Maternal alleles acquiring paternal methylation patterns in biparental complete hydatidiform moles

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We previously mapped a maternal locus responsible for biparental complete hydatidiform moles (BiCHMs) to 19q13.4. The two index patients had a total of 14 molar pregnancies, eight abortions at various developmental stages, and one 16-year-old healthy offspring. We suggested that the defective gene deregulates the expression of imprinted genes. Here, we report the methylation status of four imprinted genes in two BiCHMs from the two sisters, the 16-year-old normal offspring, and two sporadic BiCHMs from unrelated patients.

Using two bisulfite-based methods, we demonstrate a general trend of abnormal hypomethylation at the paternally expressed genes, PEG3 and SNRPN, and hypermethylation at the maternally expressed genes, NESP55 and H19, in two to four BiCHMs. Using single nucleotide polymorphisms, we provide the first evidence that SNRPN, NESP55 and H19 are abnormally methylated on the maternal alleles in BiCHMs. We show, in the BiCHMs from the two sisters, that the abnormally methylated H19 allele is inherited from either the maternal grandmother or the maternal grandfather. These data suggest that the abnormal methylation in BiCHMs is not due to an error in erasing the parental imprinting marks but rather in the re-establishment of the new maternal marks during oogenesis or their postzygotic maintenance. The defective 19q13.4 locus may have led to the development of variable degrees of ‘faulty’ paternal marks on the maternal chromosomes.

INTRODUCTION

Complete hydatidiform moles (CHMs; MIM 231090) are abnormal human pregnancies characterized by a hydropic degeneration of all villi and absence of embryo. Most of these cases are sporadic, not recurrent, and have a diploid genome. In 80% of the cases CHMs have an androgenic genome (AnCHM), while the remaining 20% have a biparental contribution to the molar genome (BiCHM) (1). Occasionally, recurrent moles have been reported in a single family member (2–5) and in a few cases in more than one family member (6–13).

We previously characterized several CHMs occurring in two sisters of family MoLb1 and showed a biparental contribution to the different molar genomes (14). We demonstrated that a maternal homozygous locus mapping to 19q13.4 is responsible for the recurrent BiCHMs in MoLb1 (15). We suggested that the defective maternal gene deregulates the expression of several imprinted genes. The exact nature of the gene deregulation underlying HMs and the tissues where the defective product is needed are unknown yet. However, the occurrence of several cases of dizygotic twin pregnancies with two separate conceptuses, a normal placenta attached to one live fetus and a hydatidiform mole to the other (16), suggests that women with HMs have normal reproductive tract. In addition, the association of the beginning and end of the female reproductive period with the increased incidence of sporadic moles (in women over 45 years and teenagers) (17–22) is in favor of a defect in the oocytes of women with HMs. The defective gene product may be needed during gametogenesis in the unfertilized oocytes, or later after fertilization during early developmental stages.

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Differentially methylated regions (DMRs) between the maternal and the paternal alleles are one of the best-studied epigenetic factors associated with the control of imprinted gene expression. Methylation analysis of several DMRs in one molar tissue demonstrated the absence of methylation at the six paternally expressed genes KCNQ1OT1, SNRPN, PEG1, PEG3, XL XlsA and XL Xlsa-antisense; a normal methylation at the maternally expressed gene, H19; and a high level of methylation at the maternally expressed NESP55 (12). However, in this study a single BiCHM was analyzed and the segregation of the disease phenotype in the analyzed family does not seem to be linked to 19q13.4, indicating possible genetic heterogeneity of this disorder. Recently, a second study demonstrated the abnormal underexpression of one maternally expressed gene, p57kip2, in a series of BiCHMs, including two from sisters in which the defect segregates with alleles at 19q13.4 markers (13).

Here, we report the methylation status of four well-characterized imprinted genes, the paternally expressed, PEG3 and SNRPN, and the maternally expressed, NESP55 and H19, in two BiCHMs from two sisters, a 16-year-old healthy girl from one of them, and two sporadic BiCHMs from unrelated patients. The aim of our study was to assess the methylation of imprinted, paternally or maternally expressed genes and investigate whether different molar tissues occurring in patients from the same family share similar patterns of abnormal methylation at identical genes. This would be expected if a specific abnormal methylation is the primary defect leading to the molar phenotype. In addition, we analyzed a set of suitable controls including two AnCHMs, and a series of first trimester chorionic villus cells and total blood from normal subjects.

We demonstrate a general trend of hypomethylation at the paternally expressed genes, PEG3 and SNRPN, and an increased level of methylation at the two maternally expressed genes, NESP55 and H19. PEG3 and SNRPN were hypomethylated in four and two BiCHMs, respectively; NESP55 and H19 were hypermethylated, respectively, in three and two BiCHMs. By analyzing SNPs, we provide evidence that the SNRPN, NESP55 and H19 genes have abnormal patterns of methylation on the maternal alleles in BiCHMs. We show, in the BiCHMs from the two sisters, that the abnormally methylated maternal H19 allele is inherited from either the maternal grandmother or the maternal grandfather. These data suggest that the abnormal methylation in BiCHMs is not simply due to an error in erasing the parental marks, but an error in the reprogramming process of the maternal marks during oogenesis. Alternatively, an abnormal postzygotic maintenance of the correctly established maternal marks may lead to the same situation.

RESULTS

Strategy of methylation analysis

DNAs were treated with bisulfite, PCR amplified and the methylation was assessed using two independent methods: the traditional cloning/sequencing method (23) and single nucleotide primer extension SNuPE followed by separation with ion pair reverse phase HPLC (SIRPH) (24). The former method provides detailed information on the methylation status of all CpG dinucleotides all over the analyzed fragment. When an informative DNA polymorphism is available, the cloning and sequencing allow the methylation patterns of the two parental alleles to be distinguished. However, this method is subject to cloning and selection bias that may affect the results, especially, in the absence of polymorphisms, unless a large number of clones (50–100) are sequenced to reach a statistical result. To solve this problem, in parallel to the cloning and sequencing method, we used the SIRPH assay, which provides an accurate quantitation of the methylation in all the PCR amplified fragments, but at selected CpG sites. The same outer and nested primers were used to amplify the same PCR products that were assessed by the two methods (Supplementary Material, Table 1). The primers used in the SNuPE are provided (Supplementary Material, Table 2). To avoid any PCR bias due to allele specific amplification or extension, we PCR-amplified and sequenced larger DNA fragments containing each of the analyzed DMRs including the outer, nested and SNuPE primer sites in untreated DNA from all the analyzed samples (Supplementary Material, Table 3). A single nucleotide polymorphism (SNP) at any of these sites could result in a bias due to a preferential annealing and amplification or extension of one of the parental alleles and would change the ratio of the maternal versus paternal alleles and consequently, the level of methylation in the final products.

The SIRPH analysis has been previously shown to be linear and quantitative (24). By SIRPH, all studied regions from normal subjects should have a theoretical 50% methylation like any imprinted DMR. SIRPH analysis of DNA from total blood and chorionic villi from normal subjects showed a methylation level around 40% for the PEG3, 40% for the SNRPN, 20–40% (site 1) and 40% (site 2) for the NESP55, and 40% for the H19 (Fig. 1). Some variations in the methylation levels were noted at the SNRPN and NESP55, but were found only in one to three CVSs (chorionic villus samples). The two samples of AnCHMs showed hypomethylation (SNRPN) or unmethylation (PEG3) at the paternally expressed genes and a clear hypermethylation (80–100%) at the two paternally repressed genes, NESP55 and H19. SIRPH analysis on sperm DNA showed, as expected, absence of methylation at the two paternally expressed PEG3 and SNRPN; hypermethylation at the paternally repressed H19; and unmethylation (site 1) or a very low level of methylation (site 2, 10%) at the paternally repressed NESP55, which is known to acquire paternal imprints in the blastocyst stage (25,26).

Methylation of paternally expressed genes in BiCHMs

Methylation of the PEG3 DMR has been shown to be a primary imprint occurring in the maternal germ line in mice (27). Analysis of the molar tissues from MoLB1, BiCHMs 9 and 16 demonstrated their hypomethylation at the PEG3 DMR by both SIRPH (10–15%; Fig. 1) and cloning/sequencing (Fig. 2). By SIRPH, BiCHMs 21 and 24 showed a slight hypomethylation on site 1 (20–25%) and a clear hypomethylation on site 2 (5–10%). A normal methylation level was observed in the normal daughter of patient MoLB1–4. The SIRPH data were in agreement with those obtained by cloning/sequencing. No polymorphisms at this DMR were detected in any of the analyzed samples.

Methylation at the SNRPN DMR 1 is a primary imprint in mice (28). However, in humans it appears to be established in
Figure 1. Summary of the quantitative SIRPH methylation analysis at selected CpG sites of the PEG3, SNRPN, NESP55 and H19 DMRs. The average methylation at each site is represented in a 100% scale. The standard deviation between different measurements is shown as vertical bars. The number of measurements at each site is shown under each column and represents the number of analyzed PCR products from two independent bisulfite treatments of each DNA.
late stages of oocyte maturation or even after fertilization (23). SIRPH analysis of the SNRPN DMR 1 showed a normal methylation level in BiCHM 9 (30–40%) and BiCHM 21 (50%), while a slight hypomethylation was noted in BiCHMs 16 (20–30%) and 24 (20%; Fig. 1). The cloning/sequencing results showed a lower level of methylation in the four BiCHMs (Fig. 2). Using cloning and sequencing, a few fully methylated clones (with normal maternal pattern of methylation) were obtained in BiCHMs 9 and 21, and none in BiCHMs 16 (in the short fragment that does not contain a SNP) and 24. To understand the differences between the levels of methylation obtained by SIRPH and cloning/sequencing, we also assessed the methylation of the same bisulfitetreated and amplified products using restriction enzyme digestion with two enzymes, Rsal and MwoI, and quantitation of the digested products using appropriate software (Quantity One, Bio-Rad). Rsal and MwoI cut only methylated CpG at sites 3 and 11–12, respectively (Fig. 2). The results of this analysis showed also some variability with 5–15% of hypomethylation at Rsal and 10–40% of hypomethylation at MwoI in all the molar samples (data not shown). This again indicates an irregular scattered, non-allelic type of methylation at this locus that could explain the differences in the level of methylation obtained by SIRPH and sequencing. The search for polymorphisms at this DMR did not reveal any in BiCHMs 9, 21 and 24. Only one SNP, a C to G, was informative to distinguish the parental chromosomes. We therefore re-amplified and re-analyzed a larger DNA fragment containing this SNP (Fig. 2). Out of the seven clones from the maternal chromosome, six were abnormally unmethylated and only one was fully methylated (normal methylation pattern). Clones from the paternal chromosome were all, as expected, unmethylated. We therefore provide a first evidence of an abnormal unmethylation at the maternal SNRPN DMR in one molar tissue from MoLb1. A normal methylation level was found in the healthy daughter of patient MoLb1–4 by the SIRPH and cloning/sequencing.

Methylation of maternally expressed genes in BiCHMs

Methylation at the NESP55 DMR is a secondary imprint that is acquired on the paternal allele in the blastocyst stage (25,26). BiCHMs 9, 16 and 24 showed a clear hypermethylation (70–80%), while BiCHM 21 displayed a normal methylation level (40%; Fig. 1). The search for polymorphisms at this DMR revealed an A to G polymorphism at position 316 (GenBank accession no. AF087017). The C to A change could be informative to distinguish the parental chromosomes. We therefore re-amplified and re-analyzed a larger DNA fragment in BiCHM 9. This SNP was informative to distinguish the parental alleles and prompted us to re-analyze a larger DNA fragment in this molar sample (Fig. 3). Out of the seven clones from the maternal chromosome, six were abnormally unmethylated and only one was fully methylated (normal methylation pattern). Clones from the paternal chromosome were all, as expected, unmethylated. We therefore provide a first evidence of an abnormal unmethylation at the maternal SNRPN DMR in one molar tissue from MoLb1. A normal methylation level was found in the healthy daughter of patient MoLb1–4 by the SIRPH and cloning/sequencing.

Methylation of one X-linked gene

The abnormal methylation of the analyzed imprinted genes in BiCHMs prompted us to investigate whether non-imprinted genes are also abnormally methylated in these tissues. We chose one non-imprinted gene, the factor VIII (F8) that we previously characterized and showed its hypermethylation in mature female and male germ cells (30). SIRPH analysis of one CpG in exon 23 of the F8 (GenBank accession no. M88645) showed a normal high level of methylation (80–90%) in all the analyzed molar and control samples (data not shown).

DISCUSSION

A homozygous maternal mutation deregulating the expression of imprinted genes is believed to underlie the pathology of familial BiCHMs. To address this hypothesis, we investigated the methylation status of four well-characterized DMRs controlling the expression of imprinted, paternal and maternally expressed genes in three conceptuses of a previously reported family MoLb1 (14), two BiCHMs from two sisters as well as a 16-year-old healthy daughter from one of them, and two sporadic BiCHMs from unrelated patients. The methylation status was assessed using two methods, bisulfitetreatment of the DNA followed by PCR amplification, cloning and
Figure 2. Summary of the methylation analysis from the isolated sequenced clones at the imprinted, paternally expressed genes *PEG3* and *SNRPN*. The accession numbers of the sequences are indicated for each gene with the extent of the analyzed region. Solid boxes represent coding regions in the vicinity of the DMR. Horizontal arrows indicate the transcriptional direction. Vertical arrows indicate CpG sites analyzed by SIRPH. Each line represents an independent original DNA strand; the solid and open circles represent methylated and unmethylated CpG, respectively. At the *SNRPN*, clones containing the SNP (at position 144772 in GenBank accession no. AC009696) are grouped together according to their maternal (M) and paternal (P) origin.
Maternally Expressed Genes

**NESP55 DMR 20q13.2-3**

AF105253
nt 106677-107019

**H19 DMR 11p15**

AF087017
nt 60986-6328

![Diagram showing methylation analysis from isolated sequenced clones at the imprinted, maternally expressed genes NESP55 and H19.](https://academic.oup.com/hmg/article-abstract/12/12/1405/602045)

**Figure 3.** Summary of the methylation analysis from the isolated sequenced clones at the imprinted, maternally expressed genes NESP55 and H19. The accession numbers of the sequences are indicated for each gene with the extent of the analyzed region. Solid boxes represent the transcripts in the vicinity of the studied region. Horizontal arrows represent the direction of transcription. Vertical arrows indicate CpG sites analyzed by SIRPH. Each line represents an independent original DNA strand; the solid and open circles represent methylated and unmethylated CpG, respectively. Clones from DNA fragments containing informative SNPs (position 316 in GenBank accession no. AF105253 for NESP55 and positions 6194, 6236, and 6325 in GenBank accession no. AF087017 for H19) are grouped together and labeled according to their maternal (M) or paternal (P) origin.

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expressed genes, with assessment of the methylation level. Here, we show a general methods are complementary; their combination allows a better sequencing or SIRPH analysis. Our data indicate that both the two paternally expressed genes, with PEG3 hypomethylated in the four BiCHMs and SNRPN hypomethylated in two BiCHMs (samples 16 and 24), and an increased level of methylation at the two maternally expressed genes, with NESP55 hypermethylated in three BiCHMs (samples 9, 16 and 24) and H19 hypermethylated in two BiCHMs (samples 16 and 24). Using informative DNA polymorphisms, we demonstrated that the maternal alleles of the three imprinted genes SNRPN, NESP55 and H19 display variable degrees of paternal methylation patterns in BiCHMs. This is in agreement with the pathology of BiCHMs, which leads to the same phenotype observed in AnCHMs. Consequently, the maternal alleles of imprinted genes, although present in the molar genome of BiCHMs, behave as their paternal homologs. Our data on the H19 and NESP55 are in agreement with the underexpression of the p57Kip2 gene in two BiCHMs from a family in which the disease phenotype is linked to 19q13.4 (13). One of our sporadic cases, BiCHM 21, showed a normal methylation level and pattern at the maternally expressed H19. A similar normal methylation at this gene was also reported in another case of BiCHM from a familial case that is not linked to 19q13.4 (13). In family MoLb1, the three patients – gene expression (31) – had a total of 14 HMs (13 complete HMs and one partial HM, see Patient materials), eight abortions of various stages, and one normal pregnancy that led to a live term baby, now a 16-year-old healthy girl whose DNA was included in this study. Analysis of the four DMRs on total blood DNA from this individual did not reveal any abnormalities in the level of DNA methylation.

Figure 4. Partial pedigree of MoLb1 family showing the grandparental origin of the abnormally methylated maternal allele transmitted to the moles at the H19 DMR. The haplotypes were constructed at the three SNPs (positions 6194, 6236 and 6325 in GenBank accession no. AF087017) assuming the absence of recombination events.

development in CHMs is arrested at an early stage, probably after the formation of the primary trophoblast and before the formation of fetal tissues. Consequently, even if different molecular defects lead to the different types of moles, the absence of differentiated fetal tissues and structures makes it very difficult to divide moles into phenotypic categories. Altogether, these data indicate that the pathology of moles may not be explained by a single mechanism, but rather by a cumulative effect of gene deregulation at multiple loci leading to early embryonic lethality and proliferation of the trophoblast. Our results on one X-linked gene, the F8, showed a normal methylation in all BiCHMs. Nevertheless, the analysis of additional non-imprinted genes is needed to reach a conclusion on their methylation status in BiCHMs.

From our data on the four imprinted DMRs in BiCHMs, there is a general tendency, in most of the samples, for a switch from the maternal methylation patterns to the paternal methylation patterns. This abnormal methylation could have happened at one of the following developmental stages: (i) in the molar tissues during the proliferation of the trophoblast; (ii) very early in the zygotes due to a failure in maintaining the correct imprinting marks; (iii) during the oocyte growth of patients with BiCHMs, as a result of an error in the reprogramming process and the re-establishment of the maternal methylation marks; (iv) or in the primordial germ cells of these patients due to a failure in erasing their parental imprinting marks. From our analysis, it is impossible to determine the exact developmental stage at which this abnormal methylation occurred. However, the presence of similar patterns of hypo- and hypermethylation at identical genes in the two moles from sisters who share the same genetic defect and in one sporadic case suggests that this abnormal methylation is not a consequence of the trophoblastic proliferation, but a primary defect leading to the molar phenotype. In addition, the inheritance of the abnormally methylated maternal H19 allele from either the maternal grandfather or grandmother (Fig. 4) suggests that the abnormal methylation is not due to an error in erasing the imprinting marks, but rather in the reprogramming process and the re-establishment of the maternal methylation marks (31). Despite the improvement of these methods and the recent successes in cloning several animal species, most of the cloned embryos die at various developmental stages and only 1–5% live to term. These latter have a normal phenotype despite widespread imprinted gene deregulation, indicating that mammalian development is tolerant to epigenetic aberrations and that normal cloned animals may or may not have changes in gene expression (31–33). In family MoLb1, the three patients had a total of 14 HMs (13 complete HMs and one partial HM, see Patient materials), eight abortions of various stages, and one normal pregnancy that led to a live term baby, now a 16-year-old healthy girl whose DNA was included in this study. Analysis of the four DMRs on total blood DNA from this individual did not reveal any abnormalities in the level of DNA methylation.
Our data on the three conceptuses of MoLb1 suggest that the defective 19q13.4 locus leads to variable degrees of imprinting deregulation on the maternal allele during oogenesis or early development. The variability of the phenotype of the conceptuses will then depend on the degree and the importance of the deregulated genes. Occasionally, critical genes for early development escape the deregulation and lead to abortion at 8 weeks of gestation. The sporadic moles were from patient MoLb1 were previously described (14). Since that time, only patient MoLb1–4 had one partial hydatidiform mole and one abortion at 8 weeks of gestation. The sporadic moles were from cases 21, 24, 23 and 28, each with a single HM. All HM DNAs were obtained from uncultured freshly dissected villi. Dissections were performed carefully under a stereomicroscope. The dissected tissues were then washed several times with 1× PBS and used to extract DNAs according to standard phenol–chloroform protocols. DNA from total blood from a 16-year-old healthy daughter of patient MoLb1–4 was also included (this offspring has never been pregnant and her phenotype status is unknown). In addition, DNAs from seven choric villus and eight total blood samples from unrelated healthy subjects were used as controls. The choric villus samples were obtained from cases referred for prenatal diagnosis for mutations in the Duchenne muscular dystrophy or the spinal muscular atrophy genes. All the DNA samples were obtained from choric villous sampling cells that were not cultured. Their respective gestational stages are indicated in Figure 1. All the analyzed HMs are of complete type according to standard pathological criteria.

Genotyping

The two molar tissues from MoLb1 (BiCHMs 9 and 16) were previously shown to be diploid with bialparental contribution (14). DNA from the four CHMs (samples 21, 24, 23 and 28) and their available parents were genotyped at six to 19 markers from at least six autosomes (34) (Supplementary Material, Table 4). Two CHMs, samples 21 and 24, were found to be bialparental. In addition, sample 21 was previously shown to be of bialparental origin by RFLP analysis of the HLA locus on Southern blots (P. Coullin, unpublished data). Two CHMs, samples 23 and 28, were found to be androgenetic monospermic. Sample 23 had also been previously reported to be androgenetic by HLA serological typing (35). The genotypes at the other markers were in agreement with either a bialparental (for cases 21 and 24) or an androgenetic monospermic contribution (for cases 23 and 28). No contamination with maternal tissues was noted in the molar DNA samples at any informative microsatellite markers and SNPs (Supplementary Material, Table 4 and Fig. 5). Because no other family members were available from the sporadic cases BiCHMs 21, 24, 23 and 28, we could not assess the segregation of the disease phenotype with markers from the HMs 19q13.4 candidate region. The analyzed markers from this region were not homozygous in the patients with BiCHMs (Supplementary Material, Table 4).

Bisulfite treatment

Bisulfite treatment of the DNA was done as previously described (36). Briefly, we digested 50–100 ng of DNA with EcoRI (total volume of 21 μl). We denatured the DNA by mixing it with 4 μl of 2 M NaOH and incubation at 50°C for 15 min. After mixing with 2% low melting agarose, we formed the DNA–agarose beads by pipeting 10 μl of this mixture in ice-cold heavy mineral oil (Sigma). After the solidification of the beads, we added 500 μl of a 2.5 M sodium metabisulfite (Merck), 125 mM hydroquinone (Sigma) pH 5.0. We incubated the tubes for 30 min on ice then for 3 h 30 min at 50°C in the dark. The beads were washed four times in 1× TE (pH 8.0), treated twice with 0.2 M NaOH for 15 min, and washed twice with 1× TE for 10 min each. Prior to PCR amplification, the beads were washed twice with H2O for 10 min each, and melted at 70°C for 10 min. Five microliters of this melted agarose were used in each 50 μl PCR reaction. We performed all PCRs in two rounds of amplification to increase the specificity for the amplification of fully bisulfite-converted templates. Most samples have been subject to two independent bisulfite treatments and were analyzed from two independent PCR products.

SIRPH analysis

We performed the SIRPH as previously described (24). Briefly, to remove the unreacted PCR oligonucleotides and excess of dNTPs, we purified the PCR product using QIAquick gel extraction kit (Qiagen). We prepared the SNuPE reaction and the dHPLC conditions are listed in Table 2 (Supplementary Material). The SIRPH analysis was performed more than once on most products.

Cloning and sequencing

We used QIAquick gel extraction kit (Qiagen) to purify the amplified products, followed by cloning in pGEM-T Easy TA vector (Promega). Five to 50 insert-containing clones were
sequenced from each DMR using vector primers on an automated ABI 3700 capillary sequencer (Montreal General Hospital, Genome Quebec).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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