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ORIGINAL ARTICLE

Diagnostic utility of p57 immunohistochemistry and DNA ploidy analysis by fluorescence in situ hybridisation in hydatidiform moles

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Abstract

Introduction: Hydatidiform moles (HMs) include complete and partial moles, are the result of abnormal fertilisation. The accurate classification of HMs and its distinction from non-molar specimens is utmost important for clinical management and risk assessment. It is diagnostically challenging if the distinction is based solely on histomorphology with poor interobserver reproducibility, especially in early gestations. This study aimed to investigate the diagnostic ability of combined p57 immunohistochemistry and DNA ploidy analysis to distinguish between complete moles, partial moles and non-molar abortus. *Materials and Methods:* We included all HMs cases diagnosed in our centre over a six-year period. p57 immunohistochemistry stain was performed. Only nuclear immunoreactivity in >50% of cytotrophoblasts and villous stromal cells was regarded as positive for p57. DNA ploidy status was determined by fluorescence in situ hybridisation. A total of 250 cells from five chorionic villi were counted and were scored as diploid or triploid if more than 10% of nuclei demonstrated two or three signals, respectively. *Results:* A total of 51 cases originally diagnosed by histomorphology as complete mole (n = 18), partial mole (n = 24) and non-molar abortus (n = 9) were recruited. The cases were reclassified based on the p57 immunostaining pattern and DNA ploidy status, into 27 complete moles (p57-/diploid), 9 partial moles (p57+/triploid) and 15 non-molar abortus (p57+/diploid). The diagnostic accuracy by histomorphological features alone in each category: complete moles, partial moles and non-molar abortus was 78.4%, 70.6% and 88.2% respectively. *Conclusion:* This study highlighted the importance of the utility of combined p57 immunostain and DNA ploidy analysis in arriving at an accurate diagnosis in HMs. An algorithmic approach utilising these ancillary techniques is advocated in routine diagnostic workup for a more refined diagnostic approach to HMs.

Keywords: DNA ploidy analysis, gestational trophoblastic diseases, hydatidiform moles, molar pregnancy, p57

INTRODUCTION

Hydatidiform moles (HMs), also known as molar pregnancy, are the commonest form of gestational trophoblastic diseases (GTDs). The incidence of HMs varies widely among countries and across different ethnic groups. Traditionally, HMs were believed to be more common in Asian, even in Asian descent who reside in the Western countries.[1] Intriguingly, a recent study demonstrated a decreasing trend in the Asian countries, while an increasing rate of GTDs was observed in the Caucasian women in some European countries, including Sweden.[2] On the contrary, Denmark showed a tremendous reduction in the incidence of HMs over a period of 15 years.[3] An explanation for this diversity in its incidence in relation to geographical distribution is still lacking, while some attributed this to the changes in

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the diagnostic methods, referral bias, lack of examination in very early molar gestations that vanished without knowledge.[3] Based on a single centre study in 2013, the incidence of HMs in Malaysia was 2.6 per 1,000 deliveries.[4]

HMs occur due to an imbalance in paternal and maternal genetic materials during fertilisation. They can be subcategorised into complete hydatidiform moles (CHMs) and partial hydatidiform moles (PHMs) based on histomorphological features and the genetic makeup. In gestational tissues following normal fertilisation, they inherit one haploid genome complement from each parent, giving rise to either a 46,XX diploid karyotype or a 46,XY diploid karyotype. In CHMs, the majority arise from monospermic fertilisation of an empty anucleated ovum, followed by endoreplication, resulting in a 46,XX diploid karyotype. Rarely, fertilisation of an empty anucleated ovum by two spermatozoa (dispermy) simultaneously may occur, which generates a 46,XX diploid karyotype or a 46,XY diploid karyotype. Thus, these androgenetic CHMs are entirely paternally derived with no maternal contribution.[5,6] On the other hand, PHMs are usually triploid (69,XXX, 69,XXY, or 69,XYY), following dispermic fertilisation of a haploid normal ovum or fertilisation of a haploid normal ovum by a spermatozoa that subsequently undergoes endoreplication. This results in two sets of paternally inherited alleles (diandric) in the genome. Unlike CHMs, PHMs have a set of maternal chromosome complements.[7] This forms the basis of the potential usage of p57, a paternally imprinted gene, in discriminating PHMs from CHMs.

Histomorphological features of CHMs and PHMs are well characterised. CHMs are characterised histologically by diffusely enlarged, irregularly shaped, hydropic chorionic villi with marked circumferential trophoblastic proliferation. On the other hand, PHMs typically show two discrete populations of large, hydropic chorionic villi and small, fibrotic chorionic villi. The former appears oedematous and enlarged with irregular scalloped borders, cistern formation and focal trophoblastic hyperplasia. Trophoblastic pseudoinclusion is a frequent finding.[8,9]

Despite the classic microscopic features between these two types of HMs, accumulating evidence demonstrated a substantial degree of histomorphological overlap between them, with considerable interobserver variability, leading to diagnostic difficulties.[10-12] Problems arise especially in the early forms of CHMs as they are less well-developed and thus lack the defining histological features. Additionally, the distinction of HMs from non-molar entities such as hydropic abortus, placental mesenchymal dysplasia, chromosomal trisomies, digynic triploidy and mosaic/chimeric conceptions can be difficult. This occurs when prominent trophoblastic hyperplasia is seen in early non-molar abortus and trisomy 21, or when abnormal villous morphology is observed in digynic triploid gestation.[13-15] Indeed, a diagnosis made based solely on histomorphological features may lead to misdiagnosis.

Imprinted genes are expressed in an exclusive parent-of-origin-specific manner, involving gene expression limited to either paternally or maternally derived allele of a gene. Cyclin-dependent kinase inhibitor p57 is an imprinted gene encoded by a maternally expressed gene *CDKN1C,* located on human chromosomal locus 11p15.5, with the paternal allele being methylated and repressed. Normal placenta, non-molar (NM) abortus and PHMs, which contain maternal genetic material, consistently demonstrate strong nuclear p57 immunoexpression in the cytotrophoblasts. Being androgenetic, immunoexpression of p57 is expected to be absent in cytotrophoblasts of CHMs. p57 immunohistochemistry is helpful in subclassifying PHMs and CHMs in the majority of cases. However, it cannot reliably differentiate PHMs from other histological mimics including non-molar hydropic abortus, trisomies, digynic triploidy as well as placental mesenchymal dysplasia.[16] DNA ploidy analysis, by flow cytometry or in situ hybridisation, karyotyping as well as polymerase chain reaction (PCR)-based microsatellite genotyping is valuable in the diagnosis of triploid gestations, and discerns them from diploid, tetraploid or aneuploid conceptuses.[15]

Since its inception in early 2000[17], p57 immunohistochemistry is now widely accepted as an important ancillary study in evaluating HMs. Nonetheless, is p57 alone sufficient for the distinction? Will additional DNA ploidy analysis help to refine the diagnosis of HMs? The aim of this study was to determine the diagnostic utility of p57 and DNA ploidy study in the diagnosis of HMs and their distinction from non-molar specimens.

MATERIALS AND METHODS

Tissue Samples

This was a retrospective study conducted in a tertiary university hospital. Ethics approval was obtained from the local medical research ethics committee (UKM PPI/111/8/JEP-2019-820). We included all HM cases diagnosed over a six-year period. The original diagnosis was made on the basis of clinical presentation, biochemical (β-hCG level at presentation) and histomorphological findings. The respective haematoxylin and eosin (H&E)-stained slides were reviewed, and one most representative section was selected for further ancillary analysis.

DNA Ploidy Analysis by Fluorescence in situ Hybridisation

Fluorescence in situ hybridisation (FISH) was performed on formalin-fixed paraffin embedded tissue sections using Dako Histology FISH Accessory Kit (Glostrup, Denmark) in accordance with the manufacturer's protocol. Briefly, the tissue sections (4 μm) were baked at 62°C for 15 minutes followed by deparaffinisation in xylene and rehydration in a serial dilution of ethanol. The sections were then pretreated in Pre-Treatment Solution, protein digested with Pepsin Solution at 37°C for 30 minutes and were evaluated for adequacy of digestion using 4′,6-diamidino-2-phenylindole (DAPI).[18]

Two sets of probes were applied onto two separate sections for each case: CytoCell® Satellite Enumeration Probes (Cambridge, UK) for (a) chromosome 11 (green) and 16 (red), and (b) chromosome X (green) and Y (red). Sections were then denatured at 76°C for 5 minutes before incubation at 37°C overnight in a ThermoBrite FISH Slide Processing System (Leica Biosystems, Illinois, US). After the washing steps using Stringent Wash Buffer and rinsing in Wash Buffer, 6μl of DAPI was applied onto each section and was coverslipped. All slides were viewed under a fluorescence microscope. A total of 250 cells from five chorionic villi (50 cells from each chorionic villus) were counted, and the ploidy status was scored as diploid or triploid if more than 10% of nuclei showed 2 or 3 signals, respectively.

p57 Immunohistochemical Analysis

A representative slide containing decidual and villous tissue was submitted for immunohistochemical analysis, using Abcam rabbit monoclonal anti-p57 antibody (Abcam, Massachusetts USA) and EnVision™ FLEX Mini Kit, High pH (Dako, Denmark), following manufacturer's guides and recommendations. Briefly, tissue sections were cut at 4 μm, mounted on poly-L-lysine coated glass slides and baked at 62°C overnight. Sections were deparaffinised in xylene and rehydrated in a serial dilution of ethanol. Antigen retrieval was performed in Target Retrieval Solution (TRS) (Code No. S1699, Dako USA) at pH 9.0, by placing it in the Decloaking Chamber™ NxGen (Ref. No: DC2012-220V, Biocare Medical California) for 30 minutes at 110°C. Endogenous peroxidase activity was blocked by a 10 minutes treatment with Peroxidase-Blocking Reagent (Code No. DM821, Dako Denmark). The sections were incubated with p57 antibody at dilution of 1:500 for 30 minutes at room temperature. Incubation with EnVision™ FLEX HRP (Code No. K8023, Dako Denmark) was carried out, followed by a treatment with 3,3'-diaminobenzidine-containing chromogenic (DAB) solution and haematoxylin counterstaining. Normal placenta was used as positive control.

The immunoexpression of p57 in villous cytotrophoblasts, syncytiotrophoblasts and villous stromal cells were analysed independently by two anatomical pathologists (G.C.T and Y.P.W) blinded to the original diagnosis and patients' clinicopathological data. Any discrepancies were resolved by consensus. Distinct nuclear staining of >50% of villous stromal cells and/or cytotrophoblasts were interpreted as positive p57 staining while no distinct staining or limited nuclear staining (<10%) of villous stromal cells and/or cytotrophoblasts were interpreted as negative for p57.[14] Staining of maternal decidua and intermediate trophoblasts were necessary for the results to be considered valid and served as internal positive control for all samples.

Algorithmic approach

The final diagnosis of CHMs, PHMs or NM abortus was reached based on the combined morphological evaluation, p57 immunoexpression and DNA ploidy result. The cases were reclassified on the basis of combined p57/ploidy status. Briefly, triploid cases with positive p57 immunoexpression (p57+/triploid) were compatible with the diagnosis of PHMs, while diploid lesions with negative p57 immunoexpression (p57-/diploid) was diagnosed as CHMs. NM abortus typically shows diploid with p57 immunopositivity (p57+/diploid) (Fig. 1). The final corrected diagnosis was then compared with the original diagnosis

made solely on histomorphology criteria alone.

Statistical Analysis

Diagnostic performance (sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy) of using histomorphology alone in the evaluation of HMs and NM gestations for various diagnostic categories were calculated. Cohen's Kappa coefficient was used to assess the agreement between original and post-ancillary testing HMs diagnosis. A kappa of 1 represents perfect agreement beyond chance, whereby a kappa of 0 indicates an agreement that is no better than chance. Diagnostic efficacy and validity of histomorphology assessment alone in the diagnosis of HMs were determined by receiver operating characteristic (ROC) curve analysis. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS version 26.0, Chicago, IL). MedCalc online calculator was used to calculate diagnostic performance. Any p value of less than 0.05 was considered to be statistically significant.

RESULTS

This study included a total of 51 cases. They were initially diagnosed on the basis of histomorphology alone, consisting of 18 CHMs, 24 PHMs and 9 NM abortus. A diagnostic algorithm for all products of conception with features suspicious for HMs, in order to refine the diagnosis of HMs and to distinguish them from non-molar gestations, was used in the current study (Fig. 1). Following the diagnostic algorithm, all cases were accurately reassigned into the respective diagnostic categories: 27 CHMs, 9 PHMs and 15 NM abortus.

Clinical Characteristics

Table 1 illustrates the clinicopathological characteristics of patients whose diagnosis were reclassified as CHMs, PHMs and NM abortus based on the proposed diagnostic algorithm. The patients with HMs aged between 21 to 51 years, with the majority (28/36, 77.8%) of patients falling into the 20 to 40 years category, with eight (22.2%) patients more than 40 years of age. The age range of patients diagnosed with CHMs was between 21 and 51 years with a mean and median of 35.3 and 32.0 years, respectively. Patients diagnosed with PHMs were slightly younger with an age range between 24 and 38 years (mean 32.1 and median 31 years, p = 0.329). The age range of patients who had NM abortus was between 21 and 42 years with a mean and median of 32.7 and 32 years, respectively. Patients with CHMs tended to present earlier

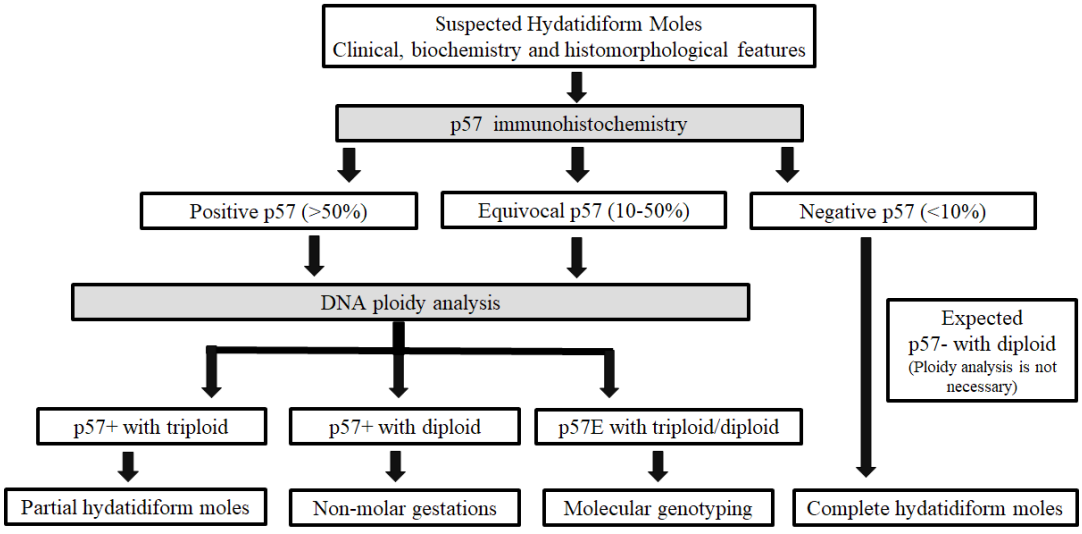

Fig. 1: An algorithmic approach to the diagnosis of hydatidiform moles based on p57 immunohistochemistry and DNA ploidy FISH study. All cases are initially evaluated by histomorphological features alone. Any potentially molar cases are triaged by p57 immunohistochemistry. A diagnosis of complete hydatidiform mole can be rendered if p57 is negative. The case is subjected to DNA ploidy analysis if p57 is positive regardless of histomorphology. Partial hydatidiform moles can be distinguished from non-molar specimens based on DNA ploidy results. In p57 equivocal cases (p57E), further molecular genotyping is needed.

Table 1: Clinical Characteristics of Hydatidiform Moles and Non-Molar Abortus

	CHMs n = 27 (%)	PHMs n = 9 (%)	NMA n = 15 (%)
Age (mean, years)	35.3	32.1	32.7
• Less than 20 years old	0 (0)	0 (0)	0 (0)
• 20 – 29 years old	9 (33.3)	2 (22.2)	5 (33.3)
• 30 – 39 years old	10 (37.0)	7 (77.8)	7 (46.7)
• 40 years old and above	8 (29.6)	0 (0)	3 (20.0)
	p = 0.329		
Gestational age (mean, weeks)	10.7	14.2	10.6
	p = 0.007#		
Ethnicity			
• Malay	23 (85.2)	9 (100.0)	13 (86.7)
• Chinese	1 (3.7)	0 (0)	2 (13.3)
• Others (Punjabi, Caucasian)	3 (11.1)	0 (0)	0 (0)
βhCG levels (mean, mIU/ml)	278,146±281,804*	18,395.1±13,479.8*	18,365.6±6,514.6

Abbreviations: CHMs – complete hydatidiform moles, PHMs – partial hydatidiform moles, NMA – non-molar abortus, *The number of cases for βhCG levels were 17 and 3 for CHMs and PHMs, respectively. #p value of <0.05 was considered as statistically significant.

in gestation, ranging from 7 to 15 weeks with a mean gestational age of 10.7 weeks, compared with that of PHMs ranged from 9 to 18 weeks and a mean of 14.2 weeks (p = 0.007). As for NM abortus, it ranged from 8 to 12 weeks, with a mean gestational age at presentation of 10.6 weeks.

As for the race distribution, the Malays accounted for 88.9% (32/36) of patients with HMs, followed by 2.8% (1/36) of Chinese, 5.5% (2/36) of Caucasian and 2.8% (1/36) of Punjabi ethnic group. Seventeen (63.0%) patients with CHMs and only three (33.3%) with PHMs had their βhCG level tested at presentation prior to surgical evacuation. In CHMs, the βhCG levels ranged from 25,000 mIU/ml to 1,000,000 mIU/ml, with a mean value of 278,146 mIU/ml, that were comparatively higher than PHMs', with a mean value of 18,395.1 mIU/ml (range from 9,515.4 to 33,906 mIU/ml) (p = 0.137).

p57 Staining Characteristics

All cases of PHMs and NM abortus demonstrated p57 staining in the nuclei of cytotrophoblasts and villous stromal cells. On the contrary, most cases (22/27, 81.5%) of CHMs showed complete p57 staining negativity in cytotrophoblasts, while remaining five cases revealed scattered (<10%) cytotrophoblast nuclear positivity. Additionally, villous stromal cells in CHMs demonstrated no immunoexpression for p57 in these cases. Immunoexpression in the decidual nuclei was noted in almost all cases of CHMs (22/23, 95.7%) and PHMs (8/9, 88.9%) and in all cases of NM abortus (15/15, 100%). Notably, p57 was consistently negative for syncytiotrophoblasts across all cases of HMs and NM abortus, while it was positive for intervillous trophoblastic islands of all cases, which served as internal control (Fig. 2). There were two cases with discordant p57 staining, in which the cytotrophoblasts were stained positive while villous stromal cells are negative for p57. These two were excluded from the current study.

DNA Ploidy Analysis by Fluorescent in situ Hybridisation

Of the 18 histologically diagnosed CHMs, all (18/18, 100%) displayed a diploid (2n) genotype. For the 24 cases of PHMs, only nine (9/24, 37.5%) demonstrated a triploid (3n) genotype, while the remaining 15 (62.5%) revealed a diploid (2n) genotype. All NM abortus (9/9, 100%) were found to be diploid (2n) (Fig. 2).

Combined p57/DNA Ploidy Analysis

Of the 18 cases that were initially diagnosed as CHMs, 17 were P57-/diploid and one was P57+/diploid; they were reassigned as 17 CHMs and 1 NM abortus, respectively. While in the 24 cases with the initial diagnosis of PHMs, 10 were p57-/diploid, 9 were p57+/triploid, and 5 were p57+/diploid; they were reassigned as 10 CHMs, 9 PHMs and 5 NM abortus, respectively.

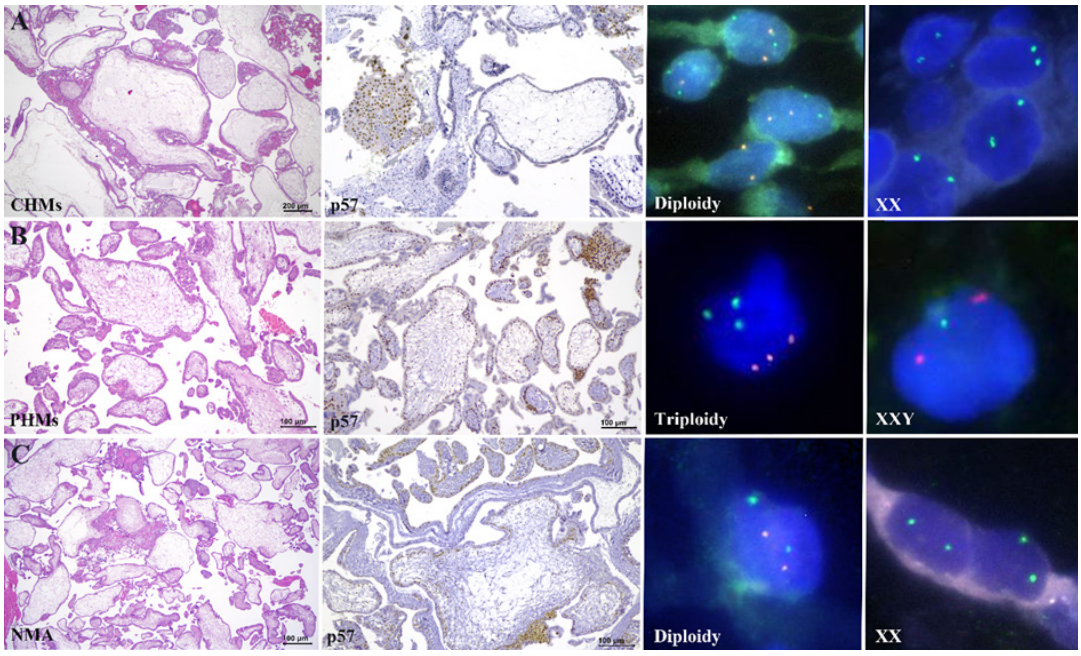

Fig. 2: Combined p57/DNA ploidy analysis by fluorescent in situ hybridisation (FISH) of complete hydatidiform mole (CHM), partial hydatidiform mole (PHM) and non-molar (NM) abortus. **(A)** A case of CHM demonstrating p57 immunonegativity, with positivity seen within intervillous trophoblasts serving as internal control (x200). Inset shows higher magnification (x400). DNA ploidy analysis reveals two green and two red signals with FISH probes for chromosome 11 (green) and chromosome 16 (red), compatible with a diploid DNA content. Two green signal patterns in the nuclei of a CHM with centromeric X (green) and Y (red) FISH probes, denoting the presence of two X chromosomes (XX). **(B)** A case of PHM showing p57 immunopositivity, with FISH analysis using probes for chromosome 11 (green) and chromosome 16 (red) shows three green and three red signals in the nuclei of cytotrophoblasts, consistent with a triploid DNA content. Centromeric XY probes demonstrate a triploidy, XXY. **(C)** A case of non-molar abortus with p57 immunopositivity and diploid DNA content with two green (chromosome 11) and two red (chromosome 16) signal patterns are observed in the nuclei. Similar pattern is seen with centromeric XY FISH probes, compatible with two X chromosomes (XX).

All the 9 cases of NM abortus were p57+/diploid. The percentage of correct diagnosis in each category on the basis of histomorphology alone for CHMs, PHMs and NM abortus were 94.4% (17/18), 37.5% (9/24) and 100% (9/9) respectively (Table 2).

Statistical Analysis

There was a moderate agreement between the original and post-ancillary testing diagnosis, with a Kappa (κ) value of 0.537 ($p<0.001$). By histomorphology alone in the evaluation of HMs, we achieved an average overall diagnostic efficacy with AUC of 0.409 (95% CI: 0.255 - 0.563) (Fig. 3), with a sensitivity of 100%, a specificity of 60.0%, a PPV of 85.7%, a NPV of 100% and a diagnostic accuracy of 88.2%. Detailed diagnostic performance of histomorphology assessment alone in each diagnostic category was depicted in Table 3.

DISCUSSION

Extreme maternal age (<20 years old or >40 years old) is a well-described risk factor for molar pregnancy. As high as 29.6% of CHMs were encountered in women aged more than 40 years in our study population, but none in this age group presented with PHMs. Similarly, a bimodal age distribution was described by Xing *et al*. (2020)[19] and Salehi et al. (2011)[20] in their large-scale studies involving 2217 and 3844 cases respectively. It was reported that the relative risk of CHMs was 1.9 times higher for teenagers and women aged 36-40 years, and 7.5 folder higher for those more than 40 years of age.[21] However, there was no reported association between women's age and PHMs.[21] Notably, CHMs were commoner in our study population, accounting for 75.0% of all HMs, in agreement to previous studies.[19,22]

Table 2: The diagnosis was reassigned based on the combined p57 immunohistochemistry and DNA ploidy results

Initial diagnosis	p57 immunohistochemistry	DNA ploidy status	Final reassigned diagnosis		
			CHMs	PHMs	NMA
CHMs n = 18	Positive	Diploid	0	0	1
		Triploid	0	0	0
	Negative	Diploid	17	0	0
		Triploid	0	0	0
PHMs n = 24	Positive	Diploid	0	0	5
		Triploid	0	9	0
	Negative	Diploid	10	0	0
		Triploid	0	0	0
NMA n = 9	Positive	Diploid	0	0	9
		Triploid	0	0	0
	Negative	Diploid	0	0	0
		Triploid	0	0	0
		Total	27	9	15

Abbreviations: CHMs – complete hydatidiform moles, PHMs – partial hydatidiform moles, NMA – non-molar abortus

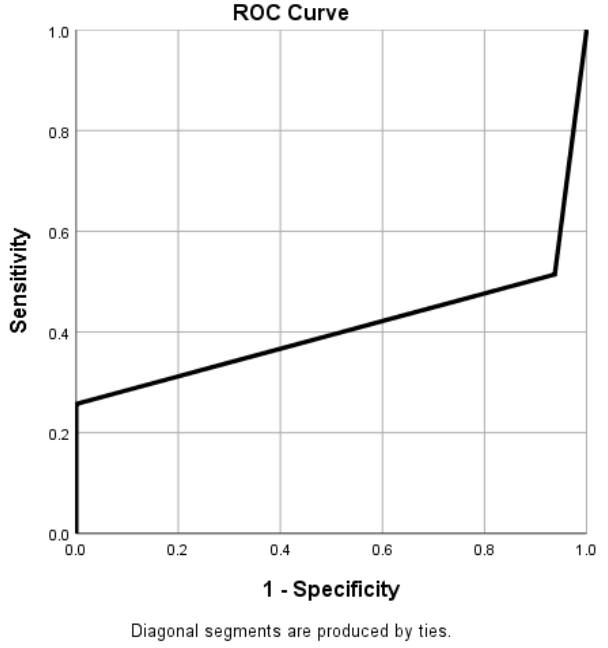

Fig. 3: Receiver operating characteristic (ROC) curve analysis of diagnostic performance of the histomorphology alone in the diagnosis of HMs and NM abortus. Area under the curve = 0.409 (95% CI: 0.255 - 0.563).

Table 3: Diagnostic Performance of Histomorphology Assessment Alone in the Diagnosis of Hydatidiform Moles and Non-Molar Abortus

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Accuracy (95% CI)
HMs	100 (90.3 – 100)	60.0 (32.3 – 83.7)	85.7 (76.4 – 92.0)	100	88.2 (76.1 – 95.6)
CHMs	63.0 (42.4-80.6)	95.8 (78.9 – 99.9)	94.4 (71.0 – 99.2)	69.7 (58.3 – 79.1)	78.4 (64.7 – 88.7)
PHMs	100 (66.4 – 100)	64.3 (48.0 – 78.5)	37.5 (28.6 – 47.4)	100	70.6 (56.2 – 82.5)
NMA	60.0 (32.3 – 83.7)	100 (90.3 – 100)	100	85.7 (76.4 – 91.8)	88.2 (76.1 – 95.6)

Abbreviations: CHMs – complete hydatidiform moles, NMA – non-molar abortus, HMs – hydatidiform moles, PHMs – partial hydatidiform molesb

Studies previously revealed a significant association between race/ethnicity and the risk of molar pregnancy, with Asian women having twice the risk for CHMs compared to the white, black and Hispanic women after age-adjustment. Similar to recent data by Zainal *et al*. (2021),[23] the majority of HMs patients were Malays. This could be attributed to the fact that the Malays being our country's largest ethnic group, accounting for 69.6% of the Malaysian population. Larger scale study involving more study subjects from all over Malaysia perhaps could reveal the true population distribution of the disease.

Presently in Malaysia due to the lack of molecular services, the majority of HMs diagnosis are made based solely on histomorphologic features alone. Several mimics and histological overlaps between PHMs, CHMs and other non-molar entities can give rise to misdiagnosis, inappropriate clinical follow up and management. The correlation with serum βhCG level is deemed necessary, especially in laboratories with limited resources. Nevertheless, serum βhCG level alone should be interpreted with caution. Sometimes, it cannot help in the diagnosis of HMs as it could be expressed by other ectopic βhCG-secreting tumours.[24] A word of caution, low level of βhCG can be seen in an infarcted CHM.

In this study, we found that the diagnostic accuracy by histomorphology assessment alone in the diagnosis of CHMs and PHMs were 78.4% and 70.6%, respectively. CHMs diagnosis was fairly specific (95.8%) but not sensitive (63.0%), with many being diagnosed as PHMs. On the other hand, PHMs diagnosis was highly sensitive (100%) but not specific (64.3%) with many eventually confirmed to be either CHMs or NM abortus. Once again, this result proves the substantial overlap in histological features between CHMs and PHMs as well as between HMs and non-molar specimens, which results in poor diagnostic accuracy and reproducibility.

Diagnosis of HMs based solely on histomorphology without the use of ancillary techniques is difficult, even for experienced gynaecological pathologists, leading to inaccurate diagnosis in at least 20% of cases.[25] Despite having relatively established morphological criteria, diagnosing an early first trimester CHMs and PHMs remains challenging due to the less well-developed key morphological features and morphological overlap with that of non-molar abortus harbouring cytogenetic abnormalities such as trisomies. For instance, abnormal villous morphology, a term that is now accepted in the WHO classification of tumours,[26] encompasses abnormal irregular villous structures, scalloping with variable hydropic changes and trophoblastic inclusions, is a feature frequently observed in trisomies,[27] while florid extravillous trophoblastic proliferation has been described in ectopic tubal pregnancy.[28]

Accurate diagnosis of HMs, classification into CHMs and PHMs, and distinguishing them from non-molar abortus are of paramount importance as clinical management differs for each diagnostic category. Notably, patients with CHMs have a higher risk to develop postmolar gestational trophoblastic neoplasia, which requires a more aggressive disease monitoring and follow-up, compared with PHMs.[29] A misdiagnosis will result in unnecessary or inappropriate clinical management in these patients.

Previous studies focused on the different types of p57 antibody[30], and standard streptavidin-biotin versus polymer methods.[31] Lund *et al*. (2020) recommended that <10% staining of p57 is considered as negative and compatible with the diagnosis of CHMs.[30] Most cytotrophoblasts in CHMs stained <1% for p57 immunohistochemistry, whereas in villous

stromal cells, staining can be seen up to 9%. A possible reason for this observation is that the paternal imprinted p57 gene is a more constant phenomenon in cytotrophoblasts than villous stromal cells. In p57 discordant villi cases demonstrating selective p57 immunoexpression in the villous stromal cells without staining the cytotrophoblasts or vice versa, mosaic molar pregnancy needs to be considered. Murphy *et al*. (2021) reported a case of CHM with discordant p57 immunoexpression. There was diffuse retention of expression in the villous stroma but was completely absent in the cytotrophoblast. Molecular study showed a diploid gestation with androgenetic lineage.[32] Our study showed complete p57 immunonegativity in the villous stromal cells for all cases of CHMs, while cytotrophoblasts revealed scattered (<10%) p57 immunoreactivity, except of two cases with p57 discordant villi. These two cases had between 50-70% cytotrophoblasts immunoreactivity but were negative in the villous stromal cells.

Vang *et al*. (2015) found that misdiagnosis of HMs was observed in diagnoses made by gynaecology pathologists by histomorphology and p57 antibody assessment, with about 20-30% misclassified, especially between PHMs and non-molar pregnancy. This finding is similar to our study. They demonstrated that correct diagnosis was better in consensus compared to diagnosis made by individual pathologists.[33]

McConnell *et al*. (2009) described a case of histomorphology typical of CHM and androgenetic diploid by molecular genotyping, with diffuse p57 expression. Interestingly, further evaluation revealed that it was androgenetic diploidy with retained maternal copies of chromosomes 6 and 11 which attributed to the aberrant p57 expression.[34] Similarly, Fisher *et al*. (2004) had found a case of CHM with strong p57 and TSSC3 expressions, due to retained maternal genetic material at chromosome 11, in a typical CHM two paternal gene copies. Using FISH analysis (chromosome 11q and centromeric probes), they found that it had XX, diploidy and trisomy 11. These are the caveats that one should be wary of when using p57 immunohistochemistry alone in evaluating HMs.[35]

Of note, chromosomes 6 and 11 are the most well-studied genes that contain imprinted domains. There are approximately 100 to 200 genes that are expressed in a parent-of-origin specific manner.[34] Other than p57, PHLDA2 (also known as TSSC3) is an imprinted gene that is located at chromosome 11. Study reported that PHLDA2 was completely absent in CHMs and diffusely positive in PHMs and non-molar gestations[36], which can serve as potential marker in the distinction.

As a corollary, ancillary techniques that examine DNA content including flow cytometry DNA analysis, in situ hybridisation and polymerase chain reaction-based genotyping are still necessary towards correct diagnosis in difficult and challenging cases. The current gold standard for HMs diagnosis is through molecular genotyping in which the genetic makeup of products of conception can be determined. Besides being capable to determine DNA content (ploidy) of the tissue specimen, molecular genotyping can help in discerning maternal and paternal chromosomal contribution, which allows further subclassification into androgenetic diploidy (CHMs), diandric triploidy (PHMs), and biparental diploidy (non-molar gestations); thus, aiding in the diagnosis of HMs.[19] This ancillary technique however is costly and may not be readily available in small laboratories in most developing countries. We showed that a simple supplementary DNA ploidy analysis by FISH would improve diagnostic accuracy.

A diagnostic algorithm workup using combined p57 immunohistochemistry and DNA ploidy analysis by FISH to improve the diagnostic accuracy of HMs was developed (Fig. 1). All initial cases with features suspicious of HMs should be triaged with p57 immunohistochemistry. Supplementary p57 immunonegativity allowed the diagnosis of CHMs to be made in the appropriate clinical settings; if p57 is positive, DNA ploidy analysis should be performed. A diagnosis of PHMs can be rendered if the result indicates a triploidy. We propose the use of this diagnostic algorithm for all products of conception with a suspicion of HMs, such as abnormal ultrasound findings, an abnormally raised βhCG level, or the presence of morphologically abnormal chorionic villi histologically. The algorithm should permit definitive diagnosis for almost all cases in routine practice. Gold standard molecular genotyping is recommended if diagnostic confusion arises in rare situations, like in cases of mosaic/chimeric conceptions.

In conclusion, the present study demonstrated that histomorphological diagnosis alone for both CHMs and PHMs lack accuracy, in particular PHMs, where half of the cases were reclassified to either CHMs or non-molar gestations. Ancillary

tests including p57 immunohistochemistry and DNA ploidy analysis should be carried out wherever feasible. It will be good to explore the potential of other imprinted genes as biomarkers in diagnosing HMs. Our findings indicate that it is important for attending pathologists to be aware that the use of ancillary study is deemed necessary in the diagnosis of HMs to avoid misdiagnosis.

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