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Utility of fluorescence in situ hybridization for ploidy and p57 immunostaining in discriminating hydatidiform moles

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ABSTRACT

Discrimination between complete moles (CMs), partial moles (PMs), and hydropic abortions (HAs) is important as the risk of persistent gestational trophoblastic disease (GTD) differs for each condition. We evaluated whether ancillary fluorescence in situ hybridization (FISH) with a set of chromosome enumeration probes (CEP) for chromosomes X, Y, and 17 and p57 immunostaining could improve the clinical diagnosis. Forty-one products of conception (POC) were reclassified according to clinical performance, morphology, p57 immunostaining results, and FISH results. The accuracy of histological examination alone was 85% for the original diagnosis. FISH analysis showed diploidy in 19 of 20 CMs and triploidy in 4 of 6 PMs. The concordance rate was 92.5% on using the CEP probes. p57 Staining was negative in all CMs and positive in all PMs and HAs. Chromosomal abnormality was detected in 3 cases of HA by using FISH. In conclusion, combined p57 immunostaining and FISH with a set of 3 CEP probes for chromosomes X, Y, and 17 could be useful in the classification of hydatidiform moles.

1. Introduction

Gestational trophoblastic disease (GTD) represents a heterogeneous group of diseases characterized by neoplastic and non-neoplastic lesions arising from different types of villous and non-villous trophoblasts. Hydatidiform mole (HM) is a subtype of GTD and result from the overgrowth of villous trophoblasts [1]. HMs are noted in approximately 1 of 120–500 pregnancies in Asian countries and in up to 1 of 125 pregnancies in Taiwan, which is 10 times the rate in the United States [2]. HMs are subclassified into two distinct entities, complete moles (CMs) and partial moles (PMs), according to clinicopathological features and karyotypic and genetic analysis. Postmolar GTD develops in 7.8–30% of CM cases and malignant transformation to choriocarcinoma is observed in up to 3% of these cases, whereas progression to persistent GTD is noted in only 2.5–7.5% of PM cases and malignant transformation is extremely rare [3]. In addition, hydropic abortions (HAs) could morphologically mimic HMs. However, follow-up serum beta-human chorionic gonadotropin (β-hCG) measurements are not essential in cases of HA, whereas these measurements form part of surveillance for persistent GTD in cases of HM. Therefore, accurate distinction of HAs from HMs and of CMs from PMs is important for appropriate clinical management.

The typical features of CMs include distended chorionic villi with multifocal to circumferential proliferation of trophoblasts on histopathological examination and androgenetic diploidy (46XX; less commonly 46XY) or tetraploidy. PMs exhibit an admixture of hydropic and normal villi with focally trophoblastic proliferation and generally show diandric triploidy (69XXX, 69XXY, and rarely 69XYY). HAs are characterized by regularly distended villi with polar proliferation of trophoblasts and nucleated red blood cells and usually demonstrate biparental diploidy [1]. Despite the differences in typical morphology, substantial histological similarities are observed between CMs, PMs, and HAs, resulting in interobserver and intraobserver variability in the classification of HMs [4].

Because of the differences in pathogenesis, a variety of techniques, including conventional cytogenetics (karyotype), flow cytometry, and molecular genotyping, have been used for the diagnosis of HMs [4–12]. Recently, fluorescence in situ hybridization (FISH) for DNA ploidy analysis of formalin-fixed paraffin-embedded tissue has been proposed, with improved accuracy [5–8]. In addition, loss of expression of p57, a protein product of the strongly paternally imprinted and maternally expressed gene *CDKNIC*, has been thought to be useful in the detection of CMs [4,5,8,9,13,14]. In this study, we evaluated the accuracy of FISH

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with a set of chromosome enumeration probes (CEPs) for the sex chromosomes and chromosome 17 as well as that of p57 staining in discriminating PMs from CMs and HAs.

2. Materials and methods

2.1. Patient specimens

Archived records of cases of CM, PM, and products of conception (POC) with hydropic villi were retrieved from the files of the Department of Pathology. In total, 41 cases were included in the study, 15 CMs, 11 PMs, and 15 HAs according to the original diagnoses. Clinical data, including gestational age, serum β-hCG level, and ultrasonography findings, were obtained from the charts. Final diagnoses were based on the combined analysis of clinical data, histological findings, and p57 immunostaining .

2.2. Histological review

All slides were independently reviewed by two pathologists and classified as CM, PM, and HA according to the main morphological findings (Table 1). The consensus diagnoses were based on morphology alone, without consideration of clinical information or the ancillary tests.

2.3. Fluorescence in situ hybridization

For the construction of CEP FISH probes, polymerase chain reaction (PCR) assays were developed to amplify alpha satellite-specific sequences of chromosomes X, Y, and 17. The 3 primer pairs were designed according to the human alpha satellite sequences [15–17]. The PCR products were ligated to the yT&A cloning system (Yeastern Biotech Co., Ltd., Taipei, Taiwan). The p-yT&A-X/Y/17 chromosome satellite clones were separately labeled using SpectrumGreen-dUTP, SpectrumRed-dUTP, and SpectrumAqua-dUTP (Abbott Molecular/Vysis, Des Plaines, IL, USA).

Representative sections of 4-μm thickness were placed on coated slides, air dried, and baked overnight at 56 °C. Sections were deparaffinized in xylene and then treated with 1 M sodium thiocyanate (Sigma–Aldrich Corp., St. Louis, MO, USA). Sections were then treated with 0.0025% pepsin (Sigma–Aldrich Corp.) for 10–20 min at 37 °C. After slide dehydration, 10 μl of probe mixture was applied to the slides in an approximately 4-cm^2 area. Slides were denatured for 5 min at 80 °C and hybridized for 16 h at 37 °C in a ThermoBrite hybridizer (Abbott Molecular, Des Plaines, IL, USA). Excess probe was washed away, and the nuclei were counterstained with 4′,6′-diamidino-2-phenylindole dihydrochloride/Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA). Slides were analyzed using a multi-filtered fluorescence microscope (Olympus BX61, Southall, Middlesex, UK).

A minimum of 50 non-overlapping nuclei were counted to determine the number of signals. For chromosomes X or 17, specimens were scored as XXX or triploid when more than 10% of nuclei showed 3 signals, and as X or monosomy 17 when more than 70% of nuclei showed only 1 signal. For chromosome Y, specimens were scored as Y when a signal was detected.

2.4. p57 Immunohistochemistry

Representative sections of 4-μm thickness were immunostained, using a Leica Bond Max Autostainer (Leica Biosystems, Wetzlar, Germany), a Bond Polymer Refine Detection/polymeric horseradish peroxidase-linker antibody conjugate system (Leica Biosystems), and lyophilized mouse monoclonal antibody (clone 25B2 at 1:50 dilution, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) against p57 protein (Kip2). The assessment of p57 staining was based on the presence or absence of nuclear staining of cytotrophoblasts and villous stromal cells. Diffuse nuclear staining of cytotrophoblasts and villous stromal cells was considered a positive result. Absent or <10% nuclear staining in the cytotrophoblasts and villous stromal cells was considered negative.

3. Results

On the basis of the combined findings of ultrasonography, serum β-hCG level measurement, histological examination, and p57 staining, the 41 cases were reclassified as CM in 20 cases, PM in 6 cases, and HA in 15 cases. One case was ultimately excluded because FISH was not performed successfully. The clinical features and the results of histological examination and p57 immunostaining are summarized in Table 2.

3.1. Patient profile

The ages of the 41 patients ranged from 20 to 50 years, with a mean age of 33.5 years. The data for the gestational age (GA) were available for 37 patients, ranging from 4 to 12 weeks, with a mean GA of 8 weeks. Laboratory data on serum β-hCG levels were available for all cases of CM and 4 cases of PM. Marked elevation of serum β-hCG levels (>100,000 mIU/mL) was observed in 13 of 20 cases of CM (65%) and in 1 of 4 cases of PM (25%). On ultrasonography, a heterogeneous mass or snowstorm pattern, a feature highly suggestive of CM, was observed in 11 cases of CM (55%). Intrauterine pregnancy with no fetal heartbeat (FHB) was observed on ultrasonography in all cases of PM. For HA, the ultrasonography findings were gestational sacs with no FHB in 7 cases, blighted ova in 5 cases, and cystic hygroma with abdominal wall defect in 1 case.

3.2. Histological examination

On comparing the original diagnoses with the final histological diagnoses after review, the results were found to be inconsistent in 5 cases (12%) (4 cases of CM previously diagnosed as PM and 1 case of CM previously diagnosed as HA). Thus, the final diagnosis was identical to the original diagnosis in 15 cases of CM (75%), 6 cases of PM (100%), and 13 cases of HA (93%) (Fig. 1), whereas a

Table 1
Main pathological criteria for the diagnosis of hydatidiform mole.

	CM	PM	HA
Villous outline	Club-shaped budding	Irregular	Smooth
Villous cistern	Varied	Varied	Absent
Trophoblastic proliferation	Multifocal, circumferential	Focal, lace-like pattern	Polar
Trophoblastic pseudoinclusion	Present	Present	Rare
Nucleated RBC	Very rare	Present	Present
Karyorrhexis	Present, increasing	Inconspicuous	Inconspicuous

CM, complete mole; PM, partial mole; HA, hydropic abortion; RBC, red blood cell.

Table 2
Summary of the clinical features and findings of histological examination and p57 immunostaining.

Case No.	Final diagnosis	MA (year)/GA (week)	Ultrasonography finding	β-hCG level	Histological diagnosis		p57
					Initial	Final	
1	CM	50/5	Mass	>100,000	CM	CM	–
2	CM	31/10	Mass	50,000–100000	CM	CM	–
3	CM	23/7	Mass	50,000–100,000	CM	CM	–
4	CM	41/8	Mass	50,000–100,000	CM	CM	–
5	CM	20/9	Thick endometrium	>100,000	CM	CM	–
6	CM	26/8	Mass	>100,000	CM	CM	–
7	CM	32/9	Heterogeneous mass	50,000–100,000	CM	CM	–
8	CM	30/8	Snowstorm	>100,000	CM	CM	–
9	CM	47/4	Thick endometrium	10,000–50,000	CM	CM	–
10	CM	36/8	Thick endometrium	>100,000	CM	CM	–
11	CM	50/8	Pelvic mass	>100,000	CM	CM	–
12	CM	31/8	No FHB, suspected molar pregnancy	50,000–100,000	CM	CM	–
13	CM	30/NA	Mass	>100,000	CM	CM	–
14	CM	30/NA	Thick endometrium	>100,000	PM	CM	–
15	CM	36/5	IUP, no FHB	>100,000	PM	CM	–
16	CM	23/11	Snowstorm	>100,000	PM	CM	–
17	CM	46/8	Suspected molar pregnancy	>100,000	PM	PM	–
18	CM	32/9	Thick endometrium	50,000–100,000	CM	CM	–
19	CM	48/NA	Honeycomb mass	>100,000	HA	CM	–
20	CM	47/5	Thick endometrium	>100,000	CM	CM	–
21	PM	34/8	IUP, no fetal pole	<1000	PM	PM	+
22	PM	25/8	Sac (+), no growth	NA	PM	CM	+
23	PM	29/12	Early pregnancy, no FHB	<1000	PM	PM	+
24	PM	40/12	IUP, no FHB	<1000	PM	PM	+
25	PM	22/8	No FHB, sac (+)	NA	PM	PM	+
26	PM	35/7	No FHB, suspected PM	>100,000	PM	PM	+
27	HA	22/5	No FHB, sac (+)	NA	HA	HA	+
28	HA	35/7	Blighted ovum	NA	HA	HA	+
29	HA	31/8	No FHB, sac (+)	NA	HA	HA	+
30	HA	29/9	No FHB	NA	HA	HA	+
31	HA	34/7	No FHB, sac (+)	NA	HA	HA	+
32	HA	34/10	No FHB	NA	HA	HA	+
33	HA	37/5	No FHB, sac (+)	NA	HA	HA	+
34	HA	35/8	N/A, unwanted	NA	HA	HA	+
35	HA	38/6	Blighted ovum	NA	HA	HA	+
36	HA	32/10	No FHB	NA	HA	HA	+
37	HA	31/11	Blighted ovum	NA	HA	HA	+
38	HA	28/NA	Blighted ovum	NA	HA	HA	+
39	HA	30/9	Blighted ovum	NA	HA	HA	+
40	HA	29/11	Cystic hygroma and abdominal defect	NA	PM	PM	+
41	HA	34/8	Blighted ovum	NA	HA	HA	+

MA, maternal age; GA, gestational age; NA, not available; FHB, fetal heartbeat; IUP, intrauterine pregnancy.

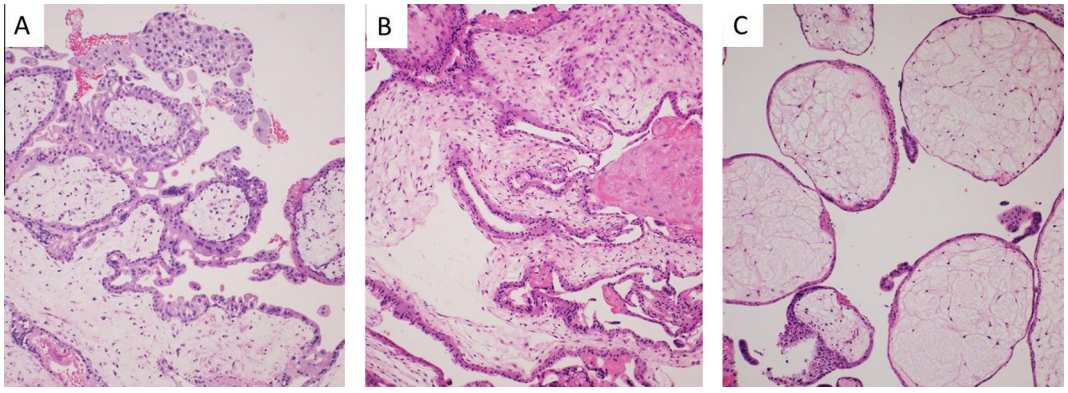

Fig. 1. Histopathology of a complete mole (CM), partial mole (PM), and hydropic abortion (HA). (A) Club-shaped budding of the villi and markedly trophoblastic hyperplasia were the typical morphologic features of CM in case 10 (100×, hematoxylin and eosin (H&E) staining). (B) The equivocal morphology for PM or CM in case 22 was an irregular villous outline with central cisterns and trophoblastic pseudoinclusions. Neither characteristic nucleated erythrocytes nor karyorrhexis was observed (100×, H&E staining) (C) in case 29, round, edematous villi with rare trophoblastic buds and poor vascularization compatible with HA were observed (100×, H&E staining).

consensus review provided an accurate diagnosis in 19 cases of CM (95%), 5 cases of PM (83%), and 13 cases of HA (93%). The overall accuracy of histological examination was 85% (34 of 40 cases) for the original diagnosis and 92.5% (37 of 40 cases) after consensus review.

3.3. Fluorescence in situ hybridization

FISH analysis was successful in 40 of the 41 cases (Table 3). No definite probe signal was detected in 1 case because of background interference. HCC tissue from a male patient showed a single X, a

Table 3
Results of fluorescence in situ hybridization analysis.

Case No.	Final diagnosis	FISH		Comment
		Chromosome X/Y	Chromosome 17	
1	CM	XX	Diploid	
2	CM	XX	Diploid	
3	CM	XX	Diploid	
4	CM	XX	Diploid	
5	CM	XX	Diploid	
6	CM	XX	Diploid	
7	CM	XX	Diploid	
8	CM	XX	Diploid	
9	CM	XX	Diploid	
10	CM	XX	Diploid	
11	CM	XX	Diploid	
12	CM	XX	Diploid	
13	CM	XX	Diploid	
14	CM	XX	Diploid	
15	CM	XX	Diploid	
16	CM	XX	Diploid	
17	CM	XX	Diploid	
18	CM	XX	Diploid	
19	CM	XX	Diploid	
20	CM	XXX	Triploid	FISH discordant
21	PM	XXX	Triploid	
22	PM	XXX	Triploid	
23	PM	XXX	Triploid	
24	PM	XXX	Triploid	
25	PM	XX	Diploid	FISH discordant
26	PM	XX	Diploid	FISH discordant
27	HA	X	Diploid	Monosomy X
28	HA	XX	Diploid	
29	HA	XX	Triploid	Trisomy 17
30	HA	X	Diploid	
31	HA	XX	Diploid	
32	HA	XY	Diploid	
33	HA	XX	Diploid	
34	HA	XY	Diploid	
35	HA	XX	Diploid	
36	HA	XX	Diploid	
37	HA	XY	Diploid	
38	HA	X	Diploid	Monosomy X
39	HA	XX	Diploid	
40	HA	XY	Diploid	Karyotype 46XY
41	HA	NA	NA	Excluded

NA, not available.

single Y, and 2 chromosome 17 signals. Of the 20 cases of CM, 19 were interpreted as diploid (a pattern of XX and 2 chromosome 17 signals) (Fig. 2A) and 1 was considered triploid (a pattern of XXX and 3 chromosome 17 signals). Of the 6 PM specimens, 4 were triploid (Fig. 2B) and 2 were diploid. In the HA group, 10 specimens were diploid (6 with a pattern of XX and 4 with a pattern of XY), 3 showed monosomy X (a pattern of X and 2 chromosome 17 signals), and 1 showed trisomy 17 (a pattern of XX and 3 chromosome 17 signals) (Fig. 2C). In 1 case of HA, the diploid status with a XY pattern was consistent with the results of cytogenetic studies (46XY). The results of FISH were discordant in 1 case of CM (triploid) and in 2 cases of PM (both diploid). The accuracy of FISH with the 3 CEPs for chromosomes X, Y, and 17 was 92.5%. FISH analysis with the three probes provided additional karyotypic information, especially in cases of HA.

3.4. p57 Immunohistochemical study

Loss of p57 expression compared to a positive internal control was observed in all 20 cases of CM, whereas p57 immunoreactivity was observed in all cases of PM and HA (Fig. 3). The accuracy of p57 immunostaining was 100% for cases of CM.

4. Discussion

Characteristic morphologic features have been proposed for the diagnosis of HM. However, the diagnosis of POC specimens on the basis of histology alone remains a challenge for pathologists. In cases of early detection of abnormal pregnancies by using advanced ultrasonography and serum β-hCG level measurement, even experienced pathologists face challenges in diagnosing HMs on the basis of subtle histological alterations during the first trimester. Many studies have also reported intraobserver and interobserver variability in the diagnosis of HM [4,18–22]. The interobserver concordance rate has been found to range from 55% to 80% [4]. In the current study, the concordance rate based on the histopathology was approximately 85% for the original diagnosis. On reviewing the slides of the discordant cases, the main problems were found to be the degree and pattern of trophoblastic proliferation, which may not be prominent in cases of CM, and the villous outline, which was sometimes similar in cases of CM and PM. Although certain characteristics such as the absence of nucleated red blood cells and karyorrhexis may be more specific for a diagnosis of CM, these may not be present in all sections, indicating the importance of ancillary studies.

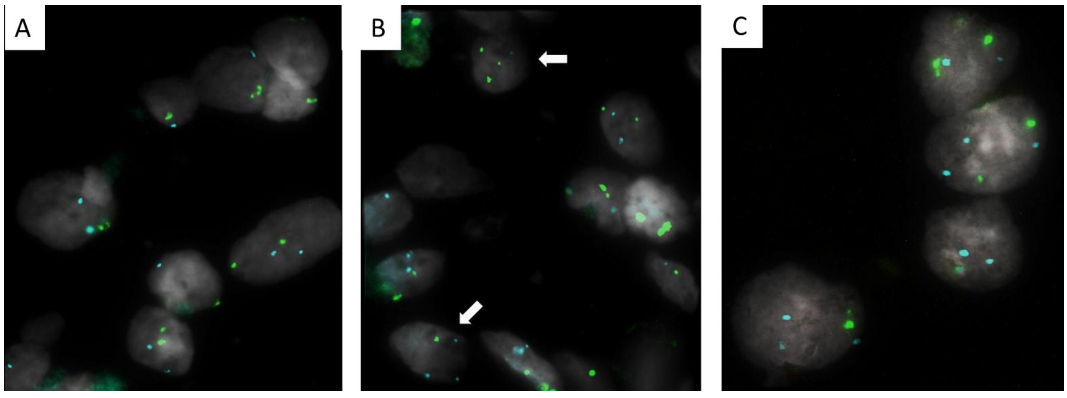

Fig. 2. DNA ploidy analysis by fluorescence in situ hybridization (FISH). FISH analysis (chromosome X: green signal, chromosome 17: blue signal) showed diploidy in CM ((A) case 10), triploidy in PM ((B) case 22), and trisomy 17 in HA ((C) case 29).

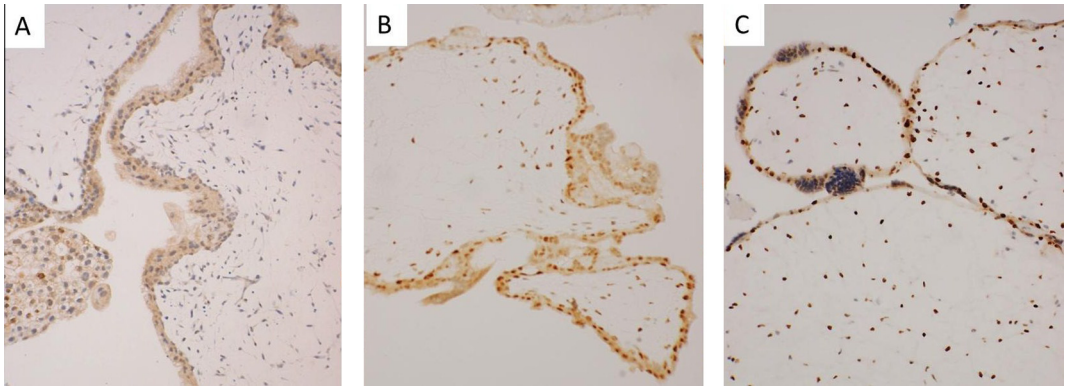

Fig. 3. Immunohistochemical staining for p57 in cases of molar pregnancy and hydropic abortion. p57 Immunostaining results were negative compared to a positive internal control in cases of CM ((A) 200×; case 10) and positive in cases of PM ((B) 200×; case 22) and HA ((C) 200×; case 29).

A few studies have advocated the use of p57, a paternally imprinted, maternally expressed gene located on chromosome 11p15.5, as a highly specific and sensitive marker of CM with extremely rare exceptions [5,8,9,13,14]. p57 is a cyclin-dependent kinase inhibitor Therefore, underexpression of p57 in cases of CM may result in loss of cell cycle control and hyperproliferation, correlated with histological features such as trophoblastic hyperplasia [8]. In our study, loss of p57 expression was identified in all cases of CM, which generally exhibited extremely elevated levels of β-hCG. Therefore, p57 immunostaining was useful in the diagnosis of CM, but unable to differentiate PMs from HAs.

FISH has been shown to be accurate in determining DNA ploidy in cases of HM [5–8]. A CM is diploid, or rarely tetraploid (in 10% of cases), with all chromosomes of paternal origin [23]. Diandric triploidy, primarily caused by dispermy, results in the appearance of PMs, whereas cases of digynic triploidy show neither clinical nor histological features of PM [24]. Although differentiation between CMs and HAs on the basis of DNA ploidy was a challenge, the inclusion of p57 staining was useful for the differential diagnosis.

Another important issue is the number of FISH probes needed to differentiate CMs from PMs. In previous studies, 1–9 FISH probes with or without sex chromosome probes were used to diagnose HMs [5–8,11]. In general, the application of a greater number of FISH probes may provide more information about the HMs. A previous study suggested that a FISH set with 3 chromosome probes was sufficient for the diagnosis of HMs [5]. In the present study, the use of 3 CEP probes (for chromosomes X, Y, and 17) was also successful in determining the DNA ploidy in a cost-effective manner. Of the 6 cases with 3 signals of chromosome 17 in our study, 5 were triploid (with an XXX pattern) and the other displayed trisomy 17 (with an XX pattern). Thus, using only 1 FISH probe could result in a misdiagnosis of HAs in cases of genetic abnormalities as PMs. Unfortunately, 2 cases, finally diagnosed as PM on the basis of clinical and morphological features and p57 immunoreactivity, were diploid on FISH analysis. Theoretically, PMs display diandric triploidy, with 1 maternal and 2 paternal chromosomal complements, and rarely tetraploidy, with 1 maternal and 3 paternal chromosomal complements [25,26]. Diploid PMs have been thought to be unlikely [27]. The discordant FISH results might probably be attributed to the scoring of the decidua rather than villi, truncation artifacts, or the variable intensity of the signal throughout the tissue specimens. Sampling of the villus-rich areas would be important in eliminating the interference of maternal decidua.

Case 20 in the current study, diagnosed as CM, displayed triploidy. This might be a case of familial recurrent hydatidiform mole (FRHM), which shows an autosomal recessive pattern of inheritance, with mutation most frequently found in *NALP7* and less frequently in *KHDC3L* [28]. Unlike general digynic triploidy, described as marked asymmetric intrauterine growth restriction in association with a small, dysmorphic, but nonmolar placenta [29], the triploid POC in FRHM still possess the morphological features of CMs and lack p57 immunoreactivity [28]. Patients with FRHM have been considered to have defects in maintaining the maternal imprint correctly, and recurrent molar pregnancies have been considered to display a biparental CM pattern. However, no repeated molar pregnancies were noted upon review of the clinical history of the patient in the current study.

Within the HA group, 4 cases displayed chromosomal abnormalities, including three cases of monosomy X and 1 case of trisomy 17, detected by FISH. Ultrasonography showed the absence of a FHB or blighted ova, and histopathological examination showed round edematous villi without trophoblastic hyperplasia or cistern formation. Some previous studies have indicated specific histopathology for genetic abnormalities such as few trophoblastic buds and poor vascularization of the hydropic villi, suggesting trisomy [7]. However, the subtle changes during the early stage (from GA of 5 to 9 weeks) make a diagnosis based on histology alone difficult. Hence, the use of FISH analysis in combination with histopathological studies could provide more information about the etiology of abortion.

In conclusion, the current study showed that the differential diagnosis of molar pregnancies was difficult based on histopathological features alone. Ancillary p57 immunostaining, which was negative in cases of CM, increased the confidence in establishing a diagnosis. Additional FISH analysis, ideally with at least 3 probes, could demonstrate triploidy in cases of PMs and probably explain the etiology of abortion in a subset of HAs.

Acknowledgments

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