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The maternally transcribed gene p57^{KIP2} (*CDNK1C*) is abnormally expressed in both androgenetic and biparental complete hydatidiform moles

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Hydatidiform mole (HM) is an abnormal gestation characterized by trophoblast hyperplasia and overgrowth of placental villi. The genetic basis in the vast majority of cases is an excess of paternal to maternal genomes, suggesting that global misexpression of imprinted genes is the common molecular mechanism underlying the genesis of this condition. Although most complete HM are androgenetic in origin, a rare, frequently familial, biparental variant has been described. Here we evaluate the expression of p57^{KIP2}, the product of *CDKN1C*, an imprinted, maternally expressed gene in a series of these rare, biparental complete HM (BiCHM). We observed dramatic underexpression of p57^{KIP2} in BiCHM, identical to that seen in complete HM of androgenetic origin (AnCHM). The series included two sisters, both of whom had BiCHM. Genotyping of this family identified a 15 cM region of homozygosity for 19q13.3–13.4 similar to that found in three other families with recurrent BiCHM. These results demonstrate that BiCHM, like AnCHM, result from abnormal expression of imprinted genes. In addition we provide further evidence for a major control gene on 19q13.3–13.4 which regulates expression of imprinted genes on other chromosomes.

INTRODUCTION

The abnormal pregnancy, hydatidiform mole (HM) represents a disorder of genomic imprinting, a phenomenon whereby genes are monoallelically expressed from the maternally or paternally-derived copy of the gene, the other being transcriptionally silent. HM are classified on the basis of histopathology and genetic origin as partial (PHM) or complete (CHM) (1). The majority of HM develop from conceptuses with an excess of paternal to maternal genomes and consequently result from the global misexpression of imprinted genes. PHM generally arise from a dispermic conception (2–4) which is consequently triploid, with one maternal and two paternal contributions to the nuclear genome. In CHM, maternal chromosomes are generally absent. The conception is diploid, but androgenetic, in that all 46 chromosomes are paternal in origin (5,6). Thus trophoblastic hyperplasia, common to both androgenetic CHM (AnCHM) and PHM, is associated with the overexpression of paternally transcribed genes. Foetal development, although abnormal, is present in PHM which have a maternal contribution to the genome. Lack of foetal development in AnCHM is therefore attributed to the absence of maternally transcribed genes in these pregnancies.

CHM may originate by dispermy (7) but are more likely to be monospermic, arising from fertilization of a functionally anucleate egg by a single sperm whose pronucleus undergoes endoreduplication to produce a diploid chromosome complement (5,6). A third and much rarer type of CHM is also diploid, but biparental (BiCHM), rather than androgenetic, in origin (8–12). Unlike AnCHM, which generally occur sporadically, BiCHM often occur in patients who have a history of multiple CHM arising in different conceptions (12,13) and more specifically, in the affected women of families in which two or more individuals have molar pregnancies (12,14,15). In cases, known to be familial, the condition appears to exhibit an autosomal recessive mode of inheritance. Although the gene(s) involved and the nature of the underlying mutation have yet to be elucidated, linkage studies have identified a region on 19q13.3–13.4 in which the gene for familial BiCHM resides (14).

BiCHM exhibit all the histopathologic hallmarks of classic AnCHM, including trophoblast hyperplasia and atypia, lack of embryonic development and abnormal villous mesenchyme.

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Indeed, BiCHM and AnCHM are histologically indistinguishable (13), requiring DNA analysis to discriminate between them. BiCHM, like AnCHM, also have a significant risk of persistent trophoblastic disease (15, unpublished data). Thus, despite their distinct genetic origins, BiCHM and AnCHM are strikingly similar in all phenotypes, suggesting that BiCHM are also the result of abnormal imprinting.

To explore this possibility we examined the expression of $p57^{KIP2}$, the product of the imprinted (maternally expressed) gene *CDKN1C* (16), in BiCHM. This protein has been previously shown to be strongly expressed in placenta and its differential pattern of expression in HM and non-molar placental villi has been well-characterized. $p57^{KIP2}$ is readily detectable in cytotrophoblast and villous mesenchyme of PHM and non-molar gestations, but is markedly under-expressed in these cell types in CHM, consistent with the absence of a maternal genome in the latter (17,18). In previous studies, the genetic origin of the CHM examined was not determined for most cases. However, given the rarity of BiCHM, it is unlikely that BiCHM were included in these studies. Here we evaluate $p57^{KIP2}$ expression in a series of genetically defined BiCHM, including molar tissue from two sisters with familial BiCHM.

RESULTS

Genotyping of chromosome 19q in family CX01

Homozygosity mapping (19) of DNA from six members of family CX01 with 14 highly polymorphic short tandem repeats established a 15 cM region of homozygosity on 19q13.3–13.4 extending from *D19S924* to the telomere of 19q in the two sisters with recurrent BiCHM. This region of homozygosity was not found in either of two sisters who had normal offspring and no molar pregnancies (Fig. 1).

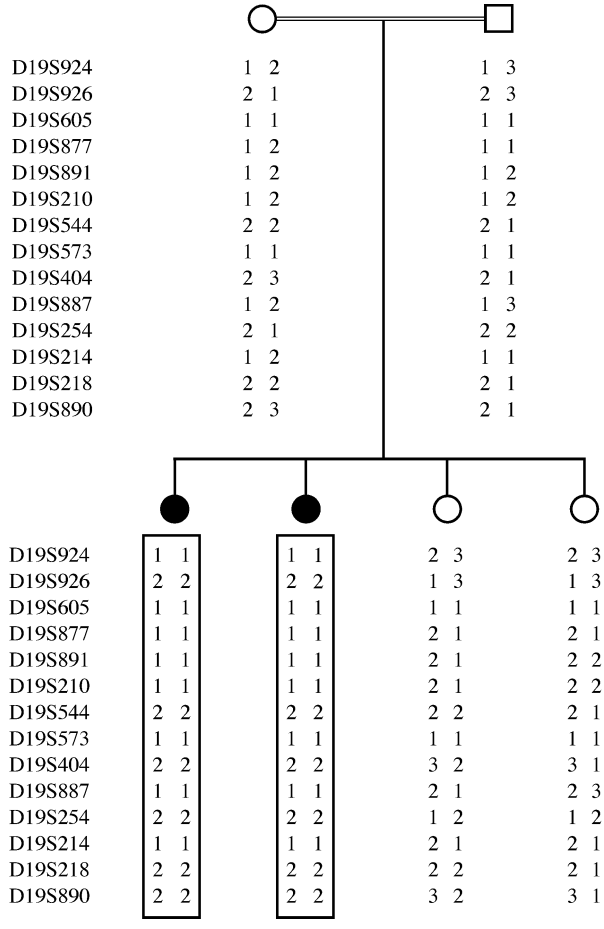

Figure 1. Pedigree of family CX01 showing haplotypes of parents, affected sisters (filled circles) and unaffected sisters (open circles). The boxed region represents a 15 cM region of homozygosity in the affected sisters. Marker order was based on data from the human genome map viewer (http://www.ensembl.org/Homo_sapiens/) and refined by overlapping sequenced chromosome 19 genomic clones (20).

$p57^{KIP2}$ expression in genetically defined hydatidiform moles

One AnCHM (case 8), one PHM (case 9) and ten BiCHM (cases 1–7) were evaluated for $p57^{KIP2}$ expression using immunohistochemistry (Table 1). In the PHM strong $p57^{KIP2}$ nuclear staining was observed in a significant percentage of CT and VM cells (Fig. 2A1–2). This pattern of $p57^{KIP2}$ expression was indistinguishable from that previously observed, by us (18) in non-CHM gestations, including PHM, normal placenta and spontaneous abortions. In contrast, the classic AnCHM showed lack of $p57^{KIP2}$ expression in CT and VM (Fig. 2B1–2), consistent with previous results for CHM (17,18). In the CHM, maternal decidua or intervillous trophoblast islands served as internal positive controls for $p57^{KIP2}$ immunostaining.

Strikingly all ten cases of BiCHM were negative for $p57^{KIP2}$ expression in CT and VM in all villi, whereas internal controls were uniformly positive (Fig. 2C1–2). Consequently, the expression pattern of $p57^{KIP2}$ in BiCHM is identical to that of AnCHM, despite their distinct genetic origins.

Table 1. Case series of genetically defined hydatidiform moles

Case	HM tested	Histopathological diagnosis[a]	Genetic diagnosis	$p57^{KIP2b}$
1	HM3	CHM	Biparental	–
2	HM1	CHM	Biparental	–
3	HM6	CHM	Biparental	–
4	HM1	CHM	Biparental	–
5	HM2, HM3	Both CHM	Biparental	–
6	HM2, HM3	Both CHM	Biparental	–
7	HM3, HM4	Both CHM	Biparental	–
8	HM1	CHM	Monospermic CHM	–
9	HM1	PHM	Dispermic PHM	+

[a]CHM, complete hydatidiform mole, PHM, partial hydatidiform mole.
[b]For the $p57^{KIP2}$ expression pattern, + indicates strong positive staining in the villous mesenchyme and cytotrophoblast cells, – indicates cases which were negative for $p57^{KIP2}$ in villous mesenchyme and cytotrophoblast cells.

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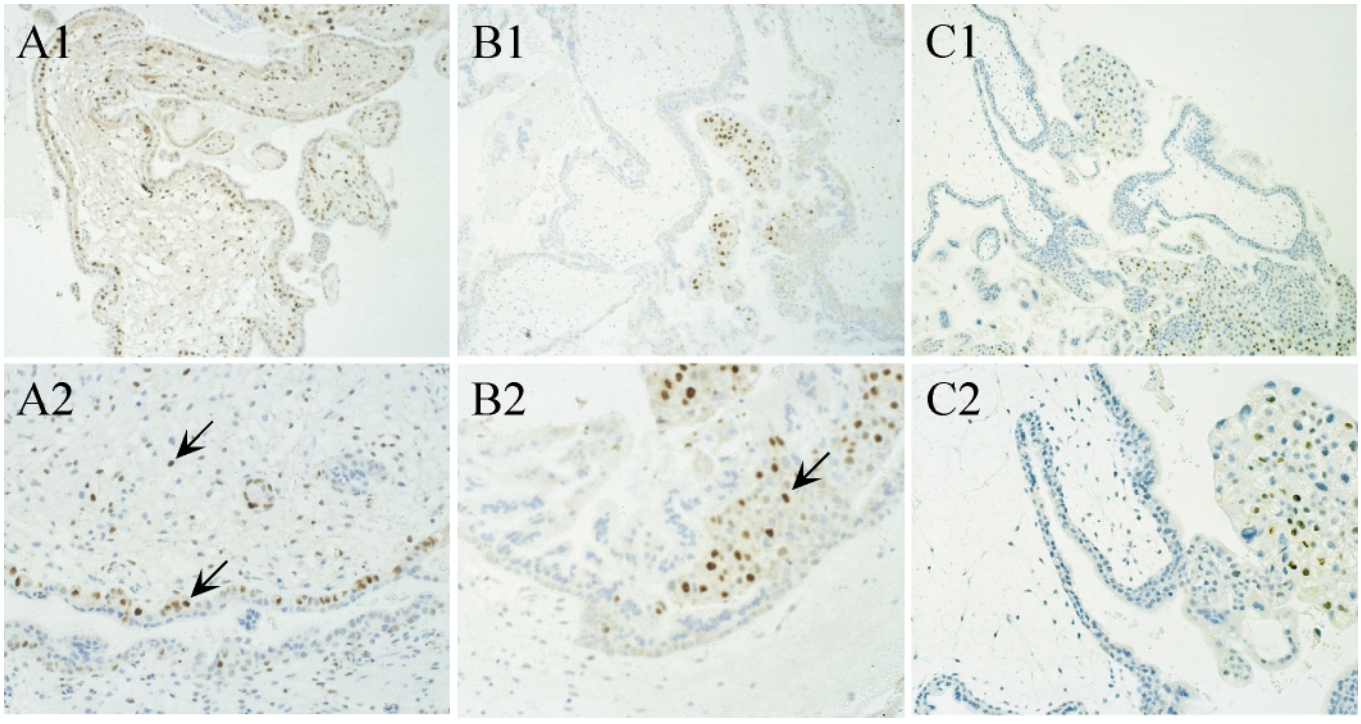

Figure 2. Examples of $p57^{KIP2}$ immunostaining in genetically defined hydatidiform moles, counterstained with hematoxylin (A1–C1 × 40; A2–C2 × 100). (**A1–2**) PHM (case 9) showing $p57^{KIP2}$ nuclear staining in CT and VM cells of chorionic villi (arrows). (**B1–2**) AnCHM (case 8) showing absence of $p57^{KIP2}$ expression in CT and VM, with positive staining of extravillous trophoblast (arrows). (**C1–2**) BiCHM (case 4), showing absence of $p57^{KIP2}$ expression in CT and VM, with positive staining of extravillous trophoblast.

DISCUSSION

The importance of genomic imprinting in normal development is underscored by the growing number of pathological conditions known to result from dysfunction of imprinted genes. Among these conditions, PHM and AnCHM are remarkable in that they result from an excess of paternal to maternal genomes and are thus likely to result from global misexpression of imprinted genes. Consistent with this view, the imprinted, paternally expressed loci *ZNF127*, *ZAC/PLAGL* and *HYMAI* have been shown to have a paternal methylation imprint in CHM (21–23) while other studies have shown abnormal expression of the imprinted, maternally transcribed genes *H19* (24,25) and *CDKN1C* (17,18) in these conceptuses. These results are consistent with the absence of a maternal nuclear genome in AnCHM.

A rare variant of CHM has occasionally been described with an apparently normal chromosome complement, being diploid with both a paternal and maternal contribution to the nuclear genome. These BiCHM are associated with recurrent CHM (12,13) and are familial in at least some cases (12,15). By analogy with AnCHM, which is histologically indistinguishable from BiCHM, the gene for familial BiCHM is likely to affect imprinting of a number of different genes. Since only females are affected and the condition may occur with more than one partner (13), the defect is likely to be in a gene involved in the establishment and/or maintenance of normal imprints in the ovum. In a recent study of a single case of BiCHM (26), bisulphite sequencing of a number of imprinted genes on different chromosomes showed them to have a paternal, rather than a maternal, epigenotype on both alleles, consistent with a failure to establish maternal imprints within the ovum.

In this study we have examined expression of the maternally transcribed gene, *CDNK1C*. We have shown expression of $p57^{KIP2}$ to be abnormal in all ten BiCHM examined, with an androgenetic pattern of expression rather than that seen in other types of biparental conceptuses. Thus we have shown that abnormal imprinting is a common mechanism underlying the development of BiCHM. In AnCHM, both overexpression of paternally transcribed genes and loss of maternally transcribed genes would be predicted. Thus loss of $p57^{KIP}$ expression in androgenetic tissues is likely to result from an absence of the maternally transcribed allele (17). More recently it has been shown that expression of *Cdnk1c*, not only requires a maternally derived allele, but is also dependent on a maternal imprint during oogenesis (27). It has now been clearly demonstrated that, in mice, a number of genes, including *Cdnk1c*, are regulated *in cis* by the maternally methylated, imprinting control centre, *KvDMR1* (28,29). Silencing of $p57^{KIP2}$ in BiCHM is consistent with both alleles of *KvDMR1* remaining demethylated and provides further evidence that the underlying defect in BiCHM is a global failure to set the maternal imprint during oogenesis.

It is unclear whether loss of $p57^{KIP2}$ plays a role in the development of CHM. *Cdkn1c* deficient mice show abnormal cell proliferation and differentiation leading to a variety of developmental defects (30) including placentomegaly (31) demonstrating that $p57^{KIP2}$ plays a role in the regulation of

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trophoblastic development in mice. In man, mutations in *CDNK1C*, or loss of $p57^{KIP2}$ expression, occur in Beckwith–Wiedemann syndrome (32) in which the placenta shares pathological features, such as villous hydrops, with hydatidiform moles (33). However, although less marked than in CHM, molar development also occurs in PHM in which $p57^{KIP2}$ is apparently normal. In addition, we have identified a single case of CHM, pathologically indistinguishable from other CHM, which, although androgenetic for other chromosomes, was trisomic for 11p15.5 and positive for $p57^{KIP2}$ staining, (D. Castrillon *et al.*, manuscript in preparation). These observations suggest that downregulation of $p57^{KIP2}$ is not essential for the development of CHM.

Since tumourigenesis may be associated with the loss of cell cycle regulators such as cyclin dependent kinases, loss of $p57^{KIP2}$ may play a role in the very high incidence of choriocarcinoma which develop after CHM compared to other types of pregnancy (34). In support of this, $p57^{KIP2}$ expression was undetectable in 3 cases of choriocarcinoma (17). Despite the small number of BiCHM described, the development of trophoblastic tumours has been observed in several cases (15, unpublished data). BiCHM, like AnCHM, appear to be a greater risk factor for trophoblastic tumour development than normal pregnancies. Larger series of BiCHM need to be examined to determine the relative risk of post-mole tumours after AnCHM or BiCHM.

The gene involved in BiCHM has not yet been identified although it is unusual in that it appears to control imprinting of genes on a number of chromosomes, *in trans* and is, therefore, unlike previously described imprinting control centres that act locally, *in cis* (32,35). In mice, loss of maternal methylation imprints in their oocytes has been reported in females homozgous for a deletion of the methyltransferase related gene product, *Dnmt3L* (36). Progeny of these mice die before mid-gestation, show defects which are common consequences of abnormalities in extra-embryonic tissues and lack expression of a number of genes dependent on a maternal imprint including *Cdnk1c* (36). They therefore share a number of characteristics with CHM. However, sequencing of all 12 exons of *DNMT3L* (37), revealed no mutations in either of the two sisters with recurrent BiCHM or two patients with sporadic BiCHM, described in this report (unpublished data). In man *DNMT3L* maps to chromosome 21q22.3 and is unlikely to be the critical gene mutated in familial BiCHM since a candidate locus has been mapped to 19q13.3–13.4 by linkage and homozygosity mapping (14,15). In this report we describe a fourth family with recurrent BiCHM with a large region of homozygosity across the19q13.3–13.4 region segregating with the disease gene. Further studies of families with this rare condition will enable the identification of one or more genes with a critical role in the control of imprinting.

Identification of genes critical in the abnormal development associated with molar pregnancies is complicated by paternal disomy of the whole nuclear genome and, in AnCHM, loss of the entire maternal chromosome complement. BiCHM however, have an equal maternal and paternal contribution to the genome and provide a unique opportunity to investigate the basic mechanisms underlying genomic imprinting and the role of imprinted genes in the early development of embryonic and extraembryonic tissues and in tumour development.

MATERIALS AND METHODS

Patient material

Patients with recurrent molar pregnancies were identified from patients registered with the Trophoblastic Tumour Screening and Treatment Unit at Charing Cross Hospital. Histopathological review of molar tissue was performed on routine sections stained with haematoxylin and eosin, to distinguish PHM from CHM, by at least two independent pathologists (38). Where available, parental blood and molar tissue were genotyped, as previously described, to confirm the diagnosis of PHM and, in case of CHM, distinguish between CHM of androgenetic or biparental origin (13). Briefly, DNA was prepared from parental blood and molar tissue and, in each case, at least 6 informative markers (Human Genome Database, http//www.gdb.org) examined in DNA from the patient, her partner and the molar tissue. HM were classified as AnCHM if at least two markers in the HM had no maternal allele and all other markers were consistent with a paternal origin. HM were classified as PHM if three alleles were present at two or more loci and the additional allele was paternal in origin. CHM were classified as BiCHM if both a maternal and a paternal marker were present for each locus tested and there was no evidence of trisomy.

Six patients with recurrent molar pregnancies of biparental origin were identified (Table 1). One patient had a sister with a single BiCHM. The five other patients had no other affected family members. Expression of $p57^{KIP2}$ was examined in tissue from a single molar pregnancy in each of the two sisters (cases 1 and 2), a single molar pregnancy from two other patients with recurrent BiCHM (cases 3 and 4) and two different pregnancies in the remaining three patients (cases 5–7). Tissue from an AnCHM (case 8) and a PHM (case 9), obtained from patients with recurrent CHM of androgenetic origin and recurrent PHM, respectively, were also examined as controls.

Genotyping of family with BiCHM

Two sisters with BiCHM in this study were the progeny of a consanguineous marriage. They had two further sisters both of whom had normal pregnancies and no history of HM. The four sisters and their parents (family CX01) were genotyped, as previously described (13), with 14 highly polymorphic microsatellite markers located within the 19q13.3–13.4 region (Fig. 1). The most likely haplotypes for the six individuals were derived by minimizing the number of recombination events.

Antibodies and immunohistochemistry

Immunocytochemistry was performed with mouse monoclonal antibodies against the $p57^{KIP2}$ protein (NeoMarkers/Lab Vision Corporation, CA) on paraffin-embedded formalin-fixed tissue. Antigen retrieval was performed at 93°C in 10 mM sodium citrate buffer pH 6.0 for 30 minutes with a 10 minute cooldown.

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The detection system was Envision (Dako Corporation, CA) with diaminobenzidine as the chromogen; slides were counter-stained with hematoxylin. Only distinct nuclear staining of similar intensity to that observed in internal controls was scored as positive. In all cases, the presence or absence of nuclear staining was assessed in villous mesenchyme, cytotrophoblast, extravillous trophoblast and decidua, blinded to the original diagnosis and independent of the H&E histologic appearance. For a case to be scored as positive for $p57^{KIP2}$ expression, staining was required in a significant proportion of villous mesenchyme (VM) and cytotrophoblast (CT) cells (>10% but typically around 30%). Sporadic $p57^{KIP2}$ expression in VM and CT cells (<1% of total cells) was not considered positive.

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