INTERPHASE CYTOGENETICS IN PARAFFIN SECTIONS OF ROUTINELY PROCESSED HYDATIDIFORM MOLES AND HYDROPIC ABORTIONS

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SUMMARY

The differential diagnosis of complete (CM) and partial (PM) hydatidiform moles and hydropic abortions (HA) can be difficult when based on histology alone. Therefore, a more objective approach of chromosome ploidy analysis as detected by in situ hybridization (ISH) was performed on 6 µm paraffin sections of seven cases, originally classified as three CM, two PM, and two HA with a histologic pattern suggestive of triploidy. Probes for repetitive DNA targets in the (peri)centromeric region of chromosomes 1 and X and in the q arm of chromosome Y were used to determine chromosome ploidy and sex chromosome composition. The findings in the three CM were consistent with diploidy: two copies of chromosomes 1 and X and none of chromosome Y. In the two HA with a histologic pattern suggestive of triploidy, three copies of chromosomes 1 and X and none of chromosome Y confirmed triploidy. Two cases originally classified as PM both appeared to have two copies of chromosome 1 with an XX pattern in one case and an XY pattern in the other case, which is consistent with diploidy instead of triploidy. After reviewing, both cases most likely represented CM. We conclude that interphase cytogenetics by ISH on paraffin sections of hydatidiform moles and hydropic abortions enables chromosome ploidy analysis with preservation of histological context. The important advantages of this procedure are (1) precise discrimination between maternal and trophoblast cells, (2) no risk of selecting certain cell types as a result of the isolation procedure, (3) detection and localization of cell subpopulations with heterogeneous DNA content, (4) determination of the contribution of a single chromosome (e.g., sex chromosome), and (5) access to archival material.

KEY WORDS—In situ hybridization, paraffin sections, hydatidiform mole, placenta, ploidy, chromosome aberrations.

INTRODUCTION

Oedema of chorionic villi is found in complete (CM) and partial (PM) hydatidiform moles and in hydropic abortions (HA). As a rule, CM have a diploid karyotype, 46,XX or 46,XY, while PM usually have a triploid karyotype, 69,XXX or 69,XXY or 69,XYY.1-4 Triploidy can also be present in HA.5-8 The three categories have a different prognosis and therapeutic approach. In at least 10 per cent of CM, persistent gestational trophoblastic disease develops,9,10 with risk of metastases and/or choriocarcinoma. In PM, persistent gestational trophoblastic disease is recognized (0–5–6–6 per cent)9,10–13 but it does not occur in HA. In CM, hormonal follow-up and contraceptives are essential for 1 year. This procedure is recommended for PM,10–13 while it is unnecessary in HA.

Histological criteria for classification of hydatidiform moles have been described.1–3 However, differential diagnosis based on histologic criteria can be difficult, as indicated by a high inter-
Table I—Results of histologic classification and in situ hybridization using specific probes for chromosomes 1, X, and Y of hydatidiform moles and abortions

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Original histologic diagnosis*</th>
<th>Chromosome copy number in the major fraction of villous stromal and villous lining cells†</th>
<th>Revised diagnosis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CM</td>
<td>2 X 2 Y</td>
<td>CM</td>
</tr>
<tr>
<td>2</td>
<td>CM</td>
<td>2 X 0 Y</td>
<td>CM</td>
</tr>
<tr>
<td>3</td>
<td>CM</td>
<td>2 X 2 Y</td>
<td>CM</td>
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<tr>
<td>4</td>
<td>PM</td>
<td>2 X 2 Y</td>
<td>CM</td>
</tr>
<tr>
<td>5</td>
<td>PM</td>
<td>2 X 1 Y</td>
<td>CM</td>
</tr>
<tr>
<td>6</td>
<td>HA</td>
<td>3 X 3 Y</td>
<td>HA</td>
</tr>
<tr>
<td>7</td>
<td>HA</td>
<td>3 X 3 Y</td>
<td>HA</td>
</tr>
</tbody>
</table>

*CM = Complete hydatidiform mole; PM = partial hydatidiform mole; HA = hydropic abortion. †Areas of trophoblast proliferations are not included. The nuclei of decidual or inflammatory cells from the mother displayed in all cases two signals for chromosomes 1 and X, and none for chromosome Y.

observer variability. Determination of DNA ploidy with the aid of more objective methods may contribute to the differential diagnosis. Karyotyping is an objective approach to detect numerical and/or structural chromosome aberrations. Nevertheless, karyotyping is difficult in a routine setting and carries a potential danger of loss and/or of selection of genetic material. Flow cytometry (FCM) has been used in the diagnosis of hydatidiform moles and abortions. However, the method is limited in the detection of heterogeneous cell sub-populations with different DNA contents and gives no information about specific chromosome aberrations. In addition, with both methods the histological context is lost.

Chromosome aberrations at the cellular level can be demonstrated by in situ hybridization (ISH) using chromosome-specific probes. Since clear results can be obtained not only in metaphase spreads, but also in non-mitotic cells, the procedure was termed 'interphase cytogenetics'. ISH using specific probes permits the screening of relatively large cell populations as well as the detection of minor cell subpopulations with heterogeneity and imbalance in chromosome copy numbers. In an earlier report and in this paper we introduce a protocol for a non-radioactive ISH procedure on paraffin-embedded tissue sections as a method to determine the copy number of specific chromosomes. In the present study, seven cases, including three CM, two PM, and two HA, were selected on the basis of the original histologic diagnosis. To determine chromosome ploidy and the contribution of the sex chromosomes, DNA probes for the (peri)centromeric regions of chromosomes 1 and X and for the q arm of chromosome Y were used.

METHODS

Patient material

A total of seven conceptuses were investigated, all obtained following curettage performed after ultrasound diagnosis of suspected molar pregnancy (cases 1, 2, 3, 5 and 6, Table I) or of missed abortion (cases 4 and 7). The period of gestation varied from 9 to 18 weeks. Embryonal or fetal parts were not found. Gross vesicles could be identified in four cases (1, 2, 3, and 6). The specimens were routinely processed (fixed in phosphate-buffered 4% formaldehyde for 12–24 h and embedded in paraffin at 58°C) and microscopically classified. A diagnosis of CM was made when diffusely, oedematous villi with central cisterns and excessive trophoblast proliferation were present. Embryonal cells (embryonic tissue or nucleated red blood cells) or amnion cells excluded a CM. A diagnosis of PM was considered when hydropically swollen villi were found intermingled with villi of normal size, while excessive trophoblast proliferation was moderate and focal. Morphologic suggestions of triploidy, such as irregular villous contours and trophoblast inclusion cysts, were often seen. Although embryonal elements are regularly present, these can be missed due to sampling error, and evidence of embryonal cells
therefore was not always considered essential for a
diagnosis of PM. In HA, varying degrees of villus
eodema could be found, sometimes with central
cristerns, but without excessive trophoblast prolifera-
tion. Scalloped villous outlines and trophoblast
inclusion cysts were features suggestive of triploidy.

**DNA probes and non-radioactive labelling**

The satellite III DNA probe for chromosome 1
(pUC 1.77) recognizes a tandem repeat of 1.77 kb in
the (peri)centromeric region (1q12) of chromosome
1. The alphoid DNA probe for chromosome X
(pBam X5) and the satellite III DNA probe for
chromosome Y (DYZ3) recognize tandem repeats
in the centromeric region of chromosome X and
in the q arm of chromosome Y, respectively.22,23
Biotinylation of the probes was performed using
Bio-11-DUTP (Sigma, St Louis, U.S.A.) in a nick
translation reaction.24,25

**In situ hybridization**

The reproducibility and validity of a protocol to
detect chromosome copy numbers in routinely pro-
cessed, paraffin-embedded tissue sections by ISH
has been tested and described elsewhere.24 A
brief description is given here. Paraffin sections
(6 μm) were mounted on 2.5 per cent glutaraldehyde-
activated poly-l-lysine (1 mg/ml; Sigma) coated
slides,26 dried at 56°C overnight, deparaffinized, and
treated with 1 per cent H2O2 in methanol (30 min),
rinsed, and air-dried. To permeabilize the tissue
sections for penetration of modified DNA probes
and antibodies, the slides were incubated with 1 M
NaSCN (Merck, Darmstadt, Germany) at 80°C (10
min), rinsed and digested with pepsin (4 mg/ml in
0.2 M HCl; Sigma P7000) at 37°C (15-40 min),
rinsed, dehydrated, air-dried, and heated at 80°C
(30 min).

ISH with chromosome-specific probes took place
in a mixture of 60 per cent formamid, 2 × SSC
(0.3 M NaCl; 30 mm Na citrate, pH 7.0), 10 per cent
dextran sulphate (Pharmacia, Uppsala, Sweden),
50 ng/μl herring sperm DNA as carrier DNA, and
50 ng/μl baker’s yeast as carrier RNA, at probe
concentrations of 1 ng/μl hybridization mixture.
Denaturation was performed at 80°C (10 min),
followed by hybridization overnight at 37°C and
successive rinsing in 60 per cent formamid,
2 × SSC containing 0.05 per cent Tween-20
(3 × 5 min; Merck) and 0.05 per cent Tween-20
in phosphate-buffered saline (PBS; 2 × 5 min).
Immunocytochemistry was performed as previously
described,19,20 with mouse anti-biotin (Dakopatts,
Glostrup, Denmark), rabbit anti-mouse peroxidase
(Dakopatts), and 3,3'-diaminobenzidine (DAB;
Sigma), 0.05 per cent H2O2 in PBS containing 0.1 M
imidazole to visualize peroxidase activity. The
slides were stained with Mayer’s haematoxylin and
mounted in Permount (Fisher Scientific, New
Jersey, U.S.A.).

**Evaluation of in situ hybridization signals**

Criteria for the evaluation of ISH signals were as
follows:26 (a) overlapping interphase nuclei were not
counted; (b) signals within one nucleus signals have
more or less the same size and intensity; (c) paired
ISH spots (split spots) were counted as one signal;
and (d) non-specific signals such as minor binding
sites were not counted. Using these criteria, the
inter-observer variability in the interpretation of
ISH signals was less than 10 per cent.

In all specimens, maternal tissue (decidua or
inflammatory cells) served as an internal control
of normal diploid XX cells (Fig. 2). Paraffin sections
of a transitional cell carcinoma of a male patient
and cell suspensions of male lymphocytes were used as
Y-chromosome controls.

**RESULTS**

**Evaluation of the ISH method**

A strong ISH signal was obtained in all cases with
preservation of acceptable nuclear, cytoplasmic,
and tissue morphology. In general, signals for
chromosomes 1 and Y were more intense than those
for chromosome X (Figs 1 and 2), probably because
the alphoid DNA probe for chromosome X recog-
nizes a less highly repetitive DNA sequence than
the satellite III DNA probes for chromosomes 1 and
Y. Within the same specimens stromal cells and
cytotrophoblast cells appeared to be less resistant
to pepsin digestion than syncytiotrophoblast cells,
which possess smaller nuclei and a more compact
chromatin. However, these differences did not
influence the analysis of ISH signals. Some
specimens had to be hybridized repeatedly under
varying conditions because of slight variability in
the optimal pepsin digestion time for paraffin-
embedded tissue in different cases. This proteolytic
digestion step is necessary to remove cytoplasmic
and nuclear proteins in order to improve penetra-
tion of the specific DNA probes as well as antibodies
used in the detection steps.22,23 In all samples, cells
with no or only one signal were seen due to nuclear
Fig. 1—*In situ* hybridization results of hydatidiform moles, using biotin labelled probes for chromosomes 1, X, and Y, on 6 μm paraffin sections, counterstained with Mayer's haematoxylin. In the villi of a complete mole (CM, case 3), the majority of nuclei showed two copies of chromosomes 1 (a) and X (b) and none of chromosome Y (not shown). The haematoxylin and cosin stained section (c) of case 5, originally classified as partial mole (PM), showed hydroptically swollen villi adjacent to villi of normal size with irregular outlines, and focal atypical trophoblast proliferations (arrow-head). In the villi, two copies of chromosome 1 (d) and one copy of chromosomes X (e) and Y (f) were found. In areas of cytotrophoblast proliferation, nuclei with three or more copies of chromosome 1 were present (g, arrows). In another case of CM (case 1), multiple copies of chromosomes 1 (h) and X (i) were found in the proliferating cytotrophoblast. (Due to truncation of nuclei or limitations in focusing, not all nuclei display the maximum chromosome copy number)

truncation, inherent to the fact that 6 μm thick sections were used.

*Applications of ISH on hydatidiform moles and hydropic abortions*

The original histologic diagnosis of the seven lesions is shown in Table 1. Cases 4, 5, 6, and 7 had morphologic features suggestive of triploidy.

In all cases histologically classified as CM, a constant pattern of two copies per nucleus was found for chromosomes 1 and X in trophoblast cells lining the villi as well as in villous stromal cells (Figs 1a and 1b). Chromosome Y was not found.

In case 4, originally diagnosed as PM with features suggestive of triploidy, two copies of chromosomes 1 and X and none of chromosome Y
were found in the main fraction of the cells. In case S, also originally diagnosed as PM, two copies of chromosome 1 and one copy of chromosomes X and Y were found in the majority of nuclei (Figs 1d, 1e and 1f). These data are consistent with a diploid DNA content rather than with triploidy. On re-examination, both cases showed normal villi adjacent to hydroptically swollen villi, many of them containing cisterns (Fig. 1c). Trophoblast proliferation with nuclear atypia was focally observed. Irregular villous contours with deep invaginations and trophoblast inclusion cysts, suggestive of triploidy, were present in these cases, but the inclusion cysts could also be explained by collapse and inversion of hydatidiform villi. Embryonic tissue, amnion, and nucleated red blood cells were not found, despite extensive tissue sampling. In case 4, 7 weeks after curettage persistent trophoblastic disease developed without metastases. The β-HCG level was 1300 ng/ml. The patient was treated with methotrexate and is in remission 2 years later. In case 5, signs of persistent trophoblastic disease were not detected after 13 months follow-up.

In some cases, cells with high chromosome copy numbers were observed in sheets of cytotrophoblast proliferation (Figs 1g, 1h, and 1i). In case 1, these polyploid or aneuploid cytotrophoblast cells showed up to 12 copies of chromosomes 1 or X. ISH signals in these areas, especially in some large nuclei, had a somewhat blurred appearance, while in other areas they were well localized.

In both cases of HA morphologically suggestive of triploidy, no copies of chromosome Y were found. The trophoblast and villous stromal cells showed three copies of chromosomes 1 and X (Figs 2a, 2b and 2c). Decidual cells displayed two copies of chromosomes 1 and X (Figs 2a and 2b).

**DISCUSSION**

Karyotyping of hydatidiform moles has established the diploid karyotype of CM (46,XX or 46,XY) and the triploid karyotype of PM (69,XXX or 69,XXY or 69,XYY)\(^1\)\(^3\)\(^-\)\(^5\). Also rare cases of diploid PM have been described\(^1\)\(^4\)\(^,\)\(^3\)\(^1\) as well as CM with trisomy 2.\(^2\)\(^2\)\(^2\). Moreover, recently one case of tetraploid CM and five cases of tetraploid PM were reported\(^3\)\(^2\)\(^-\)\(^3\)\(^7\) on the basis of karyotyping studies.

Recent investigations\(^1\)\(^9\)\(^-\)\(^2\)\(^2\)\(^,\)\(^2\)\(^6\) have demonstrated that probes to repetitive DNA sequences located predominantly in the (peri)centromeric region of a particular chromosome displayed their targets as distinct spots in interphase nuclei when hybridized...
and detected under standardized conditions. In this way, numerical chromosome aberrations could be detected by ISH in cell suspensions of solid tumours. Recently we developed a protocol to perform ISH on paraffin-embedded tissue sections from bladder cancer. A good correlation was found between chromosome copy numbers in paraffin sections and in cell suspensions of the same tumour, although truncation of nuclei caused some underestimation of the copy number in paraffin sections. In this paper we have presented data on the application of ISH on paraffin-embedded tissue sections of hydatidiform moles and abortions for the assessment of chromosome ploidy and the determination of sex chromosome constitution. Our results show three cases of CM which appeared to have two copies of chromosomes 1 and X, consistent with chromosome diploidy. In two cases of HA histologically suggestive of triploidy, three copies of chromosomes 1 and X were found, confirming chromosome triploidy.

In two cases initially classified as PM, determination of the copy numbers for chromosomes 1, X and Y excluded chromosome triploidy. In one case of PM, one copy of chromosomes X and Y was found. Although histologically PM was suggested in these cases, in retrospect a diagnosis of CM should have been considered because embryonal tissues were lacking. The incidence of diploid PM reported in the literature varies. In series with relatively high percentages of diploid PM, the presence or absence of embryonal tissues is not always mentioned. Criteria considered to be indicative of PM, such as partial hydatidiform change, and histological features suggestive of triploidy, such as irregular villous contours and trophoblast inclusion cysts, can be misleading, as illustrated by cases 4 and 5. Cases which histologically are suggestive of PM but lack evidence of an embryo and are diploid, on the basis of cytogenetic and/or flow cytometric analyses, most likely represent true CM. At the other end of the spectrum, where hydropic villous changes with evidence of an embryo are combined with triploidy, but show no obvious proliferation or atypia of the trophoblast, overdiagnosis should be avoided. Many of these cases represent hydropically degenerated abortions. In a recent study, the diagnosis of PM was confirmed in only 5 out of 11 cases following histological review and measurement of DNA ploidy. In four cases, the diagnosis was revised to CM and in two cases there was no evidence of molar pregnancy. This illustrates the value of the combined use of light microscopy and ploidy analysis in the differential diagnosis of CM, PM, and HA.

Interphase cytogenetics of tissue sections has the important advantage over karyotyping and flow cytometry (FCM) that the histological context is preserved and can be used in the evaluation. The necessity of bringing cells into culture for karyotyping increases the chance of selecting of certain cell populations or cell types (e.g., villous stromal cells), and loss of others, while also relatively few metaphases can be studied. The isolation of nuclei required for FCM causes loss of precise control over the cell types examined. The number of admixed maternal cells can influence the measured DNA index considerably and obscure the profile of the trophoblast cells, thus limiting the sensitivity for the detection of subpopulations of cells with a heterogeneous DNA content. Although with ISH the copy number of all chromosomes cannot be determined at the same time, the use of one or two chromosome-specific probes offers the opportunity to estimate chromosome ploidy with accuracy, since exact discrimination between maternal and trophoblast cells is possible and usually a large number of nuclei can be evaluated. In addition, no selection of cell subpopulations takes place. On the contrary, this method revealed the presence of cell subpopulations with high chromosome copy numbers in areas of cytotrophoblast proliferation. Sarto et al. in studies on nuclear morphology with Feulgen squash techniques as well as autoradiography, demonstrated the existence of polyplody and endoreduplication in human trophoblast and hydatidiform moles. The role of these cell subpopulations in the pathophysiology of trophoblastic disease is intriguing.

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REFERENCES

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