Complete hydatidiform mole in twin pregnancy: differentiation from partial mole with interphase cytogenetic and DNA cytometric analyses on paraffin embedded tissues

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Six cases of hydatidiform mole associated with normal chorionic villi and a normal embryo/fetus (in five cases) were investigated with interphase cytogenetic and DNA cytometric analyses for diagnostic purposes. DNA probes specific for the pericentromeric regions of chromosomes 1 and X and for the long arm of chromosome Y were used. In four cases a dizygotic twin pregnancy could be proven. In these cases, the histologically normal chorionic villi showed an XY DNA-diploid pattern, consistent with a normal male conceptus, and the molar chorionic villi a XX pattern. In the other two cases an identical sex chromosomal pattern was found in the normal and in the molar villi (XX/XX and XY/XY respectively). In all six cases the molar placental tissues showed prominent trophoblastic hyperplasia with DNA-polyploidy, consistent with a complete hydatidiform mole. In two cases persistent gestational trophoblastic disease developed. It is emphasized that twin pregnancies composed of a normal conceptus and a complete mole have a relatively high risk for the development of persistent trophoblastic disease and therefore, should be carefully differentiated from triploid partial moles with a relatively low risk of persistent gestational trophoblastic disease. These case reports indicate that additional interphase cytogenetic and DNA cytometric analyses are useful in this differential diagnosis.

Keywords: complete mole, partial mole, interphase cytogenetics, DNA ploidy

Introduction

Hydatidiform moles consist of two main entities, complete moles and partial moles1,2. Complete moles are of diploid androgenetic origin3 with a high frequency of polyploidization4 and absence of fetal parts. The characteristic histopathological changes of hydatidiform moles, villous oedema and trophoblastic hyperplasia, are uniformly present. In partial moles these changes are focal, affecting only some of the chorionic villi. A fetus is present. They are usually triploid with predominance of paternal genomic contribution (diandry)5. Partial moles, however, appear to be a heterogeneous group, since diploid cases also have been reported6.

Partial moles have to be differentiated from dizygotic twin gestations consisting of a complete mole and a normal conceptus. Based on morphology alone a diagnosis of twin pregnancy with a complete mole is very difficult, especially in cases of early gestation. Karyotyping can be useful for the diagnosis of twins, but fresh tissues are required and it is limited to unlike-sexed twins and the possibility of outgrowth by contaminating maternal cells cannot be ruled out. Analysis of cytogenetic marker polymorphism performed in fresh tissues has been successfully used in the diagnosis of twins with a diploid androgenetic complete mole7–12. In
the present study we made use of interphase cytogenetic analysis, which is a rapid method that can be performed in paraffin embedded tissue sections and therefore is of great value in routine practice\textsuperscript{4,13,14}. Six cases were investigated in which the histopathological features were suggestive of a twin pregnancy with a complete mole. In four cases a diagnosis of dizygotic twin pregnancy with complete mole (XX) and normal male conception (XY) could be proven. DNA cytometric analysis was used for further analysis of the differences in polyploidization in the molar and non-molar components.

**Case reports**

**Case 1.** A 30-year-old gravida 3, para 2, presented at nine and a half weeks' gestation with recurrent vaginal bleeding. Ultrasonography revealed a vital pregnancy with many blood clots in utero. Continued blood loss necessitated termination of the pregnancy at 13 weeks gestation. Suction curettage harvested a macroscopically normal embryo with crown-rump length of 2.5 cm, and a large amount of membranous placental tissues. The material was routinely processed and a provisional diagnosis of partial hydatidiform mole was made by the local pathologist, who subsequently referred the case to our university hospital where national mole registration takes place. The $\beta$ human chorionic gonadotrophin ($\beta$-hCG) titer was not measured at that time, but only three weeks later after the definitive diagnosis was made by the reference pathologists. The $\beta$-hCG level then was 216 ng/l and returned to normal within 5 weeks and remained normal for one year of follow up.

**Case 2.** This was the first pregnancy of a 25-year-old woman. She had an irregular menstrual cycle. Within one month after her last period she complained of fatigue, nausea and irregular vaginal bleeding, later followed by abdominal pains. Gynaecological examination revealed a painfully enlarged uterus corresponding with a pregnancy duration of 16 weeks. Ultrasonography showed a molar pregnancy. An embryo was not found. Curettage was performed, 8 weeks after her last menstrual period. The local pathologist confirmed the diagnosis of hydatidiform mole. The $\beta$-hCG levels one day and one week after curettage were 5300 and 640 ng/l, respectively. Eighteen days after curettage she again had heavy blood loss and an enlarged uterus. The $\beta$-hCG value had risen to 960 ng/l. A second curettage was performed, yielding ample amount of molar tissues. The $\beta$-hCG levels subsequently returned to normal within 2.5 months and remained normal thereafter (3 years follow up). The histological specimens of both curettages were referred.

**Case 3.** A 36-year-old woman was treated for primary infertility with ovulation induction therapy. She conceived after clomiphene and hCG therapy. The first pregnancy had resulted in the birth of a healthy girl. A second pregnancy was induced in the same way. During this pregnancy she had some vaginal bleeding and progressive nausea and vomiting. At ultrasonography a hydatidiform mole was looked for but could not be detected. Routine blood tests revealed impaired liver functions. Pathology of the gallbladder was suspected and at 18 weeks gestational age a cholecystectomy was performed for cholecystolithiasis. Two days later she developed the clinical appearance of partial placental ablation and came into immature labour. She gave birth to a normal male fetus. A manual removal of the placenta was necessary. In the placenta a sharply demarcated area of hydatidiform molar changes was macroscopically and microscopically present. The local pathologist referred the case under the diagnosis of partial hydatidiform mole. The $\beta$-hCG levels (initially postpartum 3 5000 ng/l) normalized within three months and remained normal after seven months of follow up.

**Case 4.** This case has been published previously\textsuperscript{15}. A 31-year-old female was treated for primary infertility. She became pregnant after ovulation induction with human menopausal gonadotrophin and hCG and subsequent gamete intra-Fallopian transfer of four oocytes. At 4 weeks an intact twin pregnancy was diagnosed. The pregnancy evolved uneventfully until 18 weeks when vaginal bleeding occurred. Ultrasonography revealed cystic changes of part of the placenta, suggestive of hydatidiform mole. Serum $\beta$-hCG was 327 150 IU/l. At 25 weeks intra-uterine infection induced immature labour and two karyotypically normal male (XY) fetuses were delivered. The placenta was manually removed and appeared to be bichorionic-biamniotic. Part of it had the aspect of hydatidiform mole, with XX multilocular ovarian cysts were diagnosed, which showed a spontaneous involution postpartum. During the pregnancy, at ultrasonography, there had been no
signs of molar changes of the placenta. After birth, however, the placenta showed a well demarcated area with the aspect of hydatidiform mole. The local pathologist considered partial mole or twin gestation and referred the case to our university hospital. The child lived and did well. With the mother, there were no signs of persistent trophoblastic disease.

Case 6. A 32-year-old woman, gravida 3, para 2, presented with vaginal blood loss and hyperemesis at 11 weeks gestation. Ultrasound revealed an intact intra-uterine pregnancy next to an empty gestational sac with placental changes suspect for a hydatidiform mole. Suction curettage was performed, yielding a fragmented embryo, normal placental tissue and a large molar mass. The referring pathologist made a diagnosis of twin pregnancy with complete mole. After an initial decline of the serum $\beta$-hCG (from 700 000–2000 ng/ml), a progressive rise to 12 000 ng/ml occurred three weeks after curettage. There were no indications of metastases. Methotrexate therapy was started and four courses have been given until now, upon which the $\beta$-hCG serum levels have shown a steady regression to normal. The patient is still under follow up.

Materials and methods

INTERPHASE CYTOGENETIC ANALYSIS

Interphase cytogenetic analysis was performed on 6 $\mu$m paraffin embedded tissue sections. The following chromosome specific DNA probes were used: 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 (peri)centromeric region (1q12) of chromosome 1$^{16}$, in the centromeric region of chromosome X$^{17}$ and in the q arm of chromosome Y$^{18}$, respectively. Biotinylation of the probes was performed using Bio-14-dATP (BRL: Gaithersburg, USA) according to the suppliers instructions.

The in situ hybridization procedure (ISH) on paraffin embedded tissue sections was performed as previously described$^{4,13,14}$, with minor modifications in the immunohistochemistry step: mouse anti-biotin (1:100 in PBS-tween with 5% non fat dry milk (NFDM); Dakopatts, Glostrup, Denmark) was followed by biotin labeled horse anti-mouse (1:200 in PBS-tween, 5% NFDM; Vector, Burlingame, Canada) and avidin-biotin complex (1:100 in PBS-tween, 5% NFDM; Vector).

DNA CYTOMETRIC ANALYSIS

The paraffin tissue blocks which were used for Interphase cytogenetic analyses were also taken for DNA image cytometric analyses. Intact nuclei were isolated from 50 $\mu$m thick paraffin tissue sections, as previously described$^4$. Normal appearing chorionic villi and hydatidiform degenerated molar villi were separately processed, as well as maternal decidua tissue, which served as an internal control for normal diploid cells. The DNA content of 100–200 pararosaniline-Feulgen stained intact nuclei of the normal and of the molar chorionic villi was measured using the CAS 100 System (Cell Analysis Systems, Lombard, IL, USA)$^{19}$. At least 30 rat liver cells (DNA-tetraploid) were measured as an external control for DNA content, while at least 20 decidual cells were used as an internal control. The DNA histograms were visually classified according to the previously described criteria$^4$. A brief summary is given here. A DNA-diploid pattern consisted of a distinct $G_0/G_1$ peak in the diploid (2C; DI $= 1.0 \pm 0.1$) region with a small proportion of cells in S and G2/M (4C) phases. A DNA-polyploid pattern showed distinct peaks in the diploid (2C; DI $= 1.0 \pm 0.1$) and tetraploid (4C; DI $= 2.0 \pm 0.2$) regions, or in the diploid, tetraploid and octaploid (8C; DI $= 4.0 \pm 0.4$) regions. The nuclear fraction with a DNA content exceeding the first $G_0/G_1$ peak was calculated for gestational products with a DNA-diploid or DNA-polyploid pattern using the 2.5c Exceeding Rate (DI $> 1.25$).

Results

HISTOPATHOLOGY

In all six cases normal and severely abnormal chorionic villi were present, partly intermingled, especially in the younger pregnancy products which were obtained by suction curettage (cases 1 and 2), partly well demarcated in larger clusters of normal and abnormal, molar placental tissue (Figure 1a). In the older pregnancies already at the macroscopic level there was a clear demarcation between normal part and molar part of the placenta. Transitional chorionic villi were not apparent. In the villous stroma of the normal chorionic villi capillaries filled with embryonal/fetal erythroblasts were regularly found. Villous oedema was absent except for case six in which few normal villi showed slight hydropic changes. In one of the younger pregnancies (case 1) implantation trophoblast was variably present, but atypical trophoblastic hyperplasia was not found. The abnormal chorionic villi, however, showed diffuse hydatidiform changes and abundant
Figure 1. Case 1. a Light microscopy displaying normal chorionic villi (left) adjacent to molar chorionic villi (right) with extensive trophoblast proliferation (and villous oedema which is not shown); b–g Interphase cytogenetic analysis using biotinylated probes specific for chromosomes 1, X and Y, counterstained with Mayer's haematoxylin. The normal chorionic villi show one ISH-signal per nucleus for b chromosome X and c chromosome Y, whilst the molar villi show two ISH-signals per nucleus for d chromosome X and e none for chromosome Y. The implantation trophoblast of the normal villi, in general, shows not more than two ISH-signals for chromosome 1 (f), in contrast to the extravillous trophoblast proliferation of the mole which reveals many nuclei with more than two ISH-signals (g). h DNA-histogram of the normal placenta showing DNA-diploidy. i DNA-histogram of the molar placenta showing DNA-polyploidy.
Interphase cytogenetic analysis using DNA probes specific for the sex chromosomes revealed an XY pattern in the embryo of case 1 as well as in the normal appearing chorionic villi of cases 1, 2, 3, 4 and 6 (Figure 1b, c). An XX pattern was found in the molar chorionic villi of cases 1, 2, 3 and 4, and a XY pattern in case 6 (Figure 1d, e). In case 5 a XX pattern was found in both normal and molar chorionic villi (Table 1). In all cases the normal chorionic villi showed a chromosome disomy in the implantation trophoblast (Figure 1f) with only sporadic polysomy cells, mainly in areas with fibrinoid deposits. In contrast, the extravillous trophoblastic hyperplasia of the molar placenta displayed a high frequency of nuclei with multiple chromosome copies (Figure 1g; Table 1).

DNA CYTOMETRIC ANALYSIS
Normal and molar placental tissues could be separately processed in cases 1, 3, 4, 5 and 6. In case 2 the normal villi were too intimately intermingled with the molar tissue to be separated. There was, however, ample molar tissue free from normal chorionic villi and this was measured for DNA content. In cases 1, 3, 4, 5 and 6 the normal placental tissue was found to be DNA-diploid with relatively low 2.5c Exceeding Rates (less than 40%; Figure 1h; Table 1). In all six cases the molar tissue showed a DNA-polyploidy with high 2.5c Exceeding Rates (Figure 1i and Table 1).

Discussion
Hydatidiform changes in a placenta with a co-existing fetus can be subdivided into three groups: 1 partial hydatidiform moles which are usually triploid and associated with an abnormal fetus; 2 hydropic degeneration of (part of) a diploid or less frequently triploid placenta; and 3 twin pregnancy, including a normal diploid conceptus and an in origin diploid complete hydatidiform mole. In the presented six cases twin pregnancy was suspected on the basis of the clear demarcation of normal and molar chorionic villi. Dizygosity could be proven in four of the six cases with interphase cytogenetic analysis revealing a male embryo (case 1) and XY normal placenta and XX complete hydatidiform mole. The complete hydatidiform moles were DNA-polyploid with high 2.5c exceeding rates, a finding that is fully in agreement with our previous report on complete moles showing a high frequency of polyploid cells in the extravillous trophoblast. In cases 5 and 6 dizygosity could not be proven, because normal and molar placental tissues showed an identical sex chromosomal pattern. The DNA-ploidy patterns, however, were similar to the other three cases, showing DNA-diploidy in the normal placenta and DNA-polyploidy in the mole. Therefore, it
is very likely that these cases also represent twin pregnancies with complete hydatidiform mole.

Another rare, recently described possibility is the coexistence of a normal fetus and placenta with a morphologically complete mole, but cytogenetically resulting from a single gestation with both maternal and paternal DNA contributions. The DNA-polyploidy present in our cases is consistent with the morphological aspect of complete mole with prominent and atypical trophoblastic hyperplasia. Uniparental disomy might be an explanation for these cases. The occurrence of persistent gestational trophoblastic disease in the described case as well as in one of our cases is in agreement with the expected high risk for a complete mole as compared to the triploid partial mole with a relatively low risk.

About 31 cases of hydatidiform mole with co-existent fetus have been reported in which, on morphological criteria, the possibility of a twin pregnancy was suggested. In an additional twelve recently published cases the existence of a dizygotic twin pregnancy with an androgenetic hydatidiform mole could be confirmed with cytogenetic marker polymorphism studies. In this study we used interphase cytogenetic analysis with DNA probes specific for the sex chromosomes. Although direct proof of dizygosity is limited to unlike-sexed twins, this in situ hybridization technique can rapidly be performed on paraffin embedded tissue sections with the important advantage of preservation of histological architecture, so that in small areas chromosomal aberrations can be detected and related to morphology. This was particularly important in case 2, in which normal and molar chorionic villi could not be separately processed. Using the in situ hybridization technique, triploid as well as diploid partial moles can be differentiated from twin pregnancy with complete mole, even from twins of similar sex, on basis of the high frequency of polyploid cells in the molar component.

The importance of recognition of a twin pregnancy with a complete mole component and its differentiation from a triploid partial mole and also from a hydropically degenerated placenta, is the differential risk of subsequent malignant changes. This will not occur in hydropically degenerated placentae, but follows at least 10–20% of complete moles. The risk of persistent trophoblastic disease following a partial mole is considered to be low, although the reported frequency varies from 0.5–5.5%. Not all reported cases of partial moles with subsequent persistent gestational trophoblastic disease, however, were triploid; some cases were diploid. In these cases, the possibility of a twin pregnancy with complete mole should be excluded. In case 2 the criteria of persistent gestational trophoblastic disease were met because the β-hCG level was rising again after initial decrease. Although usually this is an indication to start methotrexate therapy, in this case a second curettage was performed followed by a decrease in β-hCG level. In case 6 persistent gestational trophoblastic disease developed for which methotrexate therapy was given. In fact, in 15 of the 49 published cases in which twin gestation with complete mole was diagnosed persistent gestational trophoblastic disease developed, which is in accordance with the high frequency of malignant change in complete hydatidiform mole. Therefore, careful diagnosis and follow up of dizygotic twin molar pregnancies is needed. Although clinical follow up is advised for partial moles as well, the significant difference in relative risk of persistent gestational trophoblastic disease has implications for the prognosis in individual patients.

It is important to notice that two of our six cases and five of the cases in the literature were pregnancies induced by ovulation induction. There are only a few reports describing hydatidiform molar pregnancies after ovulation induction. As complete moles result from the fertilization of an abnormal egg, it is possible that induction resulting in multiple ovulations also increases the risk of such abnormal eggs and, therefore, the risk of twin pregnancy associated with a complete mole.

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