Clinicopathologic profile of gestational trophoblastic disease

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Klinisch-pathologisches Profil gestationsbedingter Trophoblasttumoren


Summary. Much debate exists on factors predicting the development of persistent gestational trophoblastic disease (pGTD). Diagnosis is still limited by following persistently elevated or rising postevacuation β-human chorionic gonadotropin (β-hCG) titers. The aim of the present work was to evaluate the hypothesis that the presence of c-erbB-2 oncocogene amplification and expression, in combination with parameters such as DNA-content and karyotype of the sex chromosomes, confer an increased risk of developing pGTD. Clinicopathological characteristics were evaluated in 36 cases of gestational trophoblastic diseases (GTD) and analyzed for c-erbB-2 amplification and protein p185 expression using differential polymerase chain reaction (DPCR) and immunohistochemical (IHC) techniques. The DNA-content was determined by image analysis on Feuigen stained nuclear cell preparations and karyotyping for XY chromosomes was performed by fluorescenze in situ hybridization (FISH). The data was correlated with histopathological characteristics of GTD. Seventy-five percent (n = 27) of the examined cases showed spontaneous regression after evacuation, including 2 patients who received additional chemotherapy. Twenty-five percent (n = 9) resulted in a persistent or metastatic disease. The median time between antecedent pregnancy and GTD was 45.4 months. Complete remission was achieved in all patients with pGTD after administration of chemotherapeutic agents or adjuvant surgical procedures. Cases with c-erbB-2 amplification and expression in combination with DNA hyperplodie showed higher proliferation and more aggressive behavior (2 complete hydatidiform moles with
lung and liver metastases, 2 invasive moles and 1 choriocarcinoma). XY karyotype was evident in the choriocarcinoma and in 2 complete hydatidiform moles with advanced stage and DNA hyperploidy. From these results we conclude that c-erbB-2 amplification and/or protein expression in combination with DNA-content show a significant correlation with the proliferative and aggressive potential of GTD, suggesting their combined use as a possible marker for pGTD.

**Key words:** Gestational trophoblastic disease, clinicopathologic profile, c-erbB-2 amplification/expression, DNA-content, karyotype.

**Introduction**

After uterine evacuation of a complete hydatidiform mole, about 20% of women develop persistent gestational trophoblastic disease (pGTD), which is defined as persistence of viable trophoblastic tissue in association with stable or increasing postmolar serum β-human chorionic gonadotropin (β-hCG) titers or evidence of metastatic disease. To date, none of the following factors allowed unequivocal detection of patients who will predictably develop pGTD. Various attempts have been made to correlate histologic grading [1-4], DNA-content [5-9], karyotype [10-12], oncogene and tumor suppressor protein p53 expression [13-18] to the development of persistent disease. There are no reliable genetic or molecular biological markers predicting aggressive behavior in hydatidiform moles. For now, the most reliable prognostic factor is close post-evacuation follow-up with serial serum β-hCG titers.

In a retrospective study, we evaluated clinicopathologic characteristics in 36 cases of gestational trophoblastic disease (GTD) (27 complete hydatidiform moles and 9 pGTD, including 5 complete hydatidiforms, 3 invasive moles, 1 choriocarcinoma). Furthermore, c-erbB-2 amplification and protein p185 expression were investigated on formalin-fixed and paraffin-embedded material using differential polymerase chain reaction (DPCR) and immunohistochemical (IHC) techniques. In addition, DNA-content was determined by image analysis on Feulgen stained nuclear cell preparations and karyotyping for XY chromosomes was performed by fluorescence in situ hybridization (FISH).

**Materials and methods**

**Patient characteristics (Fig. 1)**

Data on patient characteristics and response to treatment was provided by the Department of Obstetrics and Gynecology, University of Leipzig, Germany and re-evaluated at the Department of Gynecopathology, University of Vienna, Austria. Age ranged between 14 and 56 years (median age: 19.1 years). Median follow up was 4.9 years, ranging from 6 months to 13 years. Patients initially presenting vaginal bleeding were treated with suction curettage, which was performed within the first trimester of pregnancy in all 36 cases. All tissue examined was obtained from the initial evacuation of trophoblastic tumors, before initiation of chemotherapy.

**DPCR** (Fig. 2)

Described previously by Frye et al. [19] as a quantitative technique, DPCR employs the co-amplification of the c-erbB-2 gene and a reference gene (Interferon, IFN) as an internal standard. The PCR reaction was carried out in a 50 μl reaction mixture containing 5 μl of 10× PCR buffer, 0.25 μl of 1.25 U AmpliTaq DNA polymerase (Clontech Laboratories, Palo Alto, CA, USA), dNTP mix (10 mM of each), 20 μl of sense and antisense primer of neu-gene and IFN-gene (Clontech). As described in a previous report, the 35 cycles consisted of denaturation, primer annealing, and extension in a computer-controlled water bath. Electrophoresis and densitometry were performed, and the copy number of each sample was calculated according to methods and formulas reported earlier by our laboratory [20]. An incidence of twofold or greater was considered as amplification.

**Immunohistochemical (IHC) study** (Figs. 3, 4)

To determine the c-erbB-2 protein expression, an IHC study was made on the same samples. Five μm sections from paraffin blocks were cut, mounted on silane coated slides, deparaffinized and placed in 0.3% hydrogen peroxide in methanol, 0.01 M PBS (pH 7.2) to stop the endogenous peroxidase activity. The sections were washed in tap water and then PBS, followed by preincubation for 10 min with several drops of 1:4 human serum from donors with blood group AB (Behringwerke AG, Marburg, Germany) to lessen non-specific antibody binding. A monoclonal antibody for the internal domain of the c-erbB-2 oncoprotein (NCL-CB11, Molecular Biology, Medac, Germany) was used [20].

**Fig. 1.** Staging of 32 cases of complete hydatidiform moles according to FIGO classification

<table>
<thead>
<tr>
<th>FIGO stage</th>
<th>Cases</th>
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<tbody>
<tr>
<td>1a</td>
<td>16.3%</td>
</tr>
<tr>
<td>1b</td>
<td>0%</td>
</tr>
<tr>
<td>1c</td>
<td>12.5%</td>
</tr>
<tr>
<td>2a</td>
<td>31.3%</td>
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<tr>
<td>2b</td>
<td>5%</td>
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<tr>
<td>2c</td>
<td>0%</td>
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<tr>
<td>3a</td>
<td>0%</td>
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<tr>
<td>3b</td>
<td>0%</td>
</tr>
<tr>
<td>3c</td>
<td>0%</td>
</tr>
<tr>
<td>4a</td>
<td>0%</td>
</tr>
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</table>

**Fig. 2.** Differential polymerase chain reaction of amplified c-erbB-2 in persistent gestational trophoblastic diseases (lane 1 Marker x174/Hae III; lane 2 negative control; lane 3 single copy control; lane 4 high copy control, representing 5 copies of amplified c-erbB-2 gene; lanes 5–11 probes of pGTD, i.e. lane 5 single copy and lanes 6-11 more than 2 copies)
Image analysis (Fig. 5)

Nuclear DNA-content was measured by image cytometry. Nuclear cell preparations were obtained from two 50 μm sections according to Hedley et al. [21]: tissue sections were dewaxed using xylol, rehydrated in ethanol and distilled water. An enzymatic treatment was done with 0.5% pepsin (Sigma, St. Louis, USA) in 0.9% NaCl for 30 min. The nuclei were separated by filtration through a 40 μm nylon mesh filter (Small Parts, Inc., Miami, FL, USA). The suspensions were fixed on glass slides at room temperature. Staining was performed by Feulgen reaction. Lymphocytes from normal lymph nodes and trophoblast cells from non-molar placentas were used as external reference. The specimens were analyzed with an automatic densitometric video image system (Camera Sony DXC 930, microscope Zeiss Axioskop 50, software Cires 3.0, Oberkochen, Germany).

Fluorescence in situ hybridization (FISH) (Fig. 6)

Nuclear cell preparations were processed as described above and used to identify the sex chromosome composition. Five μm sections cut from the paraffin blocks were also investigated using FISH technique. Probes for chromosomes X and Y (Vysis, Spectrum CEP chromosome Enumeration System, Downers Grove, IL, USA) were applied as recommended by the manufacturer. We used a combination of 2 probes. The X probe SpectrumOrange hybridized to the centromere of human chromosome X (bands p11.1–q11.1), and the Y SpectrumGreen DNA-probe hybridized to the satellite III sequence of human chromosome Y, band Yq12. First the slides were immersed in the denaturation bath (70% formamide/2X SSC) at 75°C for 5 min, followed by a dehydration step in graded ethanol. Then the slides were placed on a 45°C slide warmer, the denatured probe solution was applied and covered with a coverslip. The slides were transferred to a humidified box for hybridization for 40 min in an incubator at 42°C. Posthybridization washings were done twice for 10 min each in 50% formamide/2X SSC and in 2X SSC. As a counterstain, a DAPI solution was applied and a coverslip added. A filter-equipped (102–104–1010, Vysis) Olympus BX60 microscope (Olympus Optical Co. Ltd., Shibuya-ku, Tokyo, Japan) was used to count the numbers of different signals. Images were captured and processed using Vysis Smart Capture System.
another one developed persistent disease after tubal extrauterine pregnancy. Two patients showed pGTD during their first pregnancy. Patients were treated with methotrexate (MTX) monotherapy. In 4 cases, therapy was switched to polychemotherapy due to resistant disease. Three patients received adjuvant surgical procedures (hysterectomy) for chemotherapy-resistant foci (Fig. 4). Complete remission was achieved in all patients with pGTD with a median duration of chemotherapy being 83.2 days (30–192 days). According to the WHO prognostic scoring system for gestational trophoblastic disease, all patients with pGTD ranged below 8, correlating to low or medium risk disease.

C-erbB-2 amplification was evident in 16 cases (44%). Six out of 9 pGTD cases showed amplified c-erbB-2 (3/5 complete hydatidiform moles, 2/3 invasive moles, 1/1 choriocarcinoma). The p185 protein was expressed in 20/36 (55%) GTD and showed in 7/9 persistent cases (4/5 complete hydatidiform moles, 2/3 invasive moles, 1/1 choriocarcinoma). Of the 36 investigated specimens, 31 (86%) were diploid. DNA hyperploidy was observed in 2/5 persistent complete hydatidiform moles, in 2/3 invasive moles and in 1/1 choriocarcinoma. These 5 hyperploid cases showed amplified c-erbB-2 and a strong p185 expression. Both hyperploid persistent complete hydatidiform moles were advanced in stage, associated with lung and liver metastases. XY karyotype was found in 2 cases (1/2 hyperploid persistent complete hydatidiform moles, 1/1 choriocarcinoma; Table 2).

Discussion

Ten to twenty percent of the patients with complete hydatidiform moles may develop persistent gestational trophoblastic disease. The introduction of methotrexate in combination with folinic acid for the management of pGTD was a major landmark in the development of cancer chemotherapy. The majority of patients receiving this drug can be cured with minimal side effects although in some cases, there is a need to switch chemotherapy to a more intense regimen. This was applied in 4 cases due to resistant pGTD. In the cases with chemoresistance, adjuvant surgical procedures were helpful to remove resistant foci (Table 2).

Several studies were performed to identify a subgroup with an increased risk for persistent disease. Hertig and Sheldon [2] were the first to link histopathological fea-

Table 1. Therapeutic procedures in 36 cases of gestational trophoblastic disease

<table>
<thead>
<tr>
<th>Procedure</th>
<th>n</th>
<th>%</th>
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<tbody>
<tr>
<td>D&amp;C†</td>
<td>25</td>
<td>70</td>
</tr>
<tr>
<td>D&amp;C plus chemotherapy (methotrexate, folinic acid)</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>switch to polychemotherapy‡</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Adjuvant surgical procedures plus chemotherapy†</td>
<td>3</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>100</td>
</tr>
</tbody>
</table>

†Dilatation and curettage; ‡etoposide, methotrexate, actinomycin D/cyclophosphamide, vincristine (EMA/CO)-regimen; †including lung biopsy, abdominal hysterectomy.

Results

Cases studied included 32 complete hydatidiform moles, 3 invasive moles and one choriocarcinoma. Twenty-seven out of 36 cases (75%) showed spontaneous remission after evacuation of complete hydatidiform moles, including 2 patients who received additional chemotherapy (Table 1). Nine out of 36 patients (25%) experienced persistent or metastatic disease. Persistent forms included 5 complete hydatidiform moles, 3 invasive moles and 1 choriocarcinoma. In accordance with the well established criteria for diagnosis of persistent and metastatic GTD, patients with pGTD were treated with chemotherapy. Median time between antecedent pregnancy and first symptoms was 45.4 months with a maximum of 320 months. In patients with pGTD, 2 had an interruption of their previous pregnancy, 3 had a term pregnancy, 1 an abortion and
tures to the risk of postmolar disease. They found a correlation between their grading system, based on trophoblastic hyperplasia and cytologic anaplasia, and the risk of malignancy. They established 6 histological grades, which were later modified to 3 grades. Elston et al. [3] examined and classified 70 trophoblastic neoplasias following hydatidiform moles, into 3 histological grades. Their findings did not confirm a relationship between histologic grade of the mole and subsequent malignancy, as many of their grade 1 moles were followed by choriocarcinoma. Genest et al. [1] studied 153 complete hydatidiforms and investigated the predictive capacity of histologic grading of the clinical outcome. No prognostic significance was found. The histopathological classification by Vassilakos et al. [22] was divided into 2 distinct groups of moles based on morphologic and cytogenetic criteria, but both were considered precursor lesions of persistent disease [10]. The pathologic diagnosis must distinguish hydropic abortus from hydatidiform moles and the low-risk partial mole from the high-risk complete hydatidiform. The clinical behavior of a molar pregnancy is still diagnosed based on serum β-hCG levels.

Overexpression and amplification of the c-erbB-2 oncogene are indicators of poor prognosis in several tumors [23]. The c-erbB-2 gene, which encodes a transmembrane growth factor receptor, is a marker of progression and bad prognosis in breast [24], epithelial ovarian [25, 26], endometrium, colon, gastric and bladder cancer. C-erbB-2 gene can be activated by point mutation, gene amplification and/or overexpression [27]. The detection of c-erbB-2 and its overexpression is currently performed using FISH and/or IHC. A review of the literature yielded one study by Cameron et al. [16], who examined 56 trophoblastic tumors for c-erbB-2 overexpression. Twenty out of 56 tumors developed into persistent disease, and only one out of these 20 showed positive immunostaining. Seven out of 56 cases were choriocarcinomas and none showed evidence of positive staining. It is noteworthy that there is no exact information on the gestational age of the material examined. Most cases of persistent gestational trophoblastic disease develop during the first trimester of pregnancy [28] at a time the placenta possesses its highest proliferative potential. As the placenta is an ageing organ, proliferative stimuli and growth receptor expression, such as EGFR or c-erbB-2, might decline over gestational age. In rare cases, GTD occurs at an advanced stage of pregnancy, which might explain the lack of evidence for c-erbB-2 oncogene product in pGTD.

We demonstrated an overexpression of the c-erbB-2 gene in 48% of complete hydatidiform moles and in 77% of cases with persistent sequela. The rate of amplification was 37% for complete hydatidiform moles and 66% for pGTD. This lower percentage of amplification might be explained by different ways of protooncogene activation (e.g. mutation). Our data is supported by Fulop et al. [17], who determined the expression of bcl-2, c-myc, c-fms and c-erbB-2 oncoproteins in normal placenta with partial and complete hydatidiform moles as well as choriocarcinomas. The expression of c-erbB-2 protein was significantly greater in complete moles and choriocarcinoma compared to normal placentas or partial moles.

Tuncer et al. [18] examined the expression of c-erbB-2 related family members EGFR, c-erbB-3 and c-erbB-4 oncogene products in normal placentas and gestational trophoblastic tumors. Strong immunostaining for EGFR and c-erbB-3 in extravillous trophoblasts of complete moles significantly correlated with the development of persistent postmolar gestational trophoblastic disease. This data suggests that increased expression of c-erbB-2 and related family members may influence the development of pGTD. Since complete hydatidiforms with spontaneous regression exhibited c-erbB-2 amplification and overexpression, this single marker might not be suitable as an early predictor in patients who may suffer from persistent disease. Interest is taken in the humanized anti-p185 antibody and the development of immunotherapeutic technologies [29].

In complete hydatidiform moles, the majority of cases are homozygous (XX) because of the replication of the haploid genome of one sperm. In about 15% of cases, heterozygous DNA is found because of dispermy [11]. Different studies [30–32] show that pGTD, including choriocarcinoma, can also develop in heterozygous complete hydatidiform moles. There have also been suggestions that heterozygous complete hydatidiform moles have a higher risk of developing persistent disease [33]. Other studies could not confirm this [34]. In our present series of 36 GTD, we identified 2 cases of XY genotype. Both belonged to the persistent disease group (the choriocarcinoma and 1 metastatic complete hydatidiform mole).

Another attempt to find a marker for a persistent course of complete hydatidiforms was to measure the DNA-content. In a study including 40 complete hydatidiforms, Martin et al. [35] observed a predictive value of DNA-aneuyploidy. They found that aneuploid tissues were associated with postmolar disease, and their aneuploid persistent moles to be non-metastatic.

DNA-content has also been related to progressive disease. Sugimori et al. [36] demonstrated a subsequent increase of DNA-content in complete hydatidiform moles.

<table>
<thead>
<tr>
<th>p185</th>
<th>Amplification of c-erb-2</th>
<th>Hyperploidy</th>
<th>Karyotype</th>
<th>n = 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHM¹</td>
<td>4x</td>
<td>3x</td>
<td>2x</td>
<td>XX, XX, XY</td>
</tr>
<tr>
<td>IM²</td>
<td>2x</td>
<td>2x</td>
<td>2x</td>
<td>XX, XX</td>
</tr>
<tr>
<td>CCA³</td>
<td>1x</td>
<td>1x</td>
<td>1x</td>
<td>XY</td>
</tr>
</tbody>
</table>

¹ Complete hydatidiform mole; ² invasive mole; ³ choriocarcinoma.
with and without progression towards destructive moles and in choriocarcinomas. Other studies [8, 37, 38] have not confirmed these findings. All of these studies were based on DNA-flow cytometric analyses. However, a previous study [33] found that DNA-flow cytometric analyses are less sensitive than DNA-image cytometry (ICM) by the detection of aberrant cell populations with increased DNA-content. Using DNA-image cytometric methods, we identified DNA-hyperploidy in persistent moles with lung and liver metastases, in 2/3 invasive moles and in 1/1 choriocarcinoma, which would recommend DNA-hyperploidy as an indicator of a tumor's proliferative and aggressive potential, although our groups were too small for statistical analysis.

Further efforts in the form of prospective studies are needed to elucidate mechanisms that play decisive roles in the progression of hydatidiform moles. In contrast to previous studies, we were able to combine several parameters such as the clinical course of the patients, c-erbB-2 amplification, p185 expression, DNA-content and karyotype in a series of 36 cases of GTD. From our results we conclude that c-erbB-2 amplification and/or p185 protein expression in combination with DNA-content show a significant correlation with the proliferative and aggressive potential of GTD, suggesting their combined use as a possible marker for persistent gestational trophoblastic disease.

References


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