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Gonadoblastoma Arising in Undifferentiated Gonadal Tissue within Dysgenetic Gonads

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Purpose: The purpose of the study was to define the histological origin of gonadoblastomas, allowing the identification of high-risk patients.

Experimental Design: Sixty paraffin-embedded gonadectomy or gonadal biopsy samples of 43 patients with gonadal dysgenesis were selected from our archives. We studied the morphology and immunohistochemical properties of the germ cells in 40 samples without neoplastic transformation and compared these findings with the morphological and immunohistochemical characteristics of 20 samples containing gonadoblastoma/dysgerminoma.

Results: The overall incidence of germ cell tumors in our patient series was 35%. In dysgenetic gonads without germ cell neoplasia, besides the presence of areas with testicular and/or ovarian differentiation, areas of undifferentiated gonadal tissue were identified in 13 of 40 samples (32.5%). A subpopulation of germ cells within these undifferentiated areas stained positive for octamer binding transcription factor (OCT)3/4, the stem cell factor receptor, placental-like alkaline phosphatase, and testis-specific protein-Y encoded. Gonadoblastoma germ cells display identical staining results. Moreover, in gonads containing gonadoblastoma, adjacent to this lesion, areas of undifferentiated gonadal tissue with identical immunohistochemical characteristics were identified in 10 of 20 samples (50%). No adjacent tissue was available in five cases, whereas in the five remaining cases, it consisted of streak tissue. In three cases, an accumulation of OCT3/4-positive germ cells in the proximity of the malignant lesions was found, suggesting clonal expansion and final organization into gonadoblastoma nests.

Conclusions: Based on these observations, we hypothesize that gonadoblastomas originate from surviving OCT3/4-positive germ cells in areas of undifferentiated gonadal tissue within the dysgenetic gonad. Supportive evidence was obtained that carcinoma *in situ* arises in regions with testicular differentiation. **(*J Clin Endocrinol Metab* 91: 2404–2413, 2006)**

GONADOBLASTOMA IS HISTOLOGICALLY defined as a tumor composed of two principal cell types: large germ cells similar to those of seminoma, and small cells resembling immature Sertoli and granulosa cells; elements resembling Leydig or lutein-like cells may also be present (1). This premalignant lesion of the dysgenetic gonad is the counterpart of the more frequent carcinoma *in situ* (CIS) lesion, which is found in well-differentiated testicular tissue (2). Gonadal dysgenesis is defined as an incomplete or defective formation of the gonads, resulting from a disturbed process of migration of the germ cells and/or their correct organization in the fetal gonadal ridge. It is caused by structural or numerical anomalies of the sex chromosomes or mutations in one of the genes involved in the formation of the urogenital ridge and sex determination of the bipotential gonad. Neoplastic transformation of germ cells in dysgenetic gonads (the formation of gonadoblastoma and/or an invasive germ cell tumor) occurs, according to literature data in 20–30% of cases and is associated with the presence of (part of) the Y chromosome in the patients' karyotype (3, 4). It is usually diagnosed at a young age (3, 5, 6). Therefore, early gonadectomy, often combined with gender reassignment and genital surgery, is mostly advocated (3, 6, 7). This safe but radical approach results definitely in infertility and lifelong dependence on hormonal replacement therapy in all patients. However, genital surgery and early gender assignment procedures have become controversial (8–11). Hormonal substitutes are sometimes considered as unphysiological, compared with endogenous hormone production. Advances in surgical techniques now allow rearing an individual born with ambiguous genitals as a male, preferably with his gonads positioned into the scrotum (9, 12). The incidence of malignancy in true hermaphroditism is estimated at 2–10% (4, 13) and is thus considerably lower than in other diagnostic groups. Interestingly, preservation of gonadal function has been described mainly in this specific group (13, 14) but also in some other patients with gonadal dysgenesis (15, 16).

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Abbreviations: AMH, Anti-Müllerian hormone; CIS, carcinoma *in situ*; c-KIT, stem cell factor receptor; DAB, diaminobenzidine; GA, gestational age; HE, hematoxylin-eosin; OCT, octamer binding transcription factor involved in the regulation of pluripotency, also referred to as POU domain class 5 transcription factor 1; PLAP, placental-like alkaline phosphatase; TSPY, testis-specific protein-Y encoded; UGT, undifferentiated gonadal tissue; VASA, human homolog of the mouse *vasa* gene.

These observations led the French group of Josso and coworkers (14) to propose a more conservative approach regarding gonadectomy in true hermaphroditism.

Dysgenetic gonads containing a germ cell tumor have been examined in more detail in several patient series (5–7, 15, 17). These combined data reveal that the gonad of origin is considered as a dysgenetic testis in 19.8% of cases and a streak in 26.1%, and that it could not be determined in 54.1% of cases. The impossibility to predict (*e.g.* from a gonadal biopsy) which gonad is prone to neoplastic transformation hampers the application of a more conservative approach relative to gonadectomy on a wider scale.

To gain insight into the nature of the gonads in which gonadoblastoma and invasive tumors may arise, we studied the histological and immunohistochemical properties of 40 dysgenetic gonads, removed as a prophylactic measure and in which no malignancy was detected on routine pathological examination, and compared them with the histological and immunohistochemical characteristics of 20 gonads containing gonadoblastoma and/or dysgerminoma lesions. Immunohistochemistry was performed with the antibodies octamer binding transcription factor (OCT) 3/4, c-KIT (the stem cell factor receptor), and placental-like alkaline phosphatase (PLAP), which are normally expressed in primordial germ cells/gonocytes and are well-established markers for the diagnosis of CIS and gonadoblastoma (18–22). Furthermore, the expression of testis-specific protein-Y encoded (TSPY), encoded by the TSPY gene and the main candidate gene responsible for the development of gonadoblastoma (19–21, 23, 24) and the human homolog of the mouse *vasa* gene (VASA), a general marker for germ cells (20, 25), was examined (Table 1). Sertoli/granulosa cells were examined for their expression of anti-Müllerian hormone (AMH).

Patients and Methods

Tissue samples

Forty-five patients with gonadal dysgenesis were retrieved from our archives. Samples of 20 dysgenetic gonads containing gonadoblastoma and/or dysgerminoma in 16 different patients and 40 dysgenetic gonads without apparent malignancy in 27 different patients were selected; three samples in two patients were excluded due to bad preservation of material. Patient and tissue characteristics are summarized in Table 2. Two fetal samples (Table 2, patients 1 and 2) obtained after induced abortion were included. Patient 5 underwent a bilateral gonadal biopsy at 4 months and a right gonadectomy 2 months later. A left gonadectomy was performed at 14 yr. Patient 26 underwent a right gonadectomy at 8 yr and a left gonadectomy at 25 yr.

Use of tissues for scientific reasons was approved by an institutional review board. The samples were used according to the Code for Proper Secondary Use of Human Tissue in The Netherlands, as developed by the Dutch Federation of Medical Scientific Societies (version 2002) (26). Samples originating from collaborating centers in Belgium, Poland, and Indonesia were treated in accordance with the above mentioned as well as local medical ethical guidelines.

Immunohistochemical staining

Tissue material was fixed in 10% formalin or Bouin's fixative, according to local fixation procedures. Slices of 3–5 μm thickness were prepared.

The antibodies used for immunohistochemistry and a schematic representation of the applied protocols are represented in Table 1. Slides were incubated with the primary antibodies at appropriate dilutions, staining was performed using 3,3′-diaminobenzidine-tetrahydrochloride dehydrate (DAB)/H_2O_2 or New Fuchsin (Fluka Chemica, Steinheim, Germany)/Naphtol ASMX (Sigma Aldrich, Zwijndrecht, The Netherlands) phosphate and counterstaining with hematoxylin. As positive controls, a normal adult male gonad for VASA, a seminoma sample for PLAP, c-KIT, TSPY and OCT3/4 and a male fetus, 8 wk gestational age (GA) for AMH were included.

Double-staining experiments were performed using the same detection methods but with different substrates: Fast Blue/Naphtol ASMX phosphate (F3378 and N500; Sigma, Steinheim, Germany) for a blue

TABLE 1. Schematic representation of origin and protocols used for the different antibodies

	Origin	Code	Dilution	Pretreatment	HIAR	Incubation	Secondary antibody	AB complex	Chromogen
Primary antibody									
VASA	Kindly provided by Dr. D. H. Castrillon[a]		1:1000	No	Yes	Overnight, 4 C	SWAR-bio	ABC-AP	New fuchsine
PLAP	Cell Marque, Hot Springs, AR	CMC203	1:200	No	Yes	Overnight, 4 C	RAM-bio	ABC-AP	New fuchsine
c-KIT	Dako-Cytomation, Glostrup, Denmark	A4502	1:500	No	Yes	Overnight, 4 C	SWAR-bio	ABC-AP	New fuchsine
TSPY	Kindly provided by Prof. C. Lau[b]		1:3000	No	No	Overnight, 4 C	SWAR-bio	ABC-AP	New fuchsine
OCT3/4	Santa Cruz Biotechnology, Santa Cruz, CA	SC8629 and SC5279	1:1000	H_2O_2 for 5′ + biotin blocking	Yes	2 h, RT	HAG-bio	ABC-HRP	DAB
AMH	Kindly provided by Prof. A. Themmen[c]		1:200	H_2O_2 for 5′ + biotin blocking	Yes	Overnight, 4 C	RAM-bio	ABC-HRP	DAB
Secondary antibody									
SWAR	Dako-Cytomation		1:200						
RAM	Dako-Cytomation		1:200						
HAG	Vector Laboratories, Burlingame, CA		1:200						

HIAR, Heat-induced antigen retrieval (40); SWAR, swine antirabbit antibody; RAM, rabbit antimouse antibody; HAG, horse antigoat antibody; bio, biotin labeled; AB complex, streptavidin-biotin complex; ABC-AP, streptavidin-biotin-alkaline phosphatase complex; ABC-HRP, streptavidin-biotin-horseradish peroxidase complex; RT, room temperature.

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TABLE 2. Overview of patient and tissue characteristics

Patient	Age	Karyotype	Phenotype	Histology					Remarks
				T	O	UGT	S	Tumor	
1	17 wk	45,X/46,XY	Male	+	−	+	−	−	
2	19 wk	45,X (7/20) /46,XY (13/20)	Male	+	+	−	−	−	
3l	1 month	46,XX	Female	+	+	−	−	−	
3r	1 month			−	+	+	−	−	
4l	4 months	46,XX	Female	−	−	−	−	Calcified GB	
4r	4 months			−	+	−	+	GB	
5r	4 months	46,XY	Female	+	−	−	−	−	r Biopsy
5l	4 months			−	−	+	−	−	l Biopsy
5r	6 months			+	−	+	−	−	r Gonadectomy
5l	14 yr			−	−	+	−	GB+DG	l Gonadectomy
6l	6 months	45,X (22/50) /46,X der(Y) (28/50)	Male	−	−	+	−	−	
6r	6 months			+	−	−	−	−	Biopsy
7l	6 months	45,X/46,X iso(Y)	Male	−	−	−	+	−	
8	11 months	46,Xyder(9) t(7;9) (q13.2;p24.2)	Male	+	−	−	−	−	
9l	1 yr	45,X/46,XY	Female	+	−	+	−	GB	
9r	1 yr			−	−	−	+	−	
10l	1 yr	46,XY	Male	+	−	+	−	−	
11	1 yr	46,XX/46,XY	Female	+	+	+	−	−	
12	1 yr	45,X (10/100) /46,XY (90/100)	Male	+	−	−	−	−	Biopsy
13l	1 yr	45,X (12/50)/46,XY (38/50)	Male	−	−	+	+	−	
14l	2 yr	45,X (53/74) /46,Xi(Yp) (21/74)	Male	+	−	+	−	−	
15	2 yr	46,XY	Female	−	−	+	−	−	
16l	3 yr	45,X/46,XY	Female	+	−	−	+	−	
17r	3 yr	46,XX (30/100) /46,XY (70/100)	Male	−	+	−	−	−	
18l	4 yr	45,X, inv(5) (q22q33.2); 46,X i(Y9), inv(5) (q22q33.2)	Female	+	−	+	−	−	
18r				+	−	+	−	−	
19l	4 yr	46,XX	Male, later	−	+	−	−	−	
19r			Female	+	+	−	−	−	T: no germ cells
20	4 yr	45,X (11/25) /46,XY (14/25)	Male	+	−	−	−	−	
21	5 yr	46,XY	Male	+	−	−	+	−	
22l	6 yr	46,XX	Male	+	+	−	+	−	T: no germ cells
23	7 yr	46,XY	Female	−	−	−	−	GB	
24r	7 yr	46,XX/46,XY	Male	−	+	−	+	−	
24l				+	−	+	−	−	
25r	7 yr	45,X/46,XY	Male	+	−	−	−	−	Biopsy, T: no germ cells
25l	8 yr			+	−	−	−	−	Biopsy, T: no germ cells
26r	8 yr	45,X/46,XY	Male	+	−	−	+	−	
26l	25 yr			−	−	−	−	GB	
27r	9 yr	46,XY	Female	−	−	+	−	Calcified GB + MT + IT	
28r	10 yr	45,X (7/12)/46,XY (5/12) - second count: 45,X (40/40)	Male	+	−	−	−	−	
29r	14 yr	46,XY	Female	−	−	−	+	GB	
29l	14 yr			−	−	−	+	−	
30r	14 yr	46,XX	Female	+	+	−	−	−	
31r	14 yr	45,X/46,XY	Female	−	−	−	+	−	
31l				−	−	−	+	−	
32r	16 yr	46,XY	Female	−	−	+	−	GB	
32l				−	−	−	+	GB	
33	16 yr	46,XY	Female	−	−	−	+	GB	
34l	17 yr	45,X/46,XY	Female	−	−	+	−	Calcified GB	
35r	17 yr	45,X (12/30) /46,XY (18/30)	Female	−	−	−	+	−	
36l	17 yr	45,X (5/50)/46,XY (45/50)	Female	−	−	−	+	−	
37r	18 yr	46,XY	Female	−	−	+	−	GB+DG	
37l				−	−	+	−	GB	
38	18 yr	46,XY	Female	−	−	+	−	GB	
39r	19 yr	46,XY	Female	−	−	+	−	GB+DG	
39l				−	−	+	−	GB	
40	19 yr	?	Female	−	−	−	+	−	
41	20 yr	45,X/46,XY	Male	−	−	−	−	GB+DG	
42l	21 yr	46,XY	Male	−	−	−	−	GB+CIS	
43	25 yr	46,XY	Male	−	−	−	+	DG	

Laterality of the gonad and karyotype are indicated if known. Age, Age at gonadectomy or biopsy; l, left; r, right; GB, gonadoblastoma; DG, dysgerminoma; MT, mature teratoma; IT, immature teratoma; T, testis; O, ovary; S, streak; wk, weeks GA.

TABLE 3. Summary of staining results in the encountered gonadal differentiation patterns and in gonadoblastoma (GB)/dysgerminoma (DG)

	Testis	UGT	Ovary	Streak	GB	DG
OCT3/4	+	+	−	−	+	+
PLAP	+	+	−	−	+	+
c-KIT	+	+	+	−	+	+
TSPY	+++	+++	−	−	+++	+/−
VASA	+	+	+	−	+	+/−

+, Positive expression; −, no expression; +++, abundant expression; +/−, expression may be present or absent.

staining and 3-amino-9-ethyl-carbazole (A.5754 and D4254, Sigma)/H_2O_2 for a red staining, without counterstaining. To reduce background signal, endogenous peroxidase activity and endogenous biotin were blocked using 3% H_2O_2 (5 min) and a blocking kit for endogenous biotin (Vector Laboratories, Burlingame CA). For a correct interpretation of histology, hematoxylin-eosin (HE) staining was performed on parallel slides.

Interpretation of results

General morphology and interpretation of the staining results were assessed by two observers with experience in germ cell pathology (M.C. and J.W.O.). Part of the results obtained in five gonadoblastoma samples were reported previously (21).

Statistical analysis

Results were analyzed using the SPSS (SPSS 11.0 for Windows; SPSS Inc., Chicago, IL) statistical program (Fisher exact test for 2 × 2 tables).

Results

Morphology and staining results in dysgenetic gonads without neoplastic transformation

Four gonadal differentiation patterns were recognized in various combinations (Tables 2 and 3 and Fig. 1).

First, tissue containing seminiferous tubules was encountered in 24 of 40 gonads (60%) and was considered as a testicular differentiation pattern. The tubules often displayed

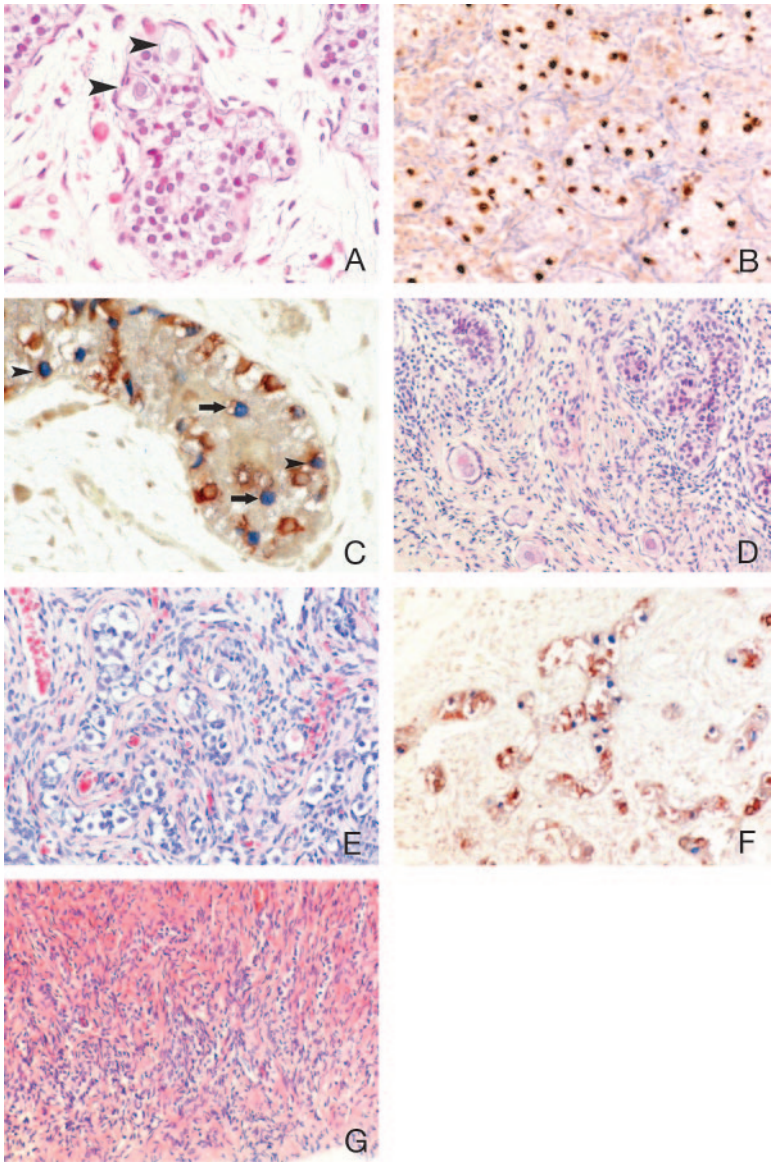

FIG. 1. Differentiation patterns encountered in dysgenetic gonads without neoplastic transformation: A, Patient 30r. Dysgenetic testicular tissue, containing germ cells (*arrowheads*). HE staining, ×400. B, Patient 18r. Testicular tissue. OCT3/4-positive cells are located in the center of the tubule but are also found on the basal lamina. OCT3/4 staining, ×200. C, Same patient. A subpopulation of germ cells (recognized by the VASA staining) expresses OCT3/4. OCT3/4-positive cells are located in the center of the tubule (*arrows*) but are also found on the basal lamina (*arrowheads*). OCT3/4 (*blue*)-VASA (*red*) double staining, ×400. D, Patient 19r. Testicular (*left part*) and ovarian (*right part*) differentiation; the testicular tissue is devoid of germ cells; ova are enclosed in primordial and primary follicles. HE staining, ×200. E, Patient 14l. Undifferentiated gonadal tissue. Germ cells align in clusters together with Sertoli/granulosa cells or lay isolated in fibrous stroma. HE staining, ×200. F, Same patient. A subpopulation of germ cells within the UGT area expresses OCT3/4. OCT3/4 (*blue*)-VASA (*red*) double staining, ×200. G, Patient 13l. Streak. Remnants of cords that have lost their germ cells and have undergone a fibromatous reaction are recognizable. HE staining, ×200.

abnormalities such as a thin basal lamina, shape irregularity, increased intertubular spaces, and branching structures, reflecting their dysgenetic nature (Fig. 1A). Germ cells were found in 20 of 24 samples (83%) and were easily recognized by positive staining for VASA and TSPY. The TSPY staining was consistently very intense, compared with normal adult and age-matched controls as well as normal fetal testicular samples (our personal observations). Staining of germ cells for OCT3/4, c-KIT, and PLAP was performed in 18 of 24 samples; in the remaining six, it could not be performed due to limited material. A subpopulation of germ cells stained positive for the three markers in 14 of 18 samples (78%). In all cases, positive cells were found centrally in the tubule, pointing at a delay in maturation, but seven of 14 patients (50%) also displayed positive germ cells on the basal lamina (Fig. 1B). OCT3/4-VASA double-staining experiments confirmed these findings. In general, OCT3/4 expression was lost as cells became positive for VASA, yet sometimes OCT3/4-VASA coexpression was observed within a single cell (Fig. 1C). Regardless of their dysgenetic aspect, in all samples the majority of the tubules displayed positive staining for AMH in Sertoli cells.

Second, an ovarian differentiation pattern was defined as gonadal tissue containing germ cells enclosed in primordial and eventually growing follicles, comparable with the ovaries of female neonates (Fig. 1D). It was encountered in 10 of 40 gonads (25%). Ova homogeneously expressed VASA and c-KIT, whereas OCT3/4-, PLAP-, or TSPY-positive ova were never found. A unique situation was found in patient 3l: follicles, containing VASA-positive and TSPY-negative ova, were enclosed in seminiferous tubules, containing VASA-positive, TSPY-positive spermatogonia (Fig. 2).

A third differentiation pattern, further referred to as undifferentiated gonadal tissue (UGT), consisted of gonadal tissue containing germ cells not enclosed in seminiferous tubules or follicles but organized together with Sertoli/granulosa cells in cord-like structures or residing without apparent organization in a background of fibrous stroma (Fig. 1E) or a combination of these two. This pattern, although present in 13 of 40 gonads (32.5%), was mostly not mentioned in the original pathology reports, or alternatively, it was referred to as a streak. Expression of markers in the germ cells within UGT was similar to that found in testicular tissue. Germ cells could easily be identified by their expression of VASA and abundant expression of TSPY. Due to limited material, OCT3/4, PLAP, and c-KIT staining could be performed in 11 of 13 samples. In nine of them (82%), a subpopulation of germ cells within UGT stained positive for these three markers. OCT3/4-VASA double staining confirmed these results (Fig. 1F). Sertoli/granulosa cells within UGT differed from their apparently more differentiated counterparts in testicular tubules: in most samples, AMH expression was totally absent, although sporadic weakly positive Sertoli/granulosa cells were found in two samples.

The fourth pattern, consisting of fibrous stroma devoid of germ cells, was referred to as streak. It was found in 14 of 40 dysgenetic gonads (35%) in an age-related manner: four of 25 cases until 4 yr of age contained streak tissue (15%), whereas it was found in 10 of 15 samples older than 4 yr (66%) ($P = 0.002$). Cord-like structures were often recognizable and might represent UGT that has lost its germ cells and has undergone a fibromatous involution (Fig. 1G). AMH expression was never observed in this tissue type.

Morphology and staining results in dysgenetic gonads containing gonadoblastoma and/or dysgerminoma (Tables 2 and 3 and Fig. 3)

Typical gonadoblastoma nests were found in 16 of 20 samples, four of them also containing an invasive dysgerminoma component and one combined with CIS. Three samples consisted of large calcifications (the so-called burnt-out gonadoblastoma), one of them in combination with mature and immature teratoma. In one sample only dysgerminoma was identified. Germ cells within gonadoblastoma stained positive for OCT3/4, c-KIT, PLAP, and TSPY. TSPY was abundantly expressed in every gonadoblastoma sample, but the expression decreased in the invasive tumor components. The expression of

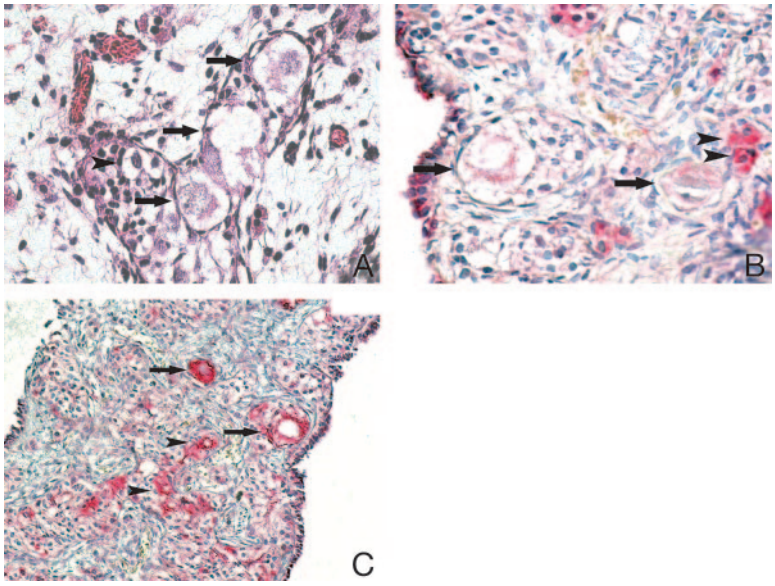

FIG. 2. A, Patient 3l. Ova in a primordial follicle (*arrows*) are enclosed in seminiferous tubules containing spermatogonia (*arrowhead*). HE staining, ×200. B, Same patient. TSPY-negative ova (*arrow*) next to TSPY-positive spermatogonia (*arrowhead*), TSPY staining, ×400. C, Same patient. Ova (*arrow*) and spermatogonia (*arrowhead*) both express VASA. VASA staining, ×200.

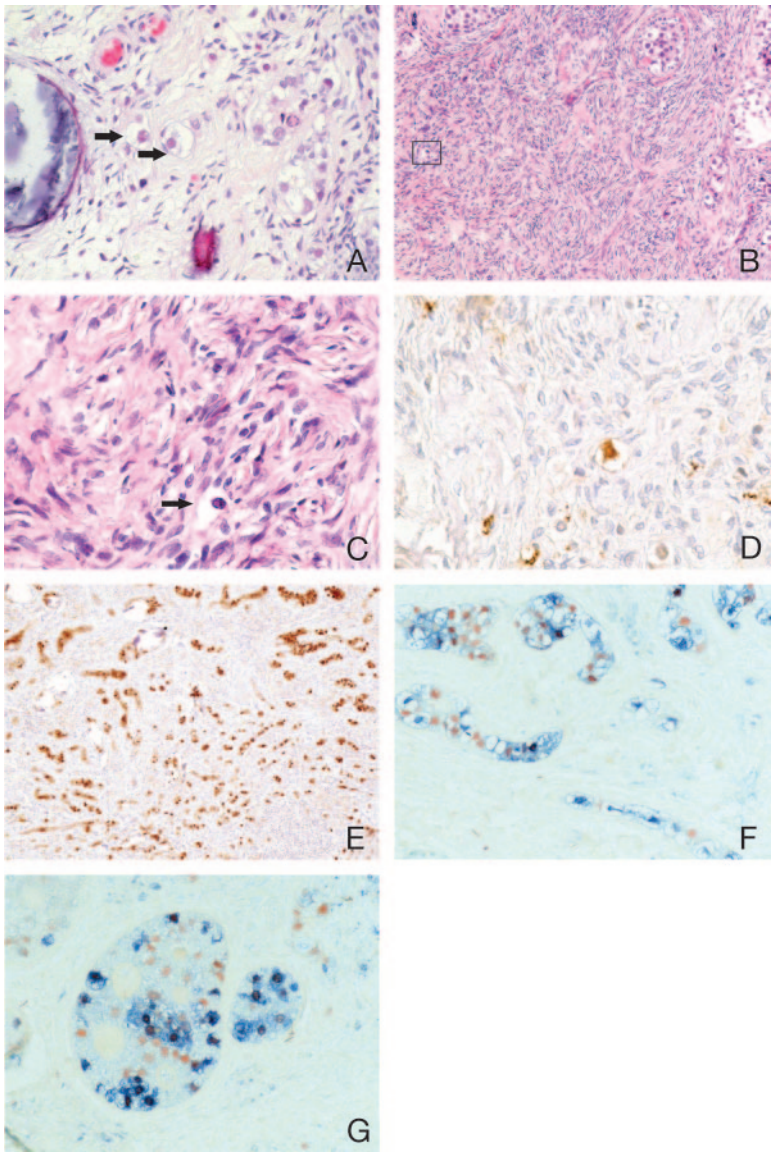

FIG. 3. A, Patient 37r. UGT with isolated germ cells (*arrows*) adjacent to gonadoblastoma lesions (*left*). HE staining, ×200. B, Patient 32r. *Rectangle*, Isolated germ cells in the tissue adjacent to gonadoblastoma. HE staining, ×100. C, Same patient, enlargement of panel B. HE staining, ×400. D, Same patient. Isolated OCT3/4-positive germ cells in UGT adjacent to gonadoblastoma. OCT3/4 staining, ×400. E, Patient 39l. Clonal expansion of OCT3/4-positive cells within UGT in the close proximity of a gonadoblastoma lesion. OCT3/4 staining, ×50. F, Same patient. A subpopulation of germ cells within UGT adjacent to gonadoblastoma expresses OCT3/4 (*red*) and TSPY (*blue*). OCT3/4-TSPY double staining, ×200. G, Same patient. Most germ cells within a gonadoblastoma lesion express OCT3/4 (*red*) and TSPY (*blue*). OCT3/4-TSPY double staining, ×200.

OCT3/4, c-KIT, and PLAP was variable: in some gonadoblastoma samples, all the germ cells stained positive, whereas in others, only a subpopulation of germ cells expressed these markers; however, in the dysgerminoma samples, these markers were homogenously present. VASA expression within the gonadoblastoma samples was variable: in 12 of 15 examined samples, germ cells were positive; the remaining three samples were negative for VASA.

Nonneoplastic gonadal tissue adjacent to the neoplastic lesions was available in 15 of 20 samples (75%). The four differentiation patterns described above were encountered: a streak was found in five of 15 cases (33%), one in combination with ovarian tissue, and UGT was present in 10 of 15 cases (67%), one in combination with testicular tissue (Fig. 3, A–C). Again, in UGT, germ cells stained positive for TSPY with a remarkably strong intensity. In all samples containing UGT, OCT3/4-, c-KIT-, and PLAP-positive germ cells were found (Fig. 3D). In patients 23 and 39 (bilaterally), in the proximity of the malignant lesions, an accumulation of OCT 3/4-positive germ cells was found, suggesting clonal expansion and final organization into gonadoblastoma nests (Fig. 3E).

These findings were confirmed in the OCT3/4-TSPY double-staining results (Fig. 3, F and G).

Discussion

The overall incidence of germ cell tumors in 45 patients with gonadal dysgenesis retrieved from our database was 35% (16 of 45 patients), with four bilateral cases. Invasive germ cell tumors were found in six of 45 patients (13%).

We studied the histological and immunohistochemical characteristics in dysgenetic gonads that have (not yet) undergone neoplastic transformation and compared these findings with the properties of the gonadal tissue adjacent to and within gonadoblastoma samples. Given the rarity of the syndrome, a relatively large patient series could be collected. However, due to limited material, some experiments were performed in selected samples.

In dysgenetic gonads, basically four patterns of gonadal differentiation (with each pattern displaying a broad spectrum of abnormalities in morphology, number, and organization of the germ cells) were found: testicular and ovarian tissue, streak, and UGT. The frequent finding of UGT was unexpected because it had not been mentioned in the original pathology reports, nor is it routinely described in literature, although some histological descriptions might suggest the presence of UGT (17, 27, 28). UGT clearly differed from the other patterns: in contrast to streak tissue, it does contain germ cells, but these are organized in neither seminiferous tubules nor follicles. In contrast, these germ cells reside apparently randomly distributed in a background of stromal cells or align in clusters, in close contact with Sertoli/granulosa cells. Without close observation and the use of specific markers, the germ cells within UGT are easily overlooked, hence its frequent classification as a streak. Alternatively, due to their presence in ovarian-type stroma, and although they are not organized in follicles, the germ cells are sometimes misinterpreted as residing in ovarian tissue. However, their correct identification is of crucial importance, as is illustrated in patient 5. In this girl, a bilateral biopsy was performed at 4 months, followed by a gonadectomy of the right testis because of its discordance with the sex of rearing. The left gonad, which was considered as ovarian tissue, was left in place. At the age of 14 yr, she developed a gonadoblastoma, already having progressed to a dysgerminoma. A reevaluation of the available biopsy material of the left gonad unequivocally demonstrated germ cells and cord-like structures (Fig. 4, A–C). A similar case is found in the literature (6).

A significantly increased incidence of streak tissue was found in patients older than 4 yr, compared with younger patients ($P = 0.002$). Cord-like structures were often recognized, suggesting a loss of germ cells in UGT, analogous to the germ cell loss that is observed in dysgenetic testicular or ovarian tissue [*e.g.* the testicular tissue in hermaphrodites (13) or the testes of patients with undervirilization syndromes (19)]. This finding is in accordance with previous reports (7).

The fate of the germ cells during normal human gonadal development was described by Gondos (29) and is largely analogous to the gonadal development and differentiation process analyzed in detail in mice (30). At their arrival in the genital ridge around 5 wk GA, the germ cells and pre-Sertoli/granulosa cells lay intermingled without specific organization in the undifferentiated gonad. The first sign of sexual differentiation (the formation of primitive cords) coincides with SRY expression, around wk 6 GA. In the absence of SRY, no changes occur in the undifferentiated gonad until the 12th week, when the germ cells enter meiosis. As stated above, germ cells within UGT either lay randomly in fibrous stroma or line up together with Sertoli/granulosa cells in cord-like structures. It is conceivable that the first pattern represents the undifferentiated state of the gonad, in which no accurate SRY expression has taken place but in which under the influence of unknown (male) characteristics, meiosis and progression along the default pathway are inhibited. The second pattern might represent early sex cords, blocked in their progression toward seminiferous tubules (Fig. 5). The differential staining results for AMH in testicular tubules and cord-like structures support this hypothesis.

Germ cells were stained with the markers TSPY, OCT3/4, c-KIT, PLAP, and VASA. TSPY expression was never encountered in ova enclosed in follicles. However, it was abundantly expressed in germ cells within dysgenetic testes and UGT, compared with the intensity of the TSPY staining in fetal and age-matched normal gonads (19, 20, 31) (and our personal observations), thereby suggesting an up-regulation of TSPY when germ cells reside in an unfavorable environment. This is in line with previous observations in gonadoblastoma (21). TSPY is thought to be related to the premeiotic proliferation of spermatogonia, although its function is not fully clarified (23). Evidence is growing that TSPY is the main candidate for the hypothetical gene in the gonadoblastoma region on the Y chromosome leading to the development of gonadoblastoma (21, 24, 32). Abnormal TSPY expression has also been related to the development of CIS in undervirilized patients (19).

OCT3/4, c-KIT, and PLAP are well-established markers

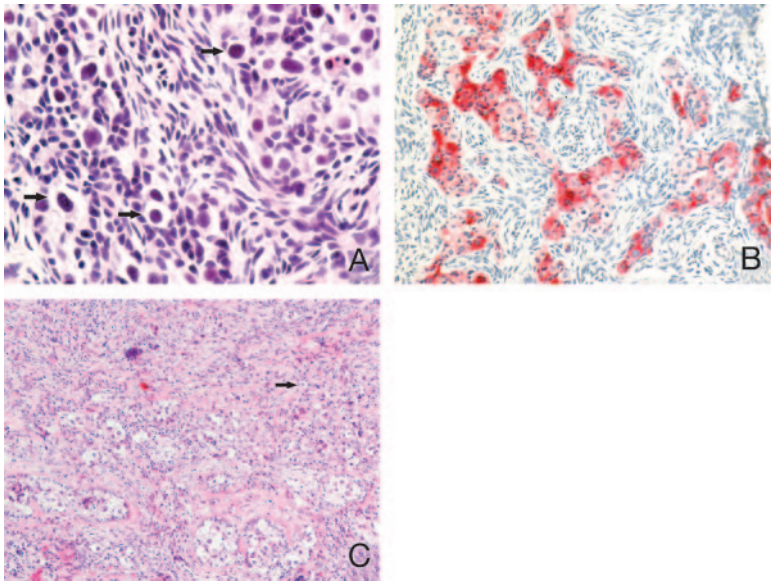

FIG. 4. A, Patient 5l, biopsy performed at 4 months. Germ cells are clearly recognizable in the biopsy sample of the left gonad (*arrows*). HE staining, ×400. B, Same patient. Germ cells enclosed in cords are encountered in a PLAP staining of the biopsy sample. PLAP staining, ×200. C, Same patient, now 14 yr old. Gonadoblastoma of the left gonad (*lower part of the image*); sporadic germ cells are still present in the UGT adjacent to the gonadoblastoma lesion (*arrow*). HE staining, ×200.

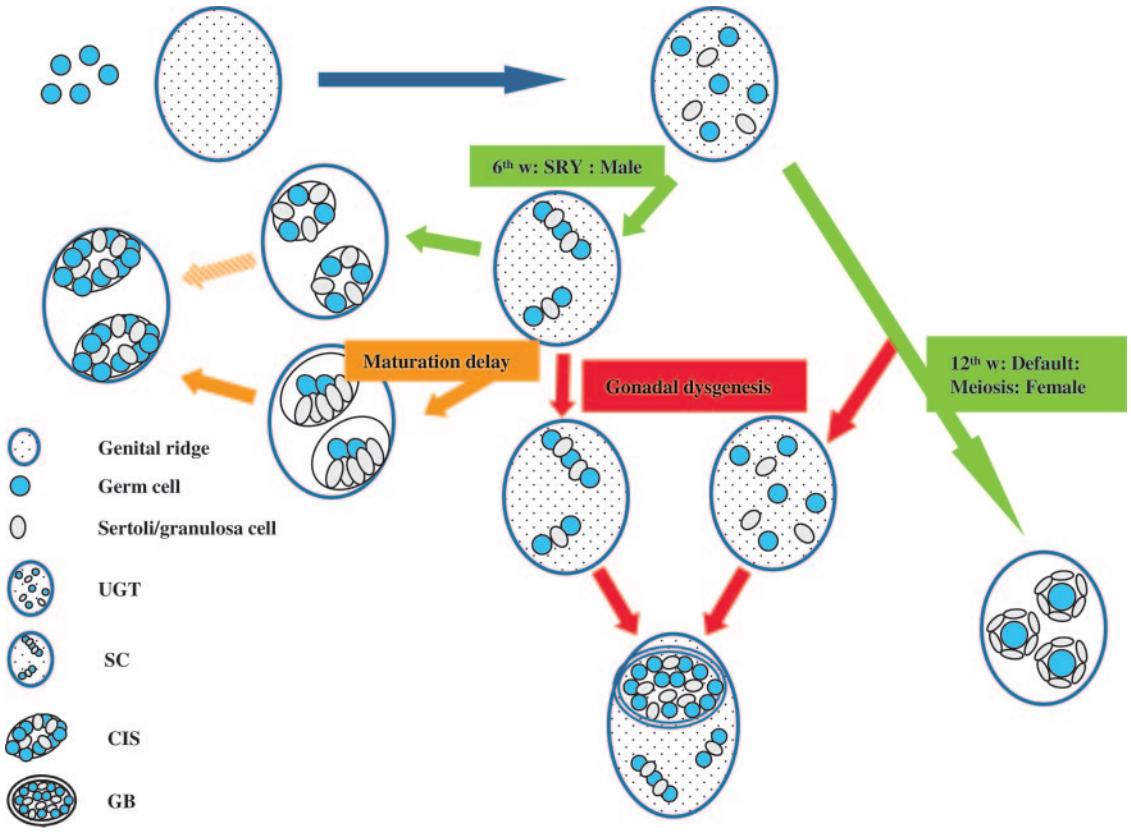

FIG. 5. Model for the development of UGT, gonadoblastoma, and CIS within the dysgenetic gonad. *Upper panel*, In the developing embryo, germ cells migrate from the yolk sac into the bipotential gonad and intermingle with pre-Sertoli/granulosa cells. *Middle panel* (from the *right* to the *left*), In the male, *SRY* expression in the sixth week GA induces organization of pre-Sertoli cells and germ cells in primitive sex cords (SC). Under the influence of other male sex-determining genes downstream of *SRY*, these sex cords differentiate into seminiferous tubules. Pathological conditions can cause a block or delay in the normal germ cell development, thereby increasing the risk for formation of intratubular germ cell neoplasia unclassified. *Lower panel*, In gonadal dysgenesis, inaccurate or absent *SRY* expression or a disturbed expression of other male-determining genes prohibits sex cord formation or further differentiation of sex cords, whereas progression along the meiosis-default pathway is also blocked (*lower panel, right*), resulting in the persistence of UGT. Surviving germ cells residing in UGT (including immature sex cords) contain a high risk for the development of GB (*lower panel, middle*). GB, Gonadoblastoma.

for the diagnosis of various germ cell tumors and are normally expressed during fetal gonadal development (20, 33). Maturation delay of germ cells, which has been described in intersex conditions and patients with chromosomal anomalies, is characterized by a prolonged expression of these markers and is considered to be a risk factor for malignant transformation (2, 19, 31, 34, 35). In the present series, these three markers revealed similar staining patterns; however, OCT3/4, resulting in a well-circumscribed and intense nuclear staining, was the most stable marker and was easiest for interpretation. OCT3/4, a transcription factor regulating pluripotency of embryonal stem cells and essential for the survival of migratory primordial germ cells (36), is consistently expressed in specific germ cell tumors and might play a pathogenetic role in their development (37, 38). In line with previously reported results (33), OCT3/4 expression was never found in ovarian follicles. In contrast, within testicular tissue and UGT of dysgenetic gonads without gonadoblastoma and tissue adjacent to gonadoblastoma, a subpopulation of germ cells expressed OCT3/4. Gonadoblastoma and dysgerminoma also consistently expressed this marker (Figs. 1 and 3). In the proximity of a gonadoblastoma lesion, an accumulation of OCT3/4-positive cells was observed, suggesting clonal expansion within UGT toward gonadoblastoma formation (Fig. 3E). Previously we demonstrated that OCT3/4-positive prespermatogonia located centrally in the seminiferous tubule reflect a state of maturation delay, whereas OCT3/4-positive cells on the basal lamina are prone to malignant transformation (19). In the present study, the latter pattern was encountered in seven samples. It is tempting to speculate that these patients were at high risk for developing CIS, eventually progressing to invasiveness within the dysgenetic testis if a gonadectomy had not been performed.

Based on our results, a model for the development of gonadoblastoma and CIS in the dysgenetic gonad containing Y chromosome material (*i.e.* the *TSPY* gene) emerges: a dysgenetic gonad may consist of different parts with various degrees of differentiation (ovarian tissue, in which the germ cells evolve along the default pathway of meiosis). These germ cells have lost OCT3/4 expression and do not express TSPY; therefore, they cannot give rise to a malignant germ cell tumor. Testicular tissue, showing variable degrees of dysgenesis, contains germ cells that abundantly express TSPY, possibly in an attempt to survive and proliferate in an unfavorable environment. Some of these germ cells maintain

OCT3/4 expression, even after having reached the spermatogonial niche due to a block in their maturation. Over time, most of these germ cells will die; however, some, possibly due to abundant TSPY levels and prolonged OCT3/4 expression, will survive and proliferate, eventually leading to clonal expansion and CIS formation. In UGT, a similar process takes place, ending in a streak in case all the remaining germ cells die or alternatively in the development of gonadoblastoma (Fig. 5). If the latter evolution takes place more rapidly than the progression toward CIS, this would explain the more frequent finding of gonadoblastoma rather than CIS in dysgenetic gonads containing both UGT and testicular tissue. Alternatively, it is conceivable that immature germ cells, blocked in their maturation but residing in UGT, which is their natural environment during early embryonic life, have increased survival chances, compared with immature germ cells residing in more differentiated testicular tissue. Our findings are not disconcordant with the hypothesis that gonadoblastomas originate from germ cells developing along the female pathway but failing to complete the meiotic prophase and organize in primordial follicles (39).

In conclusion, extrapolation to a clinical situation would suggest that a gonadal biopsy revealing the presence of UGT or testicular tissue with OCT3/4-positive cells on the basal lamina contains a high risk for germ cell tumors and should imperatively lead to gonadectomy. Ovarian tissue can safely be left in place; testicular tissue displaying maturation delay of germ cells can be left *in situ*, given that its localization allows adequate follow-up. A streak is not functional, making its preservation controversial. Evidently the decision with regard to gonadectomy is not only based on a pathological analysis of biopsy material but also must be inspired by the combined interpretation of the patients' karyotype, internal genitalia, phenotype, gender, and psychological well-being. The value, safety, and applicability of this model should be thoroughly tested in large patient series and multicenter studies before it may contribute to a more conservative approach regarding gonadectomy in selected patients with gonadal dysgenesis.

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