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Morphological and Immunohistochemical Differences between Gonadal Maturation Delay and Early Germ Cell Neoplasia in Patients with Undervirilization Syndromes

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Context: Maturation delay of germ cells and their progression into carcinoma *in situ* (CIS) frequently occurs in intersex patients. A developmentally delayed germ cell resembles a CIS cell and displays prolonged expression of immunohistochemical markers used for the diagnosis of CIS. This questions their applicability in young children.

Objective: The objective of the study was the elaboration of tools to distinguish germ cells with maturation delay and CIS.

Design: The design was a qualitative and quantitative analysis of the expression of diagnostic markers for CIS in gonads of young patients with undervirilization syndromes.

Setting: The study was conducted in the pathology department of a university center, specializing in germ cell tumor pathogenesis.

Patients: Fifty-eight formalin-fixed, paraffin-embedded testicular tissue samples of 30 undervirilized patients (1 month to 23 yr of age) were analyzed.

Interventions: Interventions included hematoxylin-eosin staining, immunohistochemistry for octamer binding transcription factor (OCT)3/4, gene encoding the stem cell factor receptor that has tyrosine kinase activity c-KIT, placental/germ alkaline phosphatase (PLAP), testis-specific protein Y encoded (TSPY), and VASA, double staining for OCT3/4 and VASA, with ploidy determination by fluorescent *in situ* hybridization.

Main Outcome Measure: Maturation delay and CIS are characterized by the staining patterns of the immunohistochemical markers.

Results: CIS was diagnosed in three of 30 patients (10%) and four of 58 gonads (6.9%). Patient age, distribution of OCT3/4-positive cells throughout the gonad, and their position within the seminiferous tubule differ between maturation delay and CIS. Abnormal OCT3/4 and testis-specific protein Y encoded expression appear to be of pathogenetic relevance in the development of these lesions.

Conclusion: The dimorphic expression of OCT3/4 allows distinction between maturation delay and CIS. Studies in larger patient series are essential before a biopsy to evaluate the neoplastic risk can eventually be proposed as an alternative for gonadectomy. (***J Clin Endocrinol Metab* 90: 5295–5303, 2005**)

UNDERVIRILIZATION SYNDROMES CAN be caused by errors in testosterone biosynthesis, testicular unresponsiveness to human chorionic gonadotrophin and LH, or defects in androgen-dependent target tissues (1). Patients have an increased risk for the development of germ cell tumors. In the complete androgen insensitivity syndrome (CAIS), this risk is estimated at 2–5% (2). In other subgroups, due to low prevalence, incomplete diagnostic information and confusing nomenclature, the incidence is actually unknown. Moreover, criteria for the correct diagnosis of early neoplasia in young intersex patients are lacking (see text below). Gonadectomy is the therapy of choice to exclude tumor development and avoid virilization (1). The optimal timing for gonadectomy is controversial, especially in CAIS patients, in whom peripheral conversion of testosterone into estradiol allows spontaneous development of secondary sexual characteristics. Patients often underscore the unphysiological bioactivity of hormonal replacement therapy, compared with their endogenous hormone production. Therefore and because the risk of malignancy seems to increase with increasing age, some authors advise to postpone gonadectomy (2–5). However, because germ cell tumors have been described in (pre)pubertal androgen insensitivity syndrome patients, others advocate to remove the gonads or at least to perform a biopsy at the time of diagnosis (6–8).

The diagnosis of carcinoma *in situ* (CIS) in children with intersex conditions is difficult. Morphologically atypical germ cells are commonly seen in their gonads (8–11) and seem to correspond with delayed maturation rather than with malignant transformation (12) (our personal observa-

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Abbreviations: CAIS, Complete androgen insensitivity syndrome; CIS, carcinoma *in situ*; c-KIT, gene encoding the stem cell factor receptor which has tyrosine kinase activity; HSD, hydroxysteroid dehydrogenase; OCT3/4, octamer binding transcription factor involved in regulation of pluripotency, also referred to as POU domain class 5 transcription factor 1 (POU5F1); PAIS, partial androgen insensitivity syndrome; PLAP, placental/germ alkaline phosphatase; TSPY, testis-specific protein Y encoded.

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tions). Immunohistochemical markers, such as octamer binding transcription factor involved in regulation of pluripotency (OCT)3/4, placental/germ alkaline phosphatase (PLAP), and the gene encoding the stem cell factor receptor that has tyrosine kinase activity (c-KIT), which are normally expressed in embryonic germ cells are well-established markers to detect CIS and some invasive germ cell tumors in adult patients (13–16). These markers are also used to demonstrate CIS in children with intersex conditions (17, 18) and even in fetal gonads (19). However, many of these markers show prolonged expression in dysgenetic gonads (20, 21) and are even normally expressed in young children shortly after birth (22). Therefore, these markers cannot be used as such to detect CIS in young patients with gonadal dysgenesis.

Germ cells in gonadal dysgenesis exhibit a developmental delay and are prone to malignant transformation if they are able to survive in their inappropriate environment (23). The aim of our study was to describe the process from maturation delay toward germ cell death or development of CIS in a series of patients with undervirilization syndromes and identify additional tools to distinguish morphological and immunohistochemical features of developmental arrest of the germ cells from a developing CIS. Ploidy determination in the CIS samples was used to gain further insight into the chronological steps leading from maturation delay toward malignancy. The ability to distinguish between these different conditions is essential before a biopsy can be proposed as a safe and temporary alternative for gonadectomy in those patients in whom preservation of proper hormone production is relevant (see above text).

Patients and Methods

Tissue samples

Gonadal tissue samples of 30 patients with undervirilization syndromes, aged 1 month to 23 yr were obtained after biopsy (5.4%) or gonadectomy (94.6%), performed in the Sophia Children's Hospital or collaborating centers. All patients had 46 XY karyotypes. The individual diagnoses were established on clinical grounds and hormonal profiles and where possible, confirmed by characterization of the underlying genetic defect (Table 1). In all patients, gonads were removed as a prophylactic measure and no cases gave rise to clinical suspicion of a tumor. Bilateral specimens were available from 28 patients and a unilateral specimen from two patients.

The gonads of eight control patients, aged 3 months to 13 yr, who suffered from sudden infant death syndrome or sudden death after trauma, were examined to compare staining and counting results.

Use of tissues for scientific reasons was approved by an institutional review board (MEC 02.981). 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).

Immunohistochemical staining

After routine fixation in 10% formalin, 5-μm-thick slices were prepared. Poorly preserved samples were excluded from the study. The antibodies used for immunohistochemistry and a schematic representation of the applied protocols are represented in Table 2. Slides were incubated with the primary antibodies in an appropriate dilution. In between the incubation steps, they were washed in a PBS-Tween 20 0.01% solution. Staining was performed using diaminobenzidine/H_2O_2 or New Fuchsin/Naphtol ASMX phosphate and counterstaining with hematoxylin. As positive controls, normal adult male gonadal tissue for VASA and seminoma for PLAP, c-KIT, testis-specific protein Y encoded (TSPY), and OCT3/4 were used.

Double-staining. After pretreatment with H_2O_2 and pressure cooking, sections were incubated with VASA (overnight) and OCT3/4 (2 h). OCT3/4 was detected using the avidin-biotin-alkaline phosphatase complex and Fast Blue/Naphtol ASMX phosphate for a blue staining. After this, free biotin was blocked using a blocking KIT (Vector Laboratories, Burlingame CA). VASA was detected, using the avidin biotin-horseradish peroxidase complex with 3-amino-9-ethyl-carbazole (Sigma, Steinheim, Germany)/H_2O_2, resulting in a red staining. A male gonad containing normal tissue and CIS served as a positive control.

Quantification of results

General morphology of tissue samples was assessed by a pathologist experienced in germ cell pathology (J.W.O.). Eventual artifacts due to the fixation procedure were not taken into account in the final analysis.

Results were quantified as follows: for each tissue sample, 500 seminiferous tubules were assessed for the staining pattern of OCT3/4, VASA, and TSPY. One tubule was considered positive for a marker if at least one germ cell in the tubule stained clearly positive. All counts were performed by the same observer (M.C.), blinded for age and origin (patient or control) of the sample. The obtained data were graphically depicted using the SPSS statistical system (SPSS, Inc., Chicago, IL).

Determination of ploidy

Ploidy of the CIS samples was determined by fluorescent *in situ* hybridization. Therefore, tissue sections of 5 μm were digested with 0.0005% pepsin (Sigma-Aldrich, Zwijndrecht, The Netherlands) in 0.01 mol/liter HCl for 1 min at 37 C, rinsed in demiwater and dehydrated. The protocol was used according to Hopman *et al.* (24). The following centromeric probes were used: chromosome 1 (pUC1.77), 12 (pα12h8) (25, 26), X (*Bam*H1), and Y (DYZ3). Probes were labeled with biotin-16-deoxyuridine 5-triphosphate (Roche, Mannheim, Germany) using a nick-translation kit (Life Technologies, Inc., Paisley, UK). After denaturation (73 C for 5 min in hybmix), they were preannealed with an excess of Cot-1 DNA (Life Technologies) added to denatured slides (3 min in 70% formamide/2 $*$ saline sodium citrate (pH 7.0), 5 min in 70% ethanol at $-$20C, and dehydrated) and hybridized for 48 h. Slides were washed in 50% formamide/2$\times$ saline sodium citrate. The hybrids were visualized with Cy3-conjugated avidin antibody (1:50; Jackson ImmunoResearch, West Grove, PA).

TABLE 1. Diagnosis of patients with undervirilization syndromes, sex of rearing, and localization of the gonads at gonadectomy

Diagnosis	Genetic defect identified	Sex of rearing	Localization		Age	CIS	
			Abdominal	Inguinal		Abdominal	Inguinal
CAIS	13/15	Female	5	10	18	1/5	0/10
PAIS	3/5	Female	1	4	13	0/1	1/4
17β-HSD deficiency	5/6	Female	0	6	4	0/0	1/6
Leydig cell hypoplasia	1/2	Female	0	2		0/0	0/2
Unknown	0/2	1 Female, 1 male	0	2		0/0	0/2
Total	22/30	29 Female, 1 male	6	24		1/6	2/24

CIS according to diagnosis, age and gonadal localization.

TABLE 2. Schematic representation of origin and protocols used for the different antibodies

	Origin	Dilution	Pretreatment	HIAR	Incubation	Secondary antibody	AB-complex	Chromogen
Primary antibody								
VASA	Kindly provided by Dr. D. H. Castrillon (Department of Pathology, NB6.452, University of Texas, Southwestern Medical Center, Dallas, TX)	1/2000	No	Yes	Overnight, 4 C	SWAR-bio	ABC-AP	New Fuchsine
PLAP	Cell Marque (Hot Springs, AR)	1/200	No	Yes	Overnight, 4 C	RAM-bio	ABC-AP	New Fuchsine
c-KIT	Dako-Cytomation (Glostrup, Denmark)	1/500	No	Yes	Overnight, 4 C	SWAR-bio	ABC-AP	New Fuchsine
TSPY	Kindly provided by Prof. C. Lau (Department of Medicine, VA Medical Center, University of California, San Francisco, CA)	1/3000	No	No	Overnight, 4 C	SWAR-bio	ABC-AP	New Fuchsine
OCT3/4	Santa Cruz Biotechnology (Santa Cruz, CA)	1/1000	H_2O_2 for 5 min	Yes	2 h, RT	HAG-bio	ABC-HRP	DAP
Secondary antibody								
SWAR	Dako-Cytomation	1/200						
RAM	Dako-Cytomation	1/200						
HAG	Vector Laboratories (Burlingame, CA)	1/200						

HIAR, Heat-induced antigen retrieval (38); SWAR-bio, swine antirabbit antibody, biotin labeled; RAM-bio, rabbit antimouse antibody, biotin labeled; HAG-bio, horse antigoat antibody, biotin labeled; AB-complex, streptavidin-biotin complex; ABC-AP, streptavidin-biotin-alkaline phosphatase complex; ABC-HRP, streptavidin-biotin-horseradish peroxidase complex; RT, room temperature; DAP, diaminobenzidine.

Results

General morphology of gonads and germ cells

The gonads of patients exhibited an immature aspect of tubules and cells. Dissociation between epididymis and testis was observed in four samples. Seminiferous tubules were small, compared with controls; branching tubules were encountered in four patients. The degree of peritubular and interstitial fibrosis and thickening of the basal membrane was variable. Apparent Leydig cell hyperplasia was found after puberty (six samples). Sertoli cell-only nodules and nodular hyperplasia of Sertoli cells were encountered in older patients (eight samples).

Germ cells were found in 28 of 30 patients and 50 of 58 gonads. They were very large and irregular of shape, with an abundant pale cytoplasm and a hyperchromatic nucleus. Multinucleated spermatogonia were often encountered. No maturation of germ cells, expected based on age, was observed, except for two pubertal partial androgen insensitivity syndrome (PAIS) patients in whom spermatocytes and spermatids were seen. Spermatozoa were never found. The number of germ cells was high in the youngest patients, but a progressive loss of germ cells was noted with advancing age. This loss presented itself as patchy: certain areas maintained an adequate number of germ cells, whereas in other areas all germ cells had disappeared. Except for the PAIS patients, the end result as could be appreciated in postpubertal patients was a Sertoli-cell only pattern in atrophic tubules throughout the gonad. On pure morphological grounds, an adult CIS pattern was discovered bilaterally in one 18-yr-old CAIS patient. In many other patients, the aberrant appearance of the germ cells raised suspicion about the presence of CIS because their morphology largely met the World Health Organization (WHO) criteria (Table 3). Immunohistochemical staining for OCT3/4, c-KIT, PLAP, TSPY, and VASA, and double staining for the combination OCT3/4-VASA was performed.

Staining results for OCT3/4, c-KIT and PLAP (Fig. 1)

OCT3/4-positive germ cells were found in all the gonads of patients younger than 9 months of age. OCT3/4 was also widely present in a control 3 months old, whereas at 5 and 6 months, only three and two of 500 tubules, respectively, contained an OCT3/4-positive germ cell. Thereafter, no OCT3/4-positive cells were identified in controls. In all but three patients, OCT3/4 expression had disappeared at 9 months: one 4-yr-old patient with 17β-hydroxysteroid dehydrogenase (HSD) deficiency, one 13-yr-old PAIS patient and the CAIS patient 18 yr old in whom CIS was discovered in the hematoxylin-eosin staining. In this patient, OCT3/4-positive cells corresponded with the CIS cells on the hematoxylin-eosin staining. In the former two patients, the morphology of the germ cells was judged as clearly aberrant (see above text). The distribution of the OCT3/4-positive cells throughout the gonad in these three patients was different from the distribution pattern in younger patients and controls: in the former, OCT3/4-positive tubules were confined

TABLE 3. WHO criteria for the diagnosis of CIS (WHO classification of tumors, international agency for research on cancer) (39)

Criteria
• Larger than normal spermatogonia
• Clear or vacuolated cytoplasm rich in glycogen
• Nuclei: large, irregular, hyperchromatic
• Nucleoli: one or more large, irregular
• (Abnormal) mitoses
• Basally located cells
• Spermatogenesis commonly absent
• Segmental involvement of tubules

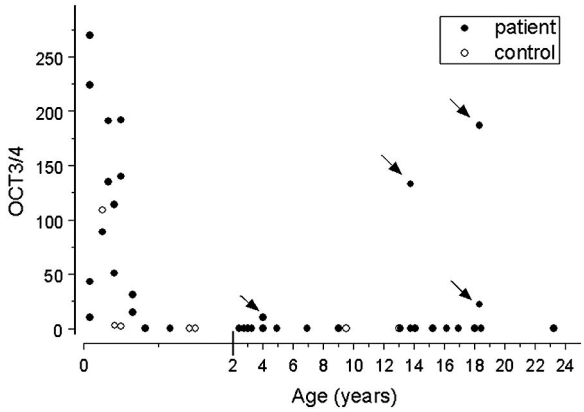

FIG. 1. Staining results for OCT3/4: number of tubules containing at least one OCT3/4-positive germ cell (*y-axis*) according to age (*x-axis*). Note the high and prolonged OCT3/4 expression in patients, compared with controls, during the first year of life. No OCT3/4-positive tubules are found anymore in the control patients above the age of 6 months and the patient population above the age of 8 months, except for the 4-yr-old patient with 17β-HSD deficiency (10 of 500 positive tubules in one gonad), the 14-yr-old PAIS patient (133 of 500 positive tubules in one gonad), and the 18-yr-old CAIS patient (187 of 500 positive tubules in one gonad and 22 of 500 positive tubules in the other gonad) (*arrows*).

to a specific area of the gonad, separated from the unstained areas by interstitial fibrous septa, whereas in young patients and controls, OCT3/4-positive cells were scattered throughout the gonad, without any preference for a certain area (Fig. 2). Also within the tubules, the distribution of OCT3/4-positive cells was different: In young patients and controls, they were found almost exclusively centrally in the tubule, separated from the basal lamina by at least one layer of Sertoli cells. In the 13-yr-old PAIS patient and the 18-yr-old CAIS patient, OCT3/4-positive cells were confined to the basal lamina, whereas in the 4-yr-old patient with 17β-HSD deficiency, OCT3/4-positive cells were equally present centrally and along the basal lamina. This was nicely demonstrated in the OCT3/4-VASA double staining (see text below).

Staining patterns for c-KIT and PLAP were comparable with the OCT3/4 staining, for both timing of disappearance of the marker and localization of positive cells within the tubules, but the color intensity was generally weaker than the OCT3/4 staining.

Staining results for VASA and TSPY (Figs. 3 and 4)

VASA is a general marker for germ cells (13) and thus allowed us to estimate the total number of germ cells throughout time in the study population relative to the controls. In the latter, VASA-positive germ cells were seen in nearly all the tubules at every age. In the study population, the number of tubules expressing VASA was comparable with controls only shortly after birth. From the 10th month onward, a rapid and progressive decline in VASA expression was observed, suggesting a massive loss of germ cells. This loss was manifest in all diagnostic groups, except in the PAIS patients, who maintained about two thirds of their germ cell population, compared with controls at the age of 15 yr.

TSPY stains spermatogonia of adult men (27) and was also observed in prespermatogonia of fetal testes during the second and third trimester of gestation (22). No data exist on the expression of TSPY in prepubertal children. In controls, TSPY expression clearly differed from VASA expression: whereas VASA stained all the germ cells, irrespective of age, TSPY expression was gradually up-regulated and reached the level of VASA expression only around puberty. In patients, however, staining of adjacent slides for TSPY and VASA demonstrated that, as for VASA, TSPY stained all the (remaining) germ cells at every age. Thus, in controls, VASA was expressed at a constant level throughout time, pointing to a continuous presence of germ cells in almost every tubular cross-section, whereas TSPY expression increased gradually. In patients, both TSPY and VASA were highly expressed during the first year of life and showed a parallel decrease thereafter. Interestingly, the intensity of the TSPY staining in the patient population was extremely high, compared with

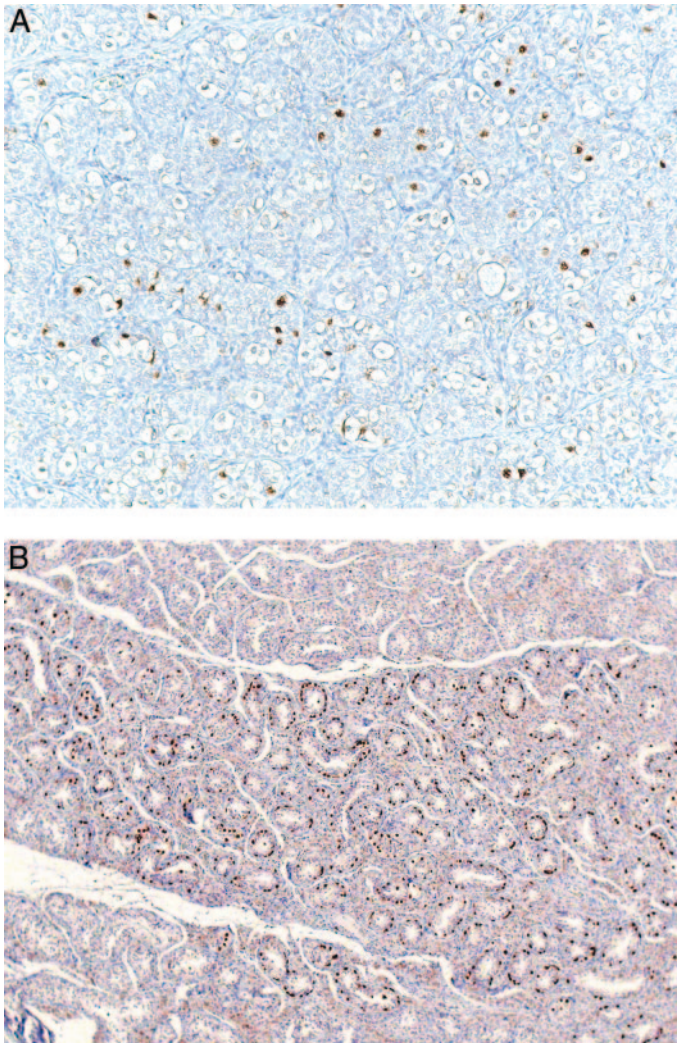

FIG. 2. A, OCT3/4-positive tubules scattered throughout the gonad in a 6-month-old patient with 17β-HSD deficiency. Note the central position of the OCT3/4-positive cells within the tubules (OCT3/4 staining; magnification, ×100). B, OCT3/4-positive tubules in a 13-yr-old PAIS patient. The OCT3/4-positive tubules are confined to one limited area of the gonad, separated from the OCT3/4-negative areas by fibrous septa (OCT3/4 staining; magnification, ×100).

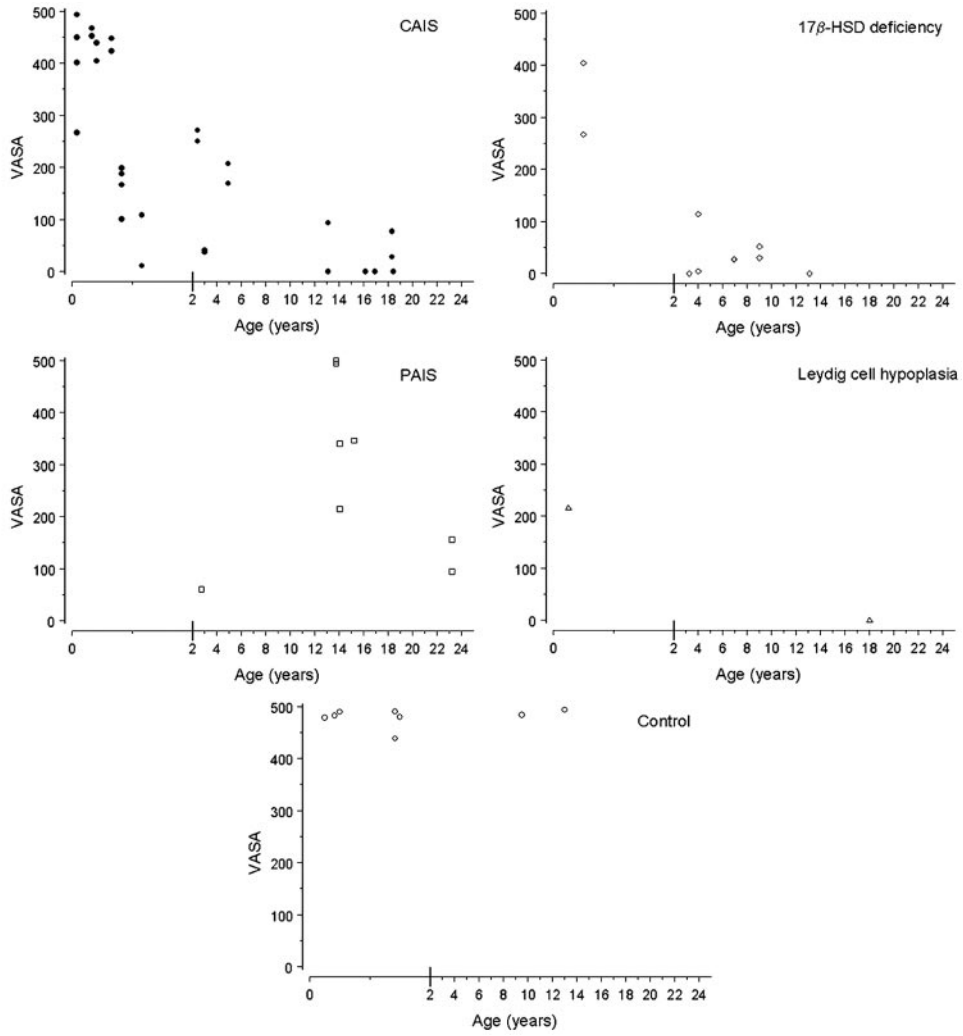

FIG. 3. Staining results for VASA. Note the loss of germ cells around puberty in all the patient groups, except for the PAIS patients and the 18-yr-old CAIS patient with bilateral OCT3/4-positive cells.

controls, a normal adult male testis, and a series of fetal testis published elsewhere (22) (Fig. 5).

OCT3/4-VASA double staining (Fig. 6)

This staining was performed to appreciate the position of OCT3/4-positive germ cells relative to the overall germ cell population and the basal lamina within the tubule.

The 3-month-old control and the patients younger than 9 months of age showed the same staining patterns: OCT3/4-positive cells were almost exclusively located centrally in the tubule, whereas VASA-positive cells lined up along the basal lamina. OCT3/4 positivity excluded VASA positivity in the same germ cell. The 13-yr-old PAIS patient and the 18-yr-old CAIS patient showed a different distribution pattern with OCT3/4-positive cells lying almost exclusively on the basal lamina. VASA expression was lost in tubules expressing OCT3/4. The 4-yr-old patient with 17β-HSD deficiency showed an intermediate pattern: OCT3/4-positive cells were found equally in the tubular center and on the basal lamina. Some of these germ cells coexpressed VASA, and VASA-positive-OCT3/4-negative cells were also seen within the same tubule.

Results of ploidy determination

The precursor CIS lesions of seminomas and nonseminomas are aneuploid (28, 29). To evaluate the ploidy of OCT3/4-positive cells in the 13-yr-old PAIS patient and the 18-yr-old CAIS patient, fluorescent *in situ* hybridization analysis with centromeric probes for chromosomes 1, 12, X, and Y was performed. In these patients, the OCT3/4-positive regions were found to be diploid.

Discussion

We studied 58 gonadectomy and gonadal biopsy samples in 30 patients with undervirilization syndromes to investigate the relationship between maturation delay and progression to CIS. To our knowledge, this represents the largest series ever published. Despite a certain heterogeneity in clinical diagnoses, we consider the group of undervirilization

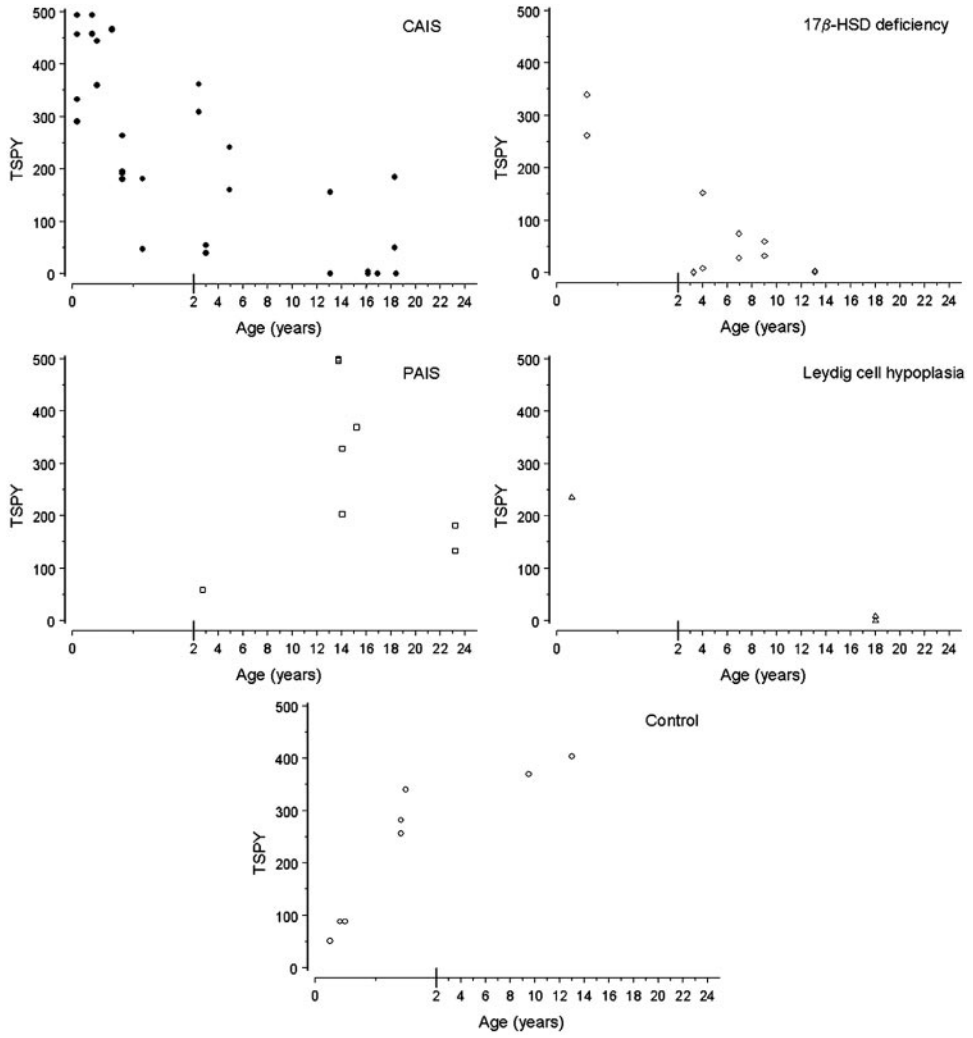

FIG. 4. Staining results for TSPY. Note the analogous pattern of germ cell loss for the different patient groups, compared with VASA, but the increase in TSPY expression in the controls, which is different from the constant VASA expression in this group.

syndromes as homogenous, taking into account the normal embryonic gonadal development and the common end result (a defective or absent action of androgens on the end organs) that characterizes these syndromes and opposes them to the gonadal dysgenesis syndromes. An important variable that we were not able to correct for is the localization of the gonads, which was abdominal in six of 30 patients (20%) and inguinal in 24 of 30 patients (80%). However, the finding that two of three CIS lesions were detected in inguinal gonads underscores the malignant potential of partially descended testes (Table 1).

The general morphology of gonads and germ cells in our patient series did not differ from previous findings (8, 9, 18), but the timing and pattern of germ cell loss are described systematically for the first time. The cryptorchid position of the gonads in our study population certainly accounts for this loss of germ cells, alone or in combination with other pathogenetic mechanisms relative to the underlying disease.

OCT3/4, PLAP, and c-KIT are well-established markers for the diagnosis of CIS and invasive germ cell tumors in adults (14–16). They are also used to detect CIS in dysgenetic gonads of young children (17, 18) and fetal gonads (19). However, the expression of these markers is not limited to malignant conditions. They have been demonstrated in the developing fetal testes, only to disappear well beyond birth (22), whereas TSPY and VASA are expressed throughout life (13, 27). High and prolonged expression of c-KIT, OCT3/4, and PLAP has been observed in dysgenetic gonads and gonads of patients with chromosomal anomalies and has been related to a degree of maturation delay (20, 21, 30) (Cools, M., F. Honecker, H. Stoop, J. Veltman, R. De Krijger, E. Steyerberg, K. Wolffenbuttel, S. L. S. Drop, and L. H. J. Looijenga, submitted for publication). This condition itself predisposes germ cells to malignancy (23).

OCT3/4 is a transcription factor, normally expressed in embryonic stem cells and embryonic germ cells, and is essential in regulating pluripotency and differentiation (14). Recently a specific role of OCT3/4 for the survival of migratory primordial germ cells has been demonstrated, preventing them from premature apoptosis (31). Moreover, OCT3/4 has been shown to be consistently present in various germ cell tumors, including CIS and gonadoblastoma (14,

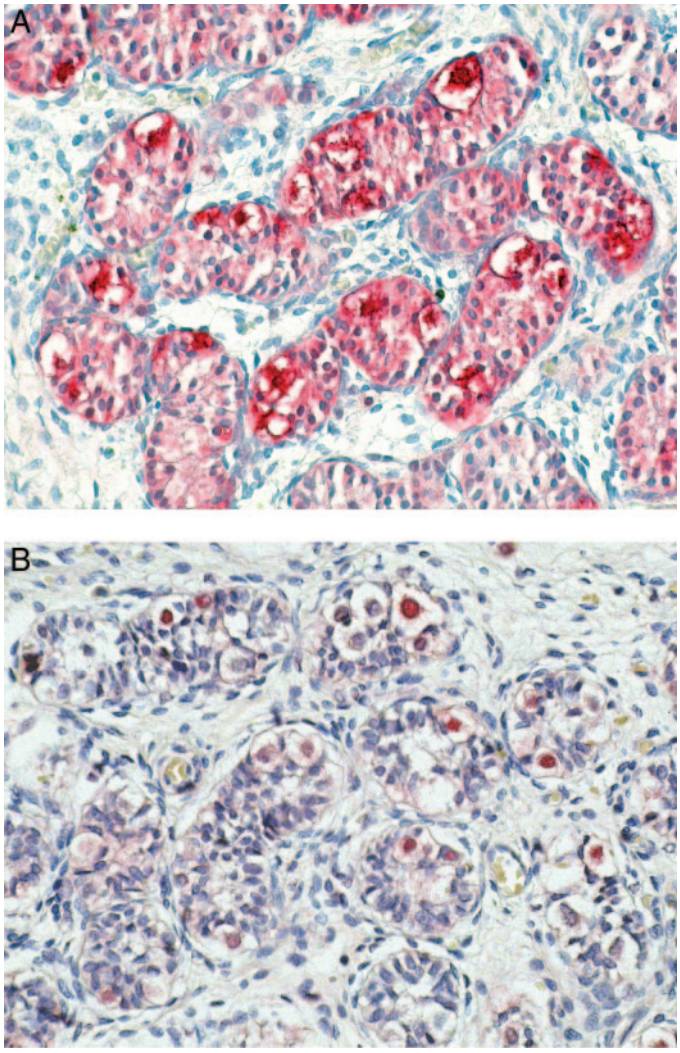

FIG. 5. TSPY staining (magnification, ×400). Abundant TSPY expression in a 10-month-old CAIS patient (A), compared with a control, 18 months of age (B).

32). By modulating the level of OCT3/4 expression *in vitro* in mice embryonic stem cell-derived tumors, the malignant phenotype of the tumor cells could be changed, suggesting that OCT3/4 is of pathogenetic relevance in the development of these tumors (33).

In our series, patients younger than 9 months of age showed a prolonged and higher OCT3/4 expression level, compared with controls. This is in line with previously published data in which OCT3/4 was detected in the gonads of a 9-month-old AIS patient but disappeared in older patients (20). OCT3/4 expression was never seen in patients older than 9 months, except for three cases. In the two oldest, in contrast to younger patients and controls, OCT3/4-positive cells were limited to one specific region of the gonad, in which the germ cells homogenously stained positive for this marker and did not express VASA. Within the tubule, OCT3/4-positive cells stuck to the basal lamina, a pattern that is generally found in CIS. The normal central position of OCT3/4-positive cells in the developing tubules, because it was encountered in young patients and controls, was de-

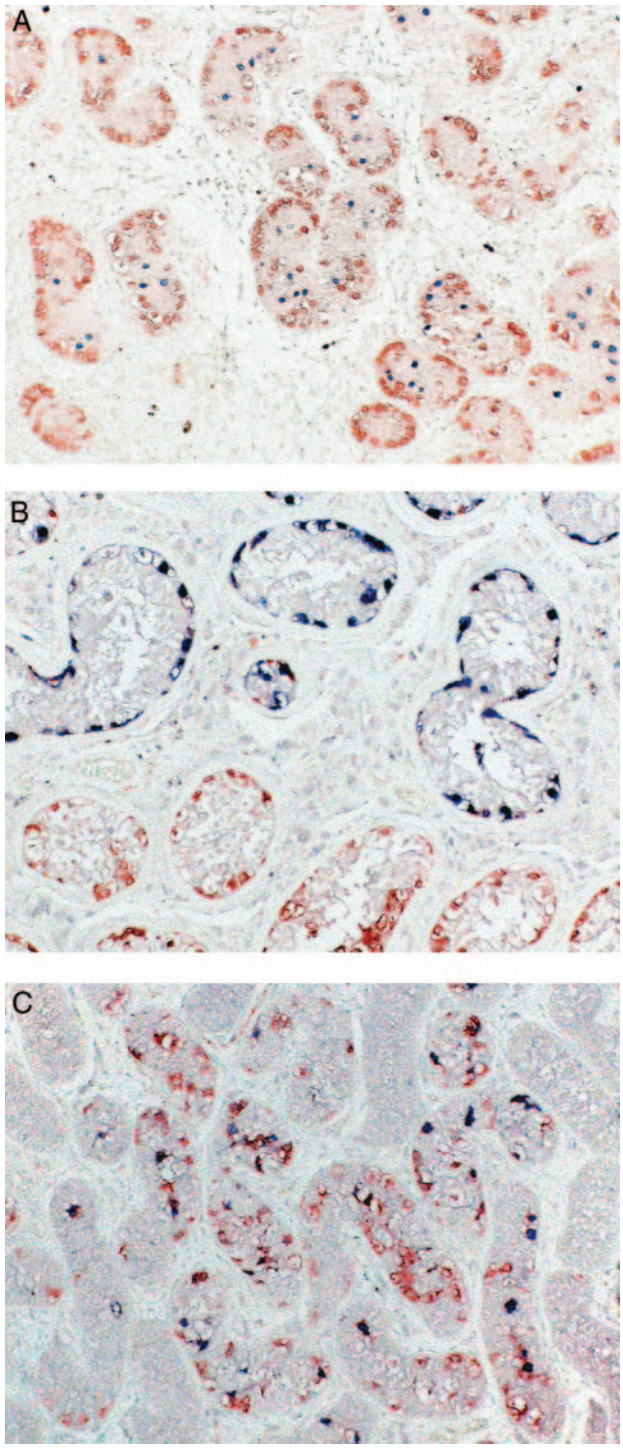

FIG. 6. A, OCT3/4 (*blue*)-VASA (*red*) double staining (×200) in a 1-month-old CAIS patient. OCT3/4-positive cells are separated from the basal lamina by at least one layer of Sertoli cells; VASA-positive cells are mainly found on the basal lamina. B, OCT3/4 (*blue*)-VASA (red) double staining (×200) in a 13-yr-old PAIS patient. OCT3/4-positive cells are found almost exclusively along the basal lamina. Tubules expressing OCT3/4 have almost completely lost VASA expression. C, OCT3/4 (*blue*)-VASA (*red*) double staining (×200) in a 4-yr-old 17β-HSD deficiency patient. OCT3/4-positive cells are found centrally in the tubule and along the basal lamina. VASA expression is maintained in OCT3/4-positive tubules. Some germ cells coexpress OCT3/4 and VASA.

scribed previously, and it was suggested that by reaching the basal lamina, early germ cells lose their pluripotency and start to differentiate (22). An intermediate pattern was found in one of six patients with 17β-HSD deficiency, 4 yr of age: OCT3/4-positive germ cells were confined to a specific region of the gonad, but within the tubule, they were found both in a peripheral and a central position. Tubules with OCT3/4-positive cells also expressed VASA, sometimes showing coexpression of these two markers in the same cell. The remaining five patients with 17β-HSD deficiency did not differ from other patients in our series, suggesting that this group does not represent a separate entity.

Thus, a possible pathogenetic mechanism for the development of germ cell neoplasia emerges: the central OCT3/4-positive germ cells, scattered throughout the gonad are delayed in maturation. By moving toward the basal lamina, they will lose their pluripotency and eventually start to differentiate. If this process does not occur in one OCT3/4-positive cell and the cell is not removed by an apoptotic or other mechanism, clonal expansion of this pluripotent cell may lead to the CIS pattern as it was encountered in the 4-yr-old patient, and at a later age to the typical CIS pattern as it is seen in adults. The expression of PLAP and c-KIT was similar to OCT3/4 but showed less consistency. Therefore, we conclude that OCT3/4 is the best marker to describe maturation delay and CIS in patients with undervirilization syndromes. Age of the patient, distribution of OCT3/4-positive cells throughout the gonad, and staining pattern within the tubule seem to be useful additional tools to differentiate between maturation delay and CIS in the young patient. Our results suggest that the presence of germ cells positive for OCT3/4, PLAP, or c-KIT in patients younger than 1 yr is in accordance with the expected maturation delay in this patient group and is thereby insufficient for the diagnosis of CIS. However, the relevance and applicability of these tools in larger patient series and other diagnostic groups must be tested before they can be widely accepted as essential diagnostic criteria.

An intriguing finding in our patient population and similar to the findings of Schnieders *et al.* (27) was the abnormal and abundant expression pattern of TSPY. The TSPY gene maps to the short arm of the Y chromosome, close to the centromeric region, in which it is highly repeated. Although its function is not fully clarified, it is thought to regulate the mitotic proliferation of spermatogonia (27). The expression of TSPY in dysgenetic gonads has been related to the development of gonadoblastoma (32, 34). It is tempting to speculate that TSPY expression is up-regulated in germ cells residing in an unfavorable environment in an attempt to survive and proliferate. The combination of maturation delay, prolonged expression of OCT3/4, and abundant TSPY expression can provide the surviving germ cell with an important proliferative advantage, rapidly leading to clonal expansion and the development of CIS.

The CIS cells in the 18-yr-old CAIS patient and the 13-yr-old PAIS patient were diploid. However, polyploidization is known to be an early event in the development of CIS in the adult testis (28, 29, 35). On the other hand, gonadoblastomas arising in dysgenetic gonads are often found to be diploid (28, 36), although aneuploidy was also described (37). It is unclear so far whether polyploidization did not take place yet in our patients, thereby representing a very early CIS stage, or alternatively, whether polyploidization is not essential in the development of CIS in the gonads of undervirilized patients.

In conclusion, we discovered CIS lesions in three of 30 patients (10%) and four of 58 gonadectomy or biopsy specimens (6.9%). Morphological criteria for the detection of CIS in adult patients and the classic use of immunohistochemical markers were insufficient to describe CIS in our patients. Based on our results, patient age, distribution pattern of OCT3/4-positive cells throughout the gonad, and their position within the seminiferous tubule can additionally be used to distinguish maturation delay from CIS in young patients with undervirilization syndromes. However, larger patient series and extension of these tools to other situations that are characterized by gonadal maturation delay and increased risk for germ cell neoplasia are essential before they can be accepted as essential criteria for the diagnosis of CIS in young patients.

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