

ARTICLE

Patients with familial biparental hydatidiform moles have normal methylation at imprinted genes

Osman El-Maarri¹, Muhieddine Seoud², Jean-Baptiste Rivière³, Johannes Oldenburg^{1,4}, Jörn Walter⁵, Guy Rouleau³ and Rima Slim^{*,6}

¹Institute of Experimental Haematology and Transfusion Medicine, Bonn, Germany; ²Department of Obstetrics and Gynecology, American University of Beirut, Beirut, Lebanon; ³Department of Medicine, Centre Hospitalier de l'Université de Montréal, Montreal, Canada; ⁴Institute of Transfusion Medicine and Immunohaematology, Frankfurt, Germany; ⁵Saarland University Biosciences Department of Genetics/Epigenetics, Saarbrücken, Germany; ⁶Department of Human Genetics and Obstetrics and Gynecology, McGill University Health Center, Montreal, Canada

In molar tissues from patients with recurrent biparental hydatidiform moles, we could previously demonstrate that differentially methylated regions (DMRs) of four imprinted genes are abnormally methylated on the maternal alleles. It remained unclear if this abnormal methylation originated *de novo* in the molar tissues or if it is even recognizable in the patient somatic tissues. To address this question, we investigated the DNA methylation of four imprinted genes in total blood from the two sister-patients. Here, we show that both patients retain normal methylation levels at the DMRs of the four genes in blood tissues. For two maternally expressed genes, we could use informative SNPs to follow the inheritance of the abnormally methylated maternal alleles in the molar tissues. We find that the transmitted abnormally methylated maternal alleles to the moles originated from the maternal grandmother and that the same alleles are not methylated in the patients. Our data suggest that the abnormal methylation in familial biparental molar tissues was acquired *de novo* in the patients' germline as a result of a false reprogramming or during the postzygotic development of the conceptuses that led to moles.

European Journal of Human Genetics (2005) 13, 486–490. doi:10.1038/sj.ejhg.5201353

Published online 12 January 2005

Keywords: hydatidiform moles; molar pregnancy; methylation analysis; SIRPH; imprinting

Introduction

Recurrent complete hydatidiform moles are a rare clinical entity in which molar tissues are diploids and have a biparental contribution to their genome (BiCHMs). These moles are undistinguishable, at both gross morphology and histopathology levels, from diploid androgenetic moles containing two sets of paternal chromosomes. Despite their diploid biparental genotype, the epigenotype

of BiCHMs is abnormal. Others and we demonstrated aberrant methylation and expression of imprinted, both maternally or paternally expressed, genes in BiCHMs.^{1–3} We showed that this aberrant methylation affects only the maternal alleles.³ To date, no studies has investigated if the methylation or expression of imprinted genes in the patients themselves is also affected, hence predisposing the patients to transmit such imprinting defects. This question is particularly interesting since patients with recurrent BiCHMs usually have miscarriages alternating with the moles and manifest during some of their molar pregnancies, complications and features of pre-eclampsia. Both recurrent pregnancy losses and pre-eclampsia are associated, in some cases, with physiological abnormalities

*Correspondence: Dr R Slim, Montreal General Hospital Research Institute, Room L12-132, 1650 Cedar Avenue, Montreal, Canada H3G 1A4, Tel: +514 934 1934 ext 44519; Fax: +514 934 8265; E-mail: rima.slim@muhc.mcgill.ca

Received 16 September 2004; revised 28 October 2004; accepted 11 November 2004

such as folate deficiency and hyperhomocystemia.^{4–9} Folate and homocysteine are important components required for DNA and histone methylation. A defect in the methionine pathway in the two patients could be responsible or contribute to the molar phenotype and may manifest in an abnormal DNA methylation in their blood tissues.

We previously reported abnormal methylation at differentially methylated region (DMRs) of four imprinted genes in two molar tissues BiCHMs 9 and 16 from two sisters with recurrent BiCHMs, patients 4 and 6 from family MoLb1.^{3,10} The aim of the present study was to follow up this investigation by analyzing the DNA methylation status at the same DMRs, but in somatic tissues (blood) from the two patients. Furthermore, using SNPs in informative samples and at informative DMRs, we analyzed the grandparental transmission of the abnormal epigenotype to examine its potential association with a particular grandparental inheritance. Altogether, our data contribute to a better understanding of the developmental events occurring during biparental molar pregnancies and suggest that germline reprogramming or/and postzygotic defects are the major cause of BiCHMs.

Results

Methylation of four imprinted genes in patients with recurrent BiCHMs

Methylation at *SNRPN*, *PEG3*, *NESP55*, and *H19* DMRs was investigated on DNAs from total fresh blood of two patients with recurrent biparental moles, members 4 and 6 from family MoLb1, by two independent bisulfite methods as described previously.³ DNA was treated with sodium bisulfite, amplified by PCR, and the methylation was assessed by single-nucleotide primer extension (SNuPE) followed by separation with ion pair reverse-phase HPLC (SIRPH)^{11,12} and by cloning and sequencing. SIRPH analysis at the four DMRs was performed at two sites within each of the *SNRPN*, *PEG3*, and *NESP55* DMRs, and at one site at the *H19* DMR as described previously.³ Our results on the four DMRs demonstrate a normal level of methylation, ranging from 35 to 45%, in the two patients similar to the methylation level found in total blood from healthy normal subjects (Figure 1).

We also used cloning and sequencing to assess the DNA methylation of several adjacent CpG by sequencing individual clones from each of the four DMRs. The results at the DMRs without polymorphisms showed normal methylation pattern with some clones being completely methylated and others completely unmethylated (see Supplementary material). DMRs containing SNPs were at the *NESP55* in patient 6 and at the *H19* in both patients 6 and 4. Analysis of sequences from these DMRs demonstrated normal methylation pattern with only clones carrying the paternal alleles being methylated (Figure 2). These data demonstrate that the two patients with

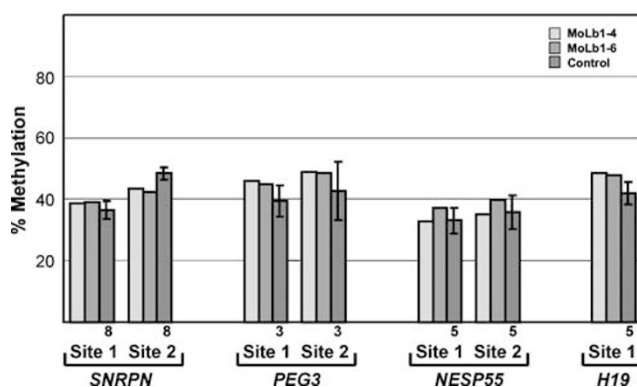


Figure 1 Quantitative methylation measurements by SIRPH analysis. Measurements were carried out at two sites at each of the *SNRPN*, *PEG3*, and *NESP55* DMRs, and at one site at the *H19* DMR. At each site, the first column from the left represents patient MoLb1-4, the second MoLb1-6 and the third is the average of measurements obtained on three to eight control DNA as indicated below each column. The different measurements represent independent samples from different subjects.

recurrent biparental moles have normal DNA methylation at the four analyzed imprinted genes.

Grandparental origin of the abnormally methylated maternal alleles in molar tissues

We previously demonstrated an abnormal methylation at the maternal *SNRPN* and *NESP55* DMRs in BiCHMs 16 and 9, respectively.³ Using microsatellite markers in proximity of the *SNRPN* and *NESP55* DMRs, cen-D15S1021-(450 kb)-D15S128-(350 kb)-*SNRPN* DMR and cen-D20S164-(400 kb)-*NESP55* DMR-(400 kb)-D20S171, respectively, we established the haplotypes carrying the abnormally methylated alleles. Haplotype analysis shows that the abnormally unmethylated maternal *SNRPN* in BiCHM 16, with C at position 144772 (GenBank Accession no. AC009696), is inherited from the maternal grandmother (haplotype 4-3-C) (Figure 3a). In BiCHM 9, the abnormally methylated maternal *NESP55* allele, with A at position 106675 (GenBank accession no. AL132655), is inherited from the maternal grandmother (haplotype 1-A-1) (Figure 3). We note that the healthy daughter of patient MoLb1-4, with normal methylation level and pattern, has inherited the same grandparental haplotype (1-A-1).

Discussion

To investigate whether two sister-patients with BiCHMs have abnormal DNA methylation at imprinted genes in their somatic tissues, we analyzed four DMRs that we previously showed are abnormally methylated/unmethylated on the maternal alleles in their molar tissues.³ Using SIRPH analysis on blood DNA from the two patients, we demonstrate normal methylation levels at the four genes (Figure 1). Cloning and sequencing data on DMRs without

Maternally Expressed Genes

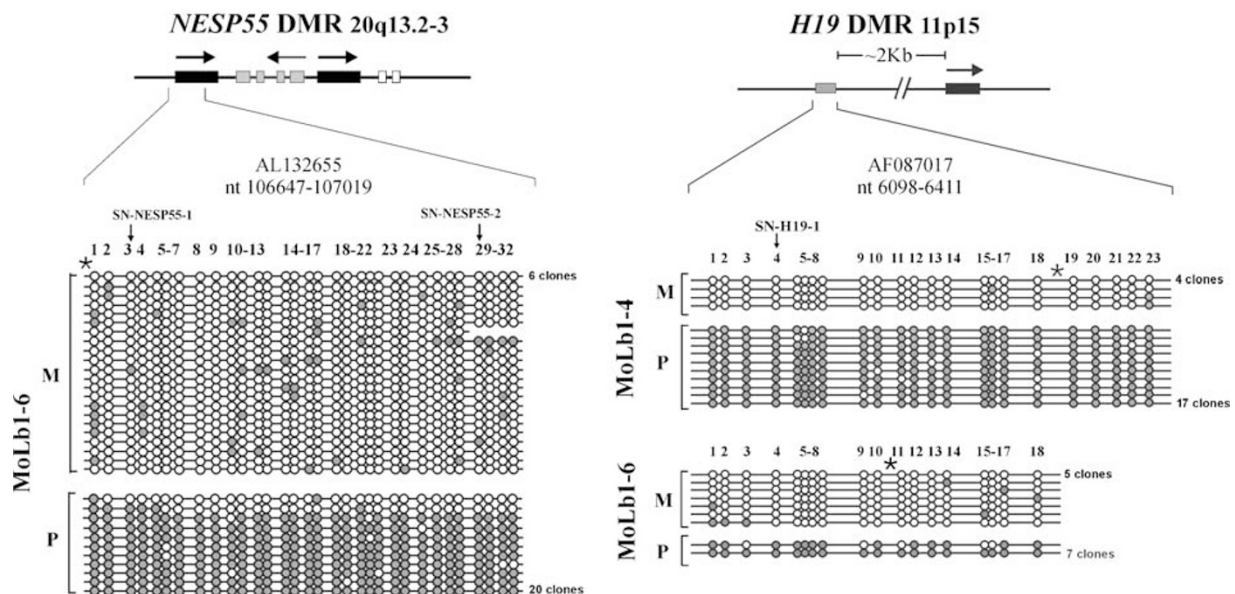


Figure 2 Summary of the methylation analysis from isolated sequenced clones at the *NESP55* and *H19* DMRs. The studied region relative to each gene is shown under the corresponding name. Each individual PCR clone is represented by a line; unmethylated and methylated CpG sites are represented by open and filled circles, respectively. The number of clones with identical methylation pattern is indicated on the right side of one representative clone. Vertical arrows indicate the sites studied by the SIRPH analysis. M: maternal; P: paternal. The position of the polymorphisms that distinguish the parental alleles is indicated by '*'. These are at position 106675 in AL132655 for *NESP55* and at positions 6225 (MoLb1-6) and 6336 (MoLb1-4) in AF087017 for *H19*.

polymorphisms are compatible with normal methylation pattern (Supplementary material). With the use of available SNPs, we demonstrate a normal methylation pattern at *NESP55* in one patient, MoLb1-6, and at *H19* in both of them (Figure 2).

We previously traced the inheritance of the abnormally methylated maternal *H19* alleles in molar tissues and found that they are inherited from the maternal grandfather to BiCHM9 and from the maternal grandmother to BiCHM16. Now, we show that the two patients have normal methylation level and pattern at *H19*, with only their paternal alleles being methylated (Figure 2). The presence of abnormal methylation marks in BiCHM16 on a nonmethylated allele in the patient's blood indicates that the abnormal methylation was acquired in the germline of the patient or during the postzygotic development of the BiCHM16. This suggests that the abnormal epigenotype in the BiCHM16 did not originate in the mothers' somatic tissues as a result of a physiological impairment, for instance, in the methylation pathway. Using microsatellite flanking the *NESP55* DMR, we show that the abnormally methylated allele in BiCHM9 is also inherited from the maternal grandmother (Figure 3b). The patient, MoLb1-4, has normal level of methylation in her total blood; beside her reproductive problem, she has a normal phenotype and is in good health. Consequently, the patient is expected to have normal methylation pattern with methylation only

on her paternal *NESP55* DMR. This demonstrates, again, at a second locus, *NESP55*, and in a second mole, BiCHM9, the acquisition of new methylation marks on a nonmethylated allele transmitted by the patient. At the *SNRPN*, we show that the abnormally unmethylated maternal allele in BiCHM16 is inherited from the maternal grandmother (Figure 3a). The patient, MoLb1-6, has normal methylation level in her blood and is expected to have methylation marks only on her maternal allele. Therefore, the abnormal absence of methylation on the *SNRPN* maternal allele in BiCHM16 could result from an incomplete erasure, an incomplete reprogramming, or abnormal postzygotic maintenance of the correctly established marks.

Of all the studies that have addressed the methylation or expression of imprinted genes in familial molar tissues, none of them allows reaching a conclusion as to whether the abnormal methylation occurred in the maternal germline or during the postzygotic development for the following reasons. First, all these studies were performed on molar tissues at 6–14 weeks of gestation in which several changes could have occurred since fertilization. Consequently, the abnormal methylation observed in these tissues may not reflect exactly the methylation marks of the parental gametes. Second, although abnormal unmethylation was reported at several imprinted, paternally expressed genes with primary imprints (known to be established in the germline), but abnormal methylation

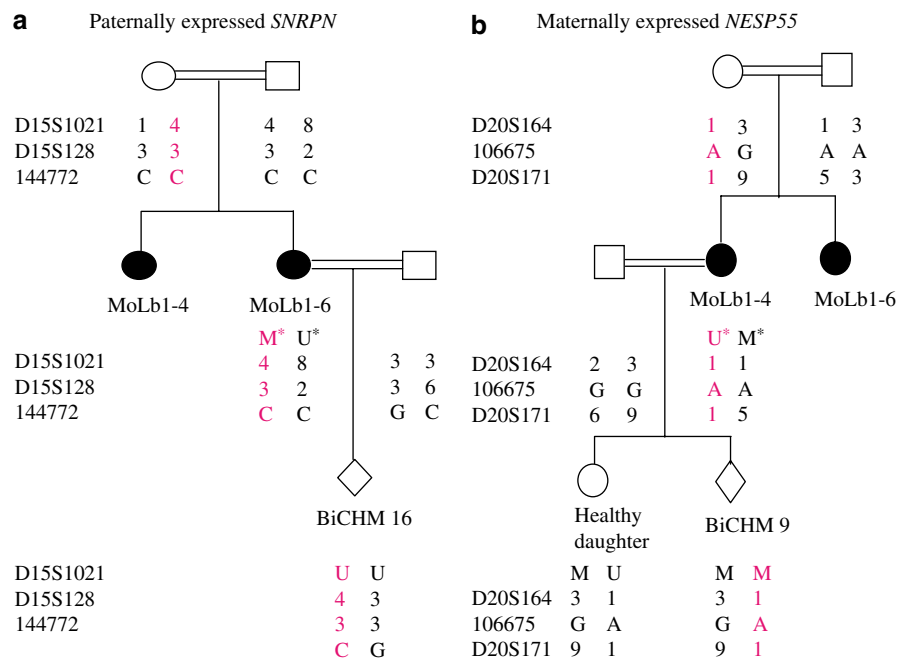


Figure 3 Segregation of the grandparental haplotypes at *SNRPN* and *NESP55* to BiCHMs. Haplotypes were constructed assuming minimal number of recombinations. The order of the markers is given from the centromere (top) to the telomere (bottom) according to the UCSC map (<http://genome.ucsc.edu>). The positions of the SNPs at the *SNRPN* and *NESP55* DMRs are given in GenBank Accession sequences AC009696 and AL132655, respectively. The haplotypes carrying the abnormally methylated alleles in molar tissues are in red, 'U' indicates haplotypes carrying the unmethylated allele and 'M' the methylated one based on the presence of informative SNPs; an asterisk '*' is added to haplotypes without informative SNPs in the patients and whose methylation status is inferred based on the normal level of methylation obtained by SIRPH and on the normal phenotype of the patients.

marks were also shown at *NESP55*,^{1,3} with secondary imprints (known to be established postzygotically around the blastocyst stage). Third, molar pregnancies are benign tumors of the trophoblast and several studies have shown gain or loss of DNA methylation at imprinted genes, including *PEG3* and *H19* (extensively studied), in a variety of tumors such as glioma, Wilms' tumor, lung carcinoma, Müllerian tumor of the uterus, hepatoblastoma, and colorectal cancer.^{13–21} In colorectal cancer and Wilms' tumors, a gain of methylation on the maternal *H19* DMR was shown.^{13,14,16,20,21} Therefore, it would be expected that at least some of the methylation changes observed in biparental molar tissues (gain or loss) have occurred during molar development and are specific for these trophoblastic tumors. This would explain the deregulation of the same genes in the same way in most analyzed biparental moles, familial and sporadic.^{2,3}

An intriguing observation in biparental moles is the loss of maternal methylation imprints on most of the analyzed paternally expressed genes while paternal methylation imprints were maintained on two analyzed maternally expressed genes. This led Judson *et al*¹ to suggest that a 'maternal' germline defect in establishing the maternal imprints underlies familial BiCHMs. Although this hypothesis is attractive and plausible, it may not be the only explanation. Another explanation that may underlie the

different behavior between maternal and paternal imprints would be due to their different properties and is supported by several observations in BWS and PWS syndromes. In case of the BiCHMs, the labile nature of maternal imprints in placental tissues during early development may explain the loss of maternal imprints.²²

Finally, the abnormal methylation patterns on the maternal alleles of maternally or paternally expressed genes in biparental molar tissues may have not occurred at all DMRs at the same time, but resulted from several events that altogether led to the molar phenotype.

Acknowledgements

We thank the patients for their cooperation and Judith Schwalbach for technical support. RS is supported by the 'Fonds de Recherche en Santé du Québec' and by an operating grant from the CIHR (MOP-67179).

References

- Judson H, Hayward BE, Sheridan E, Bonthron DT: A global disorder of imprinting in the human female germ line. *Nature* 2002; **416**: 539–542.
- Fisher RA, Hodges MD, Rees HC *et al*: The maternally transcribed gene p57(KIP2) (CDKN1C) is abnormally expressed in both androgenetic and biparental complete hydatidiform moles. *Hum Mol Genet* 2002; **11**: 3267–3272.

- 3 El-Maarri O, Seoud M, Coullin P *et al*: Maternal alleles acquiring paternal methylation patterns in biparental complete hydatidiform moles. *Hum Mol Genet* 2003; **12**: 1405–1413.
- 4 Nelen WL, Blom HJ, Steegers EA, den Heijer M, Eskes TK: Hyperhomocysteinemia and recurrent early pregnancy loss: a meta-analysis. *Fertil Steril* 2000; **74**: 1196–1199.
- 5 Nelen WL, Blom HJ, Steegers EA, den Heijer M, Thomas CM, Eskes TK: Homocysteine and folate levels as risk factors for recurrent early pregnancy loss. *Obstet Gynecol* 2000; **95**: 519–524.
- 6 Nelen WL, Bulten J, Steegers EA, Blom HJ, Hanselaar AG, Eskes TK: Maternal homocysteine and chorionic vascularization in recurrent early pregnancy loss. *Hum Reprod* 2000; **15**: 954–960.
- 7 Barro C, Ducros V, Bonaz B *et al*: Homocysteinemia and recurrent fetal losses: description of three cases. *Ann Biol Clin (Paris)* 2002; **60**: 325–326.
- 8 Kumar KS, Govindaiah V, Naushad SE, Devi RR, Jyothy A: Plasma homocysteine levels correlated to interactions between folate status and methylene tetrahydrofolate reductase gene mutation in women with unexplained recurrent pregnancy loss. *J Obstet Gynaecol* 2003; **23**: 55–58.
- 9 Reznikoff-Etievant MF, Zittoun J, Vaylet C, Pernet P, Milliez J: Low vitamin B12 level as a risk factor for very early recurrent abortion. *Obstet Gynecol* 2002; **104**: 156–159.
- 10 Moglabey YB, Kircheisen R, Seoud M, El Mogharbel N, Van den Veyver I, Slim R: Genetic mapping of a maternal locus responsible for familial hydatidiform moles. *Hum Mol Genet* 1999; **8**: 667–671.
- 11 El-Maarri O, Herbiniaux U, Walter J, Oldenburg J: A rapid, quantitative, non-radioactive bisulfite-SNuPE-IP RP HPLC assay for methylation analysis at specific CpG sites. *Nucleic Acids Res* 2002; **30**: e25.
- 12 El-Maarri O: SIRPH analysis: SNuPE with IP-RP-HPLC for quantitative measurements of DNA methylation at specific CpG sites. *Methods Mol Biol* 2004; **287**: 195–206.
- 13 Steenman MJ, Rainier S, Dobry CJ *et al*: Loss of imprinting of IGF2 is linked to reduced expression and abnormal methylation of H19 in Wilms' tumor. *Nat Genet* 1994; **7**: 433–439.
- 14 Moulton T, Crenshaw T, Hao Y *et al*: Epigenetic lesions at the H19 locus in Wilms' tumour patients. *Nat Genet* 1994; **7**: 440–447.
- 15 Kondo M, Suzuki H, Ueda R *et al*: Frequent loss of imprinting of the H19 gene is often associated with its overexpression in human lung cancers. *Oncogene* 1995; **10**: 1193–1198.
- 16 Taniguchi T, Sullivan MJ, Ogawa O, Reeve AE: Epigenetic changes encompassing the IGF2/H19 locus associated with relaxation of IGF2 imprinting and silencing of H19 in Wilms tumor. *Proc Natl Acad Sci USA* 1995; **93**: 2159–2163.
- 17 Hashimoto K, Azuma C, Tokugawa Y *et al*: Loss of H19 imprinting and up-regulation of H19 and SNRPN in a case with malignant mixed Mullerian tumor of the uterus. *Hum Pathol* 1997; **28**: 862–865.
- 18 Li X, Kigler P, Sandstedt B *et al*: Promoter-specific methylation and expression alterations of IGF2 and H19 are involved in human hepatoblastoma. *Int J Cancer* 1998; **75**: 176–180.
- 19 Maegawa S, Yoshioka H, Itaba N *et al*: Epigenetic silencing of PEG3 gene expression in human glioma cell lines. *Mol Carcinogen* 2001; **31**: 1–9.
- 20 Cui H, Niemitz EL, Ravenel JD *et al*: Loss of imprinting of insulin-like growth factor-II in Wilms' tumor commonly involves altered methylation but not mutation of CTCF or its binding site. *Cancer Res* 2001; **61**: 4947–4950.
- 21 Nakagawa H, Chadwick RB, Peltomäki P, Plass C, Nakamura Y, de la Chapelle A: Loss of imprinting of the insulin-like growth factor II gene occurs by biallelic methylation in a core region of H19-associated CTCF-binding sites on colorectal cancer. *Proc Natl Acad Sci USA* 2001; **98**: 591–596.
- 22 Mann MR, Lee SS, Doherty AS *et al*: Selective loss of imprinting in the placenta following preimplantation development in culture. *Development* 2004; **131**: 3727–3735.

Supplementary Information accompanies the paper on European Journal of Human Genetics website (<http://www.nature.com/ejhg>)