in pink (maternal) and blue (paternal) for the different alleles. In the analyzed DNA stretch, the position of the 6th CTCF binding site includes CpG 5-8, the informative SNP rs2071094 is present between CpG 10 and CpG 11 and the informative SNP rs2107425 is present between CpG 18 and CpG 19.

the maternal allele (rs2071094:T, rs2107425:C) was demethylated, whereas the paternal allele (rs2071094:G, rs2107425:T) was methylated.

Next, we investigated the spatial pattern of methylation of the *IGF2/H19* ICR1 in 4 different placental villi (and embryo) in Pregnancy 5. The density of methylation in the placental villi (excluding CpG7) was 144/194 (74.2%) in V1, 116/164 (70.7%) in V2, 12/167 (7.8%) in V3, 146/166 (88.0%) in V4 and 194/217 (89.4%) in the embryo (Fig 1B). In each of the placental villi (and embryo) analyzed, the maternal allele (rs2107425:C) was demethylated, whereas the paternal allele (rs2107425:T) was methylated.

Discussion

In humans, *H19*, but not *IGF2*, has been reported to be biallelically expressed at least in cytotrophoblasts before W10, but to become monoallelically expressed in placentas at term [4-6, 16]. In agreement, several studies have reported that the *IGF2/H19* ICR1 is normally imprinted, or slightly hypomethylated, in term placentas [5, 8-11, 17, 18]. However, of those studies only Turan and colleagues used a methylation sensitive restriction enzyme (*Mlu*I) in combination with the restriction enzyme *Cfo*I (GCGC) to identify an informative SNP rs10732516 present in the 6th CTCF binding site, being able to identify allele-specific DNA methylation and reported a spatial variation in some term placentas [11]. In a recent study, Buckberry and colleagues compared the methylation status of the *IGF2/H19* ICR1 between term placentas and first trimester placentas (W6-W12) and found no differences in mean methylation levels in the 6th CTCF binding site [5]. However, those authors used only one site of collection and although they discriminated between allele-specific expression, they were unable to discriminate between the parental alleles regarding the methylation of the *IGF2/H19* ICR1, reporting only global DNA methylation levels.

Here, we have investigated for the first time the spatial pattern of methylation of the *IGF2/H19* ICR1 in first-trimester placental villi of two pregnancies. In the placenta analyzed here, two non-CpG SNPs were informative for identification of allele-specific DNA methylation. The SNP rs2071094:G>T was previously described [2, 19] and a novel one, the SNP rs2107425:C>T which identified both paternal and maternal alleles, one methylated and the other unmethylated and therefore a normal imprint pattern in the *IGF2/H19* ICR1 in the different placental villi and embryo, in contrast to some variability encountered between villi at term [11].

It is important to report a technical bias in bisulfite sequencing data towards hypermethylation that could have resulted from one allele being preferentially amplified or cloned over the other. Therefore, we suggest the combined use of the SNPs rs2071094:G>T and rs2107425:C>T as an important tool in allele-specific DNA methylation studies because it allows discrimination of both alleles and identifying a possible bias of bisulfite sequencing data for imprinting analysis.

The *IGF2/H19* ICR1 is an important region that controls key genes for placental and embryo growth and development. The genomic imprinting in first trimester of pregnancies is

poorly understood and our results represent ongoing steps in understanding the role of epigenetic regulation during human gestation and the identification of a novel informative non-CpG SNP at *IGF2/H19* ICR1, an essential feature for determining allele-specific DNA methylation.

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Table 1. SNPs present in *IGF2/H19* ICR1 and pregnancy genotypes.

SNPs	Pregnancy 1		Pregnancy 2		Pregnancy 3		Pregnancy 4		Pregnancy 5		Pregnancy 6	
	Embryo	Mother	Embryo	Mother	Embryo	Mother	Embryo	Mother	Embryo	Mother	Embryo	Mother
rs10732516: G>A*	**GA**	**AA**	GG	GG	GG	GG	**GA**	**GA**	**GA**	**GA**	**GA**	nd
rs112620988: T>A	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	nd
rs34610866: G>-	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG
rs2071094: G>T	**GT**	**TT**	GG	GG	GG	GG	**GT**	**GT**	GG	GG	**GT**	**GT**
rs35678657: G>T	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG
rs373018220: T>A	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT	TT
rs113480908: G>A*	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG	GG
rs56125822: C>T	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC
rs2107425: C>T	**CT**	**CC**	TT	TT	**CT**	**CT**	CC	CC	**CT**	**CC**	CC	CC
rs76162918: C>A*	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC

*Informative SNPs present on a CpG; Bold, heterozygous SNP in embryo; Red bold, heterozygous SNP in embryo and homozygous in mother; nd, not determined.