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Heterogeneity of gonadoblastoma germ cells: similarities with immature germ cells, spermatogonia and testicular carcinoma *in situ* cells

N.JØRGENSEN, J.MÜLLER, F.JAUBERT*, O.P.CLAUSEN† & N.E.SKAKKEBÆK

*University Department of Growth and Reproduction, Rigshospitalet, Copenhagen, Denmark, *Service Central d'Anatomie Pathologique, Hospital Necker Enfants Malades, Paris, France and †Institute of Pathology, Rikshospitalet, Norway*

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Heterogeneity of gonadoblastoma germ cells: similarities with immature germ cells, spermatogonia and testicular carcinoma *in situ* cells

Gonadoblastoma is defined as a neoplasm containing nests of germ cells and cells resembling Sertoli cells or granulosa cells. Gonadoblastomas arise almost exclusively in dysgenetic gonads. They are associated with an increased risk of developing germ cell tumours. Testicular germ cell tumours in adults are preceded by carcinoma *in situ* cells, which are characterized by their morphology, by their immunohistochemical expression of placental-like alkaline phosphatase, the proto oncogene c-*kit* and/or epitopes for the monoclonal antibodies M2A, 43–9F and TRA-1–60, and by their aneuploid DNA content. In order to elucidate if gonadoblastomas are *in situ* neoplasms from the beginning, showing similarities with carcinoma *in situ* cells in otherwise normal testes, we investigated the germ cells in gonadoblastomas for their expression of the immunohistochemical markers of carcinoma *in situ* cells from six patients aged 8½ months to 20 years and 4 months. In addition, the DNA content of the germ cells from five of the six patients was also determined by densitometric measurement on Feulgen stained specimens. The germ cell populations were heterogeneous both within the same patient and between the patients. Expression of the testicular carcinoma *in situ* markers was detected in specimens from all the patients and germ cells with an aneuploid DNA distribution pattern in accordance with testicular carcinoma *in situ* cells were detected. However, apparently normal immature germ cells were also present in four of the patients of whom two also had germ cells with a morphology similar to normal spermatogonia. Thus, gonadoblastoma is most likely an *in situ* germ cell neoplasia from the beginning. It seems probable that the germ cell tumours associated with gonadoblastomas originate from the carcinoma *in situ* cells inside the gonadoblastoma. Our findings of carcinoma *in situ* cells in gonadoblastomas from children support the theory that the cells arose prenatally.

Keywords: gonadoblastoma, testicular carcinoma *in situ*, immature germ cells, spermatogonia

Introduction

Gonadoblastoma is defined as a neoplasm containing nests of germ cells and cells resembling immature Sertoli cells or granulosa cells. Leydig cells or lutein-type cells may or may not be present outside the nests[1]. Gonadoblastomas are usually diagnosed in patients 20–30 years old but have been observed in patients as young as 6 months old[2].

Gonadoblastomas arise almost exclusively in dysgenetic gonads[3], probably prenatally[4–7], and are associated with an increased risk of development of both classical seminoma and non-seminoma germ cell tumours[1,3]. Gonadoblastomas are usually considered to be *in situ* germ cell neoplasms from the beginning[1] but have also recently been proposed to represent a hamartomatous malformation in which there is an increased risk of

Address for correspondence: Dr N. Jørgensen, University Department of Growth and Reproduction, Righospitalet, Blegdamsvej 9, section 5064, DK-2100 Copenhagen Ø, Denmark.

developing malignancy in the germ cells[8]. A recent case report has shown the coexistence of carcinoma *in situ* cells and gonadoblastoma as well as early invasive germ cell neoplasia in a 9-year-old girl with 46,XY gonadal dysgenesis, thus favouring the former theory[9].

Adult germ cell tumours, including the classical seminoma and non-seminomas which arise in otherwise normal testes, originate from testicular carcinoma *in situ* cells[10,11], hereafter referred to as carcinoma *in situ* cells. Carcinoma *in situ* cells are large with abundant light cytoplasm and with irregular coarse clumps of chromatin in the nucleus[12,13]. The nuclear DNA content of carcinoma *in situ* cells is usually highly aneuploid, ranging from 2.0c to 18.8c with a mean ploidy value between 3.8c and 4.9c, which is not seen in non-neoplastic germ cells[14,15]. Carcinoma *in situ* cells express placental-like alkaline phosphatase (PlAP)[16,17] and the proto oncogene c-*kit*[18] and react immunohistochemically with the monoclonal antibodies TRA-1–60[19], 43–9F[20] and M2A[21]. These markers do not occur in normal adult male germ cells. Carcinoma *in situ* cells have occasionally been detected in testes of infants, the youngest being only 2 months old[22,23], and are thought to arise prenatally from primordial germ cells and rest in the testis until subsequent tumour development in post-natal life[11,24].

We investigated the germ cells in gonadoblastomas in order to elucidate if gonadoblastoma is an *in situ* neoplasm from the beginning, showing similarities with carcinoma *in situ* cells in otherwise normal testes because both gonadoblastomas in dysgenetic gonads and carcinoma *in situ* cells are associated with development of the same type of germ cell tumours.

Materials and methods

We had access to gonadectomy specimens containing gonadoblastomas from six patients. The gonads had been removed because of Y-chromosome material in a phenotypic female or an intersex condition. The specimens had been collected during the period May 1984 to September 1992. The specimens had been fixed and paraffin-embedded. The median age of the patients at time of gonadectomy was 9 years and 10 months, ranging from 8½ months to 20 years and 4 months. Information about the patients and the gonadectomy specimens are summarized in Table 1. The data on patient 3 has to some extent previously been published as a case report by Müller *et al.*[9].

For an overview of the specimens, and for identification and description of germ cells 4 μm thick sections were deparaffinized and stained with haematoxylin and eosin.

IMMUNOHISTOCHEMICAL STAINING

Sections cut at 4 μm thickness were used for immunostaining with the following primary antibodies: **1** polyclonal antibody against placental-like alkaline phosphatase (Dako, Copenhagen, DK—dilution 1:50 and 1:100), using a peroxidase-antiperoxidase (PAP) technique; **2** polyclonal anti-human c-*kit* antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA—dilution 1:10 and 1:20), using a biotin-streptavidin (ABC) technique. The antibody was raised against a peptide corresponding to residues 961–976 within the C-terminal domain of the human c-*kit* and reacts with the p145 c-*kit* protein product[25]. The sections were pre-treated for 10 min at 90°C with TUF (Target Unmasking Fluid, Kreatech, The Netherlands) for enhancement of the reaction; **3** monoclonal antibody TRA-1–60, (dilution 1:10), using an ABC technique. The antibody was raised against the human embryonal carcinoma cell line 2102 Ep cl.2Ab and recognizes a high-molecular-weight mucin-like cell surface glycoprotein[26]; **4** monoclonal antibody 43–9F, (dilution 1:5), using an ABC technique. The antibody was raised against a squamous cell lung carcinoma cell line RH-SLC-L11 and the epitope was shown to be a Le^a- X oligosaccharide determinant[20]; **5** monoclonal antibody M2A, (dilution 1:50 and 1:100), using an ABC technique. The antibody was raised against the cultured human ovarian adenocarcinoma cell line HEY and recognizes an as yet uncharacterized carbohydrate epitope on cell surfaces[21].

All antibodies were diluted in 0.05 M TRIS-buffered saline, pH 7.4. The endogenous peroxidase of the specimens was blocked by 2% H_2O_2 in methanol for 10 min. Human serum (dilution 1:4) was applied for 30 min at room temperature for blocking of non-specific background staining, followed by incubation with the primary antibody overnight at 4°C. For the PAP technique the slides were incubated with swine-anti-rabbit immunoglobulin (Dako) for 30 min at room temperature followed by peroxidase-anti-peroxidase complex (Dako) for 30 min at room temperature. For the ABC technique, the primary antibody was followed by either biotinylated goat anti-rabbit IgG or goat anti-mouse IgG (Zymed, San Francisco, CA, USA) for 30 min at room temperature and, subsequently, a streptavidin-peroxidase conjugate (Zymed or Dako) for 30 min at room temperature. 3,3′-diaminobenzidine (DAB) or aminoethyl carbazole (AEC, for the c-*kit* antibody) in the presence of H_2O_2 were used as chromogens. All the immunostained sections were counterstained with Mayer's haematoxylin for 3 min, dehydrated and mounted before examination. Sections

Table 1. Data on patients and gonadoblastoma specimens prior to investigation for characteristics of testicular carcinoma *in situ* cells

Patient No.	Age at gonadectomy	Phenotype	Karyotype	Fixative used for these specimens	Original description of gonadectomy specimens	
					Left gonad	Right gonad
1	$8\frac{1}{2}$ months	Virilized female	46,XX*	Formaldehyde	Gonadoblastoma in ovary	Gonadoblastoma in ovary
2	3 years, 8 months	Virilized female	46,XY	Formaldehyde	Gonadoblastoma in streak gonad	Streak gonad
3†	9 years, 0 months	Female	46,XY	Cleland's fluid	Gonadoblastoma, CIS‡ and invasive neoplasia	Streak gonad
4	10 years, 7 months	Female	46,XY	Cleland's fluid	Gonadoblastoma in streak gonad	Gonadoblastoma in streak gonad
5	13 years, 10 months	Female, Turner syndrome	45,X/46,XY	Stieve's fluid	Gonadoblastoma in streak gonad	Streak gonad
6	20 years, 4 months	Female, Turner syndrome	45,X/46,XY	Stieve's fluid	Gonadoblastoma in streak gonad	Streak gonad

*SRY band and localized mosaicism was detected in the gonad.
†Has been reported by Müller *et al.*[9].
‡CIS, carcinoma *in situ* cells in structures resembling seminiferous tubules described prior to the present investigations (Müller *et al.*[9]).

from adult testes with carcinoma *in situ* cells were used as positive controls. As negative controls the primary antibodies were substituted with 0.05 M TRIS-buffered saline.

Evaluation of immunostaining: The immunostained sections were evaluated by light microscopy. Germ cells were distinguished from other cells by their large size, large, round nuclei and clear cytoplasm. Positive reactions were identified as dark brown (for DAB) or red staining (for AEC) of the plasma membranes. The relative number of germ cells showing a positive immunohistochemical reaction was assessed by systematic counting of the germ cells present in a given section and expressed as the fraction of the cells positive in the total number of the germ cells present.

When interpreting the immunohistochemical results the use of different fixatives has to be kept in mind as these influence the staining reactivity. The sensitivity of carcinoma *in situ* cells reactions with the monoclonal antibodies M2 A and 43–9F is very low on formaldehyde fixed specimens, Cleland's fluid tends to give moderate staining intensity whereas specimens fixed in Stieve's fluid give a strong immunohistochemical reaction[17]. The antibody against PlAP gives a moderate to weak reaction on carcinoma *in situ* cells fixed in Cleland's fluid but strong reactivity on specimens fixed in formaldehyde or Stieve's fluid. In contrast, the antibody against the proto oncogene c-*kit*[18], and the TRA-1–60 antibody[17] react strongly on specimens fixed in either of the three used fixatives.

DENSITOMETRIC DNA MEASUREMENT

Nuclear DNA content of carcinoma *in situ* cells has previously been determined by densitometric DNA measurement on Feulgen stained sections[14,15,23,27]. By this method it is possible to measure the DNA content of single, selected cells and relate morphology to the nuclear DNA content.

Feulgen staining of 12 μm thick sections was performed as previously described[27,28]. The staining procedure included hydrolysis in 5 M HCl at 22°C for 120 min for biopsies fixed in formaldehyde, 90 min for biopsies fixed in Cleland's fluid and 75 min for biopsies fixed in Stieve's fluid. These schedules have previously been found to give the optimal staining intensities. The hydrolysis was followed by washing in distilled water, staining for 60 min in Schiff's reagent and finally washing in sulphite rinse before mounting.

Quantification of the DNA content was performed using a GIPS (General Image Processing Software) computer programme. An interference filter giving a monochromatic green light with maximum transmittance at 560 nm was used. In order to minimize the risk of measuring fragments of cell nuclei, 12 μm thick sections were used, which is approximately 120% of the median nuclear diameter of carcinoma *in situ* cells, as recommended[29,30]. Furthermore, incomplete nuclei were excluded by focusing on both the upper and lower nuclear poles before measurement[14]. In each specimen

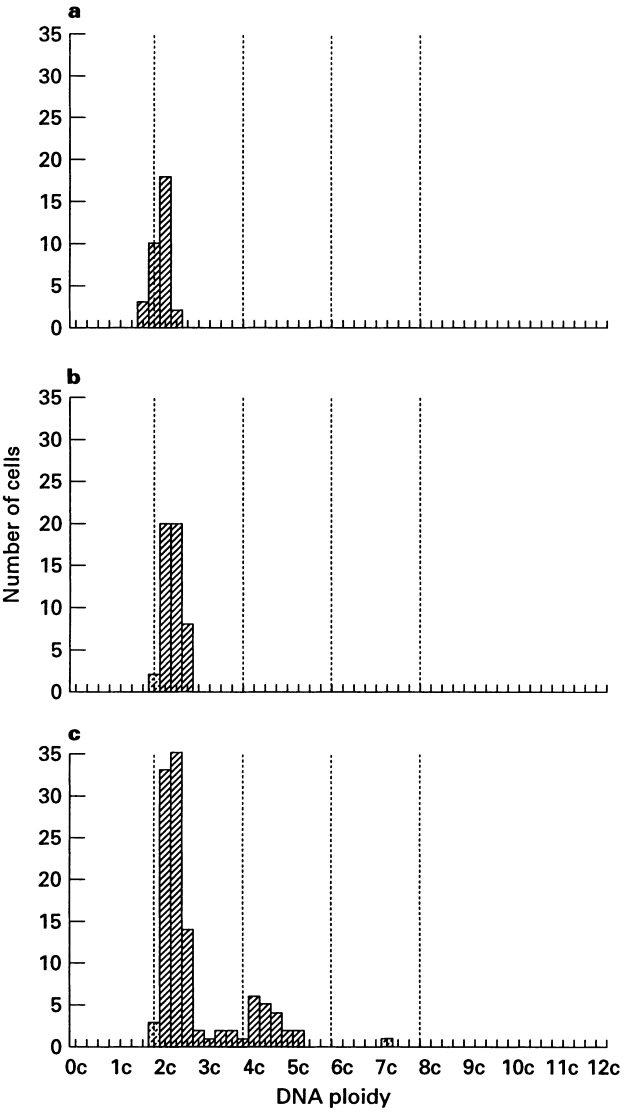

Figure 1. Nuclear DNA distribution patterns of cells from a gonadoblastoma from patient 6.**a** Sertoli-like cells in gonadoblastoma nests, **b** Leydig cells outside the nests and **c** Germ cells in gonadoblastoma nests. Note the homogeneous DNA distribution pattern of the Sertoli-like cells and Leydig cells in contrast to the heterogeneous DNA distribution pattern of the germ cells. Nuclear DNA ploidy (content) is expressed as multiples of half of the mean DNA content in Sertoli-like cell nuclei.

Table 2. Results of the investigation of gonadoblastoma germ cells

Patient No.	1	2	3	4	5	6
Gonad	Right	Left	Left	Right*	Left	Left
Immunohistochemistry						
PlAP	10/10 (100%)	39/196 (20%)	498/501 (99%)	254/345 (74%)	777/823 (94%)	264/543 (49%)
c-*kit*	ND	2/113 (2%)	294/306 (96%)	64/353 (18%)	548/704 (78%)	84/489 (17%)
TRA-1-60	9/9 (100%)	4/144 (3%)	654/744 (88%)	210/430 (49%)	798/910 (88%)	237/591 (40%)
43-9F	8/8 (100%)	0/183 (0%)†	114/282 (40%)	99/294 (34%)	856/1034 (83%)	63/441 (14%)
M2A	0/8 (0%)	0/176 (0%)†	753/768 (98%)	58/430 (13%)	648/860 (75%)	387/657 (59%)
Densitometric DNA measurement						
Median ploidy	ND	3.1c	3.8c	2.7c	4.3c	2.4c
Peak ploidy	ND	2.0c, 3.0c	2.0c, 2.8c, 3.3c	2.0c, 3.0c	3.8c	2.2c, 4.2c
Cells measured	ND	86	89	70	159	113

*Also the left gonad contained gonadoblastoma, but no tissue was available for investigation.
†Due to the fixation in formaldehyde, only a positive result can be interpreted unequivocally.
PlAP, placental alkaline phosphatase; ND, not done.

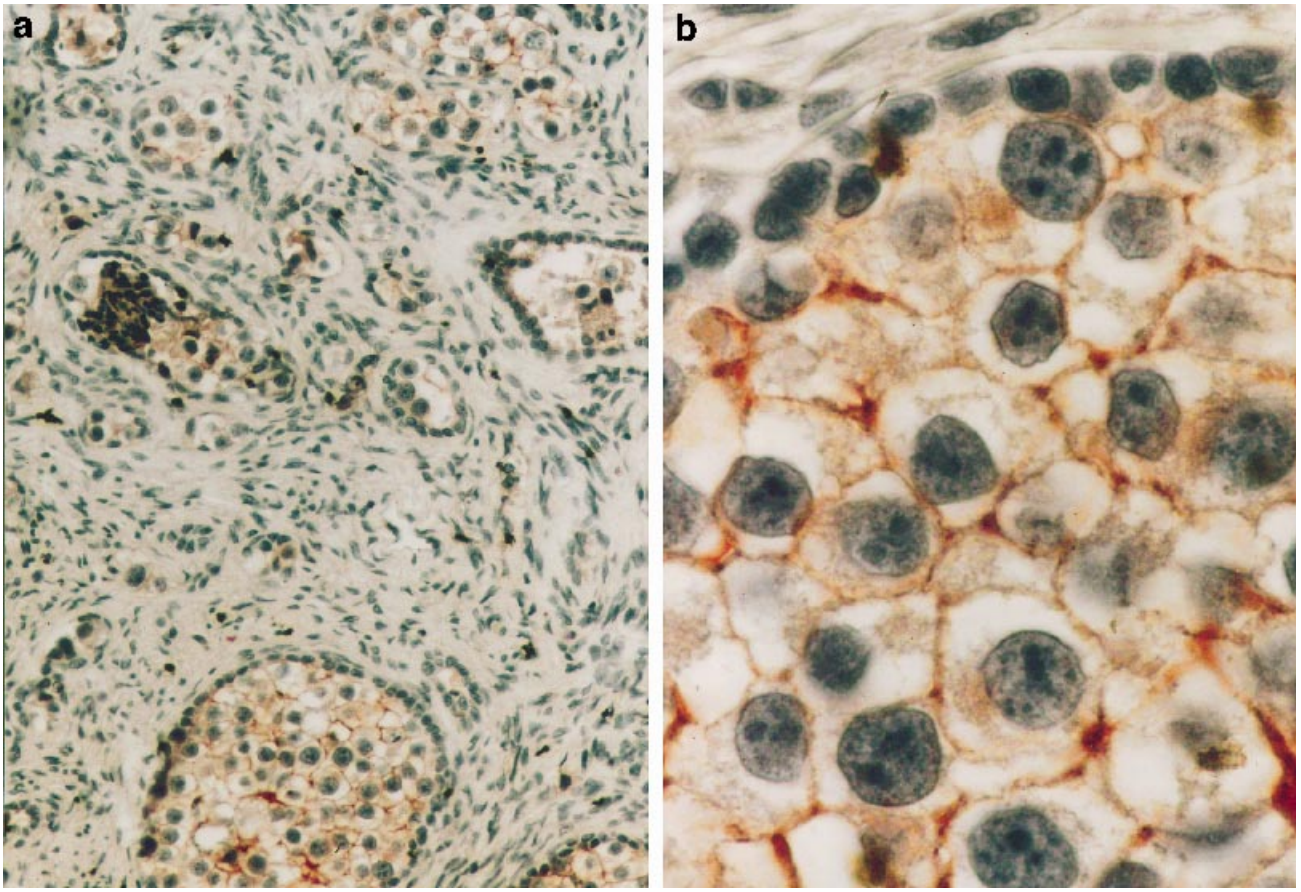

Figure 2. Immunohistochemical staining of gonadoblastoma from patient 3. Staining was performed with the testicular carcinoma *in situ* marker, the c-*kit* protein. The red staining is considered a positive reaction. **a** Overview of specimen showing gonadoblastoma nests and invasive neoplasia. Note that almost all the germ cells inside the gonadoblastoma nests and the germ cells outside the nests expressed the c-*kit* protein. **b** Same as **a**, but a higher magnification showing a gonadoblastoma nest with germ cells expressing the c-*kit* proto oncogene. The specimen was fixed in Cleland's fluid.

the mean DNA content of diploid (2c) nuclei was calculated for at least 25 Sertoli-like cell nuclei, and the DNA content of germ cells was then expressed as multiples of the calculated haploid value.

The Sertoli-like cells were chosen as the DNA diploid standard because these cells may be regarded as non-malignant in nature. Invasive malignancy or metastases associated with gonadoblastomas have only been reported to contain germ cell elements, and not Sertoli or Leydig cell elements or the histological appearance of gonadoblastomas[1,3]. In addition, in preliminary studies, the Sertoli-like cells were shown to have a homogeneous DNA distribution pattern, which is in contrast to the more heterogeneous distribution patterns, usually detected when measuring cancer cells[31] or testicular carcinoma *in situ* cells[14]. In the specimens from patient 6, Leydig cells were present outside the nests, and these cells also had a homogeneous DNA distribution pattern (median 2.2c, range 1.8c to 2.6c) in the same range as the Sertoli-like cells, indicating both types of cells had the same DNA content (Figure 1).

Results

In the specimens which originated from patient 1, a gonadoblastoma nest was present in a gonad resembling an ovary with primary follicles. Only 6–10 germ cells were present in the gonadoblastoma nests, but they all showed a morphology identical to that of carcinoma *in situ* cells. In the other five cases, the gonadoblastomas were present in streak gonads. In the gonadoblastomas from patients 3–6, some of the germ cells had a morphology like carcinoma *in situ* cells in testes; i.e. large germ cells with abundant light cytoplasm and with irregular coarse clumps of chromatin in the nuclei. In specimens from patients 3, 4 and 6 apparently normal, immature germ cells were also present. In addition, in the specimens from patients 4 and 6 smaller germ cells resembling testicular spermatogonia were present too. In the specimens from patient 3 structures resembling seminiferous tubules contained immature Sertoli cells and germ cells identical to carcinoma *in situ* cells in addition to gonadoblastoma nests. Also small foci of germ cells outside the tubular structures were observed. In the specimens from the three oldest patients (cases 4, 5 and 6) one or two mitoses were observed in the gonadoblastoma nests. A detailed morphological evaluation of the cells from patient 2 could not be performed because of poor preservation probably due to formaldehyde fixation.

The results of the immunohistochemical staining are shown in Table 2 and illustrated in Figure 2. The lack of staining reactivity with the 43–9F and M2 A antibodies in specimens from patient 2 and for M2 A in the specimens from patient 1 cannot be interpreted unequivocally since these specimens were fixed in formaldehyde. The reacting germ cells all had a morphology like testicular carcinoma *in situ* cells. The oocyte-like structures from patient 1 reacted with the monoclonal antibody 43–9F but none of the other immunohistochemical markers. Almost all the germ cells outside the gonadoblastoma nests from patient 3 reacted with all the immunohistochemical markers.

Densitometric DNA measurement: The DNA distribution patterns of the germ cells and the Sertoli-like cells are shown in Figures 3 and 4. The DNA median ploidy values and peak ploidy values of the gonadoblastoma germ cells from five of the six patients are also shown in Table 2. The median germ cell ploidy ranged from 2.4c to 4.3c and in all five cases aneuploid DNA peak values were present. In patients 2, 3, 4 and 6, diploid germ cells were also present. In patient 5, only aneuploid DNA peaks were detected. Twenty-eight germ cells outside the gonadoblastoma nests from patient 3 were suitable for analysis. They had DNA contents in the range 2.6c to 9.2c, with a median value of 4.5c (Figure 4c). The amount of tissue from patient 1 was too small to allow densitometric measurements.

Discussion

The population of germ cells in the nests of gonadoblastomas was heterogeneous. Some fulfilled the criteria for carcinoma *in situ* cells, some were non-neoplastic of which some resembled immature fetal germ cells and some resembled male spermatogonia. The carcinoma *in situ* like germ cells had a morphology similar to testicular carcinoma *in situ* cells, and expressed the immunohistochemical carcinoma *in situ* markers. Furthermore, the DNA distribution patterns for some of the germ cells were similar to the expression pattern of carcinoma *in situ* cells in testes. A detailed description of the DNA content has not been reported previously. Thus, our findings support the theory of Scully[1] that gonadoblastomas represent in-situ germ cell neoplasms, and the germ cell tumours associated with gonadoblastomas may in fact arise from these carcinoma *in situ* cells. The expression of the immunohistochemical carcinoma *in situ* markers and the finding of DNA aneuploid germ cells even in gonadoblastomas from infants indicate that the lesion is not, as suggested by Berkovitz[8], a hamartomatous malformation in which there is an increased risk of developing a malignancy in the germ cells.

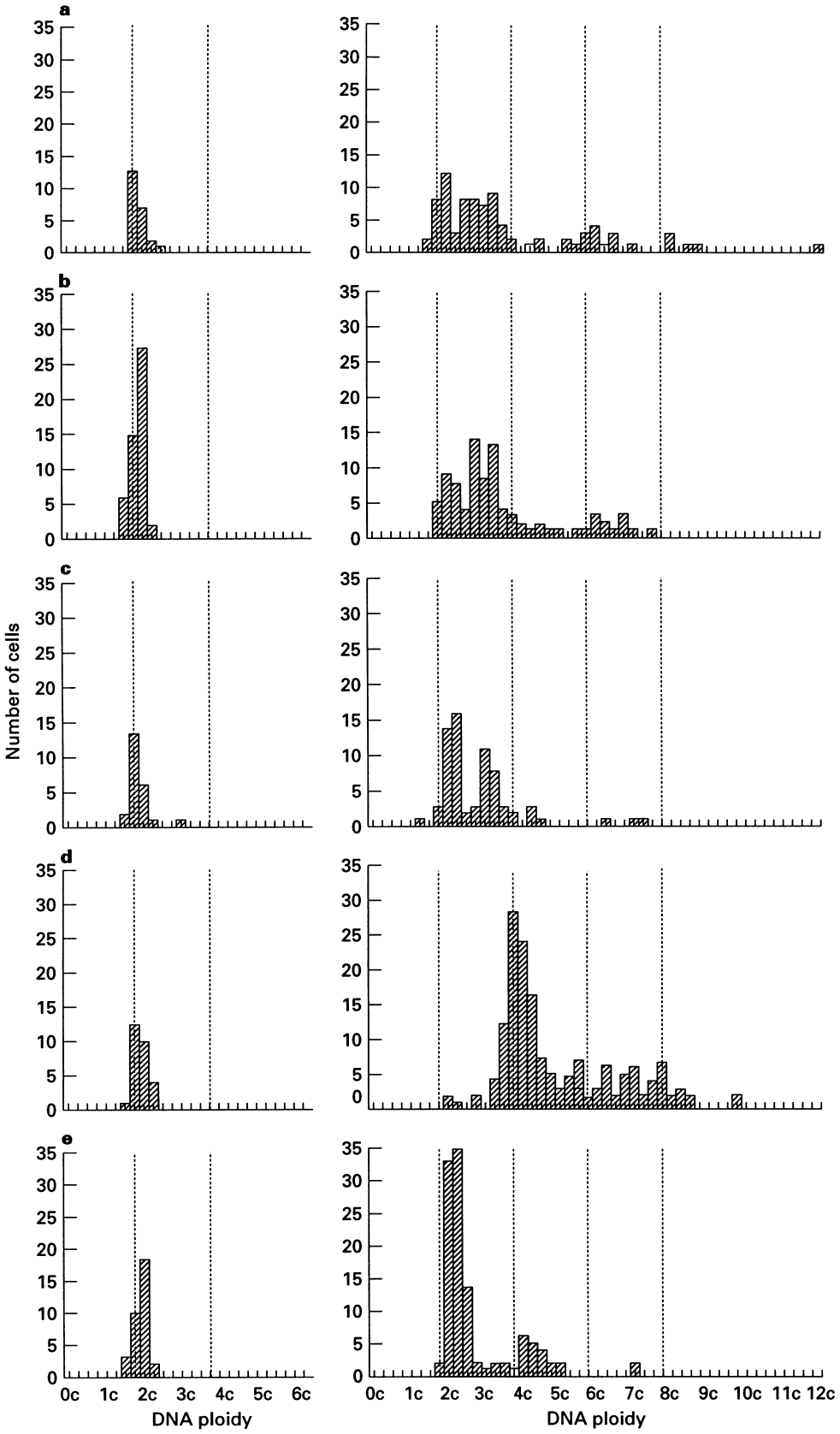

Figure 3. Nuclear DNA distribution patterns of cells inside gonadoblastoma nests. **a** patient 2; **b** patient 3; **c** patient 4; **d** patient 5; **e** patient 6. Left column; Sertoli-like cells. Right column; germ cells. Note the homogeneous DNA distribution patterns of the Sertoli-like cells and the heterogeneous DNA distribution patterns of the germ cells. Nuclear DNA ploidy (content) is expressed as multiples of half of the mean DNA content of the Sertoli-like cell nuclei.

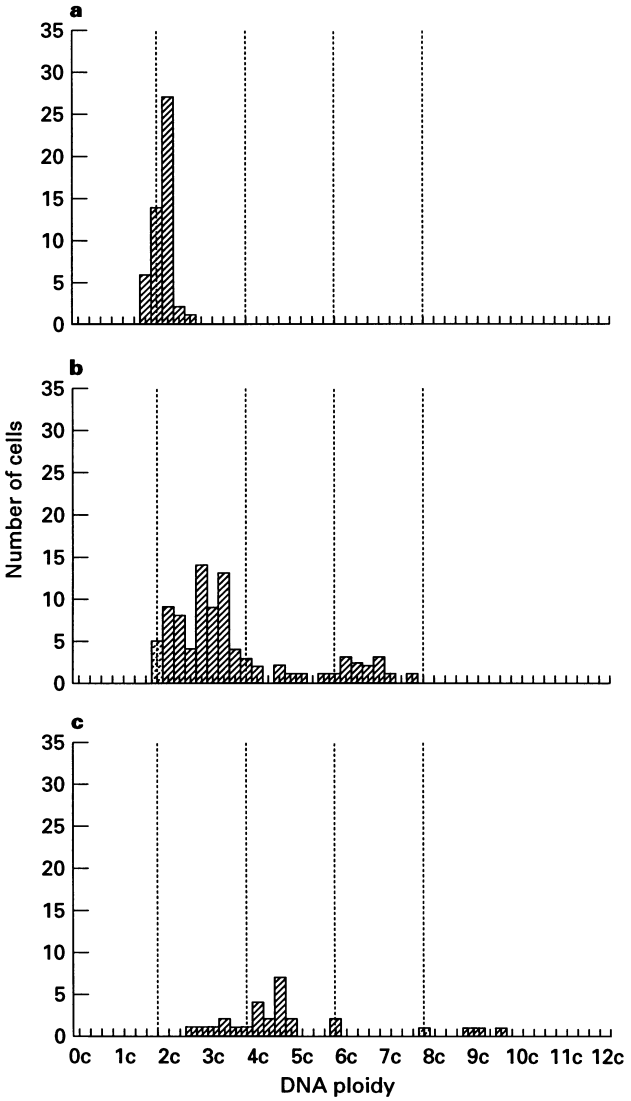

Figure 4. Nuclear DNA distribution patterns of cells in gonadectomy specimen from patient 3. **a** Sertoli-like cells in gonadoblastoma nests; **b** germ cells in gonadoblastoma nests; **c** invasive germ cells outside the gonadoblastoma nests. **a** and **b** are also shown in Figure 3b. Note the aneuploid DNA content of all the germ cells outside the gonadoblastoma nests, stressing the malignant nature of the cells. Nuclear DNA content is expressed as multiples of half of the mean DNA ploidy (content) of Sertoli-like cell nuclei.

Even though carcinoma *in situ* cells could be detected in gonadoblastomas from all the patients, the lowest number of these cells was detected in the youngest patients. This may indicate that only a few carcinoma *in situ* cells may be present in the early stages of the lesion whereafter they proliferate inside the gonadoblastomas before invasion occurs.

Non-neoplastic germ cells were also present in four of the cases. Some of the germ cells did not react with the immunohistochemical markers, and diploid DNA content of germ cells was detected. This is in line with studies of the ultrastructure of the germ cells in gonadoblastomas which showed that, in some of the cases, germ cells resembled immature fetal germ cells[32] and, in some, seminoma/dysgerminoma cells[33–35]. The finding of germ cells resembling spermatogonia in two of the patients in the present study is in line with previous reports[33,35] which described differentiation of germ cells to morphologically normal spermatogonia. Neither in our cases nor in the previously reported cases were any further stages of spermatogenesis detected.

The finding of placental alkaline phosphatase in gonadoblastoma germ cells is in agreement with previous findings by Manivel *et al.*[36], who showed that almost all germ cells in gonadoblastomas from 11 patients showed expression of this enzyme. However, the age of the patients at the time of biopsy or gonadectomy was not stated. The expression of the other immunohistochemical carcinoma *in situ* markers, which has not been shown previously, also indicated a neoplastic character of the gonadoblastoma germ cells which expressed the markers. However, the immunohistochemical carcinoma *in situ* markers can be expressed in the first year of life in normal infantile testicular germ cells[37]. After the first year of life expression in germ cells seems to be specific for a testicular germ cell neoplasia, except for c-*kit* which can be expressed in a fraction of apparently normal germ cells until puberty.

Gonadoblastomas[4–6] are believed to arise prenatally, like carcinoma *in situ* cells[11,24]. Since gonadoblastomas are also shown to be associated with Y-chromosome or Y-chromosome material in female patients[3], one can speculate that some of the events which may result in the development of neoplastic germ cells are also involved in the development of the dysgenetic gonads in which the gonadoblastomas arise, probably during the time of gonadal differentiation: Page[38] has suggested that a gene on the long arm of the Y chromosome predisposes to development of gonadoblastomas. One can speculate that an incomplete or delayed expression of müllerian inhibiting substance may lead to incomplete differentiation of the early fetal gonad and incomplete control over the developing germ cells. In line with this is the finding by Josso *et al.*[39] who showed that serum-level of müllerian inhibiting substance in infant male pseudohermaphrodites with testicular dysgenesis was significantly decreased compared with other forms of male pseudohermaphroditism.

The heterogeneity of the germ cells indicates that some of the cells escaped the events which resulted in development of the carcinoma *in situ* cells. Thus, if the speculation stated above is true, the primordial germ

cells might not be completely synchronized in their early development and only susceptible during a short period for the event(s) which are responsible for the initial transformation in the carcinoma *in situ* line. One might suspect this to be true also for otherwise normal testes with carcinoma in situ, because both carcinoma *in situ* cells and normal germ cells (including spermatozoa) may be present in the same biopsy specimen[12,40].

According to our results it seems likely that the germ cell tumours associated with gonadoblastomas arise from carcinoma *in situ* cells inside the gonadoblastoma nests. As in otherwise normal testes[41] the carcinoma *in situ* lesion seems to be widespread in the gonad.

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