Persistent Gestational Trophoblastic Disease: DNA Image Cytometry and Interphase Cytogenetics Have Limited Predictive Value

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DNA flow cytometry has shown a wider spectrum of DNA content in the complete hydatidiform mole (CM) than the originally reported diploidy. Conflicting results have been published about the relationship of DNA content and the occurrence of persistent gestational trophoblastic disease (PGTD). In the present study, 71 cases of CM and 4 cases of partial mole accompanied by PGTD and 100 cases of CM without PGTD were evaluated with DNA image cytometry for differences in DNA-ploidy pattern, expressed as the 2.5c and 5c exceeding rates. A pilot study of 20 cases of each group was performed using interphase cytogenetics to detect differences in the frequency of numerical chromosomal aberrations and in sex chromosome composition. For this purpose, DNA probes specific for the pericentromeric regions of chromosomes 1 and X and for the long arm of chromosome Y were incubated on 6-μm paraffin tissue sections. The results showed no differences between CMs with or without PGTD; DNA polyploidy occurred in 99% and 98% of cases, respectively; the 2.5c exceeding rate and 5c exceeding rate were 62.6 and 62.4, and 6.5 and 6.0, respectively. The frequency of numerical chromosomal aberrations as detected by interphase cytogenetics was 23.4 and 22.8%. An XY pattern was found in 3 of 20 cases of CM with PGTD and in 4 of 20 cases of CM without PGTD. The four cases of partial mole showed a DNA-ploidy pattern identical to that of a CM. For this reason, they would be better reclassified as CMs, despite the presence of nucleated red blood cells or amnion. Although nuclear atypia and corresponding increased DNA content is pronounced but variable in CMs, the occurrence of PGTD is not related to variations in quantitative DNA content nor to gross heterology or homology in sex chromosomes.

KEY WORDS: DNA ploidy, Hydatidiform mole, Image cytometry, In situ hybridization, Persistent trophoblastic disease.

Mod Pathol 1996;9(10):1007–1014

Persistent gestational trophoblastic disease (PGTD) requiring chemotherapy develops in 10 to 20% of patients with a complete hydatidiform mole (CM) (1, 2). Choriocarcinoma is included in this group. Before current chemotherapy regimens and β-human chorionic gonadotropin (β-hCG) monitoring, choriocarcinoma was diagnosed in 2 to 19% of patients with a CM; 50% of gestational choriocarcinomas were preceded by a CM, the other 50% by abortions or normal pregnancies (3, 4). Today, a diagnosis of postmolar choriocarcinoma is rarely made, because PGTD is detected early as a result of β-hCG monitoring (2), which makes re-evacuations for a histologic confirmation of choriocarcinoma unnecessary. If treated promptly, the prognosis of postmolar choriocarcinoma is rarely made, because PGTD is detected early as a result of β-hCG monitoring (2), which makes re-evacuations for a histologic confirmation of choriocarcinoma unnecessary. If treated promptly, the prognosis of postmolar choriocarci

For pathologists, the histologic distinction between a CM and a PM and their differentiation from hydropic abortion is not always easy. To assess prognostic and therapeutic implications in the individual patient, it is important that the pathologic

0893-3952/96/09010-1007$3.00/0 MODERN PATHOLOGY
Copyright © 1996 by The United States and Canadian Academy of Pathology, Inc.
VOL. 9, NO. 10, P. 1007, 1996 Printed in the U.S.A.
Date of acceptance: July 1, 1996.
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classification is as accurate as possible. Previous studies (7, 8) using karyotyping delineated the pathologic and cytogenetic differences between these entities. CMs have a diploid karyotype, with the entire nuclear genome being derived from the father (9). PMs are triploid, with one maternally and two paternally derived sets of DNA (1). Hydropic abortions usually have an overall diploid DNA but with a variety of individual chromosomal aberrations.

As a powerful aid in the differential diagnosis, DNA flow cytometric analysis is now widely being used. In comparison with karyotyping, however, DNA flow cytometric studies show a wider spectrum of nuclear DNA in CMs than the previously detected diploidy (10-14). A higher incidence of PGTD was initially reported in DNA-aneuploid CMs (15), but this has not yet been confirmed by other studies (11, 13, 16). In all studies, DNA flow cytometric analysis was the applied technique. In one previous study (12), however, it was found that in the detection of cell subpopulations with increased DNA content, DNA flow cytometric analysis is less sensitive than DNA image cytometric analysis (ICM) and interphase cytogenetic analysis. Using interphase cytogenetic analysis, one could establish that the aberrant cell subpopulations with high DNA content (polyploidization) were mainly located in the extravillous trophoblast, with chorionic villi being uniformly diploid.

In this study, DNA ICM was applied to 175 cases of hydatidiform mole, and interphase cytogenetic analysis was applied to a subgroup of 44 of the 175 cases for additional evaluation of the DNA ploidy and numerical chromosomal aberrations in extravillous trophoblast, with special interest in differences that could predict PGTD. For this purpose, the parameters were (1) the ratio of the cell subpopulations with increased DNA content to the DNA-diploid cell population, expressed as the 2.5c and 5c exceeding rates (ERs) using DNA ICM; and (2) the degree of numerical chromosomal aberrations in the cell subpopulation with increased DNA content using interphase cytogenetic analysis.

MATERIAL AND METHODS

Patients

For this study, 75 cases of hydatidiform mole (71 cases of CM and 4 cases of PM) that were accompanied by PGTD were compared with 100 cases of CMs without PGTD. The cases were obtained from the files of the Central Molar Registration of The Netherlands at the University Hospital of Nijmegen, Nijmegen, The Netherlands, which provides national registration of hydatidiform moles and PGTD and supraregional laboratory service for β-hCG monitoring, and from the files of The Netherlands Study Group of Trophoblastic Tumors, in which discussions and decision making concerning follow-up and treatment take place at a national level. Clinical follow-up after molar evacuation included serum β-hCG titers measured weekly until normal for 3 consecutive weeks and then monthly for 1 year. PGTD was diagnosed when β-hCG levels persisted at a plateau and/or rose for at least 3 consecutive weeks. The cases of hydatidiform mole with PGTD were selected on the occurrence of PGTD irrespective of the initial or review diagnosis. The cases of hydatidiform mole without PGTD were selected on the review diagnosis of CM. From all of the cases, the histologic slides were reviewed according to the criteria of Szulman and Surti (7, 8) before the DNA analyses.

DNA ICM

DNA ICM was performed on all of the cases, as previously described (12). Briefly, intact nuclei were isolated from 50-μm-thick paraffin tissue sections. Maternal decidual tissue was processed separately and served as the internal control for normal diploid cells. If decidual cells were not available, maternal lymphocytes were used as the alternative. After deparaffinization and rehydration, the 50-μm sections were incubated with 0.1% protease (type VII Bacillus amyloliquefaciens; Sigma, St. Louis, MO) in phosphate buffered saline (PBS) at 37°C for 20 minutes. Incubation was terminated by adding 4 to 5 ml of cold (4°C) PBS and putting the tubes on ice. After rinsing with PBS, 30,000 nuclei were counted with a Coulter Counter Model ZB1 (Coulter Electronics, Dunstable, England). After centrifugation, 200 μl of fetal calf serum (Gibco, Paisley, Scotland) were added, and this nuclear suspension was centrifuged to a glass slide using a cytocentrifuge for 10 minutes at 500 rpm (Shandon, Zeist, The Netherlands), air dried, and fixed in a mixture of methanol, 37% formaldehyde, and acetic acid (85:10:5 by volume) for 1 hour. The nuclei were then stained with pararosaniline-Feulgen. The DNA content of 200 stained and intact nuclei of trophoblast cells were selectively measured using the CAS 100 System (Cellular Imaging Systems, Becton Dickinson, Leiden, The Netherlands) (17). Leukocytes and decidual cells were not measured as diagnostic cells. At least 30 rat liver cells (DNA tetraploid) were measured as an external control for DNA content, and at least 20 decidual cells were used as an internal control.

The DNA histograms were classified as follows. A DNA-diploid pattern consisted of a distinct G0/G1 peak in the diploid (2C; DNA index (DI) = 1.0 ± 0.1) region with a small proportion of cells in S and G2/M (4C) phases, defined by a 2.5c ER of less then
A DNA-polyploid pattern showed distinct peaks in the diploid (2C; DI = 1.0 ± 0.1) and tetraploid (4C; DI = 2.0 ± 0.2) regions with a 2.5c ER of 40% or more, or in the diploid, tetraploid, and octaploid (8C; DI = 4.0 ± 0.4) regions. The 2.5c ER and the 5c ER were determined from the nuclear fractions exceeding the first 2c G0/G1 peak with a DI greater than 1.25 and the 4c peak with a DI greater than 2.5, respectively. A 2.5c ER of 40% or more seemed to be a reliable parameter for discrimination between DNA diploidy and DNA polyploidy (12).

Interphase Cytogenetic Analysis

Interphase cytogenetic analysis was performed on 6-μm-thick paraffin-embedded tissue sections in 44 of the 175 cases: 20 cases of CM with PGTD, all 4 of the cases of PM with PGTD, and 20 cases of CM without PGTD. The chromosome-specific DNA probes used were the satellite III DNA probe for chromosome 1 (pUC 1.77), the alphoid DNA probe for chromosome X (pBam X5), and the satellite III DNA probe for chromosome Y (DYZ3), recognizing tandem repeats in the pericentromeric region (1q12) of chromosome 1 (18), in the centromeric region of chromosome X (19), and in the q arm of chromosome Y (20), respectively. Biotinylation of the probes was performed using Bio-14-dATP (BRL, Gaithersburg, MD), according to the instructions of the manufacturers. The in situ hybridization procedure (ISH) in paraffin-embedded tissue sections was performed as previously described (12, 21), with minor modifications in the immunohistochemical step: mouse anti-biotin (1:100 in PBS-Tween with 5% nonfat dry milk; Dakopatts, Glostrup, Denmark) was followed by biotin-labeled horse anti-mouse (1:200 in PBS-Tween, 5% nonfat dry milk; Vector, Burlingame, CA), and avidin-biotin complex (1:100 in PBS-Tween, 5% nonfat dry milk; Vector). The frequency of ISH signals for the different chromosomes was evaluated in at least 500 nuclei according to previously reported criteria (12, 21).

**TABLE 1. Comparison of Initial Histologic Diagnosis of the Referring Pathologist with the Revised Histologic Diagnosis**

<table>
<thead>
<tr>
<th>Diagnosis after histologic review</th>
<th>No initial diagnosis (n)</th>
<th>Complete mole (n)</th>
<th>Partial mole (n)</th>
<th>Hydropic abortion (n)</th>
<th>Abortion (n)</th>
<th>Total (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moles without PGTD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete mole</td>
<td>12</td>
<td>57</td>
<td>25</td>
<td>6</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Moles with PGTD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete mole</td>
<td>5</td>
<td>50</td>
<td>14</td>
<td>1</td>
<td>1</td>
<td>71</td>
</tr>
<tr>
<td>Partial mole</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

PGTD, persistent gestational trophoblastic disease.
The findings in CHB (Tables 2, 3). DNA trypidy was
and the cases without or with PGTD (d) (Table 2). (Eleven cases within significant difference between
more ISH signals (mean 2.3% range 0.8-4.3%) (Table 2). This is comparable to diploidy. The
percentage of nuclei with three or more ISH signals in
table 3 and 4 in all of the 40 cases. The per-
PGTD (mean 2.5% ER 6.2%) The results are shown

**Figure 2.** A. Normal vessels in a C/A containing epithelium and nuclei devoid of mitoses (400x)
B. Promonochrome-bound metaphases in the neoplastic tissue of the Hamanworth strain. (400x)
C. Partially seen in some of the normal vessels (hematoxylin and eosin, original magnification 200x)
D. Promonochrome-bound metaphases in normal vessels (hematoxylin and eosin, original magnification 400x)
E. Partially seen in some of the normal vessels (hematoxylin and eosin, original magnification 400x)
F. The

**Figure 1.** A classical histological aspect of a C/A with mitoses (hematoxylin and eosin, original magnification 200x)
B. Promonochrome-bound metaphases in normal vessels (hematoxylin and eosin, original magnification 400x)
C. Partially seen in some of the normal vessels (hematoxylin and eosin, original magnification 200x)
D. Promonochrome-bound metaphases in normal vessels (hematoxylin and eosin, original magnification 400x)
E. Partially seen in some of the normal vessels (hematoxylin and eosin, original magnification 400x)
TABLE 2. 2.5c and 5c Exceeding Rates (%) in Hydatidiform Mole With and Without Persistent Gestational Trophoblastic Disease

<table>
<thead>
<tr>
<th>Exceeding rates</th>
<th>Cases with persistent gestational trophoblastic disease</th>
<th>Cases without persistent gestational trophoblastic disease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Complete mole</td>
<td>Partial mole</td>
</tr>
<tr>
<td>Total N</td>
<td>71</td>
<td>4</td>
</tr>
<tr>
<td>2.5c Exceeding rate:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>62.6</td>
<td>60.0</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>11.2</td>
<td>11.2</td>
</tr>
<tr>
<td>Range</td>
<td>31.5-89.0</td>
<td>48.0-77.9</td>
</tr>
<tr>
<td>5c Exceeding rate:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>6.5</td>
<td>5.4</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>6.3</td>
<td>2.8</td>
</tr>
<tr>
<td>Range</td>
<td>0-32.5</td>
<td>1.3-8.5</td>
</tr>
</tbody>
</table>

not found with either method. The chorionic villi showed the same sex chromosomal composition as the NRBCs, amnion, or yolk sac (Fig. 5). Statistical analysis showed no significant correlation between the 2.5c ER using ICM and the polysomic nuclear fraction using ISH.

DISCUSSION

PGTD develops in only a minority of patients with a hydatidiform mole. The lack of a reliable predictor of PGTD, however, necessitates clinical follow-up in all patients. Nevertheless, the pathologist must distinguish the low-risk (0.5%) PM from the high-risk (10–20%) CM. The degree of nuclear atypia and the DNA content of the extravillous trophoblast are significantly higher in CMs than in PMs (12), but considerable variability also exists within CMs. Because the potential for malignant progression in hydatidiform moles is thought to be inherent to molar trophoblast and because tumor aggressiveness in general correlates with increase in nuclear atypia and DNA content, it seemed logical to explore the predictive value of histologic grade and DNA content in CMs.

Histologic grade, assessed on the basis of degree of trophoblastic hyperplasia and atypia, had already been suggested to have prognostic significance by Hertig and Sheldon (22) in a six-group grading system and by Hertig and Mansell (3) in a more reproducible three-group grading system. Although some studies (23, 24) initially supported these findings, others could not (25, 3), and finally, the grading system has been abandoned.

DNA content had been related to progressive disease by Sugimori et al. (26), using microspectrophotometric analysis, which demonstrated a subsequent increase of DNA content in CMs without and with progression toward destructive mole and in choriocarcinoma. Sugimori et al. (26) also found a correlation between higher and more widely distributed DNA content and delayed β-hCG regression curves. Using DNA flow cytometric techniques, Martin et al. (15) also found a positive correlation between DNA content and the development of PGTD. PGTD developed in 45% of their series of 40 patients, involving 77% of aneuploid cases and only 30% of euploid cases. The correlation of clinical course with ploidy was significant (P < 0.01). No association was found with proliferative (mitotic) index (P > 0.05). Other studies (11, 13, 16), however, have not confirmed these findings. Hemming et al. (16) noticed a relatively high tetraploid DNA peak in most CMs, which they interpreted as a high proliferative fraction but which more likely represents the extravillous trophoblast with increased polyploidization (12); Hemming et al. (16) could not detect a correlation between this fraction and the occurrence of PGTD. Lage et al. (11) and Fuku­naga et al. (13) found a relatively high frequency of DNA-tetraploid CMs, but there was no increased incidence of PGTD in these cases as compared with their group of DNA-diploid CMs.

All of these studies were based on DNA flow cytometric analysis. A previous study (12), however, found that DNA flow cytometric analysis is less sensitive than DNA ICM and interphase cytogenetic analysis in the detection of aberrant cell subpopulations with increased DNA content. Using interphase cytogenetic methods, we demonstrated that chorionic villi in CMs are always diploid, whereas the extravillous trophoblast shows extensive polyploidization. Others confirmed this finding (14). Therefore, in the present study, both of these techniques were used for additional evaluation of the variability in DNA content in CMs and the possible relation to PGTD. Using DNA ICM, however, no such correlation could be found: almost all of

FIGURE 3. DNA ICM of a CM showing DNA polyploidy. The DNA-diploid (arrow at DI = 1), DNA-tetraploid (arrow at DI = 2), and DNA-octaploid (arrow at DI = 4) peaks represent different nuclear fractions of the molar tissue.
the cases were found to be DNA polyploid with a high 2.5c and 5c ER, which are objective numerical parameters for increased DNA content, but there was no significant difference in the height of the 2.5c and 5c ER between CMs with or without PGTD.

Interphase cytogenetic analysis performed on paraffin-embedded tissues allows the detection of numerical chromosomal aberrations at the nuclear level with conservation of histologic morphology. Therefore, a more specific evaluation of the area of interest (extravillous trophoblast) is possible. Again, however, no correlation could be found between the occurrence of PGTD and the degree of numerical chromosomal aberrations, neither with respect to the number of aberrant nuclei nor to the degree of chromosomal aberrations within the nucleus. Also, with this method, there will be an underestimation of the real degree of numerical chromosomal aberrations, because the nuclei are truncated as a result of tissue sectioning. Therefore, smaller variations could be missed.

On the basis of these results, we conclude that in CMs the degree of nuclear atypia and corresponding increase in DNA content is not correlated with PGTD. The mechanism of tumor progression in trophoblastic disease and somatic neoplasia, therefore, seems to be different. A possible explanation for these negative findings comes from experimental animal studies (27), from which it is known that during the formation of the placenta, different cycles of placental bed trophoblastic giant cells are formed, with high DNA contents present as a result of endoreduplication. This phenomenon especially occurs in the intermediate trophoblast at the placental implantation site, at which location trophoblast cells invade the uterus between decidual and myometrial cells. This giant cell transformation is

### TABLE 3. Overall Results of Interphase Cytogenetic Analysis in Extravillous Trophoblast of Hydatidiform Moles With and Without Persistent Gestational Trophoblastic Disease

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Total (N)</th>
<th>XY:XX (n:m)</th>
<th>Percentage of nuclei with in situ hybridization signals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete mole without PGTD</td>
<td>20</td>
<td>4:16</td>
<td>=2 (mean ± SD) 77.1 ± 7.8, ≥3 (mean ± SD) 22.8 ± 7.8, ≥3 (range) 11.1–40.7, ≥5 (mean ± SD) 2.8 ± 2.4</td>
</tr>
<tr>
<td>Complete mole with PGTD</td>
<td>20</td>
<td>3:17</td>
<td>=2 (mean ± SD) 76.6 ± 8.3, ≥3 (mean ± SD) 23.4 ± 8.7, ≥3 (range) 9.8–43.1, ≥5 (mean ± SD) 3.2 ± 3.6</td>
</tr>
<tr>
<td>Partial mole with PGTD</td>
<td>4</td>
<td>2:2</td>
<td>=2 (mean ± SD) 80.1 ± 10.3, ≥3 (mean ± SD) 19.8 ± 10.3, ≥3 (range) 7.1–32.3, ≥5 (mean ± SD) 2.9 ± 3.1</td>
</tr>
</tbody>
</table>

SD, standard deviation; PGTD, persistent gestational trophoblastic disease.

### TABLE 4. Mean Spectrum of In Situ Hybridization Signals per Nucleus in Extravillous Trophoblast of Hydatidiform Moles With and Without Persistent Gestational Trophoblastic Disease

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>&gt;9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>SD</td>
<td>%</td>
<td>SD</td>
<td>%</td>
<td>SD</td>
<td>%</td>
<td>SD</td>
<td>%</td>
<td>SD</td>
</tr>
<tr>
<td>Complete mole without PGTD</td>
<td>2.7</td>
<td>1.3</td>
<td>23.9</td>
<td>5.4</td>
<td>50.0</td>
<td>7.2</td>
<td>13.5</td>
<td>3.6</td>
<td>6.5</td>
<td>2.8</td>
</tr>
<tr>
<td>Complete mole with PGTD</td>
<td>3.5</td>
<td>2.2</td>
<td>23.0</td>
<td>6.4</td>
<td>50.0</td>
<td>9.5</td>
<td>13.7</td>
<td>3.6</td>
<td>6.5</td>
<td>3.1</td>
</tr>
<tr>
<td>Partial mole with PGTD</td>
<td>3.2</td>
<td>1.7</td>
<td>28.9</td>
<td>9.1</td>
<td>48.0</td>
<td>48.0</td>
<td>12.3</td>
<td>5.5</td>
<td>4.6</td>
<td>2.4</td>
</tr>
</tbody>
</table>

SD, standard deviation; PGTD, persistent gestational trophoblastic disease.

[FIGURE 4. Interphase cytogenetic analysis on 6-μm paraffin-embedded tissue sections using a biotinylated DNA-probe specific for the pericentromeric region of chromosome 1. The extravillous trophoblast shows prominent nuclear atypia with polysomy, in contrast to the villous trophoblast and stromal cells, which show disomy (upper right; counterstaining with Mayer’s hematoxylin; original magnification, 300×).]
DNA in Persistent Tropospheric Disease (C.A. van den Klaauw, et al.)

In conclusion, DNA cytometric and immunohistochemical analyses have in important application for understanding the pathogenesis of disease. The results of the present study, when compared with our previous findings, demonstrate that the use of DNA cytometric and immunohistochemical methods can provide valuable information about the complex mechanisms underlying tropospheric disease.

Figure 5: Gene expression in patients with chronic bronchitis

A: Hypothetical representation of gene expression
B: Actual gene expression
C: Control gene expression
role in differentiating the low-risk PMs, which are DNA triploid, from the high-risk CMs or from high-risk (and incorrectly classified) PMs, which are DNA polyploid. In the latter group, the information derived from these techniques has no additional predictive value in the development of PGTD. Therefore, the mechanisms that play the decisive role in the progression of hydatidiform moles remain to be elucidated.

Acknowledgments: The authors express their gratefulness to all Laboratories of Pathology and Departments of Obstetrics and Gynecology in The Netherlands that have contributed their cases to the Central Molar Registration in The Netherlands.

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