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Development of a silver *in situ* hybridisation based assay for the determination of ploidy status in molar pregnancy diagnosis

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Summary

The aim of this study was to establish a scoring method for ploidy analysis using silver *in situ* hybridisation (SISH) with a chromosome 17 centromere probe. SISH was performed using the Ventana chromosome 17 centromere probe on sections from formalin fixed, paraffin embedded archival cases of complete hydatidiform moles, partial hydatidiform moles and hydropic products of conception with previously established ploidy status (determined by flow cytometry or karyotyping). In order to determine ploidy status, a scoring method was developed based on both the average number of signals per nucleus (ASN) and the percentage of nuclei with three signals (N3S), enumerated in 50 villous cytotrophoblastic and/or stromal cells. The results of four independent observers were compared individually and collectively with previously established ploidy status. There was a highly statistically significant difference between diploid and triploid gestations for ASN (1.86 ± 0.13 and 2.70 ± 0.16 respectively, Student *t*-test, $p < 0.0001$) and for N3S (1.14 ± 1.65 and 71.59 ± 14.25 respectively, Student *t*-test, $p < 0.0001$). The sensitivity and specificity of the SISH-based assay was 99.1% and 100% respectively for ASN, and 100% and 100% respectively for N3S. A chromosome 17 centromere probe SISH-based assay can reliably distinguish between diploid and triploid gestations. This test has diagnostic utility in distinguishing partial hydatidiform moles from histological mimics.

Key words: Chromosome 17 probe, molar gestation, ploidy, silver *in situ* hybridisation.

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INTRODUCTION

Hydatidiform moles are non-neoplastic proliferations of villous trophoblast. The two subtypes of molar gestation, complete hydatidiform mole (CHM) and partial hydatidiform mole (PHM), have significant differences in the subsequent risk of developing persistent gestational trophoblastic disease (10–30% and 0.5–5%, respectively) and choriocarcinoma (0.5–5% and rarely, respectively).[1–3] Also, the prognosis and clinical follow-up for gestational trophoblastic disease and its mimics (e.g., hydropic abortus) are very different. The diagnosis of hydatidiform moles is often challenging, especially in early gestation, since CHM, PHM and some non-molar abortuses may show overlapping histological features.[1]

Genetically, CHMs are paternally derived, where in most cases an 'empty' ovum is fertilised by a single sperm that reduplicates, resulting in a diploid, homozygous 46XX genotype. Less commonly, CHMs result from two sperm fertilising an 'empty' ovum, resulting in a diploid, heterozygous 46XX or 46XY genotype; or rarely a tetraploid genotype. PHM have a triploid (diandric monogynic) genotype resulting from two sperm fertilising an ovum (90% of cases) or one sperm fertilising an ovum followed by reduplication of the paternal chromosome set (10% of cases).[4]

The genetic composition of hydatidiform moles forms the basis of ancillary diagnostic techniques to help distinguish between hydatidiform subtypes and their mimics. These include DNA ploidy determination by several techniques, including cytogenetic analysis, flow cytometry, fluorescent *in situ* hybridisation, chromogenic *in situ* hybridisation and microsatellite genotyping.[5–7] Also, p57 immunohistochemistry assists in the diagnosis of complete molar gestations.[8,9]

We sought to establish a robust scoring method for ploidy analysis by silver *in situ* hybridisation-based assay (SISH), using the Ventana chromosome 17 centromere probe, which is commonly utilised for the evaluation of HER2 gene amplification in breast and gastric carcinomas.[10,11]

METHODS

Case selection and specimens

The study was reviewed and approved as an audit activity by the Austin Health Research Ethics Committee (H2013/04940). The anatomical pathology archive of Austin Pathology was searched for reports with the terms 'hydatidiform mole', 'partial hydatidiform mole', 'complete mole', 'hydropic abortus' or 'hydropic products of conception' in the diagnostic field. Haematoxylin and eosin (H&E) slides, p57 immunostained slides (if performed) and formalin fixed, paraffin embedded (FFPE) blocks were retrieved for 50 cases that had all originally required DNA ploidy studies by flow cytometry and/or karyotyping (performed at the Royal Women's Hospital, Parkville, Victoria). In each case the same FFPE block that was tested for DNA ploidy was used for the SISH-based assay.

Chromosome 17 SISH

SISH was performed according to the manufacturer's automated procedure, using UltraView Silver ISH dinitrophenyl (DNP) detection kit on the Ventana Benchmark Ultra platform (Ventana Medical Systems, USA). Briefly, 4 μm sections from the selected FFPE blocks were incubated at 60°C for 15 min. Slides were then rinsed, deparaffinised and treated with three cycles of CC2 retrieval buffer, followed by application of ISH protease 3 for 20 min. The tissue was then hybridised with the Inform chromosome 17 probe (Ventana Medical Systems) for 6 h at 42°C. The silver signal was produced using the standard SISH

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detection method. The slides were then counterstained with haematoxylin II, dehydrated, cleared and cover-slipped. Breast tissue normally used for Her-2 SISH was used as a positive control.

Scoring

The SISH stained slides were independently reviewed by three anatomical pathologists (DSW, TYL, MN) and a pathology registrar (LAG), each blinded to the previously confirmed ploidy status, p57 immunostain and final diagnosis. The external positive control was checked for signal adequacy. In most cases maternal-derived endometrial tissue served as an internal diploid positive control. At 200× magnification, trophoblastic villi were identified and fields with adequate staining in the cytotrophoblast and/or villous stromal cells were identified. Chromosome 17 SISH signals were enumerated in these fields at either 400× or 600× magnification. Overlapping and apparently incomplete nuclei were avoided. The number of signals enumerated per nucleus in 50 villous cytotrophoblasts and/or stromal cells were used to determine the average number of signals per nucleus (ASN) and the percentage of nuclei with three signals (N3S). For cases judged to be technically inadequate for enumeration the SISH was repeated to obtain adequately stained sections.

Statistical analysis

The SISH data were checked for accuracy and statistical analysis was performed. The means and standard deviations (SD) for ASN and N3S were determined for diploid and triploid gestations. Differences between the mean values for diploids and triploids were evaluated using the Student *t*-test, with two-sided p values <0.05 considered to be statistically significant. A cut-off point to distinguish diploids from triploids, (mean – 3 SD) of the ASN and N3S values for triploid cases, was determined based upon the data.

RESULTS

Final diagnosis based on flow cytometry, karyotyping and p57 immunostain

Of the 50 cases examined, the ploidy status was previously confirmed by flow cytometry in 47 cases, by karyotyping in two cases and by both flow cytometry and karyotyping in one case (all performed at the Royal Women's Hospital, Parkville, Victoria). Twenty cases were diploid (of which 16 were hydropic products of conception and four were complete moles), 29 cases were triploid (partial hydatidiform moles). One case that was determined by flow cytometry showed a diploid population with 30.9% of cells tetraploid, of which subsequent tissue examined histologically at the Royal Women's Hospital, Parkville, Victoria, confirmed a twin gestation, one of which was molar. The p57 immunostain showed an abnormal staining pattern in the four complete molar gestations with absence of staining in cytotrophoblastic and villous stromal cells, with normal staining in all 29 partial molar gestations and 17 cases of hydropic abortus, previously confirmed by combined morphological and ploidy analyses.

SISH analysis

When the staining was adequate the nuclear signals in villous and/or stromal cells were easily identified and counted (Fig. 1). SISH was deemed to be too weak to assess in nine cases (18%). In eight of the nine inadequate cases (89%), inadequacy for assessment was concordant between two or more independent observers. All nine repeat SISH cases were re-scored and determined to be adequate for assessment by all four observers involved. In one repeated case, the interpreted ploidy status for one observer was revised from diploid to triploid.

There was a highly significant difference in the ASN of confirmed diploid and triploid gestations, 1.86 ± 0.13 and 2.70 ± 0.16, respectively (Student *t*-test, $p < 0.0001$). There was also a highly significant difference in the N3S of diploid and triploid gestations, 1.14 ± 1.65 and 71.59 ± 14.25, respectively (Student *t*-test, $p < 0.0001$).

The SISH data were used to determine suitable cut-off points for ASN and N3S to reliably distinguish between diploid and triploid gestations. Values for ASN and N3S of (mean – 3 SD) of scores for triploid cases were selected as cut-off points, with the rationale that >99.7% of expected triploid scores are expected to be above this cut-off point, assuming a normal distribution. In our series of cases, an ASN value <2.3 corresponds with diploidy and ≥2.3 with triploidy (Fig. 2 and 3). For N3S, a value of <33.6 corresponds with diploidy and ≥33.6 with triploidy (Fig. 4 and 5). Both ASN and N3S independently achieved high sensitivity, specificity, positive predictive values and negative predictive values in determining the ploidy status (Table 1). When applied in combination, the cut-points for ASN and N3S showed 100% concordance with previously established ploidy status.

DISCUSSION

A constellation of morphological features can be seen in molar gestations, which when present can lead to a confident diagnosis of PHM or CHM on morphology alone. However, the assessment of molar gestations and separating them from non-molar hydropic abortuses is often difficult, particularly for early evacuations of non-viable gestations, with inter- and intra-observer variability in histological diagnosis.[12] For example in early CHM, distinct features such as trophoblastic hyperplasia and oedema have not become fully developed, such that distinguishing between CHM and PHM, and occasionally even hydropic abortus, can be difficult.[13] PHM on the other hand can be misdiagnosed as CHM or as hydropic products of conception.[14,15] Immunohistochemistry for loss of p57 protein expression is effective in the differentiation of CHM from PHM and hydropic abortus, with important prognostic and therapeutic implications. However, p57 immunohistochemistry has no utility in differentiating PHM from hydropic products of conception, which in practice is the most commonly encountered issue for the histopathologist.

DNA ploidy analysis is helpful in distinguishing triploid PHM gestations from diploid or aneuploid conceptuses. Several studies have previously utilised fluorescent *in situ* hybridisation (FISH) or chromogenic *in situ* hybridisation (CISH) techniques

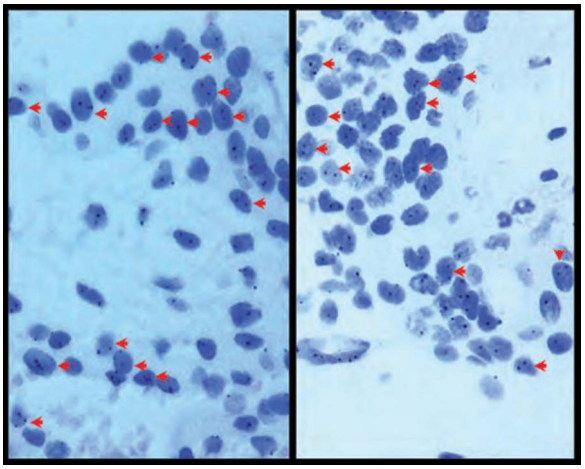

Fig. 1 (A) Chromosome 17 SISH with red arrows showing three signals in each nucleus from a triploid case and (B) red arrows showing two signals in each nucleus from a diploid case; ×600.