Genetics of Gestational Trophoblastic Disease

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Abstract

The abnormal pregnancies complete and partial hydatidiform mole are genetically unusual, being associated with two copies of the paternal genome. Typical complete hydatidiform moles are diploid and androgenetic while partial hydatidiform moles are diandric triploids. While diagnosis can usually be made on the basis of morphology, ancillary techniques that exploit their unusual genetic origin can be used to facilitate diagnosis. Genotyping and p57 immunostaining are now routinely used in the differential diagnosis of complete and partial hydatidiform moles, for investigating unusual mosaic or chimeric products of conception with a molar component and identifying the rare diploid, biparental hydatidiform moles associated with an inherited predisposition to molar pregnancies. Genotyping also plays an important role in the differential diagnosis of gestational and non-gestational trophoblastic tumours and identification of the causative pregnancy where tumours are gestational. Recent developments include the use of cell free DNA for non-invasive diagnosis of these conditions.

Key Words: Complete hydatidiform mole, partial hydatidiform mole, familial recurrent hydatidiform mole, p57 immunostaining, genotyping, trophoblastic tumour

Introduction

Gestational trophoblastic diseases (GTD) include the premalignant complete (CHM) and partial hydatidiform mole (PHM) together with the malignant conditions invasive mole, choriocarcinoma, placental site (PSTT) and epithelioid trophoblastic tumour (ETT). While morphology underlies the diagnosis of these diseases, an understanding of their genetic basis has enabled the development of ancillary techniques that are now routinely used in the differential diagnosis of both hydatidiform moles (HM) and gestational trophoblastic tumours (GTT). In addition these techniques have led to the identification of unusual subsets of molar pregnancies and enabled refinement of diagnosis, not possible on the basis of morphology alone.

Genetics of Hydatidiform Moles

(i) Complete and Partial Hydatidiform Moles

HM, or molar pregnancy, an abnormal pregnancy characterised by overgrowth of the placenta and poor fetal development, has an incidence in the UK of ~1 in 600 conceptions [1]. HM can be classified as CHM or PHM on the basis of pathology and genetics [2,3]. Morphologically CHM are characterized by chorionic villi with budding architecture, stromal karyorrhectic debris and extensive villous trophoblastic hyperplasia. In contrast, villi of PHM are variously sized with scalloped outlines, often have vessels with fetal red blood cells and trophoblastic hyperplasia is focal [4]. Genetically PHM are triploid, having an extra set of chromosomes from the father of the pregnancy. They usually result from fertilisation of an ovum by two sperm [5,6] (Figure 1a) or, very occasionally, a diploid sperm [7,8]. While PHM may be 69,XXX,

69,XXY or 69,XYY, the proportion of 69,XYY PHM in most series is considerably lower than the expected 25% [8,9]. CHM are diploid with both sets of chromosomes from the father and no contribution to the nuclear genome from the mother [10,11]. While ~ 15-25% of CHM arise by dispermy [8,12] (Figure 1b), the majority result from fertilization of an ovum by a single sperm with subsequent reduplication of the sperm genome [13,14] (Figure 1c). Since all monospermic CHM have a female, 46,XX karyotype, 46,YY conceptuses are presumed to be non-viable. How, and when, the maternal chromosomes are lost in CHM remains controversial. It has been suggested that loss results during postzygotic diploidisation of a triploid conceptus [15]. However, recent evidence from mouse models [16] supports earlier theories that the ovum may in fact be anucleate when fertilised due to loss of chromosomes or chromatids, to one of the polar bodies, during meiosis.

(ii) Recurrent Hydatidiform Moles

While most molar pregnancies are sporadic, any woman who has a HM is at increased risk of a subsequent molar pregnancy, ~1% after a single HM, increasing to ~13% after two or more HM [17]. Recurrent PHM are rare and are usually interspersed with normal pregnancies [17]. However, a small subset of women with recurrent CHM have an inherited predisposition to molar pregnancies and subsequent pregnancies are most likely to be further CHM [17]. This condition, familial recurrent hydatidiform mole (FRHM) is an autosomal recessive disorder characterized by diploid, biparental CHM (BiCHM), with a chromosomal complement from each parent (Figure 1d), in contrast to the androgenetic origin of typical CHM (AnCHM) [18,19]. Biallelic

mutations in two maternal effect genes, NLRP7 [20] and KHDC3L [21] account for ~75-80% and ~5-10% of cases of FRHM respectively. Women with this condition rarely achieve a normal pregnancy and oocyte donation is usually recommended to ensure normal embryo development [22, 23]. A smaller number of women with recurrent CHM have recurrent AnCHM [17] but these women may also have normal pregnancies in addition to the CHM, do not have other affected family members and do not have mutations in NLRP7 or KHDC3L [24]. For these women, in vitro fertilisation (IVF) with pre-implantation genetic diagnosis, to exclude androgenetic conceptions, can achieve a normal pregnancy [25].

(iii) Genomic Imprinting in Hydatidiform Moles

A small number of mammalian genes, ~100 in man [26], are unusual in that they are only transcribed from the maternally- or paternally-inherited allele. Expression of the allele inherited from the other parent is suppressed or “imprinted”, a phenomenon known as genomic imprinting. Both PHM and AnCHM, have two copies of the paternal genome, exhibit over-expression of paternally-transcribed genes and excessive trophoblastic proliferation. The relatively milder phenotype of PHM including the presence of embryonic development, virtually absent in CHM, is thought to be due to the additional expression of maternally-transcribed genes in PHM. BiCHM in women with either NLRP7 or KHDC3L mutations show loss of maternal imprinting similar to that of AnCHM [27-30] suggesting that NLRP7 and KHDC3L are required for correct imprinting during ovum development and early embryogenesis. Loss of appropriate imprinting results in a molar phenotype for any products of

conception (POC) in affected women including normal biparental conceptions and digynic triploids [31, 32], genotypes not normally associated with HM.

Differential Diagnosis of Hydatidiform Moles

A distinction between CHM, PHM and non-molar pregnancies is essential as women with a PHM or CHM have a 1.1% and 13.6% chance respectively of requiring chemotherapy for persistent disease following evacuation of the HM [1], whilst the risk for women with non-molar pregnancies is extremely low.

Improved antenatal care, earlier recognition of abnormal POC and evacuation at an earlier gestational age has made morphological diagnosis of HM more difficult in recent years and hence the need for ancillary techniques based on their genetic origin. These techniques are also important for the diagnosis of women with FRHM for whom future pregnancies are almost certain to be CHM with the associated risk of persistent disease that requires chemotherapy. Although CHM in these women can sometimes show atypical features [33-35] they are often pathologically indistinguishable from AnCHM [33]. Diagnosis relies on demonstrating that they are morphologically HM and genetically diploid and biparental, or rarely a digynic triploid, and therefore additional analyses is required for accurate diagnosis.

Ancillary Techniques in the Diagnosis of Hydatidiform Moles

A number of ancillary techniques have now been used to exploit the unusual genetic origin of HM and facilitate their diagnosis. Most significant has been the use of p57 immunostaining in the diagnosis of CHM and genotyping for the, often more difficult, differential diagnosis between PHM and non-molar

miscarriage. Genotyping has also enabled more accurate diagnosis of FRHM, mosaic conceptions with molar change and aneuploid conceptions.

(i) Immunostaining of p57 in Hydatidiform Moles

CDKN1C, a gene located on chromosome 11, is maternally-expressed in the nuclei of both the villous cytotrophoblast and stroma. Thus, immunohistochemical staining for its protein product, p57, can be used to distinguish between CHM (which lack staining due to the absence of a maternal genome) [36-37] and all other POC that express p57 in these cells (Figure 2). As CDKN1C is not imprinted in the extravillous trophoblast, expression of p57 is retained in these cells, even in CHM, providing a useful internal control for the staining procedure. While p57 immunostaining cannot distinguish PHM from non-molar miscarriages, it can play a role in the differential diagnosis of PHM from CHM with coexistent twin pregnancy. In PHM all villi show positivity but in a twin pregnancy both a positive and a negative population of villi are seen [38]. p57 immunostaining may also be useful confirmation that a diploid conception with atypical morphology is a BiCHM rather than a non-molar pregnancy since, like typical AnCHM, BiCHM are usually p57-negative [28]. p57 immunostaining and morphology may occasionally appear inconsistent and caution should be applied in these rare cases. For example POC, that are morphologically CHM, may express p57 due to retention of a maternal chromosome 11 [39, 40] while PHM may be p57-negative due to loss of the maternal copy of chromosome 11 [41].

A consequence of the wider use of p57 immunostaining has been greater observation of mosaic POC with a molar component, in particular POC comprising two populations of diploid cells, one normal and one androgenetic [42]. These androgenetic/biparental conceptions, comprising up to 2% of POC referred for suspected HM [43], show a wide range of discordant (Figure 2c-d) and divergent p57 expression within and between placental villi [44, 45]. While not often having the typical morphology of CHM it is clinically important to recognise these conceptions because of the malignant potential the presence of the androgenetic cell line confers [43, 46].

(ii) Genotyping in the Diagnosis of Hydatidiform Moles

Since early studies using chromosomal polymorphisms identified the genetic origin of HM, a number of different types of DNA polymorphisms have been used in the diagnosis of GTD. Currently, microsatellite polymorphisms, based on short tandem repeats (STR) and amenable to amplification using the polymerase chain reaction (PCR), have made rapid genotyping of DNA from formalin-fixed, paraffin-embedded tissue sections feasible. Cells of interest, usually maternal decidua and molar villi, can be identified from haematoxylin and eosin stained sections, dissected manually or by laser capture microdissection from adjacent unstained sections and used for DNA preparation. PCR amplification of the DNA using fluorescently-labelled primers that target multiple STR loci on different chromosomes and subsequent sizing of the PCR products by capillary electrophoresis can be used to identify STR in the DNA of the molar tissue. These are then

compared with those in the patient (using DNA from blood or maternal decidua) and her partner (if available) (Figure 3).

Typical AnCHM have one, or occasionally both, paternal allele(s) with no maternal contribution, at informative loci (Figure 3a). Where a DNA sample from the father is not available, diagnosis is made by demonstrating non-maternal (i.e. paternal) alleles in DNA from the molar tissue. A single allele at each locus indicates a monospermic CHM while the presence of two paternal alleles at some loci indicates the less common dispermic CHM, a distinction that cannot be made on the basis of morphology alone. The more malignant potential of dispermic CHM, initially suggested by small studies, remains controversial in larger studies [47, 48] and at present the distinction between monospermic and dispermic HM is not routinely made. Demonstration that POC with molar pathology and an absence of p57 staining are diploid and biparental (Figure 4), or rarely a digynic triploid, enables a diagnosis of FRHM and should be followed with sequencing of NLRP7 and/or KHDC3L in the patient. Identification of the specific mutations involved can play an important role in enabling screening of other family members, particularly important for nulliparous female siblings. However, absence of mutations should not exclude a diagnosis of FRHM, as ~10% of patients do not have mutations in these genes [49, 50].

With the routine use of p57 immunostaining, the more difficult differential diagnosis is between PHM and non-molar miscarriages. A number of conditions may be pathologically difficult to distinguish from PHM, including

Beckwith-Wiedemann syndrome [51, 52], placental mesenchymal dysplasia [51] and a number of chromosomal abnormalities, in particular paternal isodisomy for the short arm of chromosome 11 [53] and trisomy 13 [54, 55]. Since these conditions are essentially diploid they can be distinguished from triploid PHM that have three alleles, or two alleles of disproportionate height, at each locus (Figure 3b). Demonstrating that two of the alleles at each locus are paternally-derived, and one maternal, identifies a PHM, rather than a digynic triploid, in which the extra chromosomal complement is maternal in origin. Genotyping is now widely used to discriminate between non-molar miscarriage, PHM and CHM [8, 56] and provide accurate diagnosis in cases of probable or possible PHM [9]. Dissection and genotyping of populations of villi with divergent or discordant p57 staining patterns can also be used to differentiate PHM from CHM with coexistent twin and identify the presence of any potentially malignant, androgenetic cell line in the case of mosaic or chimeric conceptuses.

Finally genotyping can identify unusual cases that are not regular diploids or triploids. HM have occasionally been shown to be tetraploid with most tetraploid PHM being shown to arise by trispermy, rather than two sperm, one of which is diploid [8]. However, tetraploid CHM are more difficult to identify as like diploid CHM, they will usually have a single allele at each locus. In most series of HM occasional aneuploid cases, essentially CHM or PHM with loss or gain of one or more maternally- or paternally-derived chromosomes [39-41, 57-59], have been described. These aneuploid cases, which appear to be most commonly associated with dispermic CHM [60], can be resolved by

genotyping with a panel of STR, that cover loci on all chromosomes, or single nucleotide polymorphism-based microarray analysis [60]. While genotyping plays an important role in different scenarios, it must be considered in the context of morphology and p57 immunostaining to avoid misinterpretation of unusual cases. A number of algorithms for combining morphology, p57 and genotyping, in the diagnosis of suspected HM, have now been proposed [56, 61].

Genetics of Gestational Trophoblastic Tumours

GTT are characterised by trophoblastic differentiation of the tumour tissue and production of serum human chorionic gonadotropin (hCG). The tumours can arise from CHM, PHM or any non-molar pregnancy with their genetic makeup reflecting the pregnancy of origin. The proportion arising from different types of pregnancy varies across tumour subtypes. Whilst all invasive moles derive from molar pregnancies, the majority originating in CHM [48], only ~50% of choriocarcinoma and ~25% of PSTT/ETT have a molar origin [62].

Surprisingly, although ~half of the tumours arising in non-molar pregnancies would be expected to be male, for reasons that remain unexplained, the vast majority of PSTT/ETT originate from genetically female pregnancies [63-65]. Establishing the causative pregnancy, which may not be the antecedent pregnancy [66], is particularly important for a patient with PSTT or ETT, where a time interval of less or greater than four years has clear prognostic significance [67, 68].

Genetically, GTT are poorly characterised at the molecular level. Chromosomal loss or gain [69-72], complex chromosomal rearrangements [73] and rare copy number variants [74] have been described but only in a minority of cases. To date GTT have not been associated with any consistent genetic abnormalities nor has any genetic marker of tumour subtype been identified. Further investigations are needed to determine if this is due to limited investigation of these rare tumours or whether other factors drive tumorigenesis. The relatively high risk of requiring chemotherapy following a CHM and lesser, but still increased, risk after a PHM suggests aberrant genomic imprinting is likely to play a role in the development of post-mole tumours. Recent analysis of DNA from an intraplacental choriocarcinoma, revealed a very different methylation profile for the tumour and the surrounding normal term placenta, suggesting that epigenetic changes, rather than an accumulation of mutations, may also have a role in the development of tumours following non-molar pregnancies [75].

Differential Diagnosis of Trophoblastic Tumours

While raised serum hCG is the hallmark of GTT, tumours of non-gestational origin, particularly more aggressive tumours, may also secrete hCG [76]. Non-gestational trophoblastic tumours are biologically different, less chemosensitive and have a poor prognosis compared to GTT where overall survival is greater than 90% [77]. A differential diagnosis between a gestational and non-gestational tumour is therefore important for patient management. While this can usually be made on the basis of clinical criteria, it becomes more problematic where these tumours also show trophoblastic

differentiation which may be seen in some high grade carcinoma, metastatic lesions originating in different primaries and as a component of ovarian germ cell tumours [66, 78-81]. Conversely gestational choriocarcinoma and ETT may occasionally mimic non-gestational tumours [78, 82].

Ancillary Techniques in the Diagnosis of Trophoblastic Tumours

(i) Genotyping in the Diagnosis of Trophoblastic Tumours

As differential diagnosis of trophoblastic tumours relies on demonstrating the presence or absence of a paternal contribution to the tumour genome, p57 immunostaining is less relevant and STR genotyping is currently the gold standard. Manual microdissection of tumour and surrounding host tissue is usually satisfactory for DNA preparation. However, if tumours are small and are infiltrating the host tissue, or are themselves infiltrated by host lymphocytes, laser capture microdissection of the cells of interest might be considered in order to minimise contamination from host cells [71]. While analysis can be performed using pathological sections alone, analysis of DNA from the patient and her partner can be useful in confirming diagnosis. A patient sample is essential when the tissue sample is a small biopsy containing only tumour cells.

A GTT will have one or more paternal contributions to the genome, with the genotype reflecting the pregnancy of origin (Figure 5a). As with diagnosis of HM, if samples from the partner are not available, the presence of alleles in DNA from the tumour, not present in the patient, are assumed to be paternal in origin. However, when paternal samples are not available, caution should

be taken to avoid misdiagnosis. For example a non-gestational tumour in a patient with Lynch syndrome may have a high degree of instability suggesting a non-maternal component and falsely suggest an androgenetic tumour [80], as might a case where the patient has a history of IVF with a donor ovum and the maternal contribution is therefore different to that of the patient [83]. Factoring in the morphology and clinical history is recommended to avoid misdiagnosis of such unusual cases. The genotype of a non-gestational tumour reflects that of the patient with no evidence of a paternal contribution (Figure 5b). Since microdissection of tumour and host tissue for DNA preparation is performed from the same unstained sections and the genotype of the host and tumour are potentially identical at the loci examined, care must be taken to ensure that the DNA analysed is from tumour cells and not obscured by DNA from host cells, particularly important where tumours are small and there is a high degree of infiltration by host cells such as lymphocytes. Demonstrating loss of heterozygosity or allelic imbalance in the tumour DNA, compared to that of the host, can provide confirmation that the DNA analysed is tumour-derived [80] (Figure 5b).

Genotyping of tumour DNA can identify the nature of the causative pregnancy. However, additional genotyping of DNA from previous miscarriages, molar pregnancies and/or DNA from saliva of live born children is needed to identify the specific pregnancy. While choriocarcinoma have sometimes been shown to arise from an earlier pregnancy, including unrecognised CHM [66, 78, 79], with or without intervening pregnancies [66], PSTT and ETT less frequently originate in earlier pregnancies [65]. It has

recently been found that some cases of ETT and PSTT are associated with atypical placental site nodules (APSN), 10-15% of patients with APSN subsequently being diagnosed with PSTT or ETT [84]. Again the causative pregnancy of the APSN can be determined by genotyping the offspring or POC from the patient. Genotyping has also provided interesting insights into the causative pregnancy in intraplacental choriocarcinomas. These might be expected to arise from the surrounding villous tissue of the placenta in which they originated but it has now been shown they may originate in earlier, or co-existent but genetically different, pregnancies [85], or be non-gestational [80].

(ii) Non-invasive Diagnosis of Trophoblastic Tumours

While diagnosis of GTD usually relies on trophoblastic tissue it is occasionally considered safer to treat a patient with a trophoblastic tumour on the basis of a clinical diagnosis rather than perform surgery. In these cases it has been shown that cell free DNA (cfDNA), isolated from the patient's plasma, can provide a source of circulating tumour DNA (ctDNA) for genotyping [86, 87] and potentially diagnose both gestational and non-gestational tumours [87]. While these techniques currently lack sensitivity, particularly in women with a small tumour burden [87] or involve complex and time-consuming technology [88], the use of ctDNA holds considerable potential for non-invasive diagnosis of GTD and providing further resources for investigating these conditions.

Summary

p57 and genotyping are important ancillary techniques in the differential diagnosis and management of GTD. Since all types of placenta, with the

exception of CHM, show p57-positive staining of the villous cytotrophoblast and stroma, immunostaining will identify CHM. Since PHM are triploid with two paternal contributions to the genome, genotyping can differentiate PHM from other types of reproductive loss. Genotyping also enables diagnosis of FRHM by showing recurrent CHM are diploid and biparental. A combination of p57 immunostaining and genotyping can help resolve diagnosis of unusual cases of molar pregnancy with atypical pathology, particularly mosaic or chimeric conceptions with a molar component or aneuploid molar pregnancies with features of a HM. A tumour showing trophoblastic differentiation, in women with raised levels of serum human chorionic gonadotrophin, may not always be a GTT. A gestational origin can be confirmed by using genotyping to demonstrate the presence of paternally-derived DNA in the tumour. Genotyping can also be used to identify the causative pregnancy in GTT, particularly important for women with PSTT or ETT where the time interval between the causative pregnancy and tumour diagnosis has prognostic significance. Where tumour tissue is not available for investigation, circulating tumour DNA, isolated from the patient's plasma, can be used to facilitate diagnosis.

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FIGURE LEGENDS

Figure 1: Genetic origin of hydatidiform moles

(a) PHM are triploid and almost all arise by dispermy (b) Some CHM also arise by dispermy, with loss of the maternal chromosomes (c) The majority of CHM arise from fertilization of an ovum by a single sperm, loss of the maternal chromosomes and duplication of the sperm genome (d) BiCHM arise following normal fertilization of an ovum in a woman with biallelic mutations of NLRP7 or KHDC3L.

Figure 2: Photomicrographs of p57 immunostaining

(a) CHM with no p57 immunostaining in the villous cytotrophoblast (dashed arrow) or stroma (solid arrow) (b) PHM with positive nuclear staining of both villous cytotrophoblast and stroma (c) discordant p57 staining in a mosaic conception with no p57 staining of the cytotrophoblast but p57-positive staining of the villous stroma (d) discordant p57 staining in a mosaic with p57-positive staining of the cytotrophoblast but no staining of the villous stroma. Positive staining of extravillous trophoblast (arrow head) acts as an internal control for staining.

Figure 3: Genotyping of parents and molar villi

The x axis represents the size of the DNA fragments generated (alleles are annotated beneath each peak). The y axis represents arbitrary units of fluorescent intensity (a) CHM in which all alleles originate from the father of the pregnancy (blue arrowheads) and no maternal alleles are detected. A single allele at each locus is indicative of a mole derived from a single sperm.

(b) PHM in which three distinct alleles are present at the TH01 locus, one of maternal (red arrowhead) and two of paternal (blue arrowhead) origin, indicative of a dispermic, diandric triploid conception. At the other two loci, the paternal allele is detected at twice the intensity of the maternal allele, consistent with diandric triploidy.

Figure 4: Genotyping of recurrent hydatidiform moles

Genotyping of HM in a 34 year-old woman with two HM, showing atypical morphology and p57-negative immunostaining, and no other pregnancies. In both HM, each locus contains peaks of equivalent height, one peak matching the maternal sample (red or orange arrowhead) whilst the other is non-maternal (blue or black arrowhead), indicating a diploid, biparental origin and a diagnosis of FRHM. The patient was subsequently referred for sequencing of *NLRP7* and *KHDC3L*.

Figure 5. Genotyping of trophoblastic tumours

(a) PSTT from a 38-year-old patient with two prior pregnancies. At the TPOX and D18S51 loci, alleles of maternal (red arrowhead) and paternal (blue arrowhead) origin are identifiable. The VWA locus is not informative as one allele is common to both parents. (b) Case from a 39-year-old patient with one prior pregnancy (twins) presenting with a poorly differentiated lung tumour with choriocarcinomatous differentiation. All alleles present in the tumour match the maternal alleles (red arrowheads), with none of the paternal alleles detected in the tumour. Loss-of-heterozygosity is present at the D16S539 (unfilled arrowhead).