Interphase cytogenetic and AgNOR analyses of hydatidiform moles

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Abstract

Aim—To determine the potential value of interphase cytogenetic and argyrophilic nucleolar organiser region (AgNOR) analyses in the diagnosis and classification of hydatidiform moles.

Methods—Serial tissue sections from 37 hydatidiform moles, histologically classified as 11 complete and 15 partial, and from 11 hydropic abortuses were examined by in situ hybridisation using digoxigenin labelled probes specific for chromosomes 1, X, and Y, and a one step silver staining method. The percentages of diploid and triploid nuclei, and the mean number of AgNORs for each tissue were determined.

Results—Interphase cytogenetics showed that eight of the 11 cases (73%) each of complete mole and hydropic abortus had diploid pattern and the three remaining cases (27%) of each group were triploid. Two of the triploid complete moles and one of the triploid hydropic abortuses were revised to partial moles and one remaining triploid complete mole was revised to hydropic abortus. Of the 15 partial moles, nine (60%) were triploid, and six (40%) were diploid. These diploid cases were revised to three complete moles and three hydropic abortuses. There was a significant difference ($p < 0.0001$) between the mean (SD) AgNOR count in partial mole (5.11 (0.91)) versus hydropic abortus (3.79 (0.90)) and complete mole (3.39 (0.97)). The total of 15 triploid cases showed a high mean AgNOR count of 5.24 (0.73). Also, after reclassification, eight of the nine partial moles (89%) had a mean AgNOR count of ≥ 5 . The results of analyses by the two methods were closely correlated.

Conclusions—Interphase cytogenetic analysis using chromosome specific probes and AgNOR count provides a valuable approach for ploidy analysis in histological sections of hydatidiform moles and helps to resolve difficult cases.

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Keywords: hydatidiform moles; interphase cytogenetics; AgNOR count; ploidy

Molar pregnancy results from abnormal fertilisation. Hydatidiform mole occurs at an estimated rate of between 0.5 and 2.5 per 1000 pregnancies.¹ It is defined as an abnormal placenta with hydropic swelling of chorionic villi and excessive trophoblastic cell proliferation.

Hydatidiform moles are classified as complete moles and partial moles. The distinction between complete moles, partial moles, and non-molar hydropic abortuses is important because complete moles are more likely to progress to persistent trophoblastic disease than partial moles, with risk of metastases and choriocarcinoma.²

Complete mole is characterised by gross hydropic swelling of most of the chorionic villi with loss of intravillous vascularity and absence of a fetus. It occurs as a result of fertilisation of an empty egg devoid of maternal chromosomes by a single spermatozoon (in most cases) that duplicates its 23,X chromosomal content to produce 46,XX, or by two spermatozoa (about 25% of cases) to produce a dispermic 46,XX or 46,XY conceptus.³⁻⁶ Partial mole is characterised by two groups of chorionic villi, one being normal in size and the other showing hydropic swelling which is often focal. Villous capillaries containing nucleated fetal red blood cells are usually present. Other evidence of a fetus such as chorionic plate, amnion, cord, and embryonic or fetal tissue may be found. The trophoblastic cell proliferation is focal and mild. Partial mole results from the fertilisation of a normal egg by one or two spermatozoa, receiving two copies of the paternal genome to become triploid with 69,XXY in 70%, 69,XXX in 27%, and 69,XYY in 3% of cases.⁷⁻⁸ Non-molar hydropic abortuses usually do not manifest gross villous swelling. They contain oedematous villi covered by attenuated trophoblast. However, distinction between complete mole, partial mole, and non-molar hydropic abortus based on histology alone may sometimes be difficult.

Karyotype analysis has shown that complete mole is usually diploid, partial mole is mostly triploid, and hydropic abortus is often diploid,⁶⁻⁹ thus providing useful information on the classification of hydatidiform mole. Karyotype analysis, however, requires tissue culture and may result in a selective growth of wrong cell populations. Flow cytometry has been successfully used to assess nuclear ploidy in hydatidiform mole and hydropic abortus cases.¹⁰⁻¹² These methods have the intrinsic problem that they may include undesired cell populations and they lack the advantage of simultaneous assessment of tissue morphology. Recently, in situ hybridisation using chromosome specific probes has been shown to be a reproducible and valid method of detecting numerical chromosome aberrations and assessing nuclear ploidy in routinely processed, paraffin embedded tissue sections.¹³⁻¹⁶ The method had been referred to as "interphase

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Figure 1 The results of in situ hybridisation using digoxigenin labelled probes for chromosomes 1, X, and Y, and AgNOR staining in hydatidiform moles. In complete moles, the majority of villous cell nuclei showed two copies of chromosomes 1 (A) and X (B), and in one case one copy of chromosomes X and Y (C). In addition, hyperplastic trophoblast areas showed marked nuclear atypia and multiple copies of chromosomes 1 or X (D). In partial moles, the majority of villous cell nuclei showed three copies of chromosomes 1 (E) and X (F). AgNOR staining showed that in complete mole many nuclei of the villous trophoblastic cells contained three or fewer AgNOR dots (G), while in partial mole they had more than three (H). Non-molar hydropic abortus showed nuclei with mostly less than three AgNOR dots (not shown). Magnification, $\times 354$.

cytogenetics.^{17, 18} These suggest that ploidy analysis plays an important role in the diagnosis and classification of molar disease.

Nucleolar organiser regions (NOR) are segments of DNA that transcribe to ribosomal RNA and are located on the short arms of the acrocentric chromosomes 13, 14, 15, 21, and 22.^{19, 20} The number of NOR reflects the synthetic activity of cells.^{18, 19} A colloidal silver stain is generally used to visualise NOR in tissue sections; these are referred to as AgNOR.^{19, 20} Studies of AgNOR in breast

carcinoma and placental trophoblastic tissues have shown a close correlation between the mean AgNOR count and nuclear ploidy.²¹⁻²³

In this study, we examined serial tissue sections from histologically diagnosed complete moles, partial moles, and hydropic abortuses for nuclear ploidy by interphase cytogenetics using probes specific for chromosomes 1, X, and Y, and AgNOR counts. The results obtained by the two methods were then compared to determine their potential value in the diagnosis and classification of hydatidiform mole.

Table 1 Ploidy analysis of hydatidiform moles and hydropic abortuses by interphase cytogenetics using probes specific for chromosomes 1, X, and Y, and silver stained nuclear organiser region (AgNOR) count

Case No	Histological diagnosis	Chromosome ploidy (No)			Mean AgNOR count	Revised classification
		1	X	Y		
1	CM	2	2	0	3.45	CM
2	CM	2	2	0	2.31	CM
3	CM	3	3	0	3.52	HA
4	CM	2	2	0	2.60	CM
5	CM	2	2	0	4.13	CM
6	CM	3	3	0	5.11	PM
7	CM	2	2	0	2.11	CM
8	CM	2	2	0	3.20	CM
9	CM	2	1	1	4.15	CM
10	CM	3	3	0	4.32	PM
11	CM	2	2	0	2.40	CM
12	PM	2	2	0	4.21	CM
13	PM	2	2	0	5.60	HA
14	PM	3	3	0	6.22	PM
15	PM	3	2	1	5.33	PM
16	PM	2	1	1	4.52	HA
17	PM	3	3	0	5.65	PM
18	PM	3	3	0	4.86	PM
19	PM	2	2	0	3.45	CM
20	PM	3	3	0	6.20	PM
21	PM	3	3	0	5.32	PM
22	PM	2	2	0	3.40	CM
23	PM	3	3	0	5.30	PM
24	PM	3	3	0	6.25	PM
25	PM	2	1	1	4.78	HA
26	PM	3	2	1	5.65	PM
27	HA	2	2	0	2.45	HA
28	HA	2	1	1	3.55	HA
29	HA	3	3	0	5.25	PM
30	HA	2	1	1	2.86	HA
31	HA	2	2	0	3.59	HA
32	HA	3	3	0	4.75	HA
33	HA	2	2	0	2.76	HA
34	HA	2	2	0	3.80	HA
35	HA	3	3	0	4.95	HA
36	HA	2	2	0	3.90	HA
37	HA	2	2	0	3.85	HA

CM, complete mole; PM, partial mole; HA, hydropic abortus. Chromosome copy number in most nuclei from villous trophoblastic and stromal cells.

Methods

TISSUES

Tissue specimens from histologically classified complete moles ($n = 11$), partial moles ($n = 15$), and hydropic abortuses ($n = 11$) were studied. The tissues were obtained following curettage for suspected molar pregnancy or missed abortion. Gestational period varied from eight to 16 weeks. The tissues were fixed in 10% formalin and embedded in paraffin by standard methods. Serial paraffin sections of 6 μ m thicknesses were mounted on silane coated glass slides and incubated in an oven at 60°C overnight. A section from each tissue was stained with haematoxylin and eosin, and reviewed to verify the diagnosis. Histological criteria for the diagnosis were those reported in detail by Szulman and Surti^{7,8} and Szulman *et al.*,²⁴ and recently tested by Paradinas *et al.*²⁵

DNA PROBES AND ANTIBODY REAGENT

DNA probes for chromosomes 1, X, and Y were obtained from Boehringer Mannheim GmbH, Germany. The probes were labelled in vitro in the presence of digoxigenin-11-dUTP by nick translation of the plasmids pUC1.77 (isolated from human satellite III DNA) for chromosome 1,²⁶ pDMX1 for chromosome X,²⁷ and pHY2.1 for chromosome Y.²⁸ Under stringent conditions, the probes hybridise in situ to the (peri)centromeric region of chromosome 1, centromeric region of chromosome X,

and the tip of the long (q) arm of chromosome Y. The probes fragment length distribution showed a maximum of 200–500 bases. Sheep antidigoxigenin (Fab fragments) labelled with peroxidase (Boehringer Mannheim) was employed to detect digoxigenin.

IN SITU HYBRIDISATION

Interphase cytogenetic analysis by in situ hybridisation using chromosome specific probes was performed according to a protocol optimised for routine paraffin sections as described by Hopman *et al.*¹³ We incorporated microwave pretreatment of the paraffin sections to enhance signal detectability by in situ hybridisation.^{29,30} The sections were deparaffinised by immersion in two changes of xylene for 10 minutes each, followed by absolute ethanol for 10 minutes. Endogenous peroxidase activity in tissue sections was quenched by immersion in 1% H₂O₂ in methanol for 30 minutes, and the sections were rehydrated in 90%, 80%, and 70% ethanols and rinsed in Tris-HCl buffer (TBS), pH 7.6. The slides were then placed in a Coplin jar containing 0.01 M citrate buffer, pH 6.0, and heated for five minutes in a microwave oven (model MRO-M55, Hitachi, Tokyo, Japan) with operating frequency of 2450 MHz and 990 W power output. After cooling to room temperature, the slides were rinsed in TBS, and incubated in 1 M sodium thiocyanate (Sigma, St Louis, Missouri, USA) at 80°C for 10 minutes to denature proteins and permeabilise the tissue sections, rinsed in TBS, and digested with 1 mg/ml pepsin (Sigma) in 0.2 M HCl at 37°C. Pepsin digestion time was optimised for each section while preserving acceptable morphology. Most sections required five minutes' digestion with a range of three to eight minutes. Hybridisation was considered to be satisfactory when more than 90% of cells contained signals. The slides were then rinsed thoroughly in TBS, dehydrated in 70%, 80%, 90%, and absolute ethanol, air dried, and heated at 80°C in an oven for 30 minutes. The sections were prehybridised for 15 minutes at 80°C with 50 ml of a mixture of 60% formamide in 2 × SSC (0.3 M NaCl and 30 mM Na citrate, pH 7.5) containing 10% dextran sulphate, 50 ng/μl herring sperm DNA, and 50 ng/μl baker's yeast. After washes in 2 × SSC, the sections were hybridised with the same prehybridisation mixture but containing 1 ng/μl of the digoxigenin labelled DNA probe to either chromosome 1, X, or Y for 10 minutes at 80°C and overnight at 37°C in a moist chamber. The slides were then stringently washed with a solution of 60% formamide in 2 × SSC and 0.05% Tween-20 for five minutes three times, and TBS with 0.05% Tween-20 for five minutes three times to remove unbound probes. Following hybridisation, the sections were incubated with 1:20 normal sheep serum for 30 minutes to block non-specific binding sites and 1:50 peroxidase labelled sheep antidigoxigenin for one hour at room temperature, washed with TBS containing 0.05% Tween-20, and reacted with 0.05% diaminobenzidine and 0.01% H₂O₂ in TBS, pH 7.6, for 10 min-

Figure 2 Boxplot of the mean (SD) AgNOR counts for all cases (4.20 (1.19); $n = 37$) and for complete (3.39 (0.97); $n = 11$) and partial (5.11 (0.91); $n = 15$) hydatidiform moles (CM and PM), and non-molar hydropic abortus (HA; 3.79 (0.90); $n = 11$). Differences between the means for PM versus CM and HA are significant (one factor ANOVA; $p < 0.0001$) (lines through boxes indicate 50th centiles).

utes to visualise the signals. The reaction was stopped by washes with distilled water, and the sections were lightly stained with Mayer's haematoxylin (10 seconds) and mounted using Mount-Quick (Daido Sangyo Co, Tokyo, Japan).

EVALUATION OF HYBRIDISED SECTIONS

Hybridisation was considered adequate when more than 90% of cells contained signals. Hybridised sections were assessed essentially as described previously.^{13–15} Criteria for counting signals were: (1) overlapping interphase nuclei were excluded; (2) signals of nearly similar size were counted; (3) split spots were counted as one signal; and (4) weak signals were excluded. The interobserver variability in assessing the signals was less than 8%. The number of signals present in 100 nuclei from the villous trophoblastic cells and stromal cells per section was counted. The percentage of nuclei having no, one, two, three, and more than three signals for each chromosome was determined. Maternal cells such as decidual or inflammatory cells present in each tissue served as internal controls of diploid XX cells, and paraffin sections from a benign prostatic hyperplasia and male lymphocytes were used as Y chromosome controls.

NOR STAINING

Histochemical staining for NOR was performed according to a method described previously.³¹ Briefly, tissue sections were deparaffinised in xylene and rehydrated in graded ethanol series to PBS. After microwave heating of the sections, as described above for in situ hybridisation, and cooling down to room temperature, they were incubated with a standard colloidal silver staining solution consisting of one part of a 2% gelatin in 1% aqueous formic acid solution mixed with two parts of a 25% aqueous silver nitrate solution for 30 minutes at room temperature in the dark. The sections were thoroughly washed in distilled water and immersed in a 5% solution of sodium thiosulphate for five minutes to remove artefactual

silver granules. After washes in distilled water, the sections were dehydrated in ethanol series to xylene and mounted using Mount-Quick. AgNOR appeared as intranuclear black dots. Controls included endothelial and stromal cells present in each tissue section.

AgNOR QUANTIFICATION

The number of AgNOR dots was counted in 100 villous trophoblastic cell nuclei per tissue section. AgNOR were counted in comparable tissue areas as evaluated by in situ hybridisation in serial sections from the same tissues. All intranuclear silver stained dots were counted at 1000 × magnification. Careful focusing was made to resolve clustered black dots into a discernible number of discrete dots for counting. The mean and standard deviation of the number of AgNOR per nucleus for each lesion were determined. The counting procedure was performed twice and the mean inter- and intraobserver variation was less than 9%.

STATISTICAL ANALYSIS

Differences between the mean AgNOR counts of complete mole, partial mole, and hydropic abortus were assessed by one factor analysis of variance (ANOVA). A p value of < 0.05 was considered as significant. Interphase cytogenetic and AgNOR results were compared using Spearman rank correlation coefficient.

Results

INTERPHASE CYTOGENETIC ANALYSIS

In the villi of complete mole, most nuclei had two copies of chromosomes 1 (fig 1A) and X (fig 1B), and in one case one copy of chromosomes X and Y (fig 1C). The hyperplastic trophoblast areas showed marked nuclear atypia frequently associated with multiple copies of chromosomes 1 and X (fig 1D). The fraction of such nuclei ranged from 10% to 35%. In the villi of partial mole, three copies of chromosomes 1 (fig 1E), and X (fig 1F), were found. Table 1 summarises the results. Overall, the findings in eight of the 11 cases (73%) each of complete mole and hydropic abortus were consistent with diploidy. The remaining three cases (27%) of each group showed triploid patterns. Two of the triploid complete moles and one of the triploid hydropic abortuses were revised to partial mole. The remaining triploid complete mole was revised to hydropic abortus. Of the 15 cases of partial mole, nine (60%) were triploid and six (40%) were diploid. Of the diploid cases, three were revised to complete mole and three to hydropic abortus.

AgNOR ANALYSIS

In complete mole, there were usually less than four AgNOR dots in the nuclei of villous trophoblast cells (fig 1G), whereas in partial mole there were often more than four (fig 1H). The AgNOR count in hydropic abortuses closely resembled that in complete moles. The mean AgNOR count was highest in partial moles (5.11 (0.91)) followed by hydropic abortuses (3.79 (0.90)) and complete moles (3.39 (0.97)) (fig 2). The differences between mean AgNOR counts in partial mole versus

complete mole and hydropic abortus were statistically significant (one factor ANOVA; $p < 0.0001$). After consideration of the 75th centile rate of mean AgNOR counts in all cases (fig 2), a mean count of ≥ 5 was designated as a cut off limit for triploidy. Subsequently, nine of the 15 cases (60%) with partial mole had a mean AgNOR count of ≥ 5 , while 10 each of 11 cases (91%) with complete mole and hydropic abortus had a mean count of < 5 (table 1). After reclassification, eight of the nine cases (89%) with partial mole showed a mean AgNOR count of ≥ 5 .

COMPARISON OF INTERPHASE CYTOGENETIC AND AgNOR RESULTS

A high mean AgNOR count of 5.24 (0.73) was obtained in the total of 15 cases showing triploid patterns, while the count in 22 cases with diploid pattern was 3.50 (0.88). The results of ploidy analyses by interphase cytogenetics and AgNOR count were closely correlated (Spearman rank correlation coefficient: $r = 0.73$, $z = 4.39$, $p = 0.0001$).

Discussion

Nuclear ploidy analysis appears to play an important role in the diagnosis and classification of hydatidiform mole.^{9-12 24 25} In this study, we examined and compared nuclear ploidy in histological sections of complete mole, partial mole, and hydropic abortus by interphase cytogenetics, using probes specific for chromosomes 1, X, and Y, and AgNOR count. We found that the results obtained by the two methods were closely correlated and provided useful information to supplement the histological distinction between complete mole, partial mole, and hydropic abortus. Hydatidiform mole is currently diagnosed and classified on a morphological and cytogenetic basis. Recently, flow cytometry has been successfully used to assess ploidy in hydatidiform mole.¹⁰⁻¹² Flow cytometry quantifies the amount of nuclear DNA in a large fraction of particular cells relative to their normal counterparts, and is an established method of ploidy analysis. Image analysis of the nuclear DNA content of a certain cell type appears to be more accurate than flow cytometry as the cells are selected based on their morphology.^{32 33} In flow cytometry, the amount of DNA in tumour cell nuclei must show at least 4% deviation to be distinguished from normal DNA.³⁴ This amounts to the DNA content of two to three chromosomes,^{35 36} implying that two or three chromosomes have to be lost or gained before the change can be detected. Interphase cytogenetic analysis using chromosome specific probes has been successfully used to detect numerical chromosome aberrations.^{16 18} Recently, Hopman *et al* developed a simplified protocol to perform in situ hybridisation using chromosome specific probes on paraffin embedded tissue sections from bladder tumours.¹³ They found a close correlation between chromosome copy numbers in paraffin sections and in cell suspensions of the same tumours. Subsequently, Van de Kaa *et al* applied the same method on paraffin sections from a series

of hydatidiform mole and hydropic abortus for the assessment of chromosome ploidy and the determination of sex chromosome composition.^{14 15} They found a high value for this method in distinguishing partial mole from complete mole and hydropic abortus. A good correlation was also shown between the results obtained by interphase cytogenetics and DNA flow cytometry in partial mole and hydropic abortus. However, all cases with complete mole had a diploid pattern but with a high fraction of polyploid nuclei by interphase cytogenetic analysis, and also showed a polyploid pattern by DNA cytometry. Thus interphase cytogenetics can be successfully applied to paraffin sections to identify chromosomal aberrations in nuclei with minor DNA changes undetectable by DNA cytometry.

On the other hand, recent studies on AgNOR in breast carcinomas have shown a close correlation between the mean number of AgNOR and flow cytometric assessment of nuclear ploidy.^{21 22} In addition, Giri *et al* found that approximately three quarters of breast tumours with AgNOR counts of more than 3 had significant aneuploid cell populations and showed a strong correlation between the AgNOR count and nuclear ploidy.³⁷ Suresh *et al*,²³ in a study of trophoblastic tissue, showed that in non-neoplastic trophoblastic tissue AgNOR counts are clearly a reflection of ploidy rather than cell proliferation. Therefore the AgNOR count appears to be a promising tool for assessment of nuclear ploidy. These observations prompted us to evaluate formalin fixed, paraffin embedded tissue specimens from a series of complete moles, partial moles, and hydropic abortuses for nuclear ploidy by interphase cytogenetics using in situ hybridisation and AgNOR count.

In our series, the diagnosis of partial mole was confirmed in nine of 15 cases (60%) following histological examination and ploidy analyses by interphase cytogenetics and AgNOR count. In the remaining six cases, the diagnosis was revised to complete mole in three cases and hydropic abortus in three cases. Bagshawe *et al* found that in five of 11 partial mole cases (45%) the diagnosis of partial mole could be confirmed after histological examination and DNA ploidy analysis.² In the remaining six cases, the diagnosis was revised to complete mole in four and non-molar pregnancy in two cases. In another study, 55% of partial mole cases were DNA triploid.¹⁵ In the present study, eight of the 11 cases (73%) each of complete mole and hydropic abortus were confirmed to be diploid. The remaining three cases of each group were triploid. Two of the triploid complete mole and one of the triploid hydropic abortus cases were revised to partial mole. The remaining one triploid complete mole was revised to hydropic abortus. These findings signify the importance of the combined use of conventional histology and ploidy analyses in the differential diagnosis of complete mole, partial mole, and hydropic abortus.

Interphase cytogenetic and AgNOR methods do not require fresh tissue, cell culture, metaphase spread, or isolation of a certain cell

type. However, some factors inherent in paraffin sections—such as slicing through a portion of each nucleus which may result in an apparent loss of chromosomes or AgNOR dots, and nuclear overlapping—should be considered. These factors may affect the accuracy of signal¹³⁴ or AgNOR enumeration. Nevertheless, the two methods are highly reproducible and reliable for detection of nuclear ploidy in tissue sections.

In conclusion, although larger studies are required, our data suggest that interphase cytogenetics by in situ hybridisation using chromosome specific probes and AgNOR count offer useful approaches for ploidy analysis in histological sections of hydatidiform mole. In our opinion, in addition to flow cytometry, these methods are also useful in resolving unclassifiable cases of hydatidiform mole.

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