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Familial molar tissues due to mutations in the inflammatory gene, *NALP7*, have normal postzygotic DNA methylation

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ABSTRACT

An imprinting disorder has been believed to underlie the etiology of familial biparental hydatidiform moles (HMs) based on the abnormal methylation or expression of imprinted genes in molar tissues. However, the extent of the epigenetic defect in these tissues and the developmental stage at which the disorder begins have been poorly defined. In this study, we assessed the extent of abnormal DNA methylation in two HMs caused by mutations in the recently identified 19q13.4 gene, *NALP7*. We demonstrate normal postzygotic DNA methylation patterns at major repetitive and long interspersed nuclear elements (LINEs), genes on the inactive X-chromosome, three-cancer related genes, and CpG rich regions surrounding the *PEG3* differentially methylated region (DMR). Our data provide a comprehensive assessment of DNA methylation in familial molar tissues and indicate that abnormal DNA methylation in these tissues is restricted to imprinted DMRs. The known role of NALP7 in apoptosis and inflammation indicates that previously unrecognized pathways could directly or indirectly underlie the abnormal methylation of imprinted genes in molar tissues.

INTRODUCTION

Familial hydatidiform moles (HMs) are rare clinical entities where the molar tissues have a biparental contribution to their genome as opposed to sporadic androgenetic HMs that contain two copies of the paternal genome. Despite their genotypic differences, biparental and androgenetic moles are identical at the histopathology level, which led to a common belief that an imbalance in the expression of maternally and paternally imprinted genes plays a role in their pathology.

Others (Judson et al. 2002) and we (El-Maarri et al. 2003) analyzed the methylation patterns of imprinted genes in familial biparental HMs and demonstrated the absence and gain of maternal and paternal methylation marks, respectively. In our study, we analyzed two molar tissues, BiCHM9 and BiCHM16, from two sisters from family MoLb1 with defect in 19q13.4 while Judson *et al.* analyzed one tissue that is not caused by defect in 19q13.4. To investigate the origin of the abnormal DNA methylation in HM tissues caused by a defect in 19q13.4, we analyzed the methylation of the same imprinted genes, but in blood tissues from the two sisters and found a normal level and pattern of methylation (El-Maarri et al. 2005). We traced the grandparental origin of the abnormally methylated alleles in the moles and showed that the abnormal DNA methylation is not due to an abnormal erasure of the grandparental marks but was acquired *de novo* either in the maternal germline or during the postzygotic development and the proliferation of the moles. The most direct way to know when the abnormal DNA methylation began, before or after fertilization, is to examine the methylation marks in oocytes from the patients with recurrent HMs. However, in humans it is impossible to have access to oocyte materials from patients with rare reproductive diseases. In the present study, we investigated postzygotic DNA methylation in the same molar tissues from the two sisters by analyzing different CpG regions at which methylation is known to occur between fertilization and implantation or after implantation during early embryogenesis (Bird 1986; Mann and Bartolomei 2002; Reik and Walter 2001; Yoder et al. 1997). These CpGs are at repetitive elements and satellite sequences; the promoters of inactive genes on the inactive X-chromosome; and the promoters of three cancer related genes, *CDKN2A* (cyclin dependent kinase inhibitor 2A, also called *p16,*) and *CDH1* (E-cadherin*)*, and *HIC1* (hypermethylated in cancer), known to be abnormally hypermethylated in several sporadic HMs as well as other human cancers (Xue et al. 2004). Moreover, we assessed the extent of abnormal DNA methylation around the maternally expressed gene *PEG3* that is abnormally hypomethylated in these molar tissues by analyzing thirteen major CpG rich regions scattered in 762.5 kb around the *PEG3* DMR.

Altogether our data show normal DNA methylation at all the analyzed regions. This indicates, first, that postzygotic DNA demethylation and *de novo* methylation are normal in familial HMs with defects in *NALP7*; second, although a small number of cancer related genes were analyzed, no detectable and significant epigenetic changes seem to be occurring after implantation and during the proliferation of the trophoblast; and third, the abnormal DNA methylation previously observed at imprinted genes in these tissues is restricted to their DMRs and may reflect their intrinsic propriety in trophoblast tissues. Our recent identification of mutations in the inflammatory gene, *NALP7* (Murdoch et al. 2006), causing the analyzed HMs pinpoints an important pathway that may underlie the abnormal DNA methylation observed only at imprinted genes in HM tissues.

RESULTS

Methylation of repetitive elements

To investigate the DNA methylation of repetitive elements in molar tissues we used methylation sensitive restriction enzyme digestions followed by electrophoresis, Southern blotting, and restriction landmark genome scan (RLGS) on DNAs from two biparental HMs, BiCHM9 and BiCHM16, their parents; two androgenetic HM tissues, AnCHM23 and AnCHM28 (El-Maarri et al. 2003), and their parents; and five control chorionic villi obtained from therapeutic abortions of matching gestational stages. Using these approaches, we did not detect global hypo- or hypermethylation in the two familial HMs when compared to control villi. Centromeric satellite DNAs, major repetitive elements and ribosomal DNAs were all normally methylated in the two molar tissues from family MoLb1 (Fig. 1a). We noted an abnormal hypomethylation at the centromere of chromosome 8 (8q21) in only one sporadic androgenetic molar tissue, AnCHM23, that was not observed in control villi (data not shown).

We also compared the electrophoresis profiles after digestion with the methylation sensitive enzyme *Hpa*II and its methylation-insensitive isoschizomer *Msp*I before (Figure 1b) and after hybridization with a LINE consensus probe as previously described (Hansen 2003) (Fig. 1c). The results show normal global and LINE methylation in familial HMs from MoLb1 similar to that observed in normal control villi.

Methylation of the inactive X-chromosome

Methylation of the inactive X-chromosome was analyzed in BiCHM9, with a 46,XX [91]/46,XX,t(9;17)(q34;q21)[6] karyotype, at three genes known to be methylated on the inactive-X chromosome, androgen receptor (*AR*), zinc finger protein 261 (*ZNF261*), and phosphoglycerate kinase 1 (*PGK1*) (Allen et al. 1992; Beever et al. 2003; Gilbert and Sharp 1999). Using a PCR-based methylation assay, we demonstrate the presence of a methylated inactive-X at the three genes (Figure 2). As controls, we assessed the methylation of the promoters of *SMCX*, an X-linked gene that is active and not methylated on both X chromosomes (Agulnik et al. 1994), and *XIST*, known to be transcribed from the inactive X and methylated on the active X (Brown et al. 1991). Our data show the presence of an active X-chromosome with a non-methylated *SMCX* and a methylated *XIST* promoter in BiCHM9 as in normal female cells. These results indicate normal X inactivation in this biparental HM.

Methylation of three cancer related genes

To investigate methylation changes occurring after implantation and during the proliferation of trophoblast in these biparental HMs, we analyzed the promoters of three cancer related genes, *p16*, *E-Cadherin* and *HIC-1*, that were shown to be abnormally methylated in several cases of sporadic HMs. Using bisulfite treatment followed by methylation specific PCR (MSP) as described (Xue et al. 2004), we did not detect abnormal methylation at these three genes in BiCHM9 and BiCHM16: *p16* and *E-cadherin* were completely unmethylated while *HIC-1* displayed methylated and unmethylated alleles in both molar and control chorionic villi (Figure 3). Methylated *HIC-1* allele was not present in control DNA from total blood (data not shown) and seems to reflect tissue specific DNA methylation.

Methylation of thirteen CpG rich regions surrounding PEG3

We previously demonstrated an abnormal hypomethylation at the *PEG3* DMR in both BiCHM9 and BiCHM16. To investigate the extent of abnormal DNA methylation around *PEG3*, we identified 13 CpG rich regions in a 762.5 kb genomic fragment surrounding its DMR and analyzed their methylation status at two CpG sites using SIRPH (single nucleotide primer extension SNuPE followed by separation with ion pair reverse phase HPLC) as previously described (El-Maarri et al. 2002) (Supplementary Table 1). Our results at the thirteen CpG rich regions demonstrate normal levels of methylation in familial HMs similar to those observed in control villi (Figure 4). We note that one CpG (number 11) showed a different level of methylation as compared to controls, but this was observed only in one HM, BiCHM16.

DISCUSSION

Mammalian postzygotic development is characterized by a wave of demethylation followed by *de novo* methylation that is completed around the time of implantation (for review see(Mann and Bartolomei 2002; Reik and Walter 2001; Yoder et al. 1997). The active and passive demethylation waves of the paternal and maternal genomes, respectively, affect all CpGs with the exception of those at DMRs of imprinted genes (Santos et al. 2002). In female mammalian cells, DNA methylation of housekeeping genes on the inactive-X is one of the last X-inactivation steps and occurs around the blastocyst stage. To investigate whether these waves occur normally in familial biparental moles caused by a defect in 19q13.4, we used various approaches and analyzed two such molar tissues. We demonstrate the absence of abnormal methylation at the global genome level, repetitive and LINE elements, and five genes on the X-chromosome indicating that early postzygotic methylation mechanisms are properly functioning in these HMs. Beside CpG islands at the DMRs of some imprinted genes and inactive genes on the X chromosome, the methylation status of the all the remaining CpG islands (~30,000) is not known, but many are believed to be unmethylated. However, they can be methylated either in normal tissues during cellular programming and tissue differentiation (Futscher et al. 2002; Song et al. 2005) or in pathological tissues as a result of cancer where changes in DNA methylation are part of the genetic and epigenetic modifications occurring during tumorigenesis (Hatada et al. 2006; Smiraglia and Plass 2002). In cancer, these changes occur at the promoters of tumor suppressor genes and are specific for each type of tumor. Our analysis of three cancer-related genes showed their normal methylation in familial HMs.

The *PEG3* DMR was shown to be invariably and severely hypomethylated in all analyzed familial molar tissues (El-Maarri et al. 2003). The assessment of 13 CpG rich regions around the *PEG3* DMR revealed a variety of normal methylation patterns, from highly methylated to completely unmethylated, but no abnormal hypo- or hypermethylation was detected at any of the loci in the two familial HMs, demonstrating that the abnormal DNA methylation in HMs is restricted to imprinted DMRs.

NALP7 has no known role in establishing or maintaining DNA methylation and does not contain any DNA binding domain. Therefore, it is unlikely that it plays a direct role in the abnormal imprinting seen in these HMs. The abnormal DNA methylation observed in familial HMs seems to be a consequence of the defect. *NALP7* is transcribed in early oocyte stages, germinal vesicle and metaphase I (Murdoch et al. 2006). It is, thus, possible that a defective NALP7 leads to abnormal oocyte maturation and growth and subsequently abnormal establishment of maternal methylation marks on imprinted genes. *NALP7* is also known to regulate IL-1β, a pleiotropic cytokine that activates a number of inflammatory pathways required for blastocyst implantation and throughout gestation (McMaster et al. 1993; Rivier and Vale 1990; Strakova et al. 2005). HMs are characterized by the presence of swollen chorionic villi with fluid accumulation, which is one of the hallmarks of inflammation. In addition, the abundance of activated decidual T cells in the endometrium of patients with the common form of HMs (Wongweragiat et al. 1999) is indicative of a dysregulated endometrial inflammation. This suggests that a dysregulated maternal inflammation of the endometrium may also be responsible for moles caused by defects in *NALP7*. Although, at the present time, it is not clear how an inflammatory response in the mother could interfere with the DNA methylation of imprinted genes in the conceptuses, it is conceivable that it may cause an unfavourable and hostile local environment for the implanting blastocyst to maintain proper imprinting marks. A number of observations support the influence of the environment on the maintenance of imprinting marks (i) the increase in imprinting disorders in individuals born following artificial reproductive technologies (Maher et al. 2003; Wrenzycki et al. 2005), (ii) the defective imprinted gene methylation in cloned animals (Humpherys et al. 2001), in intra- and interspecies crosses (Vrana et al. 1998) and (iii) in tumors (Smiraglia and Plass 2002). In the latter, epigenetic changes on imprinted genes seem to reflect the intrinsic properties of their CpG islands, with some being methylation-prone and others resistant, in response to changes in the level of DNA methyltransferases that occur frequently in many tumors (Feltus et al. 2003).

Altogether, our data indicate an overall epigenetic stability of familial biparental molar tissues, which is in agreement with the genetic stability of HMs in general and their benign outcome.

MATERIALS AND METHODS

Subject materials

Chorionic villi from HMs and therapeutic abortions were obtained from consenting subjects. Fresh tissues were dissected under a stereomicroscope and used to extract DNA.

Restriction landmark genome scan

Genomic DNA was digested with EcoRV and NotI separated in the first dimension and *in situ* digested with *Hinf*I before the separation in the second dimension as previously described (Asakawa et al. 1995).

Methylation sensitive PCR assay

DNA was overdigested with the methylation-sensitive restriction enzymes *Hpa*II (for *AR, SMCX*, *PGK1*), *Hha*I (for *ZNF261*) and *Hha*I, *Ava*I and *Bst*UI (for the *XIST* methylation analysis). The primers flanked at least one methylation-sensitive restriction site. The primers and PCR conditions are as described (Allen et al. 1992; Beever et al. 2003; Gilbert and Sharp 1999).

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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FIGURES

Figure 1

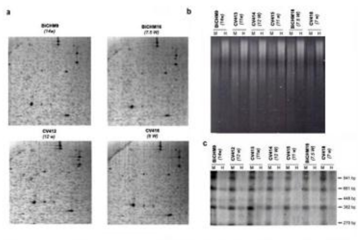


Figure 2

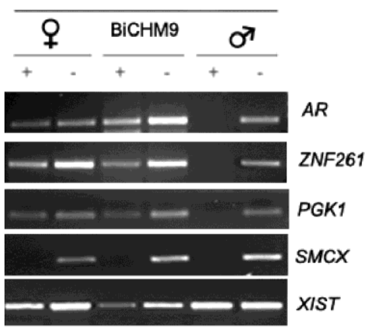


Figure 3

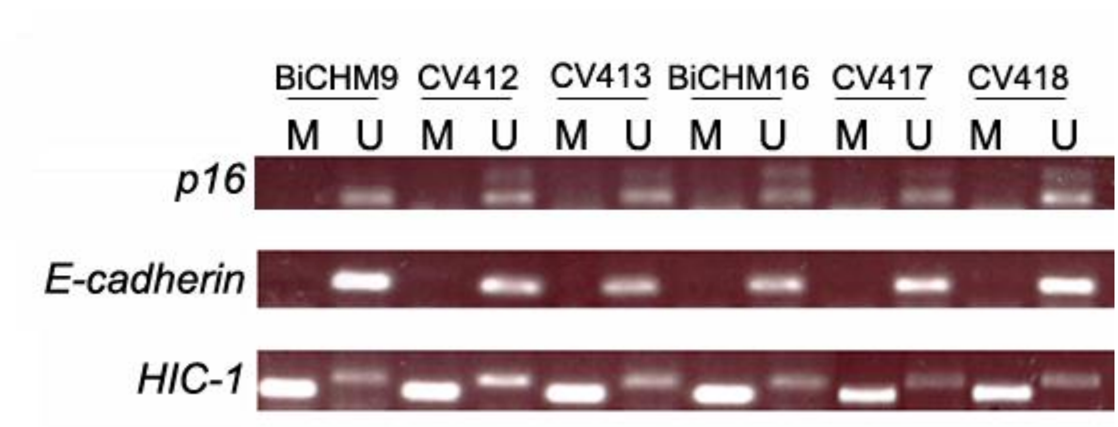
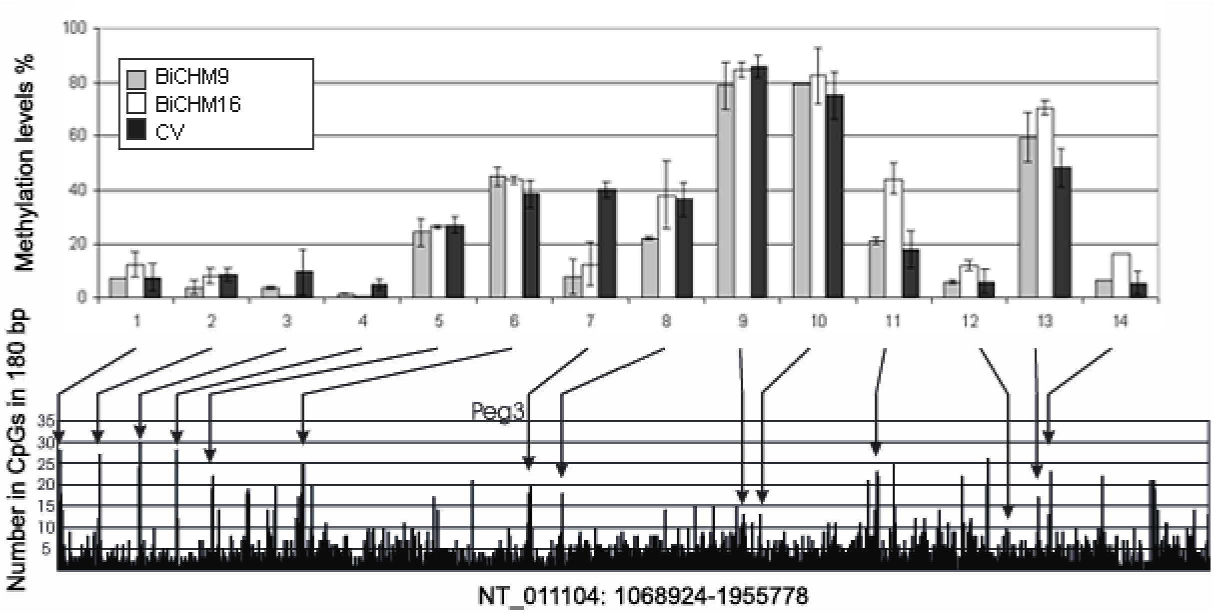


Figure 4



LEGENDS TO FIGURES

Figure 1. **Assessment of DNA methylation at the global genome level** using methylation sensitive restriction enzyme digestions in two biparental molar tissues from MoLb1, BiCHM9 and BiCHM16, and control chorionic villi of matching gestational stages, CV412 to 418. The gestational stages in weeks are between parentheses. **a**, restriction landmark genome scan (RLGS) was performed as previously described (Asakawa et al. 1995). Genomic DNA was digested with EcoRV and NotI before the separation in the first dimension and *in situ* with HinfI before the second dimension. We note the presence of two polymorphic spots from ribosomal DNA repeats with strong signals in BiCHM16 (top right) that were also observed in the parental DNA and in control villi, CV412. **b**, Genomic DNAs were digested with EcoRI and either MspI (M) or HpaII (H), separated on a 1% agarose gel containing ethidium bromide and visualized with UV. **c**, Analysis of L1 methylation. Genomic DNAs, digested as described in **b**, were

transferred into nylon membranes and hybridized with a consensus L1 probe generated by PCR amplification between primers L1t-1f:505r as previously described (Hansen 2003). The comparison of the banding patterns of the MspI and HpaII digestions indicates that L1 elements are similarly methylated in molar and normal chorionic villi.

Figure 2. Methylation analysis of X-linked genes. Genomic DNAs from BiCHM9, and its father and mother were digested with methylation-sensitive enzymes and used as a PCR template. The primers flank several methylation-sensitive restriction sites and, thus, the DNA could only be amplified if the specific CpG sites are methylated. The promoters of the genes used for the analysis are methylated only on the inactive X (*AR*, *ZNF261* and *PGK1*), only on the active X (*XIST*) or neither X-chromosome (*SMCX*).

Figure 3. Methylation analysis of cancer loci. Bisulphite treatment was carried out on DNA samples from molar tissues and control chorionic villi as previously described (Xue et al. 2004). PCR amplification was performed using primers specific for the converted methylated (M) and unmethylated (U) CpG sites in the promoter regions of *p16, E-cadherin* and *HIC-1*.

Figure 4. **Quantitative methylation analysis of a 762.5 kb region around the *PEG3* DMR.** Thirteen major CpG rich regions were investigated in both molar tissues BiCHM9 and BiCHM16 and normal chorionic villi. Each column represents the average of methylation of two individual CpG sites at each of the 13 CpG rich regions where two independent measurements were done. Each of the measurements was derived from two independent bisulfite treatments. The standard deviation between the different measurements is shown as vertical bars. The third column represents an average of 6 to 7 different chorionic villi samples. The lower part of the

graph represent a schematic diagram of the CpG densities in the 762.5 kb region around the *PEG3* DMR, the 13 major CpG regions investigated in this study are labelled with arrows that connect each of them to the corresponding measured methylation levels. The details of the studied regions, PCR conditions, and primers locations on the NT_011104 contig are summarized in Supplementary table 1.