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ARTICLE

Refined diagnosis of hydatidiform moles with p57 immunohistochemistry and molecular genotyping: updated analysis of a prospective series of 2217 cases

Deyin Xing[1,2,3] · Emily Adams[1] · Jialing Huang[1] · Brigitte M. Ronnett[1,2]

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Abstract

Immunohistochemical analysis of p57 expression and molecular genotyping accurately subclassify molar specimens into complete hydatidiform mole (CHM) and partial hydatidiform mole (PHM) and distinguish these from nonmolar specimens. Characteristics of a prospective series of potentially molar specimens analyzed in a large gynecologic pathology practice are summarized. Of 2217 cases (2160 uterine, 57 ectopic), 2080 (94%) were successfully classified: 571 CHMs (570 uterine, 1 ectopic), 498 PHMs (497 uterine, 1 ectopic), 900 nonmolar (including 147 trisomies, 19 digynic triploids, and 4 donor egg conceptions), and 56 androgenetic/biparental mosaics; 137 were complex or unsatisfactory and not definitively classified. CHMs dominated in patients aged < 21 and >45 years and were the only kind of molar conception found in the latter group. Of 564 successfully immunostained CHMs, 563 (99.8%) were p57-negative (1 p57-positive [retained maternal chromosome 11] androgenetic by genotyping). Of 153 genotyped CHMs, 148 (96.7%) were androgenetic (85% monospermic) and 5 were biparental, the latter likely familial biparental hydatidiform moles. Of 486 successfully immunostained PHMs, 481 (99%) were p57-positive (3 p57-negative [loss of maternal chromosome 11], 2 unknown mechanism). Of 497 genotyped PHMs, 484 (97%) were diandric triploid (99% dispermic) and 13 were triandric tetraploid (all at least dispermic). Of 56 androgenetic/biparental mosaics, 37 had a p57-negative complete molar component (16 confirmed as androgenetic by genotyping). p57 expression is highly correlated with genotyping, serving as a reliable marker for CHMs, and identifies molar components and androgenetic cell lines in mosaic conceptions. Correlation of morphology, p57 expression, genotyping data, and history are required to recognize familial biparental hydatidiform moles and donor egg conceptions, as the former can be misclassified as nonmolar and the latter can be misclassified as dispermic CHM on the basis of isolated genotyping results.

Introduction

Hydatidiform moles are abnormal placentas with variable degrees of trophoblastic hyperplasia and villous hydrops, with or without embryonic development [1–3]. Since the risk of persistent gestational trophoblastic disease differs for complete hydatidiform moles (CHMs), partial hydatidiform moles (PHMs), and nonmolar specimens (up to 15–20% risk for CHMs but <5% for PHMs) [4–7], correct classification of products of conception specimens into different types of hydatidiform moles and distinction of them from nonmolar specimens are important for clinical management [8–10]. Traditionally, hydatidiform moles are diagnosed based on evaluation of their morphologic features, including the degree of trophoblastic hyperplasia/proliferation, the sizes and shapes of chorionic villi, the presence of trophoblastic inclusions, and villous stromal changes [11–15]. However, it has been well demonstrated that diagnosis of hydatidiform moles based solely on morphology is subject to interobserver and intraobserver variability and that diagnostic reproducibility is suboptimal [16–19]. In fact, a reproducibility study conducted in our institution using

These author contributed equally: Deyin Xing, Emily Adams, Jialing Huang

✉ Brigitte M. Ronnett
bronnett@jhmi.edu

[1] Department of Pathology, The Johns Hopkins Medical Institutions, Baltimore, MD, USA

[2] Department of Gynecology and Obstetrics, The Johns Hopkins Medical Institutions, Baltimore, MD, USA

[3] Department of Oncology, The Johns Hopkins Medical Institutions, Baltimore, MD, USA

genotyping results for the true ("gold standard") diagnoses showed that the percentage of correct classification of all cases (CHMs, PHMs, and nonmolar specimens) by morphology ranged from 55 to 75% for individual pathologists and from 70 to 75% by consensus, with all participants being pathologists with subspecialty training in gynecologic pathology [20, 21]. This imperfect reproducibility is attributable to the fact that a variety of nonmolar entities, including those with abnormal villous morphology, early nonmolar specimens with prominent trophoblastic hyperplasia, hydropic abortuses, and androgenetic/biparental mosaic conceptions, can exhibit some features suggestive of a molar pregnancy. In addition, CHMs and PHMs can simulate each other if lacking fully developed classical morphologic features, so subtyping of hydatidiform moles can also be problematic.

To overcome morphology-based suboptimal reproducibility and correctly diagnose hydatidiform moles, ancillary studies, including immunohistochemical analysis of p57, the protein product of the *CDKN1C* imprinted gene located at chromosome 11p15.5, and molecular genotyping via polymerase chain reaction (PCR) amplification of short tandem repeat (STR) have been employed in routine practice [1, 3, 22–33]. Due to lack of maternal DNA and paternal imprinting of the p57 gene, CHMs, including early forms, which are purely androgenetic conceptions, lack p57 expression in the villous cytotrophoblast and villous stromal cells; decidua and intermediate trophoblastic cells are positive, serving as internal positive control for an immunostain that relies on a negative result. A reproducibility study using genotyped cases from our laboratory demonstrated that incorporation of p57 immunohistochemistry into the diagnostic algorithm significantly improved the diagnosis of CHMs compared with morphologic assessment alone, even for experienced gynecologic pathologists [20]. In contrast, PHMs and nonmolar specimens, including those with abnormal villous morphology and/or hydropic change as well as nonmolar digynic triploid conceptions, express p57 in villous cytotrophoblast and villous stromal cells, serving to distinguish these entities as a group from CHMs; however, p57 cannot distinguish among these entities since they all share this p57 expression pattern; thus, DNA genotyping is required to differentiate them.

In 2007, we began a prospective analysis of all potentially molar products of conception specimens encountered on the Gynecologic Pathology Consultation and In-house Services of The Johns Hopkins Hospital, Baltimore, MD, using immunohistochemical analysis of p57 expression and molecular genotyping with STR markers per a proposed diagnostic algorithm [26]. Initially, all specimens were subjected to both analyses; this was later modified to triage cases for genotyping based on p57 results: p57-negative cases were diagnosed as CHMs without genotyping, provided there was some appropriate morphology, and p57-positive cases were genotyped and diagnosed as PHMs or as nonmolar, depending on the genotyping result. In 2013, we reported the results of 6 years of analysis of 678 cases [23]. In this study, we provide an updated analysis of a larger series of 2217 cases in a single institution, with further assessment of the performance of these methods in clinical practice and expanded observations on interesting and problematic aspects.

Materials and methods

From July 2007 through April 2020, 2217 cases were analyzed, including the previously reported 678 cases. These included 1932 (87%) consultation cases and 285 (13%) routine in-house cases. According to the diagnostic algorithm we proposed previously [26] all cases encountered on the Gynecologic Pathology Consultation and In-house Services of The Johns Hopkins Hospital, Baltimore, MD, for which there was any clinical or pathological concern for a molar specimen were prospectively analyzed. Briefly, all specimens were initially subjected to both p57 immunohistochemical analysis and molecular genotyping with STR markers. This process was later modified to triage cases for genotyping based on p57 results: p57-negative cases were diagnosed as CHMs without genotyping, given the excellent performance of this assay (with some exceptions to confirm or resolve unusual or problematic cases, as detailed in the results), and all p57-positive cases were subjected to genotyping (with some exceptions depending on whether other ancillary testing resolved the diagnosis in conjunction with the p57 result—see below for further details). In addition, genotyping was performed to refine the diagnosis in certain uncommon situations that were described in detail previously [23, 26]. Since data on zygosity and sex chromosome constitutions for molar specimens was not included in the pathology reports, the Molecular Diagnostics Laboratory system database had to be accessed to retrieve these detailed data; a high proportion of the cases was retrieved but data for some could not be obtained.

p57 immunohistochemistry

Immunohistochemical analysis of p57 expression was performed using a mouse monoclonal antibody against p57 protein (predilute, Neomarkers, Fremont, CA, USA), as described previously [23]. Briefly, the presence or absence of nuclear positivity of p57 was assessed in villous stromal cells, cytotrophoblast, intermediate trophoblast, and maternal decidua. The p57 immunostain was interpreted as follows:

(1) Negative: villous stromal cells and cytotrophoblast are either entirely negative or demonstrate only limited expression (nuclear staining in <10% of these cell types) with satisfactory expression in maternal decidua and/or intermediate trophoblastic cells as internal positive control. This pattern characterizes CHMs.

(2) Positive: villous stromal cells and cytotrophoblast are both either diffusely positive (≥50%) or both types are focally positive (≥10% but <50% of both cell types in a patchy/nondiffuse pattern in villi, with no villi having only one cell type positive [see below for description of such a discordant pattern] and no sufficiently preserved villi being completely negative [extensively degenerated villi can be nonreactive]). The focal positive result had been considered an "equivocal" positive result in our initial assessments but we soon modified this to interpretation as simply positive based on our ongoing experiences with molecular genotyping showing that, with only rare exceptions, this partially positive result is essentially never encountered in CHMs. This pattern, whether diffuse or focal/patchy, characterizes PHMs and nonmolar abortuses.

(3) Discordant: positive staining in cytotrophoblast and negative staining in villous stromal cells, or vice versa, within individual villi. Discordant p57 expression characterizes androgenetic/biparental mosaic/chimeric conceptions.

(4) Divergent: two populations of villi, each with different morphologies, exhibiting two different staining patterns, e.g., a twin gestation comprised of a p57-negative androgenetic CHM and a p57-positive biparental nonmolar specimen or a mosaic specimen comprised of a p57-discordant nonmolar biparental component and a p57-negative androgenetic CHM component.

(5) Unsatisfactory: the staining result cannot be interpreted due to extensive necrosis/degenerative changes leading to loss of immunoreactivity, technical failure, or a negative preparation lacking internal positive control.

Molecular genotyping

The PCR-based STR analysis has been described in detail previously [23, 28]. Initially, PCR amplification of nine STR loci from eight different chromosomes (chromosomes 2, 3, 4, 5, 7, 11, 12, 13) and the amelogenin locus (for XY determination) was performed, with thermal cycling conditions and capillary electrophoresis carried out according to the manufacturer's instructions (AmpFlSTR Profiler kit; Applied Biosystems; Foster City, CA). An expanded analysis with PCR amplification of 15 STR loci from 13 different chromosomes (chromosomes 2–5, 7, 8, 11–13, 16, 18, 19, 21) and the amelogenin locus (for XY determination) (AmpFlSTR Identifiler kit; Applied Biosystems) replaced the initial 9-marker panel analysis since 2013. Both the maternal and villous tissues were analyzed to identify alleles at each locus. Allelic ratios (dividing the peak height of the longer allele by the peak height of the shorter allele) between 0.61 and 1.17 were considered to be consistent with diploidy. Allelic ratios between 0.33 and 0.60 or 1.5 and 2.0 or in a 1:1:1 ratio were considered to be consistent with triploidy. Every allele from the villous tissue was identified as being maternal, definitively nonmaternal (assumed paternal), or equivocal (unknown whether maternal or paternal due to shared alleles) in origin. The molecular genotyping result was interpreted as follows:

(1) the presence of only nonmaternal/novel/paternal chromosome complement(s) indicating a purely androgenetic conception was diagnosed as CHM (diploidy versus tetraploidy cannot be distinguished by genotyping);

(2) the presence of either 1 maternal and 2 novel/paternal chromosome complements indicating diandric triploidy (paternal:maternal allele ratio of 2:1), or 1 maternal and 3 novel/paternal chromosome complements indicating triandric tetraploidy (paternal:maternal allele ratio of 3:1) was diagnosed as PHM;

(3) the presence of both maternal and paternal chromosome complements with equal ratio indicating a biparental conception with allelic balance, or 2 maternal and 1 paternal chromosome complements indicating digynic triploidy (paternal:maternal allele ratio of 1:2), was diagnosed as nonmolar;

(4) p57-discordant villi, with variable paternal:maternal allele ratios usually >2:1 indicating an admixture of androgenetic and biparental cell lines within individual villi, was diagnosed as a mosaic/chimeric conception; if there was also a p57-negative purely androgenetic component this was additionally diagnosed as a CHM component.

Other ancillary tests

Additional analyses, which were not part of our diagnostic algorithm, including DNA ploidy analysis, cytogenetics (karyotyping), fluorescence in situ hybridization (FISH), and single-nucleotide polymorphism (SNP) array, were sometimes provided in the accompanying pathology reports (consultation cases) or available in the patient's laboratory information (in-house cases) and were sometimes used to guide our algorithmic approach. For example, if karyotyping demonstrated a trisomy, then the case was not genotyped and diagnosed as nonmolar, or if DNA ploidy of villous tissue was diploid and p57 was positive, then the case was also not genotyped and diagnosed as nonmolar.

Results

General information

The study included a total of 2217 cases (1932 consultation and 285 in-house) including the previous reported 678 cases.

Diagnostic categorization of cases with age distribution, p57 immunohistochemical analysis and molecular genotyping results are summarized in Table 1. The mean and median ages for all diagnostic categories were similar (29–32 years). Age ranges were similar for PHMs, nonmolar specimens, and mosaic cases (13–49 years combined) but different for CHMs for which the age range was 12–64 years. Interestingly, stratified age analysis revealed a bimodal distribution for CHMs, with a high proportion in the youngest age group (second decade) and also in those older than 45 years (Fig. 1a, b). In fact, 90% of patients (57 of 63 cases) aged > 45 years had CHMs, with only a few nonmolar specimens but no PHMs in this age group. In patients aged 50 or older there were only CHMs. In those <21 years, 48% had CHMs, 25% PHMs, and 27% nonmolar abortuses (Fig. 1b). A distribution plateau was observed for PHMs and the proportion declined with increasing age (Fig. 1b). Unlike CHMs, the peak for nonmolar abortuses was from 35 to 45 years.

Overall, 2214 of 2217 cases were subjected to p57 immunohistochemical analysis and 1633 were subjected to genotyping per our algorithm. Detailed results of immunohistochemical analysis of p57 expression and genotyping are summarized in Table 1. In all, 2181 of the 2214 cases (98.5%) subjected to p57 immunohistochemical analysis had satisfactory p57 results (Table 1), with 33 (1.5%) unsatisfactory (nonreactive) or suboptimal (limited weak/equivocal expression in the setting of suboptimal internal positive control) due to degenerative changes and/or technical factors. Also, 1595 of the 1633 cases (97.7%) subjected to genotyping yielded satisfactory results (Table 1). For uterine specimens, definitive classification obtained by either immunohistochemical analysis, genotyping, and/or occasional other ancillary tests resulted in diagnosis of 570 CHMs, 497 PHMs, 900 nonmolar abortuses, and 56 androgenetic/biparental mosaics. Among 57 ectopic pregnancies, 1 CHM and 1 PHM were diagnosed; the remainder of these was nonmolar. Of the 2217 total cases, 137 cases (6%) were categorized as nondefinitive/problematic; thus, overall 94% of cases were definitively interpreted. All 137 cases had p57 analysis but 94 did not undergo molecular genotyping. Reasons for being unable to perform genotyping included insufficient villi, villi being too intimately admixed with decidua for successful microdissection of pure tissue components, or a lack of decidua that precluded definitive interpretation even if the villous tissue was successfully analyzed (required for comparison of villous and maternal DNA patterns to determine parental sources of the chromosome complements). In these 137 cases, molecular genotyping was performed in 33 cases but the results could not be definitively interpreted due to unsuccessful PCR amplification or complex genotypes not conforming to the recognized patterns described above.

Complete hydatidiform moles

Representative examples of CHMs, including early forms, are illustrated in Figs. 2 and 3. Overall, 569 of 570 CHMs (511 consultation and 59 in-house) had p57 analysis. Of these, 535 displayed a negative p57 result in the villous cytotrophoblast and villous stromal cells, with internal positive control in decidua and/or intermediate trophoblast (Figs. 2c, d and 3c, f). Fourteen showed an overwhelmingly negative p57 result but some scattered villous stromal cells amounting to <10% of total cells were positive, as is allowed for a diagnostic of a CHM (Fig. 4); five of these were genotyped and confirmed as androgenetic and the remaining nine were accepted as CHMs based on morphology plus the overwhelmingly negative p57 result. Interpretation of p57 was suboptimal in five cases due to technical issues or lack of a valid internal positive control. These cases were confirmed by molecular genotyping, which demonstrated that these were androgenetic conceptions. The 1 p57-positive CHM was shown to be androgenetic with chromosome 11 trisomy—proven to be a retained maternal copy of chromosome 11 accounting for the retained p57 expression (Fig. 5) [29]. p57 immunostaining was not performed in one case with definitive morphologic features of invasive CHM with cervical involvement. The CHMs included 14 cases occurring in multiple gestations (12 twins, 1 triplet, 1 quintuplet), with 7 being first trimester and 7 being more mature (second and third trimester). Ten of these cases in which both components were stained displayed a divergent p57 staining pattern characterized by a p57-negative CHM and p57-positive nonmolar specimen (Fig. 6); the remaining four had only the CHM component assessed and these were negative as well. There were 28 invasive CHMs encountered in hysterectomy specimens, with 5 cases having atypical trophoblastic proliferations morphologically consistent with choriocarcinoma. In addition, choriocarcinoma was diagnosed in five cases with associated noninvasive CHMs. Most of these cases have been presented in detail in prior publications, with the molar-associated choriocarcinomas being androgenetic and most often monospermic XX, and the reader is referred to those studies for discussion and illustration of issues related to diagnosing choriocarcinoma in the setting of molar and nonmolar villi [32, 34].

We have previously reported 106 genotyped CHMs [23] and subsequently 51 more cases were analyzed. Of these 157 cases, 153 were successfully genotyped and 4 cases were not able to be precisely assessed due to technical issues. Of these 153, 148 were androgenetic and 5 were biparental. Complete genotyping data with zygosity were retrieved from the laboratory database for 144 CHMs with a pure androgenetic genotype; 122 (85%) were monospermic/homozygous (XX) and 22 (15%) were dispermic/

Table 1 Age information, p57 immunohistochemistry, and molecular genotyping in 2217 cases.

	CHM (570)	PHM (497)	NM (900)	Mosaics (56)	Ectopic (57)	Nondefinitive (137)
Age (years)						
Mean	30.3	29.9	32.1	30.2	30.0	31.9
Median	29	30	32	30	29	32
Range	12–64	13–45	13–49	16–45	18–44	17–47
P57						
Positive	1[a]	481	887	0	54	121
Negative	553[b]	5[c]	2[d]	0	1	8[e]
Discordant	0	0	0	19	0	2[e]
Divergent	10[f]	0	0	37	0	0
Unsatisfactory	5[e]	10[e]	10[e]	0	2[e]	6[e]
Not performed	1	1	1	0	0	0
STR genotyping						
Androgenetic	148	–	–	–	–	1[g]
Diandric triploidy	–	484	–	–	1	0
Triandric tetraploidy	–	13	–	–	–	–
Digynic triploidy	–	–	19	–	–	–
Biparental	5[h]		851	–	33	9
Androgenetic/ biparental (no molar component)	–	–	–	13	–	–
Androgenetic/ biparental (with androgenetic molar component)	–	–	–	16	–	–
Complex/ problematic				2		
Unsatisfactory	4[i]	–	–	–	1[i]	33[i]
Not performed	413	–	30	25	22	94

CHM complete hydatidiform mole, *PHM* partial hydatidiform mole, *NM* nonmolar, *STR* short tandem repeat.

[a]Androgenetic conception with retained maternal chromosome 11.

[b]Includes four multiple gestations with a p57-negative CHM component; includes 28 invasive CHMs.

[c]Three with loss of maternal chromosome 11 and two with unknown mechanisms.

[d]No features of familial biparental CHM; one with Beckwith–Wiedemann syndrome and one with unknown mechanisms accounting for negative p57.

[e]Degenerative villi.

[f]Ten cases of multiple gestations with p57-negative CHM component and p57-positive nonmolar component; four additional cases of twin CHM with term placenta not assessed for p57.

[g]The histologic appearance was not suggestive of a CHM.

[h]Four patients, with a total of nine specimens, including five genotyped CHMs with typical morphology, negative p57 immunostaining, and biparental genotypes, probably representing familial recurrent CHMs.

[i]Genotyping unsatisfactory due to insufficient villi, villi being too intimately admixed with decidua, no decidua, unsuccessful PCR amplification, or complex genotypes.

heterozygous (17 XY, 5 XX) (Table 2). The four patients with biparental CHMs had a total of nine specimens, with two patients having more than one confirmed CHM and five of these being available for genotyping. The first patient had five products of conception specimens over an 8-year period —three specimens were unequivocally diagnosed as CHM/early CHM by morphologic features and p57-negative immunostaining and two had overt features of CHMs but p57 analysis was not performed. The genotyping result for the most recent specimen demonstrated a biparental DNA pattern. The second patient had two products of conception specimens in a 3-year period; both were diagnosed as CHM/early CHM, were p57-negative (Fig. 7), and had biparental DNA patterns (Fig. 8). The third patient had a history of recurrent abortions but only the most current specimen was available for analysis; it was p57-negative and had a biparental DNA pattern. The last case was initially interpreted as a nonmolar conception based on a biparental

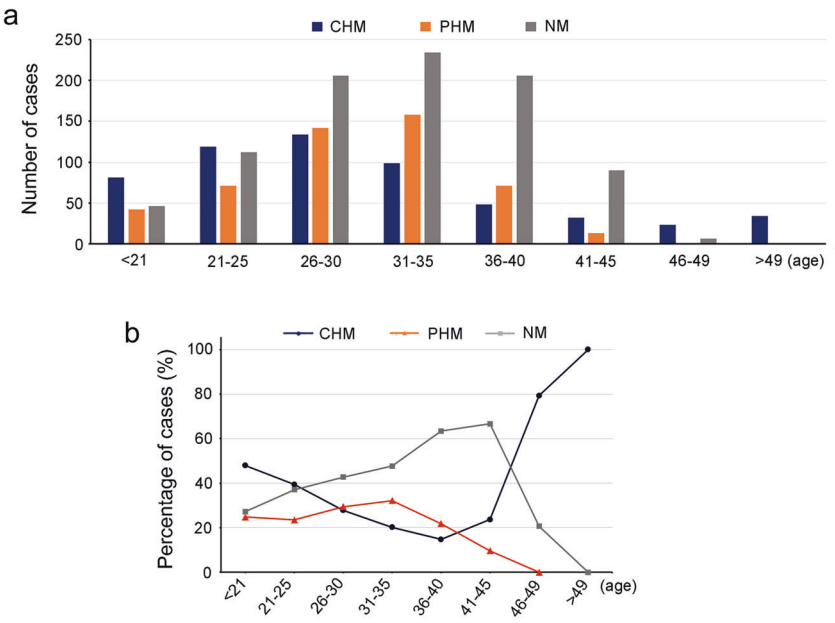

Fig. 1 Age distribution of patients with CHM, PHM, and NM. a Case number of CHM, PHM, and NM in stratified age groups. In patients aged 50 or older there were only CHMs. **b** Percentage of patients with CHM, PHM, and NM in stratified age groups. A bimodal distribution of CHMs, with highest proportions in the age group of <21 years and >45 years, is seen. CHM complete hydatidiform mole, PHM partial hydatidiform mole, NM nonmolar.

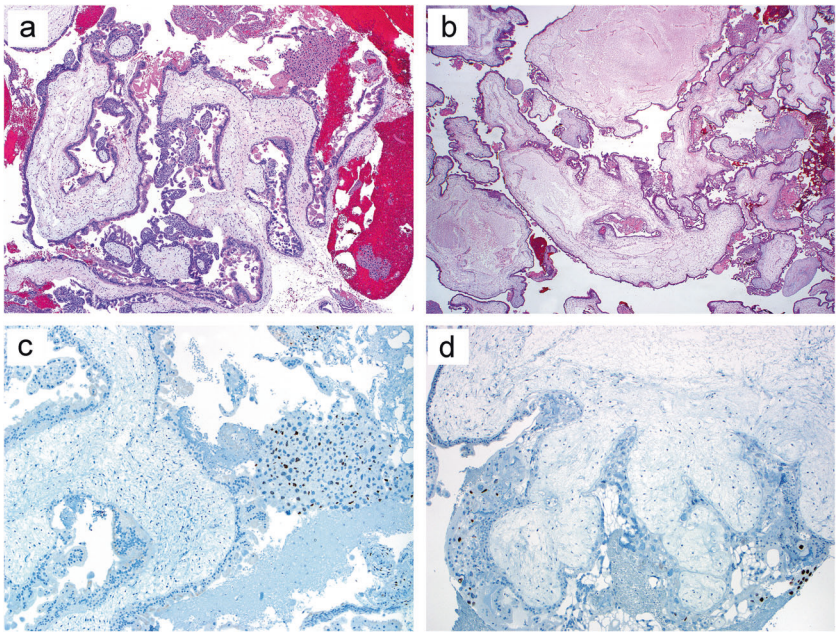

Fig. 2 Complete hydatidiform moles (two examples). a, **b** Hydropically enlarged villi have trophoblastic hyperplasia, trophoblastic inclusions, and cisterns. **c**, **d** Loss of p57 expression in villous cytotrophoblast and stromal cells confirms the diagnoses (internal positive control in intermediate trophoblastic cells). Genotyping demonstrated purely androgenetic DNA patterns for these.

genotyping result at the outside institution. A diagnosis of early CHM was rendered based on the combination of morphology and a negative p57 immunostain in our laboratory. Based on their morphology, p57 results, and biparental genotypes, these cases most likely represent familial biparental recurrent CHMs, but genetic data from the patients was not available to confirm this syndrome in these cases.

In our consultation service, we have encountered 19 CHM cases in which p57 immunostains submitted by the contributing laboratories demonstrated some degree of increased nonspecific cytoplasmic staining, raising some concern for the validity of the immunoreaction and whether a CHM was clearly excluded by the presence of nuclear expression in villous cells. In these cases, either diffuse p57 expression (Fig. 9a, b) or some degree of selective expression in one villous cell type but not the other (e.g., stromal cells but not cytotrophoblast) suggested, respectively, that the lesion was not a CHM or might be mosaic (the latter due to an apparent discordant expression pattern). However, p57 immunostains performed in our laboratory, which did not have the nonspecific cytoplasmic staining (Fig. 9c, d), showed that these cases were p57-negative and genotyping confirmed them as androgenetic conceptions.

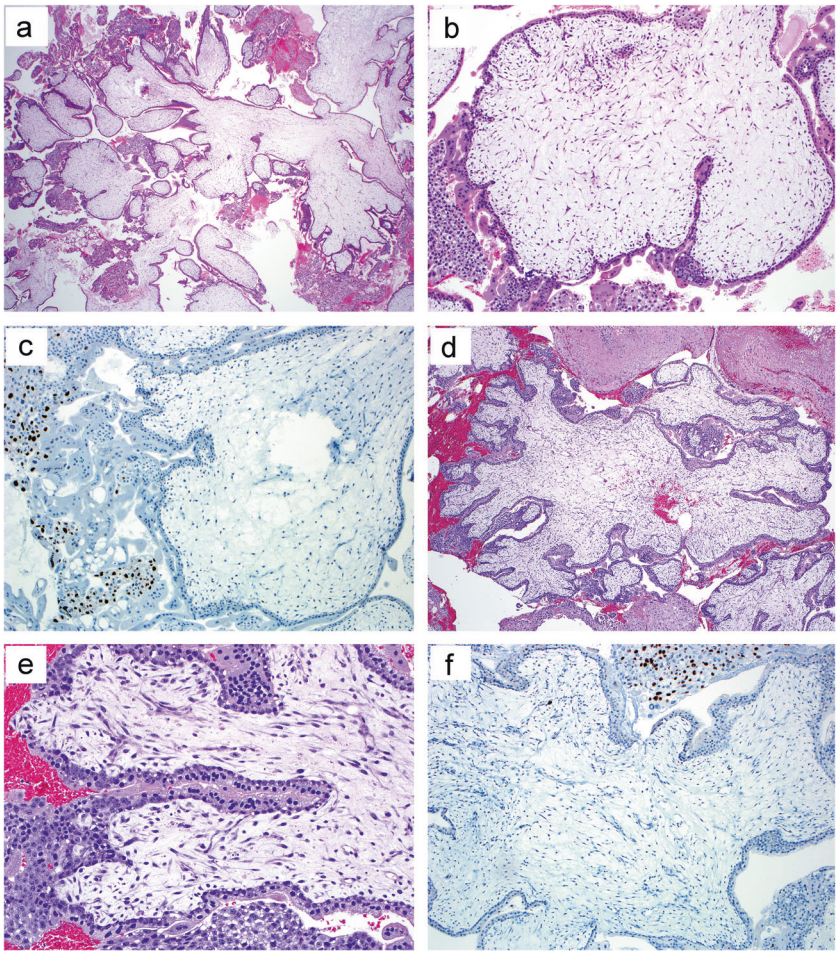

Fig. 3 Early complete hydatidiform moles (two examples). a, **b**, **d**, **e** Bulbous cauliflower-like villi, trophoblastic hyperplasia on villous tips, and slightly cellular villous stroma with canalicular vascular structures and karyorrhectic debris. **c**, **f** Loss of p57 expression in villous cytotrophoblast and stromal cells is characteristic (internal positive control in intermediate trophoblastic cells). Genotyping demonstrated purely androgenetic DNA patterns for these.

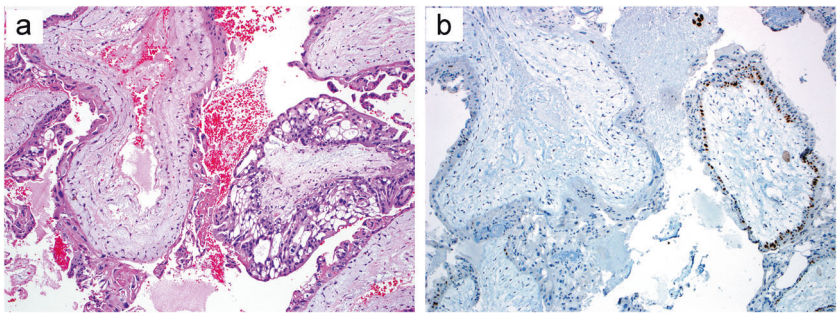

Fig. 4 Limited p57 expression in a complete hydatidiform mole. a Villi display circumferential trophoblastic hyperplasia and some cistern formation. **b** Virtually all villi lack p57 expression in villous cytotrophoblast and villous stromal cells, as seen in the left villous structure, but a rare villus has expression in the cytotrophoblastic cell layer (<10% of cells in stained section). Genotyping demonstrated a purely androgenetic DNA pattern. This limited amount of p57 expression is allowed in an otherwise p57-negative specimen with appropriate morphology for diagnosis as a complete hydatidiform mole. It is possible that such rare p57-positive villi represent a minor mosaic component (rare biparental cells in an androgenetic conception) or could be related to some focal epigenetic relaxation of imprinting.

Partial hydatidiform moles

Representative examples of PHMs are illustrated in Fig. 10. Overall, 496 of 497 PHMs (443 consultation and 54 in-house) had p57 analysis (1 did not have this analysis for a technical reason but was genotyped). Of these, 422 cases showed p57 expression in the villous cytotrophoblast and villous stromal cells with good staining quality (Fig. 10g). A total of 59 cases exhibited a weak/focal/diminished

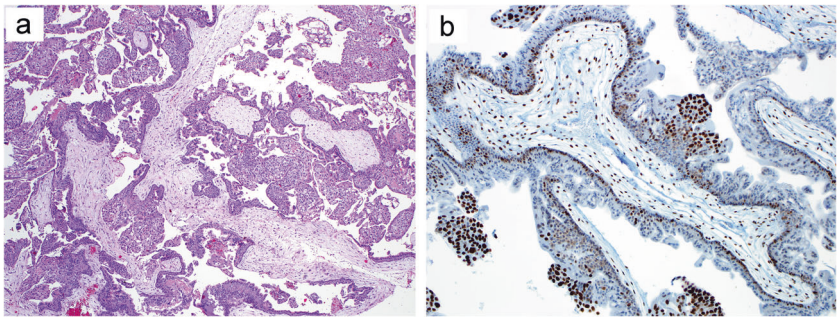

Fig. 5 Complete hydatidiform mole with aberrant p57 expression. **a** Enlarged hydropic villi with prominent trophoblastic hyperplasia are characteristic of a complete hydatidiform mole. **b** Retained p57 expression in villous cytotrophoblast and villous stromal cells led to some doubt about the diagnosis of a complete hydatidiform mole, but genotyping confirmed this as an androgenetic conception with a retained maternal chromosome 11 accounting for the p57 expression (**b**; see McConnell et al. [29] and Banet et al. [23]).

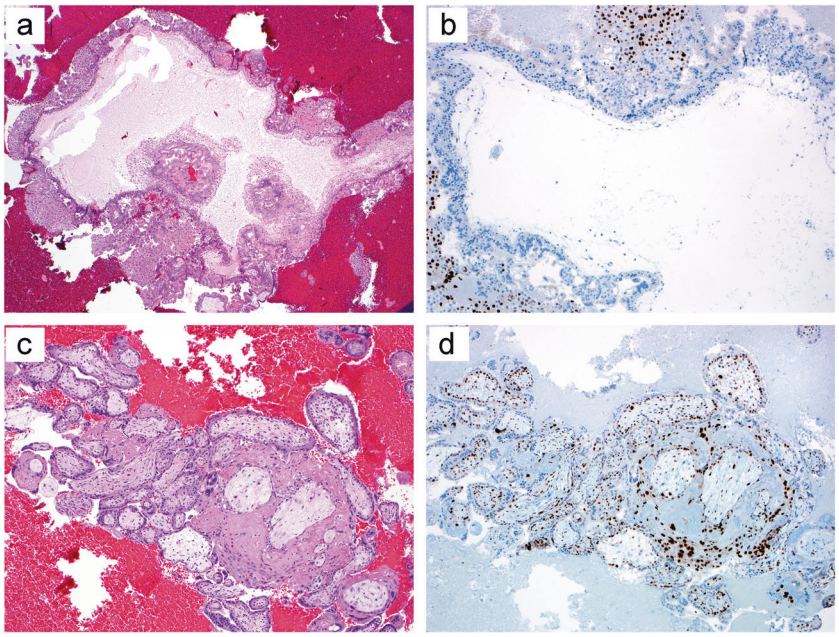

Fig. 6 Molar twin gestation. **a**, **b** A p57-negative complete hydatidiform mole component is composed of enlarged villi with trophoblastic hyperplasia, cistern formation, and trophoblastic inclusions. **c**, **d** A p57-positive nonmolar component comprised of small immature villi lacking molar features is also present in the same specimen. Genotyping confirmed the former as androgenetic and the latter as biparental.

staining pattern yet with extent of staining being more than 10% and thus interpreted as still positive (Fig. 10h). A total of 15 cases were negative/nonreactive for p57: seven due to extensive degenerative changes, three with technical issues, three with loss of maternal chromosome 11 demonstrated by genotyping (Fig. 11) (one of these was previously reported [30]), and two with unknown mechanisms.

All 497 cases were molecularly confirmed as PHMs by genotyping, with 484 having diandric triploidy and 13 having triandric tetraploidy (Fig. 11). Complete genotyping data with zygosity were retrieved from the laboratory database for 481 cases with diandric triploidy; 476 (99%) were dispermic (283 69,XXY, 160 69,XXX, 28 69,XYY, 3 68,XY, 2 68,XX) and 5 (1%) were monospermic (all XXX) (Table 2). The proportions of XXY, XXX, and XYY in these PHM cases were 59%, 35%, and 6%, respectively. Complete genotyping data retrieved from 12 of 13 triandric tetraploid PHMs demonstrated that these were at least dispermic (5 XXYY, 5 XXXY, 2 XXXX) (Table 2); genotyping does not allow for assessing more than this with regard to the number of sperm involved—further investigation via SNP array analysis to address this is provided in a recent separate publication [33].

Interestingly, 53 cases with triploidy detected by non-PCR-based ancillary analysis, which could not determine the source of the triploidy (diandric versus digynic), were further confirmed as PHMs by molecular genotyping. Two cases with tetraploidy and one with diploidy analyzed by DNA content analysis turned out to be diandric triploidy PHMs per genotyping, highlighting that DNA ploidy analysis can be unreliable and demonstrating the value of molecular genotyping. While virtually/essentially all invasive hydatidiform moles encountered in our experience have been CHMs, we encountered one PHM, confirmed by

Table 2 Zygosity data.

	CHM (androgenetic)			PHM (diandric triploidy)[a]				PHM (triandric tetraploidy)				NM (digynic triploidy)			NM (biparental tetraploidy)[b]		
	XX	XY	Total (%)	XXY	XXX	XYY	Total (%)	XXXX	XXYY	XXXY	Total (%)	XXX	XXY	Total (%)	XXYY	XXXX	Total (%)
Monospermic (homozygous)	122	0	122 (85%)	0	5	0	5 (1%)	0	0	0	0	7	7	14 (100%)	8	9	17 (100%)
Dispermic (heterozygous)	5	17	22 (15%)	283	160	28	471 (99%)	2[c]	5[c]	5[c]	12 (100%)	–	–	–	0	0	0
Total	127	17	144	283	165	28	476	2	5	5	12	7	7	14	8	9	17

CHM complete hydatidiform mole, *PHM* partial hydatidiform mole, *NM* nonmolar.

[a]Two cases with 68,XX (karyotyping and genotyping); three cases with 68,XY (genotyping).

[b]One case with 91,XXY (DNA ploidy analysis and genotyping).

[c]At least dispermic (genotyping cannot determine more than this [see Bynum et al. 2020, Ref. 33]).

molecular genotyping, with features of an invasive hydatidiform mole in the hysterectomy specimen.

Nonmolar abortuses

Representative examples of nonmolar abortuses are illustrated in Fig. 12. A total of 900 cases (780 consultation and 120 in-house) were diagnosed as nonmolar abortuses, usually with hydropic change and/or some abnormal villous morphology. Overall, 899 of 900 cases had p57 analysis (1 did not have this analysis for a technical reason but was genotyped). A total of 839 cases showed p57 expression with good staining quality. A total of 48 cases exhibited a weak/focal/diminished staining pattern yet with extent of staining being more than 10% and thus interpreted as still positive. A total of 12 cases were negative/nonreactive for p57: 7 due to extensive degenerative changes, 3 with technical issues, 1 attributable to Beckwith–Wiedemann syndrome (BWS) confirmed by outside laboratory testing of the fetus, and 1 with unknown mechanism.

Of 900 cases, 870 underwent molecular genotyping. A total of 838 were confirmed as biparental conceptions, including those with weal/focal/diminished (48 cases) or negative/nonreactive (12 cases) p57 immunostaining. A total of 13 cases were genotyped as having allelic balance, likely diploid, but not absolutely confirmed as biparental per genotyping due to having only chorionic villi available for analysis. Despite the lack of maternal (decidua) tissue for DNA analysis, these cases were diagnosed as nonmolar based on a lack of triploidy per genotyping in combination with positive p57 immunostaining. A total of 30 cases had been confirmed as diploid with (13) or without (17) trisomy by other ancillary analysis. Of note, there were 18 cases for which ploidy or karyotyping demonstrated tetraploidy, leading to consultation to address the possibility of a molar conception. These cases were p57-positive–excluding a subtle very early CHM-and were proven to be biparental per genotyping.

Digynic triploid conceptions represent nonmolar abortuses with two maternal and one paternal chromosome complements (Fig. 12a). A total of 19 cases were identified in this series. Complete genotyping data were retrieved from the laboratory database from 14 of these; 7 were XXX and 7 were XXY (Table 2). Based on the 15-marker panel, which was performed on all cases, the strength of the data for establishing that there was no evidence of diandry—that is, all loci demonstrating triploidy but with only shared alleles in double dosage, and no evidence of paternal alleles in double dosage (Fig. 13)—has been enhanced, and as such, the chances of diandric triploidy become extremely small (absolute proof requires analysis of the paternal DNA pattern but that is not available for these cases).

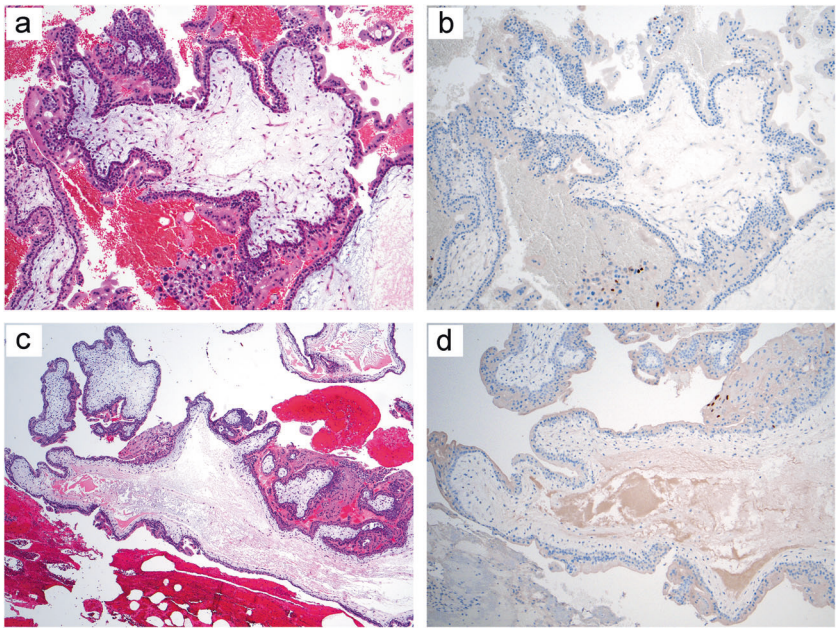

Fig. 7 Familial biparental hydatidiform mole. Early complete hydatidiform mole with cauliflower-like villous shape, myxoid bluish stroma with small canalicular vessels, and trophoblastic hyperplasia, demonstrates loss of p57 expression (**a**, **b**). Another p57-negative complete hydatidiform mole 3 years later in the same patient (**c**, **d**). Genotyping confirmed these as biparental conceptions, providing evidence to suggest the syndrome of familial biparental hydatidiform mole.

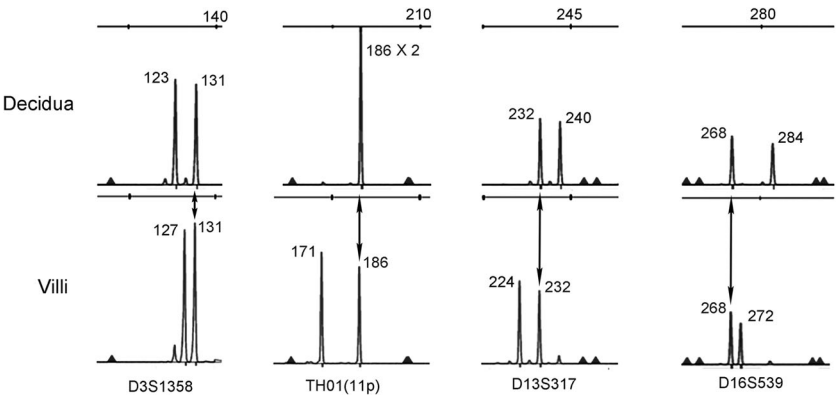

Fig. 8 Genotyping result for the familial biparental hydatidiform mole example in Fig. 7. All loci from villous tissue demonstrate a biparental pattern with allelic balance (both maternal [PCR product peak with bidirectional arrow] and nonmaternal [PCR product peak without arrow, presumed paternal] chromosome complements present, with equal ratio).

Tetraploidy in a products of conception specimen is a nonspecific finding and could indicate a pure androgenetic CHM, a triandric tetraploidy PHM, or a biparental tetraploid nonmolar conception. A total of 18 tetraploid cases in this series (12 by DNA content analysis and 6 by karyotyping) demonstrated biparental genotyping results and were classified as nonmolar tetraploid conceptions. Complete genotyping data retrieved from 17 of 18 cases demonstrated that these were all monospermic (9,XXXX, 8 XXYY) (Table 2).

Among the nonmolar specimens, 147 cases with trisomy/trisomies were identified by molecular genotyping (134 cases) and/or karyotyping (13 cases), including: 135 single trisomies involving chromosomes 2 (5 cases), 3 (2 cases), 4 (3 cases), 5 (2 cases), 6 (1 case), 7 (15 cases), 8 (3 cases), 11 (1 case), 12 (3 case), 13 (21 cases), 15 (1 case), 16 (42 cases), 18 (12 cases), 21 (22 cases), 22 (1 case), XYY (1 case); 10 double trisomies involving chromosomes 4 and 7 (2 cases), 16 and 21 (2 cases, Fig. 12b), 2 and 11 (1 case), 2 and 12 (1 case), 2 and 16 (1 case), 7 and 13 (1 case), 13 and X (1 case) and 15 and 16 (1 case); and 2 triple trisomies involving chromosomes 7, 13, 20 (1 case, [Fig. 12c]) and 12, 16, 21 (1 case). Trisomy 16, 21, 13, 7, and 18 were among the most common single trisomy cases, with trisomy 16 accounting for 31% of cases (Fig. 14a). The peak age for trisomy was from 36 to 40 years (Fig. 14b). Of note, trisomy results for chromosome 6, 15, and 22 were obtained from karyotyping but not molecular genotyping since no STR markers for these chromosomes were present in the kit.

Four cases with nonmaternal egg donation were diagnosed as nonmolar hydropic abortuses. All four cases were provided with a history of in vitro fertilization-assisted pregnancy, with two cases being clinically suspicious for a molar pregnancy. Microscopically, the features in these cases favored a nonmolar abortus but some mild abnormal villous morphology suggested the possibility of a subtle form of PHM. All cases were positive for p57 in villous cells and were subjected to molecular genotyping. In two cases, the genotyping data were initially assessed without

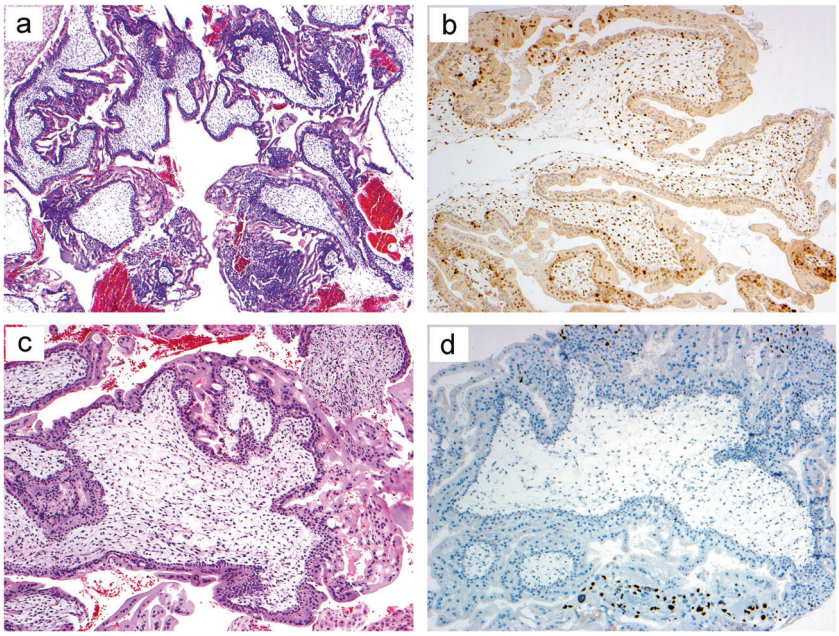

Fig. 9 Optimization of p57 immunostaining for diagnosis of complete hydatidiform moles. a Complete hydatidiform mole with features of the early form, including variably bulbous villous shapes, trophoblastic hyperplasia, and myxoid slightly cellular villous stroma. **b** Villous stromal cells and some cytotrophoblastic cells demonstrate p57 expression on a preparation from an outside laboratory, raising doubt about the morphologic impression, but there is an inappropriate level of nonspecific cytoplasmic staining. **c**, **d** Preparations performed in our laboratory show the typical morphologic features of an early complete hydatidiform mole (**c**), with lack of p57 expression in villous cytotrophoblast and stromal cells (**d**), without any nonspecific cytoplasmic staining yet robust internal positive control in intermediate trophoblastic cells to assure an adequate reaction. Genotyping confirmed this as an androgenetic conception.

knowledge of the clinical history, morphologic impression, and p57 results, and the results were interpreted as consistent with a dispermic/heterozygous androgenetic conception, indicating a CHM. However, discussion between the pathologist and the molecular laboratory in view of the discordance between the combined morphologic impression and p57 results, which argued against CHMs, established that these were actually nonmolar biparental conceptions with the DNA pattern of the villous tissue explained by the donor egg situation.

Two cases of choriocarcinoma associated with a scant amount of nonmolar chorionic villi, one with focal p57 expression and one with heterogeneous/mosaic p57 expression, were encountered. Molecular genotyping demonstrated biparental gestational choriocarcinomas related to/derived from the identified villous component (the reason[s] for the p57 results could not be determined).

Androgenetic/biparental mosaic/chimeric conceptions

Representative examples of androgenetic/biparental mosaic/chimeric conceptions are illustrated in Fig. 15. A total of 56 cases (52 consultation and 4 in-house) were classified as forms of androgenetic/biparental mosaic specimens; 11 of these have been previously reported in detail [35]. These included fundamentally two types of cases: nonmolar mosaic conceptions and molar mosaic conceptions in which the molar component was in this series essentially always a CHM/early CHM. There were 19 uniformly mosaic specimens characterized by villi that were variably hydropic with some trophoblastic inclusions, cisterns, and some villi with stromal hypercellularity, but all villi lacked trophoblastic hyperplasia and had discordant p57 expression in villous stromal cells and cytotrophoblast throughout the villi. A separate molar component with trophoblastic hyperplasia or lack of p57 expression was not identified in these. Genotyping performed in 13 cases demonstrated an excess of androgenetic alleles with variable paternal:maternal allele ratios ≥ 2:1, indicating admixtures of androgenetic and biparental cell lines within individual villi. These probably represent/are consistent with early forms of placental mesenchymal dysplasia [36, 37].

There were 37 androgenetic/biparental mosaic specimens that had two distinct components within each case, representing molar mosaic conceptions. One component was characterized by p57-discordant hydropic villi lacking trophoblastic hyperplasia—the mosaic component—and the other component was characterized by p57-negative villi with trophoblastic hyperplasia—the molar component, morphologically typical of a CHM or early CHM. Genotyping performed in 16 cases demonstrated an excess of

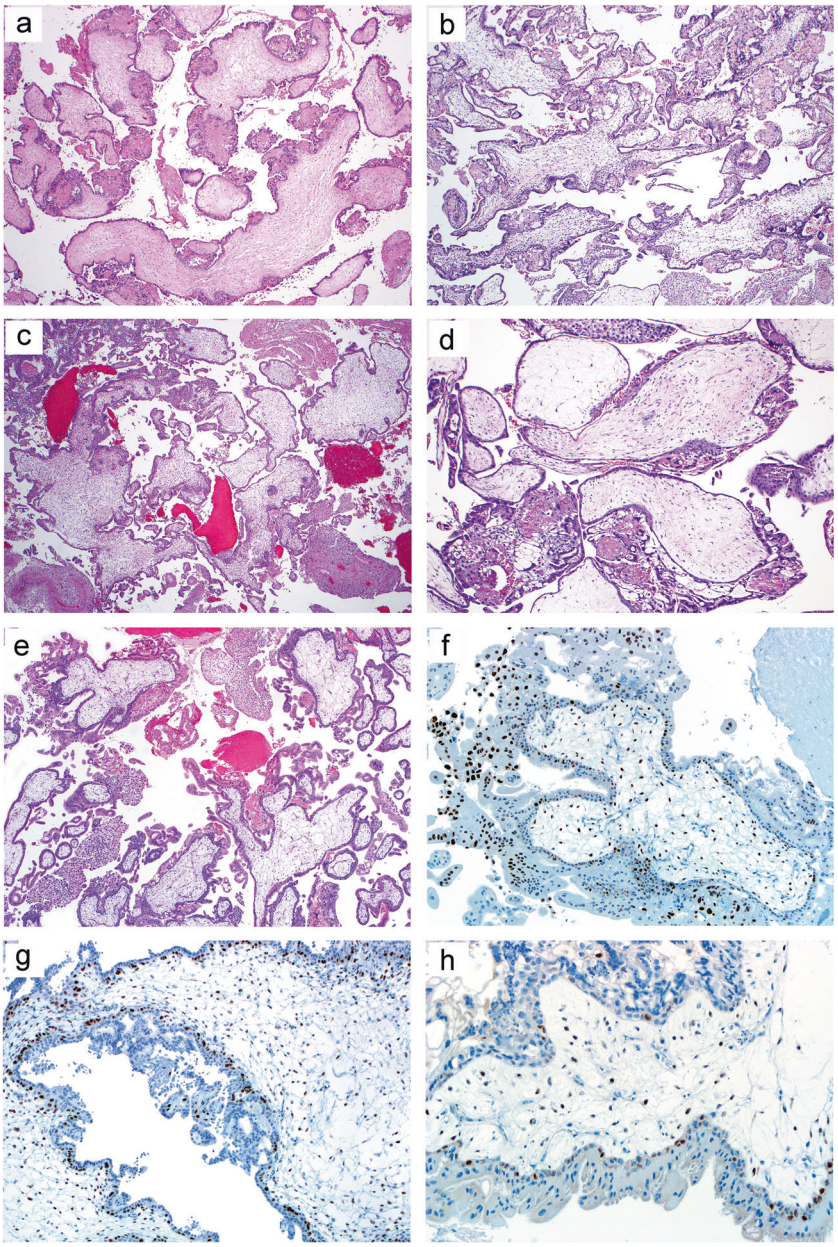

Fig. 10 The morphologic spectrum of partial hydatidiform moles. a–c Three different examples demonstrate the more typical spectrum of villous morphology, with classical enlarged fibrotic to hydropic and irregularly scalloped villi with variable trophoblastic hyperplasia and some trophoblastic inclusions (all were p57-positive [not shown]). **d** Another example with rounded hydropic villi having more trophoblastic hyperplasia suggests a complete hydatidiform mole but p57 was positive (not shown). **e**, **f** Bulbous smaller villi with trophoblastic hyperplasia on villous tips and myxoid stroma with canalicular vessels suggest an early complete hydatidiform mole but p57 expression is present in villous cytotrophoblast and stromal cells. Typical diffuse (**g**) and focal (**h**) p57 staining patterns in partial hydatidiform moles. Essentially all villous cytotrophoblast and stromal cells are positive in **g** but only some cells are positive in **h** (overall this was less than 50% in the stained section). All cases demonstrated diandric triploidy per genotyping.

androgenetic alleles with variable paternal:maternal allele ratios >2:1 in the nonmolar mosaic component and purely androgenetic genotypes in the molar components (see [1, 3, 35] for examples of genotyping data). The details of multiprobe FISH analysis of a subset of these cases are provided in our prior study [35]. Briefly, this analysis demonstrated that most of the analyzed cases were uniformly diploid in all cell types of both the nonmolar mosaic and molar components, with only some cases having aberrant triploid and/or tetraploid results only in cytotrophoblast of the nonmolar component. Of note, it is the diploid FISH results in most cases that assist in interpreting the allele ratios, which have an androgenetic excess in the nonmolar mosaic cases or components and are purely androgenetic if there is a molar component, with the excess attributable to a mixture of p57-negative androgenetic diploid and p57-positive biparental diploid cells rather than as a result of aberrant ploidy. The minority of cases with aberrant mixtures of ploidy results in the different cell types are more complicated to interpret but in our experience these have had nonmolar mosaic components characterized by p57-negative androgenetic diploid stromal cells and p57-positive biparental cytotrophoblast with triploid and/or tetraploid results. The molar components, when present, have been uniformly p57-negative and androgenetic. One case with a p57-negative androgenetic CHM and p57-discordant mosaic components also had a mature villous component that was biparental, consistent with a

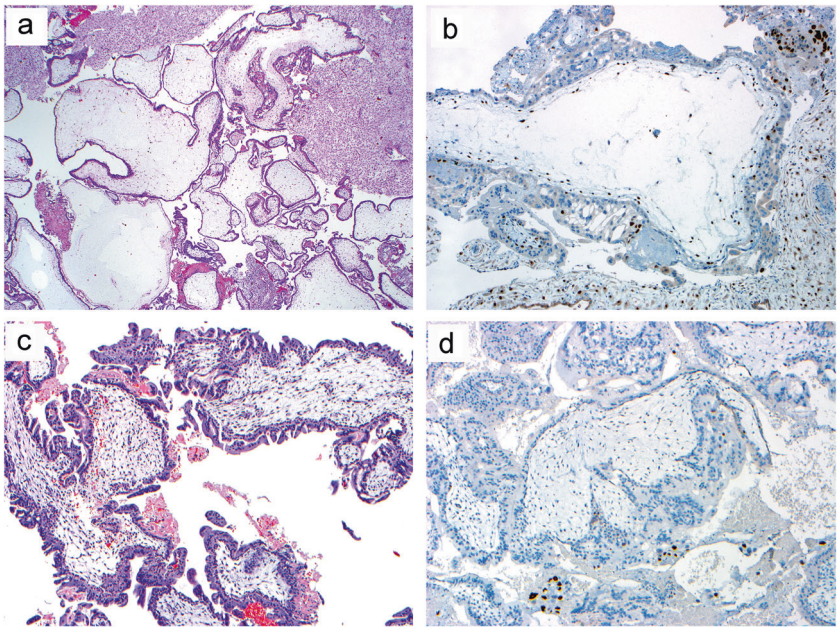

Fig. 11 Variants of partial hydatidiform moles. Hydropically enlarged villi with some trophoblastic hyperplasia, trophoblastic inclusions and cistern formation suggest a complete hydatidiform mole (**a**), as did the finding of tetraploidy by DNA content analysis, but p57 expression was present in villous cytotrophoblast and stromal cells (**b**) and genotyping confirmed this as a triandric tetraploid conception. **c**, **d** Villi with some trophoblastic hyperplasia and slightly cellular villous stroma suggest an early complete hydatidiform mole (**a**), and the loss of p57 expression in villous cytotrophoblast and stromal cells would usually support that assessment. However, genotyping demonstrated a diandric triploid conception, supporting diagnosis as partial hydatidiform mole, with the lack of p57 expression attributable to loss of the maternal copy of chromosome 11, which was demonstrated by genotyping.

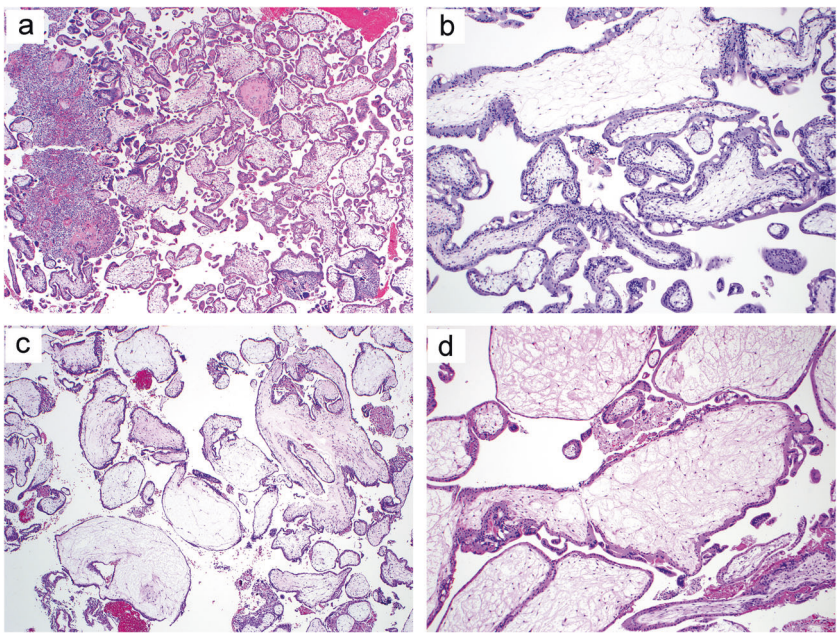

Fig. 12 Nonmolar conceptions. a Nonmolar digynic triploid conception has some mild irregular villous shapes, with some syncytiotrophoblastic snouts and trophoblastic islands suggesting trophoblastic hyperplasia, but villi with polarized trophoblast are present (lower right). **b**–**d** Three examples of nonmolar biparental conceptions with some abnormal villous morphology demonstrate a spectrum of abnormal irregular villous structures with some variable hydropic change, scalloping, mild trophoblastic hyperplasia, and trophoblastic inclusions, suggesting partial hydatidiform moles (**b**, double trisomy 16 and 21; **c**, triple trisomy 7, 13, and 20, see Norris-Kirby et al. [59]; **d**, no trisomy identified per genotyping).

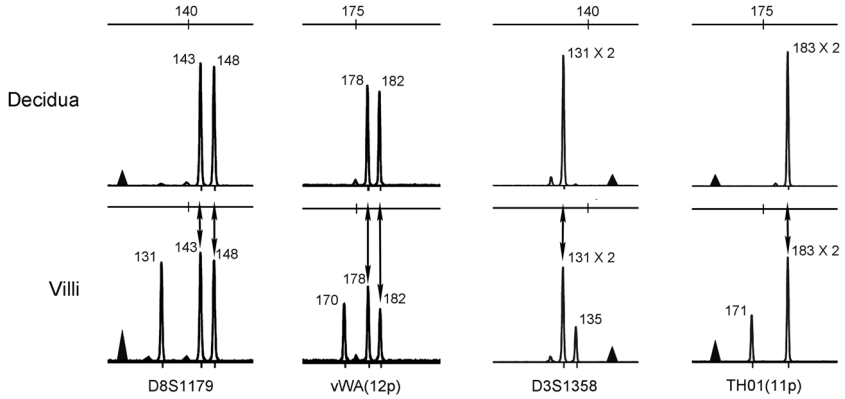

Fig. 13 Genotyping result for the digynic triploidy example in Fig. 12a. Analysis of DNA polymorphic markers (microsatellites) demonstrates that the DNA pattern from villous tissue is consistent with triploidy. None of the markers demonstrates two unique (paternal) alleles (PCR product peak without arrow). All informative markers demonstrate a pattern consistent with an additional maternal chromosome complement (PCR product peak with bidirectional arrow) with paternal:maternal ratio 1:2, consistent with digynic triploidy.

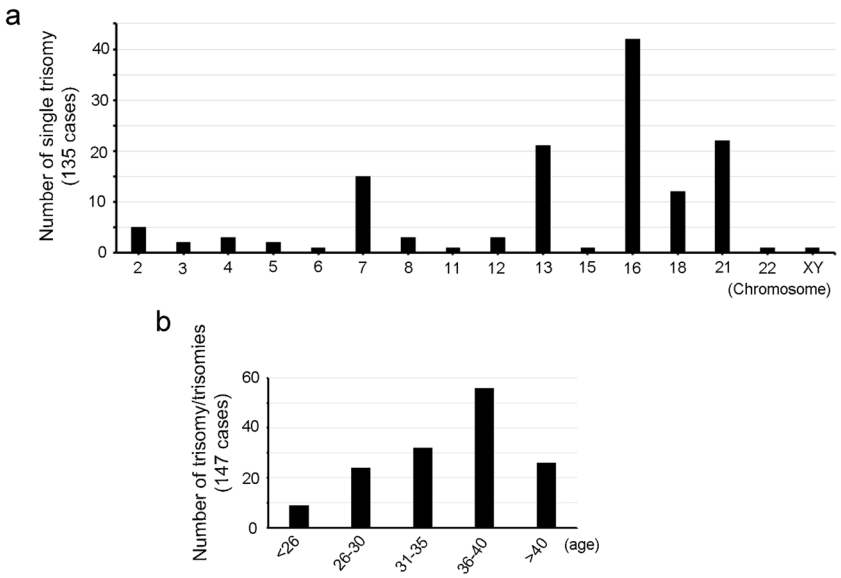

Fig. 14 Distribution of trisomy in nonmolar abortus specimens. a Single trisomy case numbers for different chromosomes. Trisomy 16, 21, 13, 7, and 18 were among the most common single trisomy cases, with trisomy 16 accounting for 31% of cases. **b** Trisomy case numbers in stratified age groups. The peak age for trisomy was 36–40 years.

nonmolar placental component of a twin gestation. Of three additional cases with mosaic p57 patterns and some molar features, two had complex genotypes that could not be clearly interpreted and one had genotyping interpreted as consistent with diandric triploidy, favoring a PHM. However, this result was considered inconclusive based on some other experiences we have had. In particular, several of the other nonmolar mosaics with typical discordant p57 expression with genotyping results initially interpreted as either diandric triploidy or triandric tetraploidy required revision to androgenetic/biparental when further analysis by FISH demonstrated uniform diploidy in all villous cell types. Since this case suggesting a mosaic form of PHM was not further analyzed with FISH to establish its ploidy and more sophisticated analysis for potential mosaic loss of the maternal chromosome 11 to explain the p57 result was not available, it was classified as mosaic but complex. Of note, others have reported mosaic PHM cases (see "Discussion") [38]. A total of 19 cases were diagnosed by morphology in conjunction with discordant or discordant and divergent p57 expression, respectively, without genotyping. This approach was taken in some cases based on our published [35] and ongoing practice experiences with these specimens indicating that the combination of morphology and p57 results is sufficient to establish a diagnosis of either a nonmolar androgenetic/biparental mosaic conception or a CHM arising in an androgenetic/biparental mosaic conception and the fact that microdissection and interpretation of genotyping in some cases can be challenging even with experience.

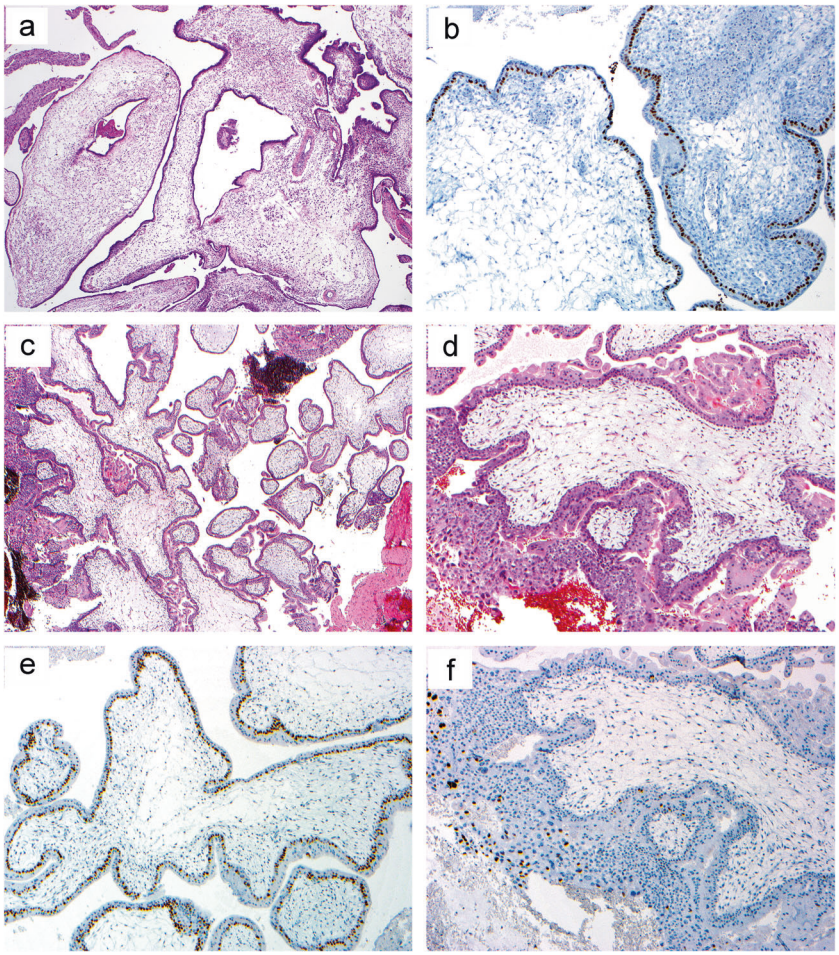

Fig. 15 Mosaic conceptions. **a** Nonmolar mosaic conception is composed of hydropically enlarged villi that have somewhat cellular stroma and trophoblastic inclusions but lack trophoblastic hyperplasia. **b** Villi demonstrate a discordant pattern of p57 expression, with positive cytotrophoblastic cells and negative villous stromal cells (this was seen throughout the stained section). Genotyping demonstrated paternal:maternal allele ratios enriched for paternal alleles. **c**, **d** Molar mosaic conception has two distinct villous components. Villi with features of an early complete hydatidiform mole are focally present (**c**, left portion and **d**), whereas other villi have some irregular shapes and hydropic change but lack trophoblastic hyperplasia (**c**, right portion). **e**, **f** p57 immunostain highlights the different components, with discordant p57 expression in nonmolar mosaic component manifested as p57-positive cytotrophoblastic cells and p57-negative villous stromal cells (**e**) and lack of p57 expression in molar villi (**f**) (see Lewis et al. [35] for examples of genotyping data).

Ectopic pregnancy

A total of 57 cases (47 consultation and 10 in-house) were ectopic pregnancies with locations as follows: 30 in right fallopian tube, 22 in left fallopian tube, 1 in a fallopian tube of unknown laterality, 2 in uterine cornu, 1 in left ovary, and 1 in liver. It is not uncommon that immature chorionic villi in an ectopic location display some abnormal morphology including irregular shape, variably hydropic changes, and appreciable trophoblastic hyperplasia. These features can suggest a hydatidiform mole and often lead to consultation.

All 57 cases had p57 analysis, and of these, 54 cases had p57 expression in the villous cytotrophoblast and villous stromal cells with good staining quality, arguing against a diagnosis of CHM. One case (left fallopian tube) displayed a negative p57 result in the villous cytotrophoblast and villous stromal cells, with internal positive control. In combination with morphology, the case was diagnosed as an early CHM. Two cases were negative/nonreactive for p57. One of these was a right tubal ectopic with primitive trophoblast and only very rare immature villi (not recognizable as early CHM) that was biparental per genotyping and was favored to be a very early nonmolar abortus with trophoblastic hyperplasia, although the earliest form of a rare ectopic biparental choriocarcinoma could not be absolutely excluded. The other was an unusual ectopic in the liver that lacked diagnostic features of a hydatidiform mole and was also biparental per genotyping.

Overall, 35 of 57 cases underwent molecular genotyping. A total of 33 cases were confirmed as nonmolar biparental ectopic pregnancies with allelic balance. One case (left fallopian tube) was diagnosed as a PHM based on the finding of diandric triploidy. Molecular genotyping for one case failed due to an uninterpretable result. Two cases with DNA ploidy analysis and one case with FISH analysis showed diploid results, and these results in conjunction with positive p57 immunostaining were sufficient to establish diagnosis as nonmolar ectopic conceptions. Molecular genotyping was not performed for a variety of predominantly technical reasons in 18 cases that had positive p57 results. Based on the morphology, the likelihood of PHM in these cases was assessed as very low, and a diagnosis of nonmolar conception was favored for each of these.

Nondefinitive, problematic cases

All 137 cases (99 consultation and 38 in-house) in this category had p57 analysis. A total of 94 cases did not undergo molecular genotyping for several reasons, including insufficient villi, villi being too intimately admixed with decidua to successfully microdissect pure tissue components, lack of decidua that precluded definitive interpretation, and declination by the contributing pathologist. Of note, the "microdissection" process is actually a "macrodissection" process in that marked areas on a stained slide are used to localize the villous and decidual tissues on unstained serial sections using naked eye visualization and tissue removal from the slides rather than laser capture microdissection. The latter is not used in our diagnostic laboratory for this assay but is a technique that could be used to improve the success rate for those cases in which villous tissue is too intimately admixed with decidua for the macrodissection method. In some cases, most often in-house cases, the assessment at consensus conference was that the suspicion for a hydatidiform mole was sufficiently low to forego genotyping but these were nonetheless subjected to p57 analysis to exclude the possibility of a subtle form of very early CHM. In these 94 cases, 85 cases had p57 expression with good staining quality. Eight cases exhibited a weak/focal/diminished staining pattern that was still sufficient for interpretation as positive. In conjunction with the morphology, the pattern of p57 expression in 93 cases argued against a diagnosis of a CHM. One case showed no expression of p57, but there was no internal positive control to determine whether this was a true negative result or failure of the assay due to the extensive degenerative changes. Thus, in virtually all of these, a CHM was excluded but a more definitive diagnosis could not be established.

Molecular genotyping was attempted in 29 cases but the results could not be definitively interpreted due to either failed PCR amplification or complex genotypes that were not readily classifiable. In these 29 cases, 24 cases showed p57 expression with good staining quality (21 cases) or weak/focal/diminished staining (3 cases), arguing against diagnoses of CHMs. Three cases displayed a discordant pattern of p57 immunostaining and two cases showed essentially negative p57 immunostaining with internal positive control. However, the morphologic and immunohistochemical evaluation was limited due to the scant amount of villi. Thus, in most of these as well, a CHM was excluded but a more definitive diagnosis could not be established.

Fourteen cases were successfully genotyped. Nine cases were biparental conceptions with allelic balance and either negative p57 immunostaining (five cases, with internal positive control) or nonreactive/diminished p57 immunostaining (four cases, inadequate internal positive control and/or inadequate immunoreactivity in villi). Thus, the differential diagnosis included nonmolar biparental conceptions with aberrant loss of p57 expression due to other (nonmolar) genetic mechanisms versus very early CHMs of the rare familial biparental type. Definitive diagnoses could not be rendered without other ancillary tests, especially high-resolution SNP array for chromosomal deletions or germline mutational analysis. Four cases had p57 expression in villous cytotrophoblast and villous stromal cells and were molecularly confirmed as triploid. However, the parental origin of these chromosome complements could not be determined due to lack of maternal decidual tissue for comparison and thus, definitive diagnosis as PHMs versus nonmolar digynic triploid conceptions could not be established.

Discussion

We have previously reported 678 cases of potentially molar products of conception specimens analyzed with p57 immunohistochemistry and STR genotyping, providing a comprehensive summary of the characteristics of molar and nonmolar specimens assessed with these techniques [23]. In the current study, we provide an updated summary of a larger prospective series of 2217 cases, with emphasis on expanded observations obtained over a 13-year experience not discussed in detail in our prior analysis.

In our prior analysis, 22 cases of CHMs were encountered in women aged >45 years and none of the women in this age group had PHMs or nonmolar specimens. Likewise, our updated series showed that 57 of 63 cases (90%) in women aged >45 years had CHMs; the remaining cases in this age group were nonmolar specimens and thus, again none were PHMs. In a stratified age distribution analysis, we observed a bimodal distribution of CHMs, with highest proportions in the age groups of ≤20 years and >45 years. Consistent with this finding, a population-based study in Sweden involving 3844 unique cases of molar pregnancy demonstrated the incidence of hydatidiform mole was characterized by a bimodal pattern with distinctive peaks in the youngest (below 20 years of age) and oldest (above 39 years of age) women of reproductive age [39]. Unlike that study, which did not specify the type of hydatidiform moles, we found that CHMs specifically, and not PHMs, accounted for this bimodal distribution. This distinct predilection toward CHM over PHM in women under and over particular ages has been observed in previous studies [40, 41]. Specifically, it has been demonstrated that very young or advanced maternal age has consistently correlated with higher rates of CHM. Compared with women aged 21–35 years, the risk of CHM is 1.9 times higher for both

teenagers and women > 35 years as well as 7.5 times higher for women > 40 years [40]. There was no association between age and frequency of PHM. The pathogenetic mechanism of this phenomenon remains unknown.

One limitation of our study is potential selection bias in that the vast majority of the cases were derived from our gynecologic pathology consultation service, which is clearly different from a population-based epidemiological study. Despite this, we believe the study set (2217 cases) is comprised of a wide spectrum of cases, including a sufficient number of hydatidiform moles and a variety of non-molar entities, suggesting that the database is not unduly biased. Of note, our molar cases still include more CHMs than PHMs (570 versus 497), similar to what we observed in our prior study. This is contrary to what one might expect for biased consultation cases focused on more difficult cases and also contrary to an increasing incidence of PHMs among molar gestations observed in some recent studies [42–44]. One consequence of having such a large proportion of consultation cases is that obtaining follow-up information is exceedingly difficult, making it quite challenging and even prohibitive (given the regulatory aspects of seeking clinical follow-up) to use this large series to ascertain risk of persistent gestational trophoblastic disease associated with the subtypes of hydatidiform moles. Thus, while follow-up data for a large series of genotyped cases is desirable, the nature of our cases is not conducive to such analysis. Of note, one recent sizeable study using cases from a single institution has provided such analysis [44].

Our ongoing analysis of cases has established that immunohistochemical analysis of p57 expression is highly correlated with genotyping results. In this updated series, only 6 of the 1067 hydatidiform moles (0.6%) had aberrant p57 expression that was contrary to the expected result for the diagnostic category, further demonstrating that p57 immunohistochemistry is extremely reliable for diagnosis of CHMs. These included one p57-positive CHM attributable to a retained maternal chromosome 11, and five p57-negative PHMs, with three being attributable to loss of maternal chromosome 11 and two with unknown mechanisms. Thus, genotyping of CHMs is not necessary in routine practice for diagnosis, particularly if genotyping is not available or cost-prohibitive in limited-resource settings, and can be reserved for problematic cases, such as when p57 immunostaining is suboptimal or unsatisfactory or when morphology and p57 results appear discrepant. Interestingly, a recent study demonstrated that dispermic/heterozygous CHMs are clinically more aggressive, with a significantly higher risk for development of post-molar gestational trophoblastic disease compared with monospermic/homozygous CHMs [44]. Thus, while genotyping of CHMs may not be required for routine diagnosis, the zygosity data obtained via genotyping can provide important prognostic information, which can guide patient management.

One important issue we encountered in our consultation cases is the importance of optimization of p57 immunohistochemical staining. P57 preparations with increased nonspecific cytoplasmic staining, often evident in decidual cells and intermediate trophoblastic cells, can lead to misinterpretation of CHMs as non-CHMs. Occasionally, very limited p57 nuclear staining can be seen in the villous cytotrophoblast and villous stromal cells of CHMs, usually <10% of these cell types, but this degree of expression is allowed and such cases have been confirmed as androgenetic CHMs in our experiences. Unlike such very focal/limited discrete nuclear p57 staining in CHMs, an overstained p57 preparation is characterized by both strong nuclear and variable cytoplasmic staining rather than a clean pure nuclear labeling. When such nonspecific staining is observed, several steps can be taken to address the validity of the result, including critical evaluation of the morphology, modification of the staining protocol with repetition of the assay under refined conditions (ideally, using a definitive CHM with good internal positive control to establish a good control for a negative result), and genotyping to achieve a definitive diagnosis (the latter via consultation if not available in the originating laboratory).

Familial biparental hydatidiform moles (FBHM) are a pure maternal-effect recessive disorder resulting, in most cases, from mutations of *NLRP7* or *C6orf221* [45–49]. FBHMs have morphologic features of a CHM/early CHM and are p57-negative but demonstrate a biparental rather than androgenetic genotype. These cases are reliably diagnosed as CHMs by concordant morphology and p57 results, but their familial/inherited nature is only established by identifying their biparental nature per genotyping. In our previous study, only one potential familial biparental hydatidiform mole was described. In the current study, we encountered four patients with molar specimens that were consistent with FBHMs based on morphology, negative p57 immunostains, and biparental DNA patterns per genotyping. These cases highlight the importance of correlating morphology, p57 results, and genotyping data to avoid misinterpretation as nonmolar conceptions based on the biparental genotyping results, which is important for patient management. Further genetic testing of the patients was not provided to us to establish a diagnosis of the syndrome of FBHM for these cases.

The issue of negative or nonreactive p57 results in cases that do not appear to be a CHM/early CHM and have biparental genotyping results is an uncommon but problematic issue because genotyping does not address many/most of the possible mechanisms for these results. Loss of p57 expression in a biparental conception can occur in FBHM, but these are rare and are expected to have the

morphology of a CHM/early CHM. Loss of p57 expression in a specimen lacking a better developed spectrum of molar features yet sometimes having hydropic enlargement, leading to consideration of the possibility of a hydatidiform mole, can be due to other kinds of nonmolar genetic alterations affecting the p57 gene on chromosome 11. These include:

(1) loss of the entire maternal copy of chromosome 11 (the chromosome where the p57 gene is located and from which p57 expression is derived),

(2) loss of a portion of the maternal chromosome 11 containing or disrupting the p57 gene,

(3) paternal uniparental disomy affecting chromosome 11 (which could be regional or complete) [50], and

(4) other alterations affecting p57 expression (e.g., point mutations, epigenetic changes).

One entity included in these is BWS, a disorder associated with various epigenetic and/or genetic alterations that dysregulate the imprinted genes on chromosome 11p15 [51]. The genotyping assay cannot address most of these kinds of alterations and if the marker on chromosome 11 (which is near the location of the p57 gene on chromosome 11) demonstrates a single shared allele in all analyzed tissues, the possibility of paternal uniparental disomy affecting chromosome 11 cannot be addressed. Thus, other kinds of analyses are required to resolve some of these p57-negative/nonreactive cases.

In our consultation service, we encountered some cases for which ancillary testing, such as ploidy analysis/flow cytometry or cytogenetics/karyotyping, demonstrated evidence of triploidy or tetraploidy. While triploidy by itself can suggest a diagnosis of a PHM, particularly when there is abnormal villous morphology, a triploid result obtained by methods that do not specifically determine the parental source of the extra chromosome complement cannot distinguish the diandric triploidy of a PHM from the digynic triploidy that occurs in some nonmolar abortuses. Digynic triploid abortuses do not exhibit the characteristic morphologic features of a PHM but rarely some focal abnormal villous morphology can be present which might suggest that possibility. In addition, some PHMs can have minimally developed morphologic alterations. Thus, morphology is not reliable for distinction of these entities and the finding of some abnormal villous morphology and triploidy of undetermined origin does not absolutely guarantee that a specimen is a PHM [52]. In the current study, of 61 cases with nonspecific triploidy detected by non-PCR-based ancillary analysis, 53 were confirmed as diandric triploid PHMs and 8 were confirmed as digynic triploid nonmolar abortuses by genotyping. In total, 19 cases with digynic triploidy were identified in the current study, accounting for 3.8% of the total confirmed triploid specimens (503 triploid cases, including 484 diandric triploid PHMs and 19 digynic triploid nonmolar abortuses).

While tetraploidy in a molar conception is traditionally thought to favor a CHM, ploidy by itself does not determine that a specimen is molar and also does not determine the parental origins of the chromosome complements. Thus, ploidy and karyotype analysis cannot distinguish the purely androgenetic tetraploidy (four paternal and no maternal chromosome complements) of some CHMs from the triandric tetraploidy (three paternal and one maternal chromosome complements) of some PHMs or the biparental tetraploidy of some nonmolar conceptions. While the vast majority of PHMs are characterized by diandric triploidy, triandric tetraploidy occurs in a small subset. Both types are virtually always p57-positive but rare examples of each type can have loss of p57 expression, attributable to loss of the maternal copy of chromosome 11 or other mechanisms affecting expression of this gene. Conversely, some CHMs are tetraploid and rare CHMs can have aberrant (retained) p57 expression attributable to a retained maternal copy of chromosome 11. Therefore, additional more specific ancillary testing, including p57 immunohistochemistry and molecular genotyping, is required to distinguish an androgenetic tetraploid CHM from a triandric tetraploid PHM or a biparental tetraploid nonmolar abortus. It is interesting to note that all triandric tetraploid PHMs were at least dispermic, whereas all biparental tetraploid nonmolar conceptions were monospermic. Genotyping cannot determine exactly how many sperm are involved in these tetraploid PHMs but subsequent investigation by our laboratory using SNP array analysis has demonstrated evidence for involvement of three sperm in these conceptions [33].

Mosaic conceptions pose several diagnostic and interpretive challenges. These have been described in detail in prior studies and the reader is referred to those for illustrations of genotyping data as well as discussion of potential mechanisms by which they arise [35, 53] One issue is that some examples can have an early CHM component that is limited/focal and difficult to recognize on routine hematoxylin and eosin-stained sections and without the assistance of p57 immunostains. Any areas with any degree of trophoblastic proliferation should be subjected to p57 immunohistochemical analysis to identify such focal molar components, particularly since microdissection of these foci can be difficult or impossible due to their small size and admixture with mosaic components. For this reason, and because interpretation of genotyping results for mosaic specimens is in itself challenging, we often diagnose pure mosaic and molar mosaics by morphology and p57 immunostaining without genotyping. Identifying a p57-negative molar component is important because the subset of mosaic conceptions with a molar component has some risk of persistent gestational trophoblastic disease, including subsequent development of

choriocarcinoma [35]. One other problematic issue we have encountered in a few cases concerns distinction of PHMs and mosaic conceptions. These are uncommon and unusual cases in which FISH analysis is actually useful to assist in resolution of an apparent discordance between combined morphology and p57 results versus genotyping results. In our experiences, most nonmolar mosaic conceptions are uniformly diploid per FISH analysis, although a small subset can have other ploidy results and even different ploidies in different cell lines within individual villi [35] We have seen 3 cases (unpublished observations) in which morphology and p57 expression patterns were typical of nonmolar mosaic conceptions but genotyping suggested diandric triploidy or triandric tetraploidy (paternal:maternal allele ratios hovering around the 2:1 or 3:1 ratios, respectively). However, FISH analysis, which was pursued for investigational purposes to address the discordance between the morphologic/immunohistochemical impression and genotyping, demonstrated only diploid signals in villous cytotrophoblast and stromal cells. Further assessment of the genotyping data determined that the allele ratios were generally within the range of those ratios (2:1 or 3:1) but with some variability just beyond the allowed ranges. The combined findings were then reassessed as most consistent with androgenetic/biparental mosaic conceptions. These odd cases demonstrate that even genotyping has certain interpretive challenges and that correlation with morphology and p57 results, and at times also ancillary testing to determine actual ploidy within individual cells, is required for correct interpretation.

To assess the sex chromosome constitutions in PHMs, genotyping data were retrieved from 481 diandric triploidy PHMs. The frequencies for the sex chromosome constitutions XXY, XXX, and XYY were 59%, 35%, and 6%, respectively, which were similar to what we reported previously with analysis of 155 PHMs [23]. Consistent with our findings, several studies have observed a very low frequency of XYY conceptuses in diandric triploids compared with XXY and XXX [7, 54–57]. PHMs are characterized by diandric triploidy (two paternal and one maternal chromosome complements), with most arising by fertilization of an ovum by two sperm (dispermy; ~99%), as shown in previous studies [44, 57, 58] and our data. Theoretically, the frequency of pregnancies with the various karyotypes would be 25% 69,XYY, 50% 69, XXY, and 25% 69,XXX. A frequency very far from the expected 25% for XYY karyotype suggests that a conceptus with the sex chromosome constitution XYY is less likely to survive to the point of recognition as a missed abortion than a conceptus with the sex chromosome constitution XXX or XXY. It has been speculated that excess paternal Y contribution to the zygote may have a more adverse effect on placental implantation and development [56].

Abnormal villous morphology is a term used to describe a nonmolar abortus having some morphologic features suggestive of a hydatidiform mole, usually a PHM but sometimes an early CHM, but lacking the specific genetic profiles that define these molar entities (diandric triploidy for PHMs, androgenetic conception for early CHMs). Abnormal villous morphology can be associated with other genetic abnormalities, such as trisomy [59–61]. In the current study, 147 cases with trisomy/trisomies were identified by molecular genotyping (134 cases) and/or karyotyping (13 cases), including 135 single trisomies. Trisomy 16, 21, 13, 7, and 18 were among the most common single trisomy cases, with trisomy 16 accounting for 31% of cases. Consistent with our findings, it has been reported that trisomy 16 is the most frequent autosomal anomaly seen in early spontaneous abortions, accounting for 14-18% of all chromosomally abnormal early/first trimester spontaneous abortions [62–64]. It is worth noting that, although the 15-marker panel includes markers for those chromosomes most commonly affected by trisomy in first trimester spontaneous abortions, not all chromosomes are covered by this analysis. Consequently, a complete picture of the spectrum of trisomy in this series cannot be provided. In fact, in the current study, trisomy results for chromosomes 6, 15, and 22 were obtained from karyotyping but not molecular genotyping since no STR markers on these chromosomes are included in the kit.

It has been documented that hydatidiform moles are commonly overdiagnosed in ectopic locations [65, 66]. Sheets of florid extravillous trophoblast may be prominent in tubal ectopic gestations because tubal pregnancies fail earlier or are diagnosed earlier than intrauterine pregnancies. Similar to implantation sites in intrauterine pregnancies, ectopic pregnancies may be associated with local invasion of surrounding tissues by intermediate trophoblast. These features can raise concern for a hydatidiform mole or even a gestational trophoblastic neoplasm. Indeed, the current series included 57 cases of ectopic pregnancy, including 47 consultation cases for which the main reason for a second opinion was concern for a molar pregnancy. Of these 57 cases with p57 analysis, only 1 (1.8%) was diagnosed as an early CHM and only 1 (3%) of 34 successfully genotyped cases was diagnosed as a PHM. Our study highlights the value of p57 analysis and/or molecular genotyping to avoid overdiagnosis of ectopic nonmolar pregnancies as molar entities.

The current study included four cases of donor egg products of conception specimens that highlight a potential diagnostic pitfall. Misclassification of the allelic pattern of a donor egg specimen as a CHM has been reported in the literature [67]. In fact, in the absence of this history and without knowledge of the p57 result, the genotyping result of a donor egg conception will be misinterpreted as a

dispermic/heterozygous form of purely androgenetic conception. This is because the conception has donor maternal and paternal allele patterns that do not match the carrier maternal allele pattern, yielding only novel alleles at informative loci. Thus, analysis of morphology, the p57 result, and history is required to properly interpret genotyping results in these cases. Interestingly, a recent study demonstrated that assessment of allele zygosity ratio might be helpful for the evaluation of donor egg products of conception specimens, with a certain level of predominance of heterozygous alleles relative to homozygous alleles favoring a nonmolar abortus [68].

In summary, the current updated series of 2217 potentially molar products of conception specimens further supports that the modern approach to diagnosis of hydatidiform moles is best accomplished with integration of ancillary techniques, particularly p57 immunohistochemistry and DNA genotyping, into routine practice as much as possible. The goals of using these techniques are to provide refined diagnosis so that the risk of persistent gestational trophoblastic disease associated with different subtypes of hydatidiform moles can be accurately assessed and to guide clinical management.

Note added in proof

Following submission of this manuscript, we identified another molar twin gestation, encountered during the study time frame, that had not been captured in our case collection process. This was a twin gestation comprised of a PHM and a non-molar third trimester (~37 week) placenta for which the molar component had focal p57 expression and genotyping demonstrated diandric triploidy. All other twin/multiple gestations encountered in the series had a CHM as the molar component, so we wanted to include this case to document the rare occurrence of a PHM in a twin gestation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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