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GENETICS*Helen Dearden and Rosemary A Fisher***2.1. GENETICS OF GESTATIONAL TROPHOBLASTIC NEOPLASIA**

Gestational trophoblastic neoplasia (GTN) includes the abnormal pregnancies complete (CHM) and partial hydatidiform mole (PHM) and the malignant gestational trophoblastic tumours (GTT) invasive mole (IM), choriocarcinoma, placental site trophoblastic tumour (PSTT) and epithelioid trophoblastic tumour (ETT). While many genetic studies have been performed in both CHM and PHM, similar studies of trophoblastic tumours have been limited by the availability of fresh material. The introduction of genetic techniques, in particular those which can be used in conjunction with fixed material, has facilitated both diagnosis and further studies of the pathogenesis of this unusual group of diseases.

2.1.1 HYDATIDIFORM MOLES**2.1.1.1 Karyotypes of Complete and Partial Hydatidiform Moles**

The genetics of HM are of interest because of their potential to progress to GTT. In the UK 13.6% percent of women with a CHM and 1.1% percent of those with a PHM will go on to require chemotherapy for GTN [1]. The first genetic studies of HM hinted that they might be genetically unusual when most HM were found to have Barr bodies, darkly staining bodies found in the interphase nuclei of female cells [2]. Cytogenetic studies went on to confirm that most HM had a 46,XX karyotype and were therefore female [3-6]. Some of the early studies also revealed a small number of HM with triploid karyotypes [7, 8]. Correlation of the morphology of HM with their karyotype led Vassilakos and his colleagues [9, 10] to suggest that the two pathological entities CHM and PHM were genetically distinct. They found all CHM to have a diploid 46,XX karyotype while PHM were mainly triploid or trisomic for a single chromosome [10]. PHM were classified as conceptuses with a fetus, cord or amniotic membranes and a placenta comprising both normal and cystic villi. Hyperplasia, present in some cases, was not marked. When hyperplasia was included as a prerequisite for the pathological diagnosis of PHM [11, 12], it became apparent that PHM are almost always triploid [13-15] confirming that PHM and CHM are distinct entities.

2.1.1.2 Parental Origin of Complete Hydatidiform Moles

Although CHM have a normal chromosome number they are androgenetic ie. all 46 chromosomes in the molar tissue are paternally derived. This was initially demonstrated by showing that all cytogenetic polymorphisms in the molar tissue were paternally derived [13, 16-21]. These investigations also showed that homologous chromosome pairs were homozygous for all informative markers examined [16, 17]. Subsequent studies that included enzyme polymorphisms, located more distally on the chromosomes, showed similar homozygosity providing evidence that CHM arise as a result of duplication of a haploid sperm rather than fertilization of an egg by a diploid sperm [13, 14, 20] (Figure 2.1a).

Although most CHM arise following duplication of a haploid sperm a minority result from fertilization of an anucleate egg by two sperm. In 1981 Ohama et al described four androgenetic CHM with a 46,XY karyotype in which only half the informative polymorphisms studied were homozygous and suggested that these 46,XY CHM arose by dispermy [22] (Figure 2.1b). Several other examples of both 46,XY and 46,XX CHM arising by dispermy were subsequently reported [23-27]. While 46,XY CHM can be easily identified by the presence of a Y chromosome, a number of informative polymorphisms may need to be examined in order to identify heterozygosity in a 46,XX CHM [28]. When more informative molecular genetic markers became available to examine the origin of CHM, the incidence of dispermic CHM was shown to be in the region of 20-25% [28-30]. Since CHM with a 46,YY karyotype have not been reported, androgenetic conceptuses without an X chromosome are presumed to be non-viable.

Despite the androgenetic nature of the nuclear genome in CHM, analysis of DNA polymorphisms has shown the origin of the mitochondrial DNA to be the same as that in a normal pregnancy and to be maternally derived [31-33]. The fate of the maternal chromosomes in CHM is unclear. Extrusion of both maternal sets of chromosomes or chromatids into one of the polar bodies during meiosis could give rise to an anucleate egg. More recently it has been argued that evidence for a reservoir of anuclear oocytes is lacking and that CHM and other abnormal pregnancies might arise as a result of postzygotic diploidisation of a triploid conceptus [34].

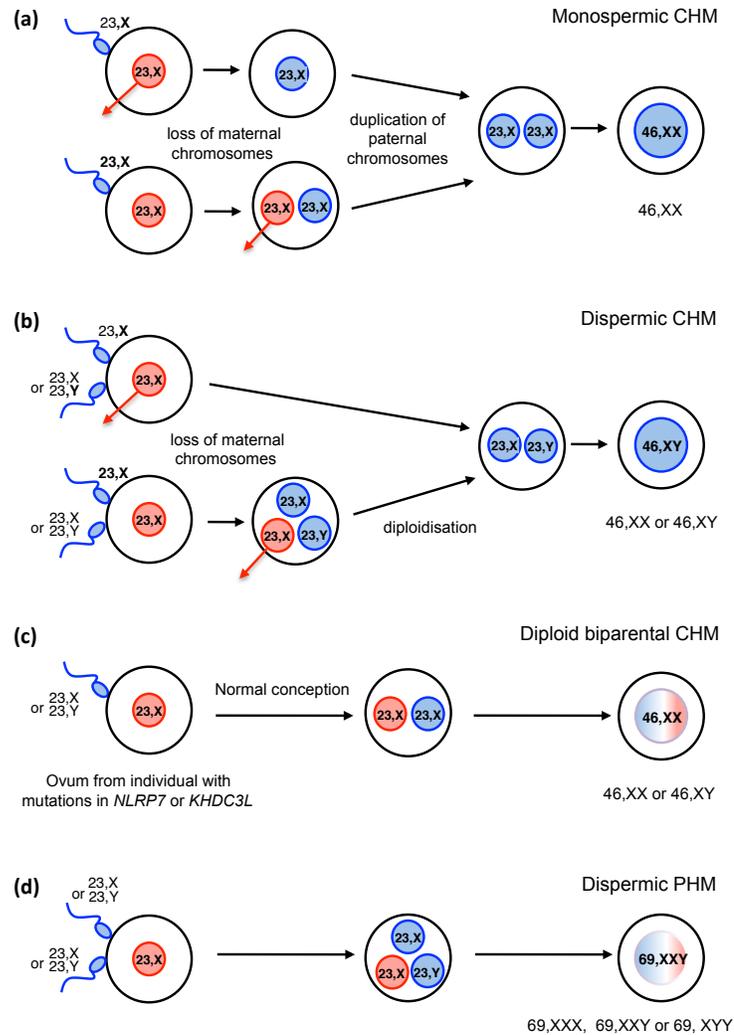


Figure 2.1. Genetic origin of molar pregnancies. (a) Monospermic CHM arise from fertilisation of an ovum by a single sperm. The maternal nuclear genome is lost, pre- or post-fertilisation, while the paternal genome duplicates resulting in a homozygous CHM. (b) Dispermic CHM arise as a result of two sperm fertilising an anucleate ovum or post-fertilisation loss of the maternal nuclear genome following fertilisation of an ovum by two sperm. The resulting diploid conceptus is genetically heterozygous and may be 46,XX or 46,XY. (c) Biparental CHM result from a normal conception involving an ovum from a female who is homozygous, or a compound heterozygote, for mutations in *NLRP7* or *KHDC3L*. These biparental conceptuses are phenotypically CHM and may be 46,XX or 46,XY. (d) Most PHM are dispermic and arise as a result of fertilization of a single ovum by two sperm. These diandric triploid conceptions may be 69,XXX, 69,XXY, or 69,XYY.

Post zygotic diploidisation of a triploid conceptus explains the formation of both homozygous and heterozygous complete hydatidiform moles and may also account for the creation of more

unusual molar/twin conceptions, and cases of mosaicism and chimerism. However, it does not explain the 4:1 ratio of homozygous to heterozygous CHM [35].

2.1.1.3 Parental Origin of Partial Hydatidiform Moles

PHM are distinct from CHM in that they are triploid and may have a 69,XXX, 69,XXY or 69,XYY karyotype [11, 13-15]. Investigation of tissue from spontaneous abortions has identified several mechanisms that can account for triploidy [36-39]. The most commonly observed mechanisms are failure at the first or second meiotic division during gametogenesis to produce a diploid sperm or ovum or fertilisation of an ovum by two sperm. Studies of cytogenetic and enzyme polymorphisms in molar tissue have confirmed that PHM are triploid and, in almost all cases, diandric the additional chromosome set being paternally derived [14, 30, 40, 41]. The majority of these diandric PHM have been shown to arise by dispermy (Figure 2.1d) although occasional cases have been described in which the most likely origin is fertilisation of an egg by a diploid sperm [41, 42]. While PHM may be 69,XXX, 69,XXY, or 69,XYY, the proportion of 69,XYY PHM in most series is found to be lower than expected [40, 41, 43, 44].

2.1.1.4 Genetically Unusual Hydatidiform Moles

Although most HM can be classified as androgenetic diploids or diandric triploids, occasional HM do not fit either category. Amongst CHM rare triploid and tetraploid cases have been reported. These are still generally androgenetic in origin having three or four paternal sets of chromosomes [45] although two unusual digynic CHM have been described in association with the rare inherited condition, familial recurrent hydatidiform mole (FRHM) [46]. Tetraploid PHM have also been reported [30, 45, 47-49], and, like triploid PHM, usually have an excess of paternal genomes although two cases with apparently equal contributions from each parent have also been described [30, 50]. The most likely origin of tetraploid PHM is fertilisation of an egg by three sperm or two sperm one of which is diploid. The morphology of the few aneuploid HM that have been described [14, 15, 35, 45, 51-56] generally reflects their basic karyotype. Hypo- or hypertriploid HM are PHM while hypo- or hyperdiploid HM are generally androgenetic and morphologically CHM.

2.1.1.5 Mosaics and Chimeras with Molar Pathology

Occasionally molar change may be seen in the placenta of an otherwise normal pregnancy. These rare cases, which have often

been found to comprise two genetically different cell lines, are likely to represent unusual forms of mosaic or chimeric conceptions. A small number of pregnancies in which the placenta appears to have features of PHM, while the fetus is apparently normal, have been shown to be mosaics with a population of triploid cells in the placenta while the fetus is diploid [57-60]. Other mosaic pregnancies with molar changes of the placenta and apparently normal fetuses involve the presence of two different diploid cell lines, one androgenetic and one a normal biparental line, admixed in the placenta. This is in contrast to a twin pregnancy with a CHM and coexistent normal fetus in which two distinct populations of placental villi are present. A number of cases of biparental/androgenetic mosaicism (both the androgenetic and biparental lines derived from the same zygote) or biparental/androgenetic chimeras (fusion of two genetically different zygotes) have now been described [61-69]. Some cases may represent confined placental mosaicism with biparental cells confined to morphologically normal areas of placenta while the androgenetic cells are confined to areas that are pathologically CHM [62]. In others the biparental or androgenetic lines may be almost exclusively restricted to the trophoblastic or stromal component of the villi [63, 66, 69] while some are reported to contain villi with varying distributions of the two cell lines [66]. Investigation of these rare cases suggests that the morphological phenotype of the placental tissue may reflect the distribution of the two cell lines. Where the cytotrophoblast are androgenetic, trophoblastic hyperplasia is marked [62, 69] but where the cytotrophoblast is derived from normal biparental cells trophoblastic hyperplasia may be absent and the phenotype that of placental mesenchymal dysplasia (PMD), a condition characterised by morphologically normal terminal villi admixed with hydropic stem villi but lacking trophoblastic hyperplasia [70] rather than CHM [63]. Clinically, it is important to recognize the presence of the molar component in these mosaic/chimeric conceptions because of the propensity of the androgenetic line to progress to GTN [65, 68, 69].

2.1.1.6 Genomic Imprinting in Hydatidiform Moles

A small number of genes are unusual in that they are transcribed only from the maternally or paternally inherited allele, the allele inherited from the other parent being “imprinted” or silent. This phenomenon, known as genomic imprinting, underlies the abnormal pathology seen in HM. Several lines of evidence suggest that the characteristic morphological features shared by androgenetic CHM and PHM are due to the presence of two paternal genomes. Ovarian teratomas genetically analogous to CHM, but with a maternally derived diploid genome [71], have a very different pathology to CHM with the development of embryonic tissues being favoured in this condition. Similarly digynic triploids, which have two maternal contributions

to the nuclear genome, are not generally associated with molar pathology but have an abnormally small placenta and growth-retarded fetus [72, 73]. Where only paternal chromosomes are present a CHM develops, trophoblastic hyperplasia is marked and no fetus is present. In PHM the presence of a maternal genome is associated with more moderate trophoblastic hyperplasia and fetal development. Thus both overexpression of paternally transcribed genes and loss of maternally transcribed genes are likely to play a role in molar development.

Further evidence for the association between the paternal genome and trophoblastic development comes from mouse models [74, 75]. Experiments that involve nuclear transfer in mice have shown that normal development during mouse embryogenesis requires both maternal and paternal contributions to the genome. In these models relatively good trophoblast differentiation occurs in androgenetic embryos with two male pronuclei compared with gynogenetic embryos having two female pronuclei [75] thus demonstrating an association between the paternal genome and extraembryonic development analogous to that seen in CHM.

Several imprinted genes have now been identified in man [76] and some examined in molar tissue. Products of two genes that are normally transcribed from the maternally inherited allele, p57^{KIP2} the product of *CDKN1C* (cyclin-dependent kinase inhibitor 1C) [77] and IPL, (imprinted in placenta and liver), the product of *IPL* (also known as *TSSC3* - tumor suppressing subtransferable candidate 3 and *PHLDA* - pleckstrin homology-like domain, family A, member 2) and show high levels of expression in normal human placenta, have been shown to be absent from villous cytotrophoblast in CHM [78-82]. While expression of p57^{KIP2} is lost from the villous mesenchyme and trophoblast in CHM, the gene is not imprinted in the extravillous trophoblast and expression is retained providing an internal control such that immunohistochemical staining for p57^{KIP2} has proved to be a reliable diagnostic discriminator between CHM and other types of pregnancies [79, 83-88]. The role of genes such as *CDKN1C*, that are located in the imprinted domain of chromosome 11, in molar development is unclear. Two unusual cases of CHM that had retained a maternally derived chromosome 11 in addition to two paternal chromosome complements and therefore expressed p57^{KIP2} were pathologically indistinguishable from typical androgenetic CHM [53, 55]. In contrast to this a case of PHM with loss of the maternal copy of chromosome 11 and absence of p57^{KIP2} expression was reported to have morphological features of early CHM [89]. The imprinting status of a number of genes has recently been examined in the context of androgenetic CHM

(AnCHM) and familial recurrent hydatidiform moles (FRHM) that are unusual in being diploid but biparental in origin (Figure 2.1c).

2.1.1.7 Diploid, Biparental Hydatidiform Moles

A number of diploid PHM have been reported in the literature. Many of these may represent misdiagnosis of diploid hydropic abortion, cases of mosaicism or CHM, particularly early CHM [90-92]. However, some cases of diploid PHM may in fact fall into a rare but extremely interesting subgroup of HM that, although diploid, have a normal or biparental genotype with a genetic contribution from both parents in all cells. In many series of CHM, in which genetic studies have been performed, occasional cases of diploid, biparental CHM (BiCHM) have been described [15, 29, 93-96]. Subsequent studies have shown that these rare BiCHM are frequently associated with patients who have recurrent CHM [97-99] and occur in families in which two or more individuals have molar pregnancies [97, 99, 100]. BiCHM are now recognized as a clinically important subgroup of HM.

2.1.1.8 Familial Recurrent Hydatidiform Moles

Familial clustering of GTN is rare. The first report of familial HM described three unrelated Indian kindreds in which two or more individuals presented with molar pregnancies [101]. Two Italian kindreds each with two affected sisters were subsequently described, one pair of sisters being monozygotic twins [102, 103]. Three further families were then reported in the literature, the first a Lebanese family in which 2 sisters and a cousin [104, 105] were affected, the second, a German family in which three sisters had HM [106, 107] and the third two Italian sisters with recurrent HM [99].

Genetic studies of the CHM in some of these families showed them to be quite unusual in that, although diploid, they were biparental in origin rather than androgenetic with a chromosomal complement from each parent [97, 99, 100]. These families thus appeared to have an inherited condition predisposing to pregnancies with molar pathology despite the placental tissue appearing genetically normal. The pattern of inheritance in these families suggested FRHM to be an autosomal recessive condition. Although most pregnancies in women with FRHM are pathologically CHM they may present with other types of pregnancy including PHM, non-molar miscarriage, stillbirth and, very occasionally, normal pregnancy [97, 108, 109]. Where PHM have been investigated in these families they have also been found to be diploid and biparental, rather than triploid [105, 110, 111].

While ‘atypical’ features can be identified in a few cases of BiCHM, the majority are pathologically indistinguishable from AnCHM [112]. BiCHM also show similar imprinting defects to those in AnCHM. They show an identical pattern of p57^{KIP2} expression [113] while several studies investigating differentially methylated regions of imprinted genes have shown a number of maternally imprinted genes to have a paternal methylation pattern in BiCHM [114-117]. Interestingly the extent of the imprinting defect appears to vary between different pregnancies within the same family. A rare full term pregnancy in which the offspring developed normally was found to have normal methylation patterns while molar pregnancies in the same family showed aberrant imprinting [117].

Mutations in one of two maternal-effect genes have now been found to be responsible for the majority of cases of FRHM. Homozygous or compound heterozygous mutations in *NLRP7*, the first gene to be identified [118] have now been found in approximately 75% of cases and encompass a wide variety of protein-truncating mutations including deletions, insertions and duplications, and pathological variants [115, 116, 118-128]. Clustering of the pathological variants in the leucine rich region of the gene (Figure 2.2) suggests this is the important functional domain. More recently mutations in a second gene *KHDC3L* have been shown to be responsible for FRHM in a minority of cases [129, 130]. No mutations in either gene have been found in approximately 20% of women with BiCHM. It remains to be seen whether these result from mutations in non-coding regions of *NLRP7* or *KHDC3L* or whether novel genes are still to be identified in association with this condition. The mechanism by which mutations in either *NLRP7* or *KHDC3L* result in a molar phenotype remains unclear. Mutations in both genes appear to be specifically associated with FRHM, mutations in neither gene having been shown to be associated with androgenetic CHM, miscarriages of unknown aetiology or unexplained infertility [126, 131-136].

NLRP7 is a member of the NACHT, leucine rich repeat, and PYD containing (NLRP) protein family and as such is a member of a family of proteins implicated in the activation of proinflammatory caspases through multiprotein complexes called inflammasomes. Since *NLRP7* itself has been reported to act as a feedback regulator of caspase-1-dependent interleukin 1-beta secretion [137, 138] it has been suggested that women with defective *NLRP7* fail to mount an appropriate immune response during pregnancy [128].

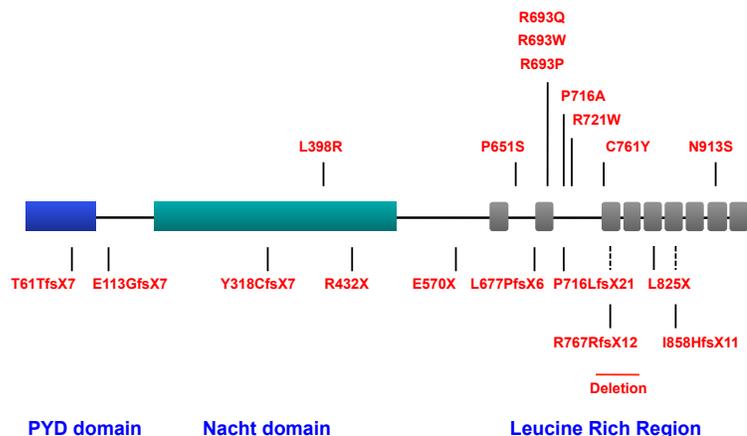


Figure 2.2. Schematic representation of NLRP7 showing typical protein truncating mutations (bottom panel) and pathological variants (upper panel) found in women with FRHM [121, 131].

A different role for NLRP7 is suggested by the similar aberrant expression of imprinted genes observed in BiCHM and AnCHM. In AnCHM aberrant imprinting results from the presence of two paternal copies of the genome. In BiCHM, the paternal epigenotype observed for genes that are normally maternally imprinted [114], suggests that NLRP7 might be important in establishing the correct maternal imprint in the oocyte or maintaining the maternal imprint during preimplantation development. Loss of NLRP7 would then result in a phenotype similar to that of an AnCHM. NLRP7 has been demonstrated in all follicular stages in the ovary [121] while at least four successful pregnancies with ovum donation confirm that that it is the role of NLRP7 in the oocyte that is important in enabling normal embryonic development [139][unpublished observations]. Recent studies have started to unravel this apparent contradiction by showing that reduced levels of NLRP7 in embryonic stem cells can alter DNA methylation and accelerate trophoblast lineage differentiation, novel functions for this family of genes [140].

The second gene associated with FRHM, *KHDC3L* belongs to the KHDC1 family, members of which contain an atypical KH domain. Mutations in this gene, like those in *NLRP7*, result in BiCHM with a phenotype indistinguishable from that seen in AnCHM [46] suggesting the two genes function in the same pathway. This is supported by the observation that *KHDC3L* shows a pattern of expression in oocytes similar to that of *NLRP7* [129] and recent studies which show they co-localise to the oocyte cytoskeleton [141].

Further studies are needed to elucidate the role of these two proteins in the development of BiCHM.

2.1.2 GESTATIONAL TROPHOBLASTIC TUMOURS

2.1.2.1 Invasive Moles

In the UK 13.6% of CHM and 1.1% of PHM go on to develop persistent GTN [1], most of which will be invasive HM (IM). These tumours are identified by serum human chorionic gonadotropin (hCG) monitoring and treated with chemotherapy and hence tumour tissue, for investigation, is rare. Consequently there have been few genetic investigations of IM. Cytogenetic studies that have been done have shown the majority to be diploid with a high proportion of cells in the tetraploid range [5, 142]. A detailed study of four IM by Wake et al [26] found them to be diploid, three 46,XX and one 46,XY, reflecting their origin from CHM. In one case where the antecedent HM was also examined it was shown to be identical to the IM for all markers examined. All four IM appeared to be derived from dispermic CHM rather than the more common monospermic type.

2.1.2.2 Gestational Choriocarcinoma

Gestational choriocarcinoma may follow any type of pregnancy; approximately equal numbers following molar or non-molar pregnancies [143]. The nature of the antecedent pregnancy will determine the genetic make up of the tumour. Tumours that result from term pregnancies, non-molar abortions or PHM will have both maternal and paternal chromosomes while those derived from CHM will be androgenetic in origin. Again these tumours are often treated with chemotherapy, without surgical intervention, and tumour tissue is rarely available for study. Where cytogenetic analysis of choriocarcinoma cell lines and tumour tissue has been performed they have revealed a more aberrant karyotype than that of IM with considerable variation in karyotype. Most choriocarcinoma are aneuploid with modes in the hyperdiploid and hypotetraploid range irrespective of whether they follow term births, HM or spontaneous abortion [5, 144-151]. Although karyotypes of choriocarcinomas show a range of abnormalities, including chromosomal gains, losses and rearrangements, cytogenetic studies have not revealed any consistent chromosomal abnormalities. Molecular genetic studies, using comparative genome hybridisation (CGH) and microsatellite genotyping, have demonstrated loss of chromosome 7p12-q11.2 and 8p12-p21 [152-154] in some cases, suggesting that these are chromosomal regions in which tumour suppressor genes, involved in the development of GTT might be located. However, the specific

genes involved have yet to be identified. One interesting tumour suppressor gene that may be involved in the development of GTT is NECC1 (not expressed in choriocarcinoma clone 1), located on human chromosome 4q11-q12 [155]. Abundantly expressed in normal placental villi, NECC1 expression is absent in all choriocarcinoma cell lines examined and most choriocarcinoma tissue samples. Transfection of NECC1 into choriocarcinoma cell lines results in altered cell morphology and suppression of *in vivo* tumorigenesis suggesting that loss of NECC1 expression is involved in the malignant progression of trophoblast cells. Less is known about the role of oncogenes in the development of GTT although amplification of 7q21-q31 observed by Ahmed et al [153] in a series of choriocarcinoma suggests a role for oncogenes located in this region. Most of these early observations of loss or gain of chromosomal regions were found in post-term rather than post-mole GTT [153, 154]. This observation has been confirmed in a more recent molecular genetic investigation using high resolution array CGH, in which post-mole choriocarcinomas were found to exhibit only simple chromosomal abnormalities or normal profiles while cell lines, derived from post-term tumours, showed complex chromosomal aberrations [156]. Whether these changes have arisen during evolution of the cell line or were present in the primary tumour, they involve regions with high numbers of microRNA clusters and imprinted genes which is of interest given the high propensity of GTN that follow CHM with their inherent imprinting defects.

2.1.2.3 Placental Site / Epithelioid Trophoblastic Tumours

Few data are currently available on the genetics of PSTT or ETT, and like those for choriocarcinoma there is considerable variation between the results of different studies. Although these tumours are reported to be less often associated with an antecedent molar pregnancy than choriocarcinoma, DNA analysis has confirmed that PSTT and ETT may arise from either HM or normal term pregnancy [157, 158], [unpublished observations]. Ploidy analysis, performed on only a small number of PSTT, has shown the majority to be diploid [159-162] or occasionally tetraploid [161-163]. Chromosomal gain of 21q has been observed in two of four cases [164], loss of 7p12-q11.2 and 8p12-p21 in two and one of six cases of PSTT respectively [154] and a balanced chromosomal profile in a further three cases of ETT [165]. One of the more interesting observations in relation to these tumours is that PSTT and ETT are usually female [166, 167] suggesting that pathogenesis of PSTT and ETT may require the presence of a paternally inherited X chromosome or that more of these tumours originate in CHM than the 13% reported to develop from CHM based on the nature of the antecedent pregnancy [168].

2.1.2.4 Genomic Imprinting in Gestational Trophoblastic Tumours

The greatest risk factor for the development of a GTT is a molar pregnancy, in particular a CHM [169]. Despite this, the majority of CHM still resolve spontaneously. One of the important questions in the management of patients with HM is predicting which will progress to GTN. Since early studies of CHM showed them to be homozygous it was suggested that development of GTN might be related to homozygosity for deleterious recessive genes [16]. Dispermic CHM, homozygous for only some loci, would be expected to show less frequent progression to GTT. However, there is currently no evidence that dispermic CHM are less likely to progress to GTN and it may be the paternal nature of the genome and consequent deregulation of genomic imprinting, rather than homozygosity for specific genes, which is the important factor in post-mole tumourigenesis. This hypothesis is consistent with the more malignant potential of CHM, which show a greater degree of aberrant imprinting, compared to the potentially less malignant PHM. Further support for this is provided by the similar risk of GTN after BiCHM to that found for AnCHM [53][unpublished observations] that share similar patterns of aberrant imprinting.

Association of excess paternal genes with tumourigenesis has been implicated in the Beckwith-Wiedemann syndrome (BWS) in that individuals with BWS, which may result from unipaternal disomy or paternal duplication of part of chromosome 11, show a predisposition to embryonal tumours [170]. More direct evidence of a role for imprinted genes in tumourigenesis comes from studies showing loss or gain of normal imprinting in tumour tissue. Following the original reports of loss of imprinting, for both the maternally imprinted *IGF2* and the paternally imprinted *H19* genes, in Wilms tumour [171, 172] there is now considerable evidence for deregulation of imprinted genes in a variety of tumour types [173].

Studies of *H19* and *IGF2* expression in choriocarcinoma have shown both to be expressed in tumours derived from CHM [174-176] despite *H19* being a maternally expressed gene. Investigation of *H19* and *IGF2* expression in choriocarcinoma cell lines derived from tumours following term pregnancies has also shown frequent biallelic expression of one or both genes [174, 176]. Thus deregulation of imprinting may be a factor in the development of GTT from molar and non-molar pregnancies. Further studies of epigenetic changes involving imprinted genes, particularly in tumours following non-molar pregnancies, are now needed to provide a greater understanding of the development of GTN

2.2. GENETIC DIAGNOSIS OF GESTATIONAL TROPHOBLASTIC NEOPLASIA

Correct diagnosis of molar pregnancies and GTN is important for the appropriate management of women with GTN. Diagnosis can usually be made by a pathologist experienced in trophoblastic disease. However, there are situations in which a differential diagnosis is difficult and for these cases ancillary techniques can prove useful. The development of a range of techniques that can be used routinely for analysis of DNA from formalin-fixed paraffin-embedded material, has made ancillary tests, based on the genetics of GTN, a useful adjunct to routine pathological diagnosis in the diagnosis of molar pregnancy and GTN.

2.2.1 DIFFERENTIAL DIAGNOSIS OF HYDATIDIFORM MOLES

2.2.1.1 Hydropic Abortion, Partial or Complete Hydatidiform Mole

In the UK women with HM are registered and screened following evacuation of their molar pregnancy because of the high risk of GTN. It is therefore important to distinguish HM from non-molar miscarriages for which the risk of GTN is likely to be less than 1 in 50,000 [177]. While the risk of GTN following PHM has been difficult to assess accurately, due to the small size of many early studies, inaccurate diagnosis of PHM, the possibility that some PHM may be mosaics with an androgenetic population and the fact that many PHM may go undiagnosed [90, 178], a large UK study in which most cases had been reviewed by an experienced pathologist recently estimated this to be approximately 1% [1]. Although the definitive risk of developing GTN after a PHM is still unknown it is clearly very much lower than that for CHM and it is therefore important to distinguish between these conceptions particularly if different screening and/or follow up protocols are in place for the two entities.

A differential diagnosis of molar pregnancy is usually possible on the basis of morphology. However, poor sampling or necrosis in a HM that has been retained for a long period can make a pathological diagnosis difficult [90]. The introduction of ultrasound scanning and earlier termination of suspected molar pregnancies has also presented diagnostic problems. At earlier gestational age the distinction between CHM and PHM is less marked [90]. Fetal tissue such as nucleated red blood cells, endothelial cells, stromal macrophages, amnion and yolk sac, characteristics once thought to be diagnostic of PHM, can be present in early CHM [96, 179-181] and different criteria need to be addressed for accurate diagnosis [182]. Other conditions may be pathologically difficult to distinguish

from PHM, but which would be genetically diploid, include BWS [91, 183] and placental angiomatous malformation [91]. The differential diagnosis between HM and PMD may also be difficult in cases of mosaicism or chimerism involving an androgenic and normal biparental cell line. In any of these circumstances ancillary genetic investigations can play an important role in the differential diagnosis.

2.2.1.2 Twin Pregnancy with Complete Hydatidiform Mole and Coexistent Normal Fetus

PHM are characterised by a range of villi from normal to cystic and show evidence of the presence of a fetus or fetal development, generally in the form of fetal red blood cells in the villi. Twin pregnancies consisting of normal twin and CHM may show similar characteristics [184]. It has been suggested that a patient with CHM in a twin pregnancy has a significantly greater risk of GTN than with a singleton CHM and that the disease tends to be more aggressive [185-188]. However, this has not been substantiated by subsequent studies [189, 190]. In a large study of CHM with co-existing normal twin 40% of women successfully delivered a normal baby without increased risk of GTN [189]. In any event both the clinical outcome, which may be a liveborn baby, and the risk of GTN is likely to be very different for a twin pregnancy with CHM than a PHM. It is therefore important to make the distinction between these two entities for appropriate management of the pregnancy and subsequent follow up.

2.2.1.3 Monospermic, Dispermic or Biparental CHM

A distinction between monospermic and dispermic CHM can only be made on the basis of genetic polymorphisms since there is no morphological difference between these two conditions [191]. At present the clinical significance of this distinction is not clear. Studies of choriocarcinoma tissue and cell lines have suggested that dispermic CHM have the more malignant potential [26, 93, 192-195]. However, this has not been shown to be the case in other series [30, 196, 197]. This topic remains controversial and larger studies are still necessary to resolve this question.

The rare BiCHM are usually pathologically indistinguishable from AnCHM and present an important diagnostic problem. In a review of 152 pregnancies in 37 affected individuals from 14 families with recurrent HM, 113 (74%) were CHM, 26 (17%) were miscarriages, six (4%) were PHM and only 7 (5%) were normal pregnancies [108]. Despite the occurrence of other types of pregnancy in these families over 70% of pregnancies in affected women were CHM, a figure likely to be higher since some HM may be missed where miscarriage

tissue is not available for examination by a pathologist. More important for counselling women with FRHM is the less than 5% chance that they will have a normal pregnancy.

In addition to the small number of families with recurrent BiCHM several isolated cases of women with recurrent CHM have been reported. Demonstration that CHM in these women can also be BiCHM [98] and may have mutations in *NLRP7* [121, 131] confirms that these women are single affected individuals with the same autosomal recessive condition. However, in the absence of a family history it is important to carry out genetic testing of molar tissue to confirm diagnosis since women with three or more CHM can also have typical androgenetic CHM [122, 131, 198].

For some women with recurrent molar pregnancies IVF may be an appropriate strategy to achieve normal pregnancy. Intracytoplasmic sperm injection can be used to ensure only a single sperm enters the egg thus preventing dispermic CHM or PHM. Following fertilisation, rejection of 46,XX embryos in favour of 46,XY embryos will eliminate monospermic CHM that arise by doubling of a haploid sperm [199]. Alternatively preimplantation genetic diagnosis can be used to specifically identify embryos with a biparental genotype [200]. However, these strategies will not be effective for women with FRHM since current diagnostic tests would not distinguish between a normal conceptus and a BiCHM. Fortunately there are now options for women with FRHM since the first successful case of a full term normal pregnancy with ovum donation [139]. In this case a patient who was a compound heterozygote for mutations in *NLRP7* achieved a successful pregnancy following ovum donation from her cousin. Since then a further three women with FRHM have gone on to have successful pregnancies using ovum donation.

2.2.2 DIFFERENTIAL DIAGNOSIS OF TROPHOBLASTIC TUMOURS

2.2.2.1 Gestational or Non-gestational Trophoblastic Tumour

GTT are a unique group of tumours in that they are allografts arising not from the patient's own tissue but from a genetically distinct conceptus. They are also unique in their response to cytotoxic drugs. GTT are characterised morphologically by the presence of cytotrophoblast and syncytiotrophoblast cells and biochemically by the production of hCG. However, a number of other tumour types, particularly metastatic lesions, may occasionally show trophoblastic differentiation and hCG production [201, 202]. In cases of hCG-producing metastases of unknown primary or unusual clinical

presentation a non-gestational origin should be considered. Conversely, an unusual presentation such as age or long interval since last known pregnancy should not exclude a diagnosis of GTT [203]. A distinction between a GTT and a non-gestational trophoblastic tumour is important. While a patient with a non-gestational tumour may respond to current chemotherapy, long-term survival is uncommon.

Using genetic techniques it is now possible to determine the gestational or non-gestational origin of trophoblastic tumours. A tumour that is gestational in origin will have a genome that reflects that of the pregnancy in which it arose. If it derives from a normal pregnancy or spontaneous abortion the tumour will have paternal and maternal DNA. If it originates in a CHM the DNA will usually be entirely of paternal origin. In any case the presence of paternal genes will distinguish a GTT from a non-gestational tumour that will contain only DNA from the host. Genetic studies in a wide variety of tissues including brain, lung and ovary have shown that hCG-producing tumours with trophoblastic differentiation may be non-gestational in origin [203-207]. Conversely a small number of tumours, initially thought to be primary tumours of the ovary, have been shown to be gestational in origin [204, 208].

2.2.2.2 The Causative Pregnancy in Gestational Trophoblastic Tumours

The type of pregnancy in which a tumour arises cannot be determined morphologically but may be clinically relevant as tumours that arise following a HM have a more favourable prognosis than those that arise from a term pregnancy or non-molar abortion. The time interval between a pregnancy and the diagnosis of a tumour is also a factor in determining the appropriate chemotherapeutic regime [209] and determining prognosis for women with PSTT/ETT [168]. Therefore, ascertainment of the nature of the causative pregnancy and the time interval between that pregnancy and the diagnosis of the tumour may enable better patient management. Clinically the immediately antecedent pregnancy is perceived as the causative pregnancy. However, in a patient with multiple pregnancies it is not possible to be sure that the last recognised pregnancy is the one in which the tumour arose. A number of genetic studies have now shown that the antecedent pregnancy is not always the causative pregnancy in cases of GTT [194, 203-205, 210, 211]. These investigations have identified tumours that are clearly androgenetic in origin where the antecedent pregnancy was non-molar [194, 212]. Less common are the occasional choriocarcinoma that derive from a normal fertilization although the history is an antecedent molar pregnancy, or tumours in which the sex chromosome complement is different to that of the previous

pregnancy [194]. In some cases it has been shown that the causative pregnancy may in fact be a much earlier pregnancy. One patient has recently been described with two intervening pregnancies between the HM in which the tumour originated and the subsequent development of choriocarcinoma ten years later [203].

2.2.3 DIAGNOSTIC TECHNIQUES

A number of genetic techniques have been used to examine both HM and GTT. While early studies relied on karyotyping of cultured cells from fresh tissue, a number of techniques for examining ploidy and parental origin of archival material have now been developed and used to both investigate and facilitate diagnosis of GTN.

2.2.3.1 Determination of Cell Ploidy

Ploidy of both HM and GTT has been determined by karyotype analysis. However, these techniques generally require cell culture and good quality metaphase spreads for analysis. An assessment of ploidy can be made by other techniques. Flow cytometry, a technique based on the level of fluorescence emitted from labelled nuclei, has been extensively used to distinguish diploid CHM from triploid PHM [91, 213-216] (Figure 2.3) and for identifying the more unusual tetraploids [50]. Flow cytometry has the advantage that it can be applied to cells recovered from formalin-fixed, paraffin-embedded blocks [217]. Other techniques which have been used to determine cell ploidy in the differential diagnosis of molar pregnancies involve fluorochrome staining of interphase nuclei [218] or *in situ* hybridisation with chromosome-specific probes [219-223]. Triploid cells are distinguished from diploid by the presence of three spots rather than two for each probe used.

Flow cytometry and *in situ* hybridisation have revealed more heterogeneity in HM tissue than previously found in most cytogenetic studies. Single cases of an apparently haploid CHM [30] and apparently haploid PHM [224] have been identified by flow cytometry while other studies of cell ploidy have identified aneuploid or polyploid populations of cells in CHM [216, 219, 224, 225]. Flow cytometry has also demonstrated higher proliferative activity in CHM than non-molar placenta. However, neither ploidy nor proliferative activity has been found to be a useful predictor of prognosis in CHM [214, 216, 225, 226]. Flow cytometry has been used to determine ploidy of both choriocarcinoma and PSTT [160, 161, 227-230] and generally shown both to be diploid despite the varied karyotypes identified in choriocarcinomas by cytogenetic analysis. In some cases this may reflect the high proportion of infiltrating host cells present in pathological blocks of tumour tissue

that will be diploid or the fact that the tumours were post-mole GTT since more recent molecular genetic studies suggest this particular group of tumours show few chromosomal abnormalities[156].

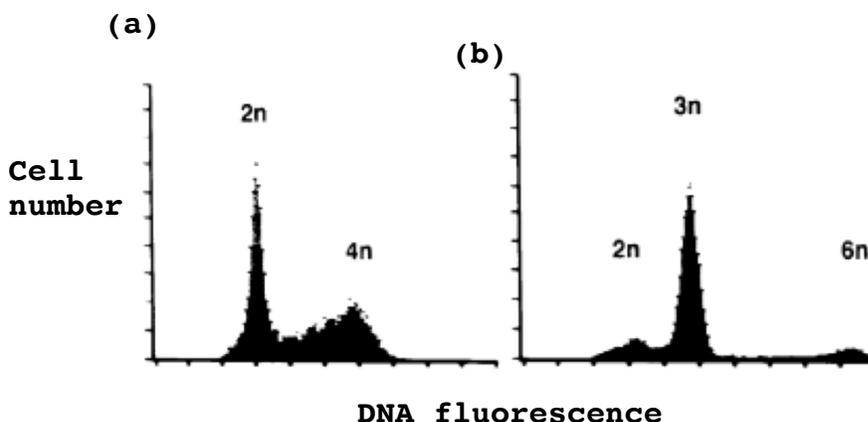


Figure 2.3 Differential diagnosis of CHM and PHM using flow cytometry (a) CHM and (b) PHM tissue. In the CHM a major diploid (2n) peak is observed with a smaller tetraploid (4n) peak representing CHM cells undergoing division. In PHM a small diploid peak is presented which corresponds to contaminating host cells while the major triploid peak (3n) and smaller 6n peak represent resting and dividing cells of the PHM respectively.

While cell ploidy can be very useful for establishing a differential diagnosis between CHM and PHM or PHM and its pathological mimics in problem cases [91, 231] these techniques are unable to distinguish between molar and non-molar triploids. Nor can they generally be used to determine the origin or causative pregnancy in GTT. Analysis of polymorphic markers is required to answer these questions.

2.2.3.2 Molecular Genetic Techniques

Major advances in the diagnosis and classification of GTN came with the introduction of molecular genetic techniques that used DNA polymorphisms as a basis for determining the parental origin of molar or tumour tissue. These polymorphisms, in particular the hypervariable minisatellite polymorphisms [232-234], were very much more informative than the cytogenetic or enzyme polymorphisms previously used [28, 30]. Restriction fragment length polymorphisms [95], DNA fingerprinting [29, 235, 236] and the locus specific minisatellite probes [28-30, 237, 238] have all been used to confirm the androgenetic origin of CHM, to distinguish monospermic from dispermic CHM and confirm the diandric origin of PHM. Several cases have also been reported where these

techniques have been used to identify CHM in twin [239] or multiple pregnancies [240, 241]. In the diagnosis of trophoblastic tumours, restriction fragment length polymorphisms of DNA [157, 204, 205, 242-244] PCR amplification of minisatellite polymorphisms [210] and sex chromosome-specific sequences [205, 212] have all been used to distinguish GTT from non-gestational tumours and identify the causative pregnancy in cases of GTT.

Shorter and more numerous, the microsatellite polymorphisms [245, 246] were subsequently identified. These short repetitive sequences are highly polymorphic, widely dispersed throughout the genome and stably inherited making them ideal markers to study the origin of GTN. Used in conjunction with the polymerase chain reaction (PCR), a technique for amplifying small amounts of DNA, these markers have enabled rapid diagnosis from even small amounts of tissue. Since these microsatellite markers are short enough to be amplified even in degraded DNA prepared from pathological blocks [211, 247, 248] it has become feasible to carry out diagnosis using tissue from formalin-fixed, paraffin-embedded tissue sections. Preparation of DNA from pathological blocks has the advantage over DNA prepared from fresh tissue in that manual or laser capture microdissection of the cells of interest can easily be performed prior to DNA preparation thus minimising any contamination from host cells [154].

More recent modifications of PCR based techniques including the incorporation of fluorescently-labeled primers into the PCR reaction followed by automated sizing of the PCR products and the development of kits that allow multiplexing of the PCR reaction [42, 44, 203] have allowed a number of polymorphisms from different regions of the genome to be examined simultaneously and provide a rapid means of determining genetic origin of both molar and tumour tissue.

More commonly occurring polymorphisms, the single nucleotide polymorphisms (SNPs), have recently been identified. These involve single base differences in the DNA sequence of different individuals [249]. Although genotyping of CHM with a panel of SNPs has confirmed their androgenetic origin [250], individually SNPs (which usually have only two alternative alleles) are much less informative than the microsatellite polymorphisms (which usually have several different alleles) and these remain the marker of choice in the diagnosis of GTN.

2.2.3.3 Genetic Diagnosis of Hydatidiform Moles

One of the major advances in the diagnosis of complete hydatidiform moles has been the introduction of p57^{KIP2} immunostaining which

has enabled CHM to be distinguished from all other types of placental tissue, based on their androgenetic origin [79, 83-88, 124, 251], although caution should be applied in rare cases that are histologically CHM but express p57^{KIP2}, due to retention of maternal chromosome 11 [53, 251]. This technique is also useful for distinguishing different cell populations in the case of CHM with twin and for examining the distribution of androgenetic and normal diploid cells in mosaic conceptions [66, 69]. Fluorescence in situ hybridisation (FISH) may also be useful in determining the distribution of different cell population where these populations have a different sex chromosome complement, or carry a chromosomal abnormality [66].

The more difficult differential diagnosis for molar pregnancies is between PHM and non-molar miscarriage. While FISH can be used to determine ploidy in these cases [252, 253], and has the advantage over flow cytometry that it enables analysis of single cells in situ and is therefore not affected by contaminating maternal cells, these techniques alone cannot distinguish between maternal, non-molar triploids, and PHM with two paternal copies of the genome. In these cases molecular genotyping can provide the additional information to make a definitive diagnosis (Figure 2.4).

In practice parental blood is rarely available and molecular genotyping is performed on DNA prepared from maternal and villous tissue microdissected from the same section. By defining non-maternal alleles identified in the villous tissue as paternal, the parental origin of the tissue can then be established and a diagnosis made. Using fluorescent microsatellite genotyping it has now been possible to discriminate between non-molar miscarriage and PHM in large series of products of conception [42, 254-256] and provide accurate diagnosis in cases of probable or possible PHM [44]. These large series have also demonstrated the power of genotyping in distinguishing between monospermic and dispermic CHM, in identifying tetraploids with 3 paternal chromosome sets and delineating different populations in mosaics or chimeras.

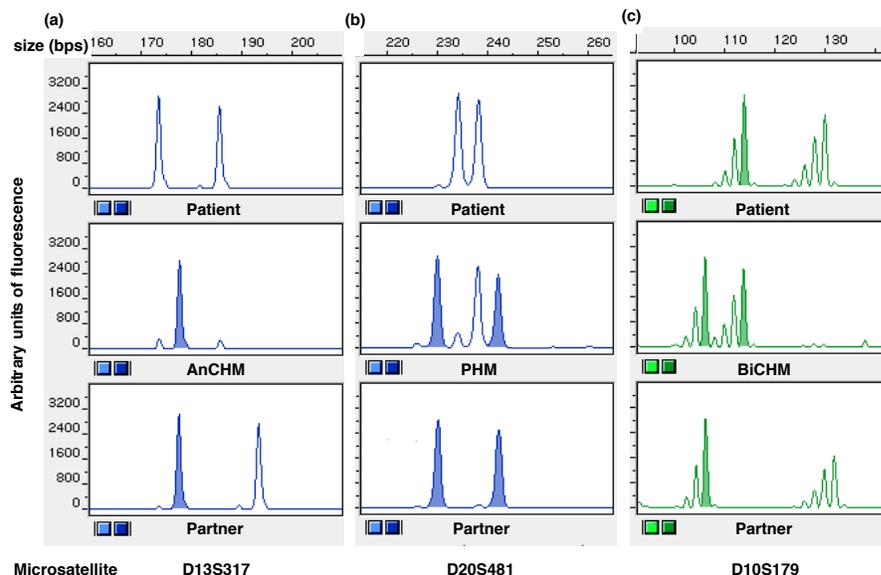


Figure 2.4 Differential diagnosis of HM based on microsatellite polymorphisms identified in DNA from parental blood and molar tissue following PCR amplification with fluorescently labeled primers. The X axis represents the sizes of the DNA fragments generated (in base pairs). (a) An AnCHM, homozygous for the D13S317 microsatellite marker, having a single allele that is the same size as one of the paternal alleles (shaded). No maternal DNA is present indicating that the HM is androgenetic. (b) A PHM, trisomic for the D20S481 locus. The molar DNA has one allele from the patient and two alleles (shaded) from the father, consistent with a diandric triploid conception. (c) A BiCHM that is disomic but has one allele from the patient (shaded) and one from the partner (shaded), consistent with a biparental origin.

Recently molecular genetic diagnosis has played an important role in identifying the rare BiCHM associated with FRHM. Most cases of FRHM have been shown to result from mutations in *NLRP7* or *KHDC3L* [118, 129] with affected women being homozygous or compound heterozygotes for a variety of different mutations including insertions, duplications, deletions and amino acid substitutions. However, there are a small number of women that have diploid BiCHM and no evidence of mutations in either gene and therefore definitive diagnosis relies on the demonstration that recurrent moles are diploid and biparental (Figure 2.4). DNA sequencing of *NLRP7* or *KHDC3L* can then be used to confirm diagnosis by demonstrating the presence of mutations in one of these genes. In addition, identification of the mutations in an affected individual enables screening and counselling of other family members to determine their status (Figure 2.5). This is particularly important for nulliparous women in affected families who wish to become pregnant.

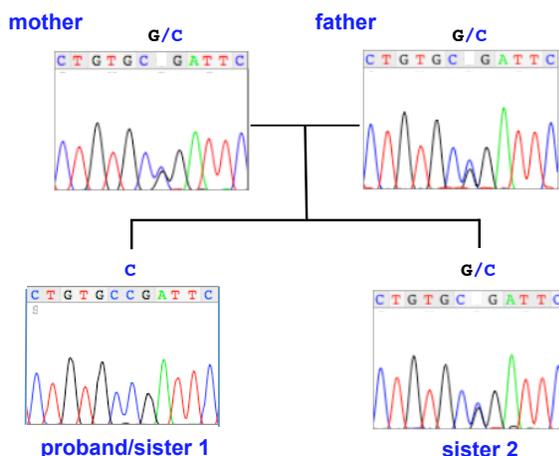


Figure 2.5 Sequence traces from exon 5 of *NLRP7* in a family carrying a 2078G>C (R693P) mutation. Both parents are heterozygous at position 20778, having both a normal G allele and a mutated C allele, and are unaffected. The proband and one sister are homozygous for the mutation having inherited a mutated copy of the gene from each parent. Both have had several CHM and no normal pregnancies. However, their younger sister is heterozygous, having inherited a mutated allele from only one parent, and is unlikely to be affected by the condition.

2.2.3.4 Molecular Genetic Diagnosis of Gestational Trophoblastic Tumours

Genotyping using microsatellite polymorphisms have confirmed the diagnosis of GTT in a variety of tissues including lung [257, 258], ovary [208, 259-264], fallopian tube [258, 265], abdomen [266], kidney [267], and uterus [261, 268] to identify the causative pregnancy in unusual cases of GTT [212, 269] and confirm the origin of intraplacental choriocarcinoma in a mother and infant [270].

The use of microsatellite markers has facilitated two large studies of the genetic origin of trophoblastic tumours [194, 203]. In the first approximately 10% of the cases were found to be non-gestational. Of those found to be GTT half originated in CHM and half in hydropic miscarriages or term pregnancy [194, 205]. However, in at least 8 of the 33 cases of GTT the causative pregnancy was not the clinically antecedent pregnancy. In three the immediately antecedent pregnancy was a CHM while the tumour resulted from a normal conception despite the fact that the last normal pregnancy in one case was 16 years previously. In five cases, where the previous pregnancy was non-molar, two of the tumours had originated in CHM while 3 were found to be of different sex to the antecedent

pregnancy. In a further series of trophoblastic tumours, selected on the basis of uncertain origin [203], a higher proportion of tumours (37%) were found to be non-gestational (Figure 2.6) and a higher proportion of the GTT (83%) to have arisen in non-molar pregnancies.

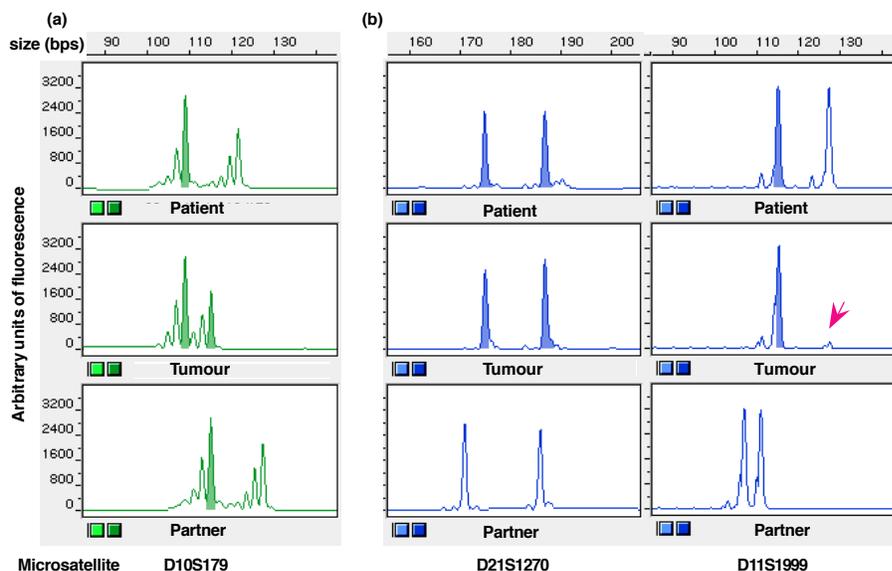


Figure 2.6 Typical, informative microsatellite polymorphisms identified in DNA from parental blood and trophoblastic tumour tissue following PCR amplification with fluorescently labeled primers. (a) A GTT with two alleles, one derived from each parent (shaded). (b) A non-gestational tumour with two alleles identical to those in the patient (shaded) for the D21S1270 locus. For the D11S1999 locus, loss one of the two maternal alleles (arrowed) confirms that the tissue examined is tumour and not contaminating host cells.

While most cases of non-gestational trophoblastic tumours produce high levels of hCG and have pathology resembling choriocarcinoma, one non-gestational tumour with the pathology of a PSTT was found in this series. As in the previous series, a number of patients in this study were found to have post-mole tumours despite the antecedent pregnancy being non-molar [203] (Figure 2.7). Although all post-mole tumours in both these series, and in a more recent study in Chinese patients [261], originated in CHM, genetic analysis has previously shown that both choriocarcinoma and PSTT can arise in PHM [158, 259, 271]. Molecular genotyping is clearly a valuable tool for the differential diagnosis of gestational and non-gestational GTN and for identifying the causative pregnancy, which is not possible on the basis of histopathology alone.

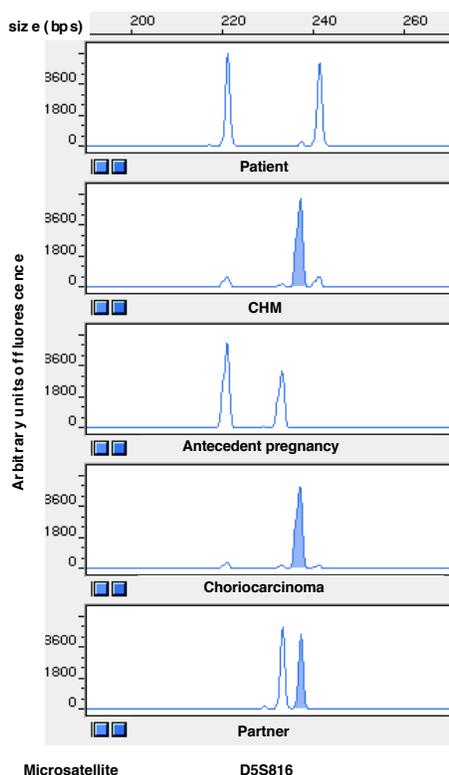


Figure 2.7 Fluorescently labelled products identified following amplification of microsatellite markers in DNA from a patient, her partner, tumour and antecedent pregnancy. The pregnancy antecedent to the development of the choriocarcinoma was shown to have two alleles, one from each parent. The genotype of the tumour was shown to be different to the antecedent pregnancy and to have only a single paternally derived allele (shaded) which was different to the paternal allele in the antecedent pregnancy. The genotype of the tumour was consistent with an origin in an androgenetic conceptus and was subsequently shown to be identical to that of the CHM in a previous twin pregnancy with CHM and co-existent live born infant.

At present many of the specific changes giving rise to tumour development have yet to be identified. Use of molecular genetic techniques together with novel technologies such as microarray analysis, next generation sequencing and analysis of epigenetic changes should facilitate these investigations and enable biomarkers, that that can predict the outcome of molar pregnancies, to be identified.

2.3. SUMMARY

CHM are generally diploid and androgenetic in origin while PHM are diandric triploids differences that can be exploited to facilitate correct diagnosis. The introduction of p57^{KIP2} immunostaining has greatly facilitated the differential diagnosis of PHM and CHM but genotyping can still be helpful in making a differential diagnosis between PHM or non-molar miscarriage. p57^{KIP2} immunostaining and molecular genotyping also important for investigating the unusual mosaics and chimeras, where genetic analysis can identify both the origin and distribution of the different cell lines present, and identification of BiCHM, a diagnosis that cannot be made by the pathologist. Recent identification of *NLRP7*, and *KHDC3L* as the genes mutated in most cases of FRHM, have enabled confirmation of diagnosis and screening of nulliparous women in affected families. Further studies are needed to identify the mechanisms by which mutations in *NLRP7* and *KHDC3L* lead to the aberrant imprinting seen in BiCHM.

Genotyping has also proved important in the management of patients with trophoblastic tumours, in particular the differential diagnosis between GTT and non-gestational trophoblastic tumours for which there is a very different prognosis. Current challenges are to find markers of malignancy in molar tissue that would enable early identification of those women who will go on to develop GTN and markers of resistant disease for women receiving chemotherapy.

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