Brazilian Journal of Medical and Biological Research (2013) 46: 916-919, http://dx.doi.org/10.1590/1414-431X20133035
ISSN 1414-431X Short Communication

Placental hydroxymethylation *vs* methylation at the imprinting control region 2 on chromosome 11p15.5

H.R. Magalhães[1], S.B.P. Leite[1], C.C.P. de Paz[1], G. Duarte[2] and E.S. Ramos[1,2]

[1]Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil
[2]Departamento de Ginecologia e Obstetrícia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil

Abstract

In addition to methylated cytosines (5-mCs), hydroxymethylcytosines (5-hmCs) are present in CpG dinucleotide-enriched regions and some transcription regulator binding sites. Unlike methylation, hydroxymethylation does not result in silencing of gene expression, and the most commonly used methods to study methylation, such as techniques based on restriction enzymatic digestion and/or bisulfite modification, are unable to distinguish between them. Genomic imprinting is a process of gene regulation where only one member of an allelic pair is expressed depending on the parental origin. Chromosome 11p15.5 has an imprinting control region (ICR2) that includes a differentially methylated region (KvDMR1) that guarantees parent-specific gene expression. The objective of the present study was to determine the presence of 5-hmC at the KvDMR1 in human placentas. We analyzed 16 third-trimester normal human placentas (chorionic villi). We compared two different methods based on real-time PCR after enzymatic digestion. The first method distinguished methylation from hydroxymethylation, while the other method did not. Unlike other methylation studies, subtle variations of methylation in ICRs could represent a drastic deregulation of the expression of imprinted genes, leading to important phenotypic consequences, and the presence of hydroxymethylation could interfere with the results of many studies. We observed agreement between the results of both methods, indicating the absence of hydroxymethylation at the KvDMR1 in third-trimester placentas. To the best of our knowledge, this is the first study describing the investigation of hydroxymethylation in human placenta using a genomic imprinting model.

Key words: Methylation; Hydroxymethylation; Placenta; Genomic Imprinting; ICR2; KvDMR1

Introduction

Genomic imprinting is an epigenetic process involved in the control of gene expression in a parent-of-origin-specific manner (1). This functionally haploid state of imprinted genes is essential for normal development of both fetus and placenta. The monoallelic expression of imprinted genes is due to epigenetic mechanisms such as methylation, which consists of the covalent addition of methyl groups by DNA methyltransferases, preferentially at CpG dinucleotides (2). The differentially methylated regions (DMRs), where the parental specific methylation takes place, are located within regulatory regions known as imprinting control regions (ICRs). Several recent studies have shown that, in addition to methylated cytosines (5-mCs), hydroxymethylcytosines (5-hmCs) are also present in DMRs and some transcription regulator binding sites (3-5). The 5-hmC is formed from the oxidation of 5-mC by enzymes of the ten-eleven translocation family and, unlike methylation, it does not result in silencing of gene expression (3). 5-hmCs represent approximately 5% of all cytosines in embryonic stem cells (4), and 20% of all CpGs present in cerebellar Purkinje cells in mammals (5).

Imprinted genes have been considered to be more dose sensitive than other genes, mainly because of their monoallelic expression. This means that subtle variations of methylation in ICRs could represent a drastic deregulation of the expression of imprinted genes, leading to important phenotypic consequences, as observed in the Beckwith-Wiedemann syndrome (6). This implies that a more sensitive method that is capable of distinguishing

Correspondence: E.S. Ramos, Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, USP, Av. Bandeirantes, 3900, 14049-900 Ribeirão Preto, SP, Brasil. Fax: +55-16-3602-4915. E-mail: esramos@fmrp.usp.br

Received May 16, 2013. Accepted July 23, 2013. First published online October 22, 2013.

between methylation and hydroxymethylation is therefore necessary to exclude the interference of 5-hmC when quantifying methylation (2).

Techniques based on DNA modification by sodium bisulfite are unable to distinguish between methylation and hydroxymethylation, since both 5-mC and 5-hmC do not undergo deamination after reacting with sodium bisulfite, and 5-mC and 5-hmC contents are mixed up in the final results. In addition, most methylation-sensitive restriction enzymes are unable to distinguish between these two modified bases (7).

The main objective of this study was to determine the occurrence of 5-hmC at KvDMR1 (ICR2), an imprinted region of 11p15.5, which could possibly influence the results derived from techniques based on restriction enzymatic digestion and/or bisulfite modification, comparing two different methods.

Material and Methods

Patients and sample collections

The present study was approved by the National Committee of Ethics in Research (Process #25000.080902/2004-11; CAAE-0091.0.004.000-04). All patients signed a written informed consent form. We collected 15 third-trimester normal human placentas immediately after birth. Placental samples of 0.5 cm^3 were biopsied, near the cord insertion, from the fetal side of each placenta. Chorionic villus tissue was obtained for DNA extraction after washing with PBS to eliminate residual blood.

Molecular analysis

DNA extraction was performed using standard techniques (8). We used two different methods (Methods I and II) based on DNA digestion by *Hpa*II (a methylation-sensitive enzyme) and *Msp*I (a methylation-insensitive isoschizomer enzyme) followed by real-time PCR amplification to quantify methylation. The assays were carried out independently and at least in duplicate. Additionally, Method I has a glycosylation step in which the DNA is treated with T4 β-glucosyltransferase and UDP-glucose (Treatments 1-3; Table 1). Glycosylation protects the 5-hmC from *Msp*I digestion, making it possible to quantify hydroxymethylation by real-time PCR. Therefore, Method I is capable of distinguishing 5-hmC from 5-mC, whereas Method II is not. Method I was carried out using the Epimark 5-hmC and 5-mC analysis kit (New England Biolabs, USA, catalog #E3317S), according to the manufacturer's manual. Method II was carried out as previously described (9), and corresponds to Treatments 4 to 6 of Method I (Table 1), but without the use of a commercial kit. The digested material obtained by both methods was analyzed by real-time PCR [comparative Ct method (Figure 1)] (10) using the StepOne system (Applied Biosystems, USA), and the results were normalized by the formula $(1/2)^{a-b}$, where "a" corresponds to the Ct value resulting from treatments numbered from one to six of Method I (individually), and "b" corresponds to the Ct value from Treatment number 6 (Table 1) (9). Only samples with standard deviations in the replicate groups (Ct SD values) lower than 0.3 were considered. The normalized data were applied to the formulas provided by the kit's manual to quantify methylation and hydroxymethylation at KvDMR1. Using the comparative Ct method, samples were normalized by setting the control reaction (Tube 6) as the calibrator. This normalization gave an approximate percentage of methylated [*Hpa*II-digested samples (Tubes 2 and 5)] and hydroxymethylated (Tube 1) alleles.

Table 1. Method I: treatments and quantification.

Tube	Treatment	Result
1	T4-BGT + *Msp*I	M2 (5-hmC)
2	T4-BGT + *Hpa*II	H2 (5-hmC + 5-mC)
3	T4-BGT (uncut)	C2 (Total DNA)
4	*Msp*I	M1 (No product detected)
5	*Hpa*II	H1 (5-hmC + 5-mC)
6	Uncut	C1 (Total DNA)

Formulas: 5-hmC = [M2*(C1/C2)–M1]/C1; 5-mC = [H1–M2*(C1/C2)]/C1; C = (C1–H1)/C1. T4-BGT: T4 phage β-glucosyltransferase; 5-hmC: 5-hydroxymethylcytosines; 5-mC: 5-methylcytosines.

Positive and negative control reactions were performed with the undigested (mock) material, and the material was digested by the *Msp*I enzyme from the same sample (internal controls), and normal, hyper- and hypomethylated samples were analyzed in other studies using Multiplex Ligation-dependent Probe Amplification (MLPA; data not shown). In addition, for Method I, we also used material (unmodified, methylated and hydroxymethylated DNA) provided by the kit as controls.

Data analysis

Statistical analysis was performed using the general linear model (GLM) procedure for analysis of variance in SAS (SAS System for Windows, Version 9.1, 2003, SAS Institute, Inc., USA).

Results

The results obtained by both methods are summarized in Table 2. In a previous study, we determined the normal methylation values between 34 and 58% for KvDMR1, using Method II (data not shown). Hypomethylation was found in 6 of 15 cases (Samples 7, 8, 9, 10, 13, and 15) and hypermethylation in 2 of 15 cases (Samples 5 and 6) by both methods (Table 2). The effect of the method, verified by the SAS GLM procedure, was not significant with respect to the variable percentage of methylation (F value = 0.84; Pr>F = 0.3664). This indicates that the

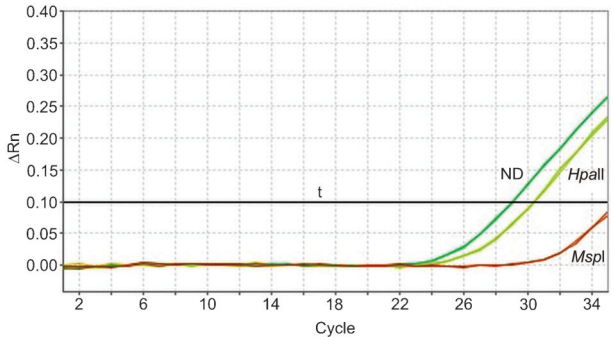

Figure 1. Amplification plot obtained by the StepOne system software using Method II. ND: non-digested sample; *Hpa*II: sample digested with *Hpa*II, a methylation-sensitive restriction enzyme; *Msp*I: sample digested with *Msp*I, an isoschizomer of *Hpa*II, which is insensitive to methylation; t: threshold.

glycosylation step performed additionally in Method I did not interfere with the results of methylation. Since the values found for 5-hmC were very low (sometimes negative, as an artifact of the equations used for normalization) and the values for 5-mC from both methods were statistically similar, we considered that the methylation percentage found by Method II is reliable and that 5-hmC was absent at KvDMR1 in these placenta samples.

Discussion

We compared two methods used for methylation quantification. In both methods, the DNA was digested by *Hpa*II (a methylation-sensitive enzyme) and *Msp*I (a methylation-insensitive isoschizomer enzyme) and analyzed by real-time PCR amplification. Additionally, Method I has a glycosylation step in which the DNA is previously treated with T4 β-glucosyltransferase and UDP-glucose. In both methods, the unmodified DNA (C) is digested by *Hpa*II and *Msp*I and the methylated DNA (5-mC) is digested only by *Msp*I. The hydroxymethylated DNA (5-hmC) is digested by *Msp*I but not by *Hpa*II in Method II, since it behaves as 5-mC. Therefore, Method I is capable of distinguishing 5-hmC from 5-mC while Method II is not. Although *Msp*I activity could be dramatically decreased by symmetrical hydroxymethylation of its recognition sequence and it is partly inhibited by hemi-hydroxymethylation, according to a recent study, a very long incubation of the genomic DNA with a large excess amount of *Msp*I, as used in both methods, could overcome the inhibition (11).

In a whole methylation analysis, Li and Liu (12) found very low levels of global 5-hmC in human placenta, but abundant levels in brain, kidney, colon, rectum, and liver tissues. We observed agreement between the results of both methods (I and II), indicating the absence of hydroxymethylation at the KvDMR1 in third-trimester normal human placentas (chorionic villi).

To the best of our knowledge, this is the first study describing a hydroxymethylation and methylation comparative analysis at KvDMR1 (ICR2) in human placenta and in a genomic imprinting model.

Acknowledgments

We thank Adriane A. Oliveira, Karine Coelho, and Francielle M. Araujo for placenta sampling. Research

Table 2. Quantification of hydroxymethylation and methylation in the human placenta.

Sample	5-hmC (I)	5-mC (I)	C (I)	5-mC (II)
1	0.007416	0.499841	0.481524	0.518476
2	–0.029524	0.459395	0.462821	0.537179
3	–0.012199	0.495749	0.490825	0.509175
4	–0.000409	0.373749	0.624579	0.375421
5	–0.101249	0.71016	0.170625	0.829375
6	–0.034123	0.93993	–0.060206	1.060206
7	–0.017234	0.307043	0.670357	0.329643
8	–0.092131	0.187068	0.661791	0.338209
9	–0.003755	0.264666	0.720809	0.279191
10	–0.008515	0.276197	0.706463	0.293537
11	–0.001995	0.432473	0.545786	0.454214
12	0.000543	0.460348	0.527548	0.472452
13	0.034326	0.307892	0.63424	0.36576
14	–0.00522	0.455683	0.54225	0.45775
15	–0.002939	0.27194	0.720514	0.279486

5-hmC (I): 5-hydroxymethylcytosines (Method I); 5-mC (I): 5-methylcytosines (Method I); C (I): unmodified cytosines (Method I); 5-mC (II): 5-methylcytosines (Method II).

supported by FAPESP (#2009/08313-2 and #2010/03519-9), CNPq (#08856/2006-8 and #478353/2010-3), CAPES-PROEX, and FAEPA.

References

1. Reik W, Walter J. Genomic imprinting: parental influence on the genome. *Nat Rev Genet* 2001; 2: 21-32, doi: 10.1038/35047554.
2. Ivanova E, Chen JH, Segonds-Pichon A, Ozanne SE, Kelsey G. DNA methylation at differentially methylated regions of imprinted genes is resistant to developmental programming by maternal nutrition. *Epigenetics* 2012; 7: 1200-1210, doi: 10.4161/epi.22141.
3. Ficz G, Branco MR, Seisenberger S, Santos F, Krueger F, Hore TA, et al. Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. *Nature* 2011; 473: 398-402, doi: 10.1038/nature10008.
4. Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* 2009; 324: 930-935, doi: 10.1126/science.1170116.
5. Kriaucionis S, Heintz N. The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. *Science* 2009; 324: 929-930, doi: 10.1126/science.1169786.
6. Maher ER, Reik W. Beckwith-Wiedemann syndrome: imprinting in clusters revisited. *J Clin Invest* 2000; 105: 247-252, doi: 10.1172/JCI9340.
7. Huang Y, Pastor WA, Shen Y, Tahiliani M, Liu DR, Rao A. The behaviour of 5-hydroxymethylcytosine in bisulfite sequencing. *PLoS One* 2010; 5: e8888, doi: 10.1371/journal.pone.0008888.
8. Olerup O, Zetterquist H. HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. *Tissue Antigens* 1992; 39: 225-235, doi: 10.1111/j.1399-0039.1992.tb01940.x.
9. Gomes MV, Gomes CC, Pinto W Jr, Ramos ES. Methylation pattern at the KvDMR in a child with Beckwith-Wiedemann syndrome conceived by ICSI. *Am J Med Genet A* 2007; 143: 625-629.
10. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; 25: 402-408, doi: 10.1006/meth.2001.1262.
11. Ichiyanagi K. Inhibition of *Msp*I cleavage activity by hydroxymethylation of the CpG site: a concern for DNA modification studies using restriction endonucleases. *Epigenetics* 2012; 7: 131-136, doi: 10.4161/epi.7.2.18909.
12. Li W, Liu M. Distribution of 5-hydroxymethylcytosine in different human tissues. *J Nucleic Acids* 2011; 2011: 870726, doi: 10.4061/2011/870726.