Malignant Germ Cell Tumors and Disorders of Sex Development: Towards Clinical Implication

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The research described in this thesis was done at the Department of Pathology, Erasmus Medical Center, Josephine Nefkens Institute, Daniel den Hoed Cancer Center Rotterdam, The Netherlands.

The research was made possible by a research fellowship grant from the European Society for Pediatric Endocrinology (ESPE), sponsored by Novo Nordisk A/S.

ISBN: 978-94-6299-071-5
Cover: Ridderprint BV, the Netherlands
Printed by: Ridderprint BV, Ridderkerk, the Netherlands

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Maligne kiemceltumoren en stoornissen in de seks ontwikkeling: Klinische implicaties

Proefschrift

ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam

op gezag van de rector magnificus Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 6 mei 2015 om 13.30 uur

door

Jana Kaprová - Pleskačová

geboren te Praag, Tsjechische Republiek

Erasmus University Rotterdam

Erasmus

Promotiecommissie:

Promotor:	Prof.dr. L.H.J. Looijenga
Overige leden:	Prof.dr. C.H. Bangma Prof.dr. C.M.A. van Raavenswaaij-Arts Prof.dr. F.J. van Kemenade

“Equipped with his five sences,
man explores the universe around him
and calls the adventure science.”

E.P. Hubble

Mým rodičům Evě a Jiřímu
Robertovi a Toníkovi

Contents

Chapter 1

General introduction

Introduction

Year 2005 represents a milestone in the history of Disorders of Sex Development (DSD). In that year, 50 specialists recruited from ESPE (European Society for Pediatric Endocrinology) and LWPES (Lawson Wilkins Pediatric Endocrinology Society) proposed changes in terminology and created a new classification of the Disorders. The act known as Chicago Consensus symbolizes an increasing interest in these rare yet important conditions [1-4].

DSD cover a group of rare to very rare inborn disorders with remarkably heterogeneous etiology that are defined as congenital conditions in which chromosomal, gonadal or anatomical sex is atypical [1]. According to the new classification, they are divided into three basic subgroups respecting karyotype: 1) Disorders caused by aberrations of sex chromosomes, 2) Disorders with normal male karyotype (46,XY DSD), and 3) Disorders with normal female karyotype (46,XX DSD) [1-4]. For more details see Table 1.

Vast part of the disorders is connected not only with anomalies of the genital system but also with other health problems such as congenital defects of other organs, increased risk of autoimmune diseases and tumor development, mental retardation, etc. [5-16] Therefore, every individual with DSD should be provided with care of a well trained multidisciplinary team consisting of (pediatric) endocrinologist, surgeon/urologist, gynecologist, geneticist, psychologist and other specialists when necessary.

DSD offer many interesting issues from both clinical and scientific point of view. These embrace among others revealing yet unknown etiology of monogenic disorders (cause remains unclear in an important number of cases) [17-19], improving techniques of genital surgery and their timing [20,21], evaluating long term outcome of the treatment and quality of life [22,23], optimizing hormonal therapy [24], searching for fertility options [25], and last but not least increasing the accuracy of diagnostics and prediction of gonadal germ cell cancer that may develop in up to 15-40% of patients in selected disorders [26,27]. The later is a subject matter of the thesis.

Table 1: Classification of disorders of sex development (DSD) according to Chicago consensus 2005 (adopted from Hughes *et al.* 2006).

DSD WITH DISORDERS OF SEX CHROMOSOMES	46,XY DSD	46,XX DSD
A. 47, XXY (Klinefelter syndrome and variants)	**A. Disorders of testicular development**	**A. Disorders of ovarian development**
	1. **Complete or partial gonadal dysgenesis** (e.g. *SRY,SOX9, SF1, WT1, DHH,* etc.)	1. **Gonadal dysgenesis**
	2. **Ovotesticular DSD**	2. **Ovotesticular DSD**
	3. **Testicular regression**	3. **Testicular DSD** (e.g. *SRY+, dup SOX9, RSPO1, WNT4*)
B. 45,X (Turner syndrome and variants)	**B. Disorders of androgen synthesis and action**	**B. Excess of androgens**
	1. Disorders of synthesis - mutation of LH receptor - Smith-Lemli-Opitz sy - mutation of StAR (Steroid acute regulatory protein) - mutation of enzyme cleaving side-chain of cholesterol (*CYP11A1*) - mutation of 3β-hydroxysteroid dehydrogenase 2 (*HSD3B2*) - mutation of 17α-hydroxylase/ 17,20-lyase (*CYP17*) - mutation of p450 Oxidoreductase (*POR*) - mutation 17β-hydroxysteroid dehydrogenase (*HSD17B3*) - mutation 5α-reductase 2 (*SRD5A2*) - ect.	**1. Fetal origin** - mutation of 3β-hydroxysteroid dehydrogenase 2 (*HSD3B2*) - mutation of 21-hydroxylase (*CYP21A2*) - mutation of p450 oxidoreductase (*POR*) - mutation of 11β-hydroxylase (*CYP11B1*) - mutation of glucocorticoid receptor - etc.
	2. Disorders of action - androgene insensitivity syndrome (*AR*) - drugs and environmental disruptors influencing the sex development	**2. Fetoplacental origin** - mutation of aromatase (*CYP 19*) - mutation of oxidoreductase (*POR*)
		3. Maternal origin - tumors with androgen production - drugs with androgen action
C. 45,X/46,XY (Mixed gonadal dysgenesis)	**C. Others**	**C. Others**
	1. Disorder as a part of a syndrome (e.g. cloacal anomalies, Robinow sy, Aarskog sy, Hand-Foot-Genitalia sy, atd.)	**1. Disorder as a part of a syndrome** (e.g. cloacal anomalies)
	2. Syndrome of persisting müllerian ducts	2. **Agenesis of müllerian duct structures**
	3. **Vanishing testis syndrome**	3. **Uterus anomalies** (e.g. MODY5)
	4. **Isolated hypospadia**	4. **Vaginal atresia**
	5. **Congenital hypogonadotropic hypogonadism**	5. **Labial adhesions**
	6. **Cryptorchidism**	
	7. **Environmental influences**	
D. 46,XX/46,XY (Chimerism)		

Germ cell tumors: classification and terminology

Germ cell tumors (GCT) constitute a group of neoplasms that originate from germ cells and may occur throughout the whole life, from neonatal period to the old age. They are classified into five groups according to their origin and biological characteristics (Table 2) [28]. The thesis will be focused on so called Type II GCT, also referred to as germ cell cancer (GCC), that most frequently reside in gonads and account for the most prevalent solid tumors in Caucasian men between 20 and 40 years of age [29]. Lifetime risk for GCC development reaches 1% in the Danish men, in whom the prevalence is the highest [30,31]. However, the occurrence of the Type II GCT is even more frequent in DSD patients with a specific part of Y chromosome in their karyotype (to be discussed hereunder) [26, 32].

Table 2: Classification of germ cell tumors (adopted from Oosterhuis and Looijenga, 2005).

Type	Anatomical site	Phenotype	Age	Originating cell	Genomic imprinting	Genotype
I	testis/ovary/ sacral region/ retroperito-neum/ mediastinum/ neck/middle brain/ other rare sites	(Immature) teratoma yolk-sac tumor	Neonates and children	Early primordial germ cell/ gonocyte	Biparental, partially erased	Diploid (teratoma) Aneuploid (yolk-sac tumor): gain of 1q, 12(p 13) and 20q; loss of 1p,4 and 6q
II	Testis Ovary Dysgenetic gonad Mediastinum Middle brain	Seminoma/ non-seminoma Dysgerminoma/ non-dysgerminoma Dysgerminoma/ non-dysgerminoma Seminoma/ non-seminoma Germinoma/ non-germinoma	> 15 years > 4 years Congenital Adolescents Children	Primordial germ cell/ gonocyte	Erased	Aneuploid (+/- triploid): gain of X, 7, 8, 12p, 21; loss of Y, 1p, 11, 13, 18 Aneuploid Diploid/tetraploid Diploid/tri-tetraploid Diploid/tri-tetraploid
III	Testis	Spermatocytic seminoma	> 50 years	Spermato-gonium/ spermato-cyte	Partially complete maternal	Aneuploid: gain of 9
IV	Ovary	Dermoid cyst	Children/ adults	Oogonia/ oocyte	Partially complete maternal	(Near) diploid/ diploid/ tetraploid (gain of X,7,12,15)
V	Placenta/ uterus	Hydatiform mole	Fertile period	Empty ovum/ spermatozoa	Completely paternal	Diploid (XX and XY)

Type II germ cell tumors of the testis are histologically and clinically divided into seminoma and non-seminomas (i.e. embryonal carcinoma, yolk sac tumor, choriocarcinoma, and teratoma); their counterparts in dysgenetic gonad are termed

as dysgerminoma and non-dysgerminomas [26,28]. Carcinoma *in situ* (CIS, also intratubular germ cell neoplasia unclassified, IGCNU) is a germ cell non-invasive tumor precursor in the testis whereas gonadoblastoma represents the non-invasive lesion in dysgenetic gonad [26,28,33,34]. Concerning the terminology of non-invasive testicular precursor, IGCNU is stated in WHO classification [31]; CIS is a historical term that is, however, still widely used, especially in European literature [35]. Because chapters 2 to 6 are based on manuscripts published in or submitted to both American and European journals, IGCNU as well as CIS are to be cited.

Germ cell tumors: etiology

Despite the significant effort, etiology of GCC has not been fully elucidated. Nevertheless, co-existence of environmental and genetic factors is suspected from multiple studies performed on both animals and humans [36-43]. Similar morphology, expression profile and epigenetic status of neoplastic germ cells and early fetal germ cells as well as relatively young age of patients suffering from GCC (mean age 25-35 years in general population, and even early childhood in some DSD individuals) suggest that tumorous germ cells arise from fetal germ cells arrested in an early developmental stage [26,28,44-50]. Environmental pollutants in combination with genetic changes (SNPs, chromosomal aberrations, gene mutations) supposedly contribute to an impairment of the microenvironment of fetal gonad (testis). Survival of immature germ cells with malignant potential, disturbed spermatogenesis, disrupted testicular descent, and insufficient masculinization of external genitalia (e.g. hypospadias) represent possible consequences of the situation (for overview see Figure 1) [36,38]. Niels Skakkebaek proposed an umbrella term Testicular Dysgenesis Syndrome (TDS) that encompasses the above listed phenomena which frequently coincide in a single individual [51,52]. In fact, DSD cases could be considered as an extreme form of TDS with strong genetic background.

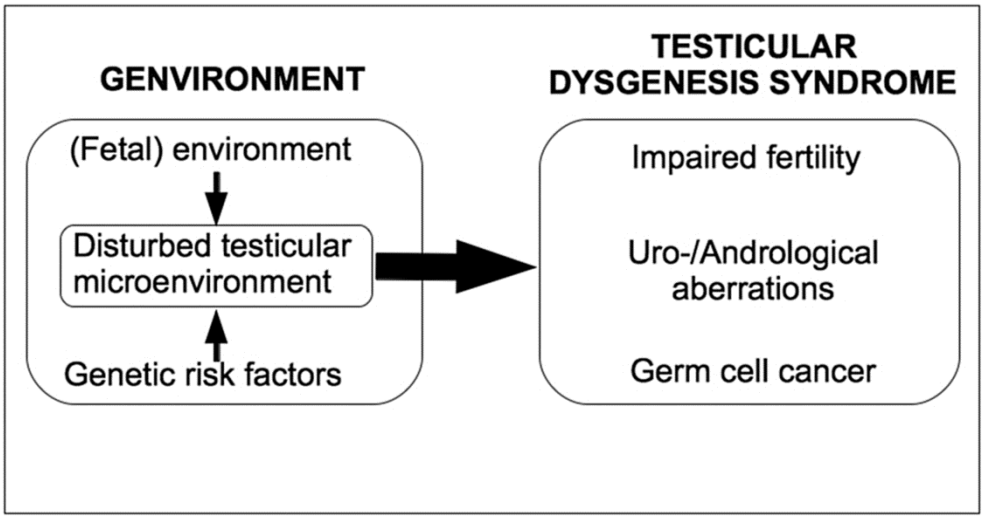

Figure 1: Genvironmental hypothesis of gonadal GCC (adopted from Rijlaarsdam and Looijenga 2014).

Germ cell tumors: diagnostics

Suspicion of gonadal tumor is usually based on finding of visible gonadal swelling or sometimes on symptoms caused by compression of neighboring organs if the gonad is not situated in the scrotum. Less frequently, disease presents through symptoms caused by metastatic progress. Imaging methods are only supportive in case of GCC and final diagnosis is made in a bioptical sample on a histological basis [31,53]. Several non-invasive screening methods are under development. Historically oldest measurement of serum levels of Alpha-fetoprotein (AFP) or human chorionic gonadotropin (hCG) is not sensitive enough for detection of germ cell neoplasia in general, however, elevated values indicate that at least a part of the tumor is very likely constituted of yolk sac tumor or choriocarcinoma, respectively [54]. Immunohistochemical detection of neoplastic cells in semen and analysis of GCC specific microRNAs in serum are available only in several centers and still need further refinement [55,56].

Recently, an important improvement has been achieved in histological diagnostics of germ cell tumors. Assessment of overall and cellular morphology may be further supported by immunohistochemical analysis of germ cell tumor markers [57,58]. Range of these significantly widened during the past decade. Detection of PLAP (Placental alkaline phosphatase) [59] is being gradually replaced by detection of

OCT3/4 (Octamer-binding Transcription Factor 3/4, other term POU5F1 is used in Chapter 3 and 6) that is expressed in pluripotent neoplastic germ cells (i.e. non-invasive lesions, seminoma, and embryonal carcinoma) [60-62]. Several other markers with similar use (c-KIT, NANOG, AP-2gamma) were also introduced [63-66]. Presence of SOX2 or SOX17 may be supportive for diagnosis of embryonal carcinoma and seminoma, respectively [67]. KITLG (c-KIT ligand, also Stem cell factor, SCF) occupies a unique position among other markers distinguishing between gonadal tissue containing neoplastic germ cells and tissue with germ cells delayed in maturation [68]. This is helpful especially in patients with DSD in whom both delayed development and early malignant changes (pre-stage of CIS/IGCNU) of germ cells are frequently present [69,70]. Details about the markers and their relation to germ cell tumor pathogenesis with an emphasis on situation in DSD are further described in Chapter 2.

Germ cell tumors and DSD

Existence of noticeable relationship between DSD with (a part of) Y chromosome in the karyotype and high risk of GCC development has been known for several decades. During the "pioneer era" in this field, retrospective cohort studies and case reports of gonadal tumors in both adult and children were published [71-73]. Subsequently, awareness of the tumor risk led to a(n almost) generalized implementation of prophylactic gonadectomy in DSD patients with Y chromosome. Thus, later publications have been dealing with series of pediatric patients who underwent preventive gonadectomy and in whom rather non-invasive tumor precursors than invasive cancer were observed [69,70,74-78].

Interestingly, Kolesinska et al. recently described a clearly rising tendency of male gender assignment in individuals with remarkable masculinization of external genitalia (median external masculinization score 6/12), especially during last decade [79,80]. This trend is linked to gradual changes in management of patients assigned as males. Indeed, prophylactic gonadectomy prevents them from inner sex hormones production and in some cases even from fertility. Therefore, more conservative approach is currently undertaken [81].

Generally, there exist several facts which allow us to spare gonads or at least to postpone the time of gonadectomy after the end of pubertal development in selected

patients. First, only patients bearing a specific area around centromere of Y chromosome (so called GonadoBlastoma locus on the Y, GBY) in the karyotype are endangered [26,32,82]. Second, meta-analyses of numerous studies suggest that level of risk differs significantly between different disorders (range 0.8-60%) [26,83-85]. Third, (histological) diagnostics of germ cell cancer, germ cell non-invasive neoplasia (CIS/IGCNU), and even of its pre-stage has amazingly improved during the last decade [62,68,86]. However, there is still a space for further refinement of prediction of the tumor risk in individual patients. This may be realized through several different actions indicated below.

Patients with 46,XY gonadal dysgenesis in general bear one of the highest tumor risks (app. 30%), however there are some hints of differences existing between patients with diverse molecular background. For example, patients with mutations in genes involved in early gonadal formation (i.e. SRY, WT1) seem to be highly endangered [87-89]. As mentioned above, cause of the disorder in many patients with 46,XY gonadal dysgenesis is currently not identified [17]. Increase of clarified cases would enable us to group the patients and supposedly to calculate tumor risk for particular mutated genes. With respect to very low frequency of the disorders, international cooperation is indispensable to achieve enough large series of patients.

Current tools of immunohistochemical analysis allow us to reveal not only a presence of non-invasive tumor precursors, but even a pre-stage of them (further termed as pre-CIS/pre-IGCNU) which is believed to be a developmental step between delayed maturation and non-invasive neoplasia [86]. Based on the comparison of incidence of CIS/IGCNU in men after sudden death and overall occurrence of GCC, it is suggested that CIS/IGCNU eventually progresses to an invasive tumor in 100% of cases in general population [53,90]. Whether this is true also in DSD patients remains unclear as well as a rate of further malignant progression of pre-CIS/pre-IGCNU or of germ cells with delayed maturation. To elucidate this, prospective studies on sufficiently large series of patients are needed.

Whereas several clinical predictors of increased risk of GCC development (e.g. personal or family history of GCC, sub/infertility, cryptorchidism, hypospadias, and testicular microlithiasis) are known in general population [91-97], clinical features which would either positively or negatively correlate with GCC development practically do not exist in DSD patients. Determination of such indicators in this group of disorders would enable us to target the patients at risk more precisely.

Aims of the thesis

Using currently available and well established histological methods and comparing the results with clinical data, the thesis aims to search for factors connected to GCC development in selected types of DSD and thus to widen our possibilities in diagnostics and management of patients endangered by gonadal germ cell malignancy as indicated previously.

Chapter 2 reviews current knowledge of the relationship between DSD and GCC with an emphasis on GCC development and actual immunohistochemical possibilities in diagnostics. This information is applied in subsequent parts.

Chapter 3 deals with individuals with complete form of androgen insensitivity syndrome (CAIS). Two factors which may possibly influence tumor development, i.e. gonadal position and level of androgen receptor (AR) activity, are tested in this group of patients.

Chapter 4 is targeted on individuals with 45,X/46,XY gonadal dysgenesis. We hypothesized that level of virilization of external genitalia may reflect severity of gonadal (testicular) dysgenesis and therefore also survival of germ cells and their tendency to malignization.

Chapter 5 represents a case report of an infant with 45,X/46,X,psu dic(Y) mosaicism in whom both cell lines had a clear impact on final phenotype including a predisposition for GCC development. This case also illustrates a complexity of DSD topic.

Chapter 6 aims to test a hypothetic association between KITLG staining pattern and number of abnormal germ cells in testicular tissue of DSD patients. Results of the analysis may possibly allow us to propose criteria for delayed maturation and pre-CIS/pre-IGCNU based on OCT3/4 staining when compared with results obtained from fetal gonads.

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Chapter 2

Tumor risk in disorders of sex development (DSD)

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Sex Dev 2010;4(4-5):259-69.

Abstract

Certain patients with Disorders of Sex Development (DSD), who bear Y chromosome material in their karyotype, are at increased risk for development of gonadal tumors. The majority of malignancies in these patients is represented by so called type II germ cell tumors (GCT), which arise from early fetal germ cells. DSD gonads frequently harbor immature germ cells which express early fetal germ cell markers. Some of them (e.g. OCT3/4 and NANOG) seem to be of pathogenetic relevance in GCT development providing cells with the ability of pluripotency, proliferation and apoptosis suppression. Also TSPY (Testis-Specific Protein Y-encoded), the main candidate for the so-called gonadoblastoma locus on Y chromosome, is overexpressed in germ cells of DSD patients and possibly contributes to their survival and proliferation. Nowadays, the use of immunohistochemical methods is highly relevant in identifying DSD gonads at risk. The risk for GCT development varies. While the prevalence of GCT is 15% in patients with partial androgen insensitivity syndrome, it may reach more than 30% in patients with gonadal dysgenesis. Patients with complete androgen insensitivity syndrome and ovotesticular DSD develop malignancies in 0.8% and 2.6% of cases, respectively. However, these data may be biased for various reasons. To better estimate the risk in individual groups of DSD, further investigations on large patient series are needed.

Introduction

Disorders of sex development (DSD) are defined as congenital conditions in which development of chromosomal, gonadal or anatomical sex is atypical [1]. The overall occurrence is estimated to be one in 4,500 life births [1]. DSD represent a group of disorders with heterogeneous genetic background bringing a need of a uniform classification. Several attempts have been made in the past. The very last proposed classification is a result of consensus made by members of European Society for Pediatric Endocrinology/Lawson Wilkins Pediatric Endocrinology Society (ESPE/LWPES) in Chicago in 2005 [1].

Nowadays, every DSD patient should be provided with care of a skilled multidisciplinary team. Only under these circumstences, all specific issues of the different aspects of DSD are covered. Among these the most pronounced are sex assignment, psychological support, hormonal substitution, and reconstruction of external genitalia. In addition, in specific subgroups of DSD patients, namely those bearing Y chromosome material in their karyotype, prophylactic gonadectomy may be indicated due to an increased risk of gonadal tumor development [1-3]. Gonadectomy is an invasive and psychologically stressing procedure and may in some cases prevent the spontaneous course of puberty [4]. The reason for increased tumor risk in DSD, the actual risk in the various subgroups and how to identify gonads at risk at an early stage are the topics of this review.

Gonadal tumors in DSD patients

The overwhelming majority of gonadal neoplasias in DSD patients is represented by so called type II germ cell tumors (GCT), namely classic seminoma in the testis and its counterpart dysgerminoma in the dysgenetic gonad, as well as various types of nonseminoma/nondysgerminoma [4,5]. Stromal cell tumors and epithelial tumors may also develop, although these are very rare [6]. In this review we will focus on the most frequent tumors, i.e. seminoma/nonseminoma, dysgerminoma/nondysgerminoma and their non-invasive precursors, carcinoma in situ (CIS) [7] and gonadoblastoma (GB), respectively [8].

Based on their noticeable similarity in morphology and protein expression, it was suggested that the neoplastic cells (CIS and GB) are derived from fetal germ cells (primordial germ cells/gonocytes) arrested in an early stage of development [9-11].

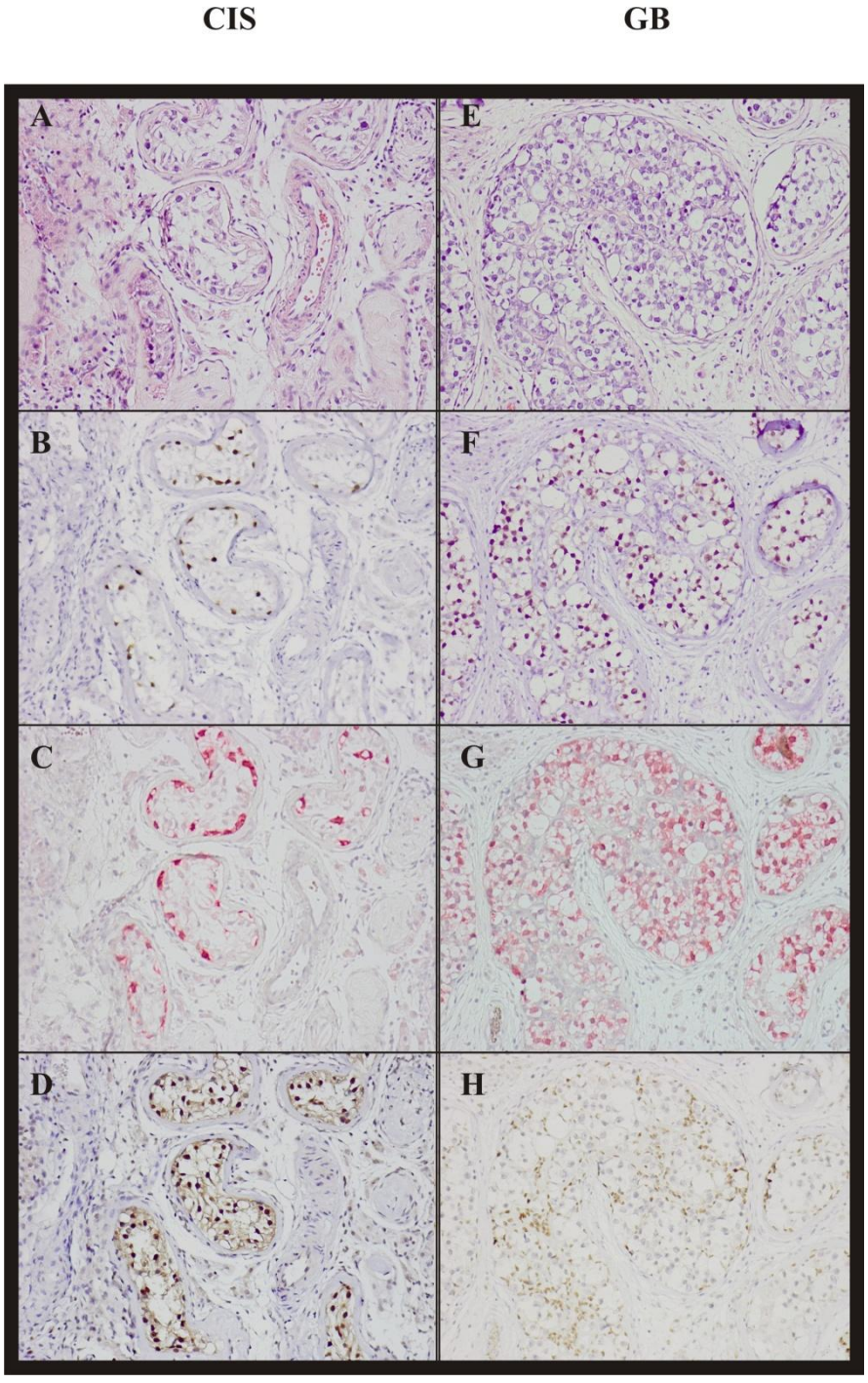

Figure1: Carcinoma *in situ* (CIS) and gonadoblastoma (GB), 100x magnification. A - CIS - HE staining; B - CIS - OCT3/4 positive malignant cells; C - CIS - TSPY positive (malignant) germ cells; D - CIS - SOX9 positive supporting (Sertoli) cells; E - GB - HE staining GB; F - OCT3/4 positive malignant cells; G - GB – TSPY positive (malignant) germ cells; H - GB - FOXL2 positive supporting (granulosa) cells.

While CIS is made up of neoplastic cells located within the context of seminiferous tubules, GB is composed of a mixture of different cell types including malignant germ cells and supporting (granulosa) cells arranged in well circumscribed nests (see Figure 1) [12]. Therefore, it was initially hypothesized that CIS and GB were 2 basically distinct entities. However, immunohistochemical profiling of both lesions indicates a tight similarity of their malignant cells. The difference in overall morphology may be only a result of a different gonadal microenvironment (see below for further discussion). Moreover, the theory of a common origin of CIS and GB is also supported by the resemblance of the subsequent invasive tumors, seminoma and dysgerminoma respectively, in morphology, mRNA expression and miRNA profiling [12-15].

Normal gonadal and germ cell development

To explain the relationship between DSD and GCT development more precisely, it is necessary to understand specific basic issues about the development of gonadal tissue and germ cells themselves. Our knowledge in this field is mainly based on research in mice but is, with some exceptions (see below), also applicable to humans.

Primordial germ cells (PGC) arise from embryonic stem cells (ESC) and can be detected in humans for the first time at 5-6 weeks of gestation in the yolk sac as they stain positive for PLAP (Placental Alkaline Phosphatase) and OCT3/4 (Octamer-binding Transcription Factor, also termed POU5F1), amongst others [13,15]. OCT3/4 is a transcription factor which is physiologically expressed only in ESC and PGC to provide them with the ability of pluripotency, survival and proliferation [16]. NANOG (Homeobox Transcription Factor NANOG), another pluripotency regulatory transcription factor, shows similar function and temporospatial expression as OCT3/4 [17,18]. Moreover, OCT3/4, as well as NANOG, prevents PGC from apoptosis [19,20].

PGC migrate from the yolk sac along the hindgut towards the genital ridges. Among others, SCF (Stem Cell Factor) and its receptor c-KIT are responsible for the proper migration. c-KIT is expressed in germ cells while SCF serves as chemo-attractant [13,21]. As soon as the PGC arrive at the genital ridges the structures are called

indifferent gonads while PGC are termed gonocytes despite the unchanged morphology and expression profile [22].

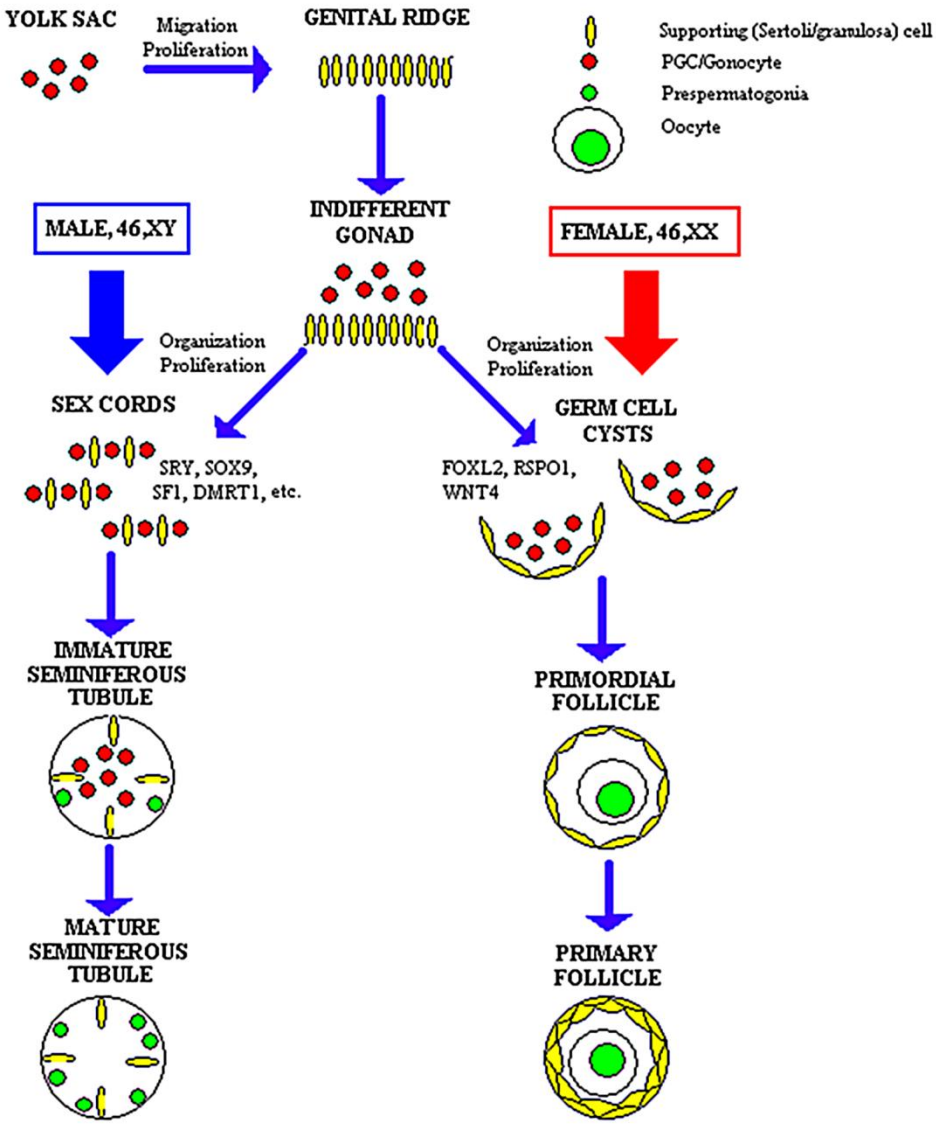

Figure 2: Normal male and female gonadal differentiation. Primordial germ cells migrate from the yolk sac to the gonadal ridge which is then called indifferent gonad. Subsequent development can follow either a male (sex cords, seminiferous tubules in the testicle) or a female (germ cell cysts, follicles in the ovary) differentiation pathway according to the chromosomal constitution and genes expressed.

The indifferent gonad has a potential to develop either as a testis or as an ovary. Its fate depends on the genetic constitution, i.e. the combination of sex chromosomes (XY in males and XX in females) [23]. In the presence of the Y chromosome, SRY (Sex-determining region on Y, also TDF, i.e. Testis Determining Factor) expression occurs during human embryogenesis at week 7 of gestation in supportive (pre-Sertoli) cells and initiates the expression of a cascade of down-stream genes (among others SOX9) which orchestrate testicular differentiation [23,24]. During this process pre-Sertoli cells and gonocytes form cord-like structures (sex cords), and eventually seminiferous tubules [23]. Initially, germ cells, still with all the characteristics of gonocytes, are located in the centre of the tubules while Sertoli cells are situated in the periphery. Germ cells then start migrating gradually towards the periphery. Once they reach the basal lamina, they mature to pre-spermatogonia, their morphology changes and expression of gonocyte markers (OCT3/4, NANOG, AP-2gamma, PLAP, c-KIT, etc.) ceases (see Figure 2) [18,25-27]. Testicular tissue of normal neonates hardly shows any OCT3/4 positive cells and none of these cells can be detected in 4-months old infants [28,29]. In mice, however, differences exist in expression pattern of the markers mentioned before in spermatogonia compared to humans [30].

In females, absence of SRY expression allows the differentiation towards an ovary [23,24]. Germ cells (oogonia) arrange in cyst-like structures with supportive (pre-granulosa) cells, then enter the first step of meiosis, and form primitive follicles. At that time they lose OCT3/4 expression, and from then on they are called oocytes [23,29,31]. The process is no more believed to be a simple default pathway, since several indispensable genes (FOXL2, WNT4, RSPO1) have been identified [32-34]. Recently, the importance of FOXL2 in maintenance, instead of only induction, of ovarian phenotype of the gonad in mice was demonstrated, as the loss of FOXL2 expression leads to up-regulation of SOX9 and subsequently to a change of gonadal morphology, i.e. testis formation. FOXL2 and SOX9 are expressed in supporting cells (i.e. in granulosa and Sertoli cells respectively) in a mutually exclusive manner [34,35].

Pathogenesis of germ cell tumors in DSD

In gonads of DSD patients immature germ cells which resemble PGC/gonocytes frequently persist as the insufficiently differentiated supporting cells (Sertoli/granulosa

cells) are not able to provide a satisfactory milieu to induce maturation to either pre-spermatogonia or oocytes. Immature germ cells can be identified by the positivity for factors typically expressed in early fetal germ cells (OCT3/4, PLAP, etc.) [36]. The prolonged expression of OCT3/4 is believed to be one of the crucial factors in GCT development as it allows germ cells to survive and proliferate [37]. Thanks to its similar function and temporospatial expression pattern, NANOG supposedly has the same impact on tumorigenesis as OCT3/4.

Another piece of the GCT pathogenetic puzzle seems to be an abundant expression of TSPY (Testis-Specific Protein Y-encoded) in germ cells in the DSD gonad. The physiological function of TSPY is not fully understood, but it is thought to be involved in control of germ cell mitotic proliferation in normal testis [38]. Therefore, TSPY overexpression in germ cells may also contribute to their survival and proliferation in an unfavorable environment which would otherwise result in a depletion of the germ cell population. Interestingly, TSPY has no functional homologue in mice and no type II GCT has been reported in this species so far [5]. However, TSPY transgenic mice develop tumors of the pituitary gland and adrenals more frequently but not gonadal tumors [39]. *In vitro* TSPY potentiates cell proliferation by promoting cell cycle progression via cyclin B [40]. A special role of TSPY in GCT development in general, but in DSD patients specifically, is depicted by the fact that it is the most likely candidate for the GBY region (Gonadoblastoma locus on Y chromosome). Existence of such a gene was previously postulated by Page who based his hypothesis on genetic studies in DSD patients with GB or invasive tumor [41]. As mentioned above, only patients with this part of the Y chromosome in their karyotype have a higher risk to develop a type II GCT.

The gonad in which the CIS/GB or GCT arise may be of various phenotypes. CIS and (non)seminoma develop in the context of testicular tissue both in patients with hypovirilization syndromes and with gonadal dysgenesis. GB seems to develop mainly in the context of undifferentiated gonadal tissue (UGT), which remarkably resembles the developmental stage of sex cords. Also streak tissue can harbor GB [8]. Interestingly, supporting cells in GB display significant FOXL2 (a marker for granulosa cells) and no or very low SOX9 (Sertoli cell marker) expression, indicating that GB most likely arises in the gonads which failed to follow the male differentiation pathway (Figure 1) [35]. The highest risk for GCT development is attributed to UGT and to the testis displaying dysplastic changes (see Figure 3)[37].

The forms of DSD with increased risk for GCT development could be considered as an extremely escalated case of testicular dysgenesis syndrome (TDS). TDS was postulated as an umbrella entity for phenomena as testicular cancer, poor semen quality, maldescended testicles and hypospadias which frequently coincide in a single patient. The rapid increase of incidence of reproductive disorders indicates that environmental factors are the likely cause in most of the cases, although it is supposed that some genetic aberrations or polymorphisms might be involved [42,43]. Indeed, genetic background in DSD patients seems to play a major role.

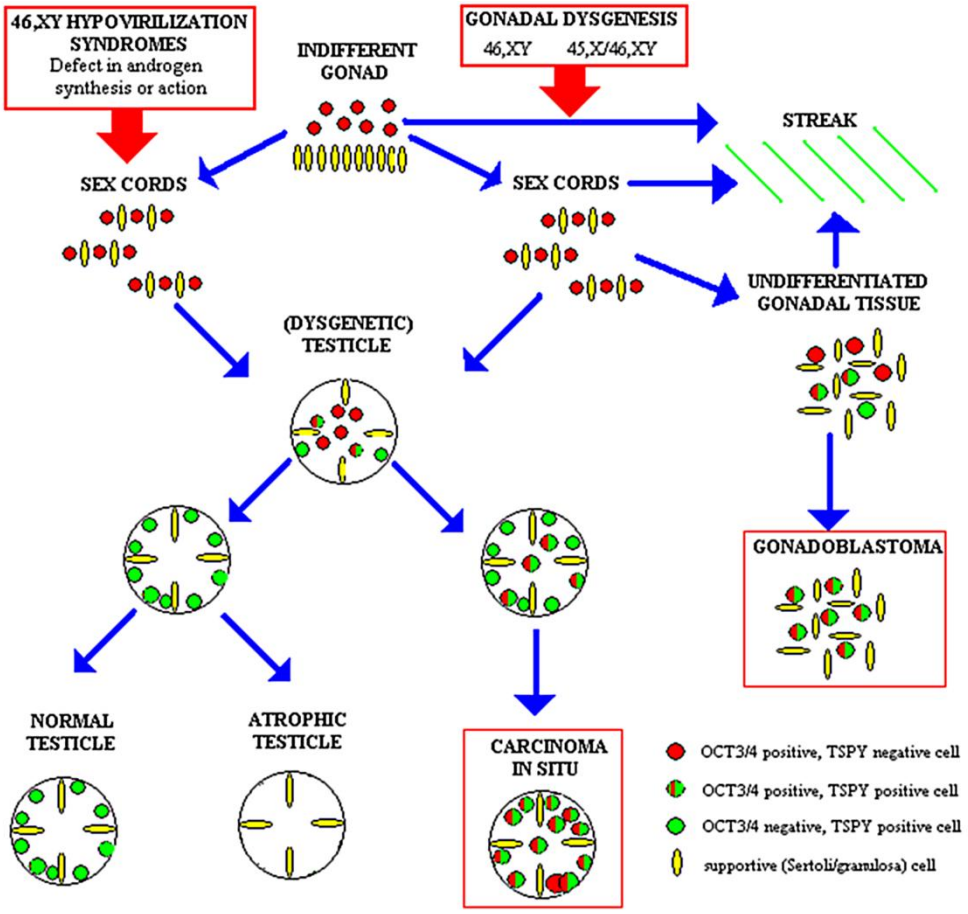

Figure 3: Carcinoma *in situ* (CIS) and gonadoblastoma (GB) development. In hypovirilization syndromes the gonad develops as a testicle, whereas in case of gonadal dysgenesis final morphology can vary from streak, undifferentiated gonadal tissue (UGT) to a dysgenetic testicle according to the step of differentiation in which the development was disrupted. CIS arises from testicular tissue in which OCT3/4 positive germ cells persist and escape normal development while expressing TSPY at the same time. Similar persisting cells in UGT give rise to gonadoblastoma. Supporting cells in CIS and GB have characteristics of Sertoli and granulosa cells, respectively.

The risk for GCT development varies among different disorders

It is possible to estimate the risk for tumor development in various forms of DSD by analyzing previously reported patient series. However, in this approach we have to reckon with bias due to i) inaccurate DSD classification ii) heterogeneous criteria for CIS/GB diagnosis iii) possibly preferential reporting of tumor cases iv) lower incidence in later series due to early prophylactic gonadectomy.

In the last few years, the most extensive meta-analysis was performed by Cools and colleagues [4]. They divided DSD patients into several risk groups for development of type II GCT. Patients with hypovirilization syndromes have a normal male karyotype and their gonads developed into normal testicles. These disorders are caused by a defect in either synthesis or action of androgens. The most numerous are patients with androgen insensitivity syndrome. The overall prevalence of CIS and invasive type II GCT (seminoma and nonseminoma) in this group is estimated to 5.5%. There is however an important difference between patients with complete and partial androgen insensitivity syndrome in whom malignancies occur in 0.8% and 15%, respectively [4]. Other hypovirilization syndromes are very rare. Malignancies in 17% of patients with 17β-hydroxysteroid dehydrogenase were mentioned in one small series [36], while only one case of seminoma in a patient with 5α-reductase deficiency and no tumors in patients with Leydig cell hypoplasia has been reported so far [36,44]. However, this needs confirmation in larger series.

Patients with gonadal dysgenesis (with either a 46,XY or 45,X/46,XY karyotype) seem to be the most endangered subgroup, although the prevalence in different series is rather incoherent being reported in 15-100% of all cases [4,45]. After the rational interpretation of available data, Cools *et al.* rated the total occurence at 12%, and possibly more than 30% if gonadectomy had not been performed. Particularly in patients with mosaic karyotype the prevalence ranges between 15 and 40%, while in those with 46,XY karyotype it achieves approximately 30% [4]. In a series of patients with a defect of the *WT1* gene, malignancies were reported in 60% of patients with Frasier syndrome [46] and in 40% of patients with Denys-Drash syndrome [47]. This knowledge is important because of the limitations on the use of chemotherapeutics for treatment of these patients based on their suboptimal kidney function.

In patients with ovotesticular DSD, previously referred to as true hermaphroditism, the occurence of neoplasia is estimated at 2.6% (see Table 1 for summary) [4].

Patients with XX karyotype do not display a higher risk for development of GCT [37].

Table 1: Prevalence of type II GCT in various forms of DSD.

Risk	Type of DSD	Prevalence
High	GD in general	12%*
	46,XY GD	30%
	Frasier syndrome	60%
	Denys-Drash syndrome	40%
	45,X/46,XY GD	15-40%
Intermediate	PAIS	15%
	17β-hydroxysteroid dehydrogenase defficiency	17%
Low	CAIS	0.8%
	Ovotesticular DSD	2.6%
Unknown	5α-reductase	?
	Leydig cell hypoplasia	?

GD – gonadal dysgenesis; CAIS – complete androgen insensitivity syndrome; PAIS – partial androgen insensitivity syndrome. * might reach more than 30%, if gonadectomy would not be performed.

How to identify a gonad at risk for development of malignancy? The role of OCT3/4, TSPY and SCF

From a clinical point of view, early diagnosis of gonads with increased risk of malignant transformation is mandatory, especially in patients with retained gonads. Beside close clinical follow-up, gonadal biopsy is of high relevance, although its representativeness for the complete gonad in DSD patients is a matter of debate [37]. It is not expected that a GCT will arise in gonadal tissue in the absence of germ cells, as is the case in streak gonad and testis strictly formed by Sertoli cell only tubules. But even the gonad harboring germ cells may not be necessarily endangered if these do not display any sign of neoplastic changes (see Figure 4) [22]. The crucial diagnostic point then is to identify these cells at risk within the abnormally developed gonad.

In the last decades several markers for detection of malignant germ cells in CIS/GB and GCT have been established (e.g. PLAP, c-KIT, OCT3/4, AP-2gamma, NANOG) [18,25,26,28,48-50]. Among these markers, OCT3/4 appears to be the most reliable

for detection of CIS/GB in DSD gonads thanks to its consistent strong nuclear staining with weak or no background [12,36]. OCT3/4 is a transcription factor indispensable for maintenance of pluripotency in ESC and PGC. As discussed above, it seems to have an anti-apoptotic function in PGC [16,19]. It serves as a highly specific and sensitive marker for both CIS/GB and pluripotent types of GCT (i.e. seminoma/dysgerminoma and embryonal carcinoma) in which its expression is possibly of pathogenetic relevance [28,37].

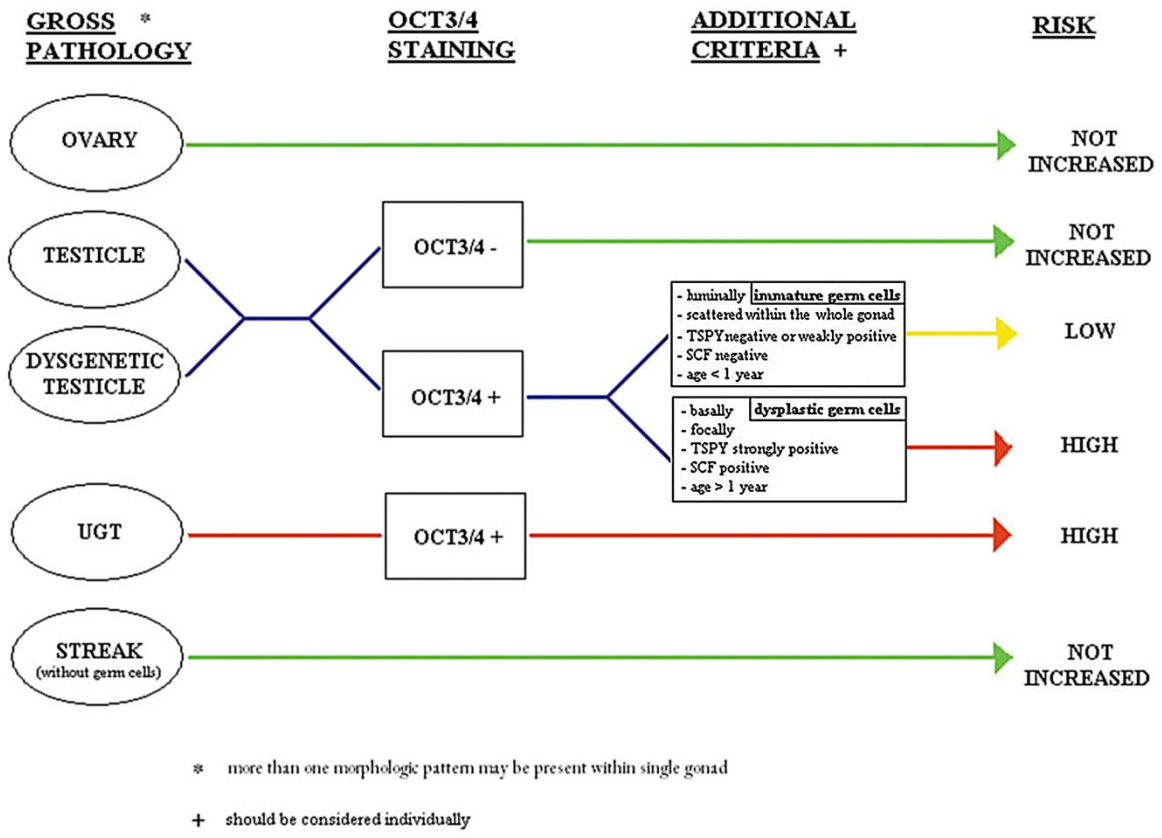

Figure 4: Assessment of gonads at risk based on gross pathology and immunohistochemical staining. The risk for development of GCT is related to the pattern of gonadal differentiation. OCT3/4 positive germ cells in testis may be concidered as immature or dysplastic according to the additional criteria. UGT – undifferentiated gonadal tissue, according to our experience, OCT3/4 positive cells are always being found within this morphologic pattern.

When assessing gonadal tissue in DSD patients, a more cautious approach should be applied due to possible maturation delay of germ cells which frequently occurs in DSD patients and may lead to overdiagnosis and overtreatment [36]. Immature germ

cells express similar markers as early fetal germ cells (PGC/gonocytes) and as CIS/GB cells which are believed to develop from them [9,10,36]. Cools *et al.* proposed additional criteria which should help to distinguish between maturation delay and early neoplasia in patients with hypovirilization syndromes [36]. The criteria are based on knowledge of fetal germ cell development. OCT3/4 positive cells which are located in the centre of the seminiferous tubules and are scattered throughout the whole gonad are believed to be delayed in their maturation, especially in patients younger than 1 year (see Figure 5AD) [36].

As mentioned above, TSPY is the most probable candidate for GBY and is abundantly expressed in germ cells in DSD gonads and in CIS/GB cells [12,36,38,51]. During normal gonadal development TSPY expression is almost exclusively restricted to (pre-) spermatogonia, while OCT3/4 is expressed in gonocytes [26,27]. When migrating from the lumen towards the basal lamina of seminiferous tubules, germ cells gradually lose OCT3/4 expression whereas expression of TSPY is becoming more intense during the maturation process. Double-staining for OCT3/4 and TSPY is informative for the detection of dysplastic cells, which escape normal development [12]. These cells are attached to basal lamina, and keep strongly expressing both OCT3/4 and TSPY (as CIS/GB cells) [4]. Double positive cells in the luminal site are likely to be only delayed in their maturation (Figure 5BE).

Moreover, SCF seems to be a useful additional tool in distinguishing between immature germ cells and early malignant cells [52]. SCF is a ligand of the proto-oncogene c-KIT which acts as a tyrosine kinase [53]. The SCF/c-KIT system plays an important role, not only in fetal germ cell migration as mentioned above, but also in their proliferation and apoptosis as well as in regulation of adult spermatogonia proliferation and maintenance [49,52-55]. In the adult testis SCF is synthesized by Sertoli cells in two splice variants - the membrane-bound form acts in establishing and maintaining germ cells, and the soluble form induces the testosterone production in Leydig cells [55]. c-KIT mutations have been identified in numerous GCT [49,56-58], and most recently, an association between specific polymorphisms within SCF and the risk for type II GCT has been reported [59,60], but still the detailed pathogenetic role of SCF in tumorigenesis remains to be elucidated. SCF is undetectable in germ cells of both normal fetal and postnatal testes using immunohistochemical methods. SCF staining in seminoma/dysgerminoma is

heterogeneous and inconsistent in nonseminomas. However, SCF positivity in CIS/GB cells is convincing [52,61]. Interestingly, in the DSD gonads, germ cells displaying OCT3/4 positivity but not fulfilling the criteria for CIS cells, i.e. cells with maturation delay, are SCF negative [52]. This gives SCF a unique position among other CIS/GB markers (see Figure 5CF).

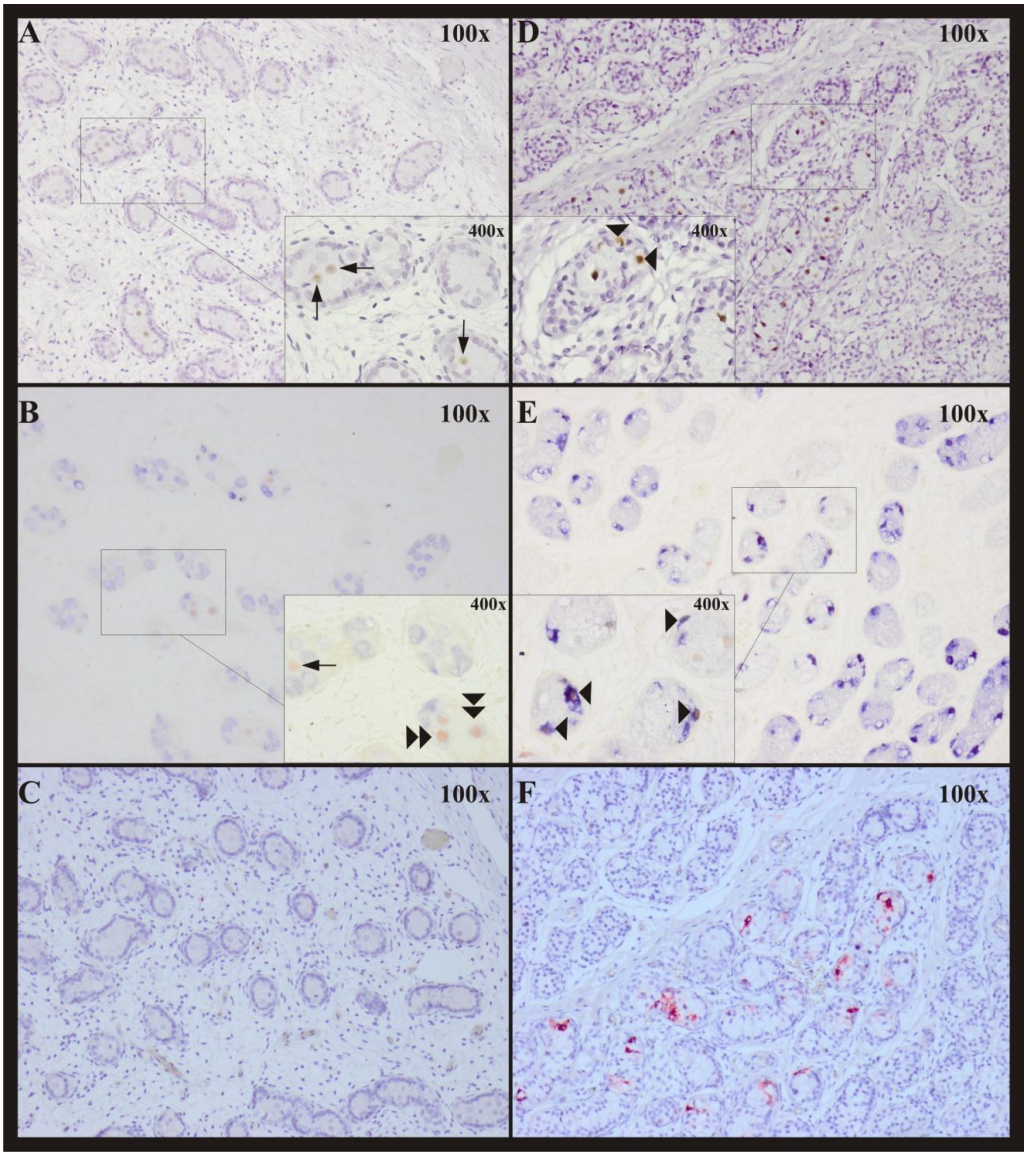

Figure 5: Detection of dysplastic cells in a DSD gonad using immunohistochemistry. A) OCT3/4 positivity in luminally located germ cells scattered within the gonad of a 16-month old 46,XY female; B) same area with luminally located OCT3/4 (orange) positive cells showing no (arrow) or weak (double arrow head) TSPY (blue) positivity; C) same area displaying SCF negativity; D) area of OCT3/4 positive basally located germ cells (arrow head) in a 9-year old 46,XY female; E) same area with OCT3/4 (orange) and TSPY (blue) double positive germ cells attached to the basal lamina (arrow head); F) same area displaying SCF positivity.

Looking towards the future

Presently available tools allow us to assess gonadal tissue of DSD patients and identify gonads at risk for GCT development, i.e. gonads containing dysplastic cells or non-invasive neoplasia. This ability together with precise diagnosis of DSD cases based on molecular-genetic methods may facilitate a more accurate estimation of the tumor risk in various forms of DSD. With that knowledge we might be able to preserve gonads in selected patients. Of course large series of patients are required for such an ambitious vision. As DSD is relatively rare, multi-centric studies and international cooperation are indispensable.

Acknowledgements

This work was supported in part by ESPE Research Fellowship, sponsored by Novo Nordisk A/S (JP), as well as the Translational Research Grant Erasmus MC 2006 (RH) and The Flanders Research Foundation – FWO (MC).

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Chapter 3

Complete androgen insensitivity syndrome: factors influencing gonadal histology including germ cell pathology

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Mod Pathol 2014;27(5):721-30.

Abstract

Patients with complete androgen insensitivity syndrome are at an increased risk for the development of gonadal germ cell cancer. Residual androgen receptor (AR) activity and abnormal gonadal location may influence the survival of atypical germ cells and the development of other histopathological features. To assess this, we evaluated 37 gonads from 19 patients with complete androgen insensitivity (ranging in age from 3 months to 18 years). Histological abnormalities were examined using hematoxylin and eosin-stained sections and sections stained for POU5F1 and KITLG, markers of early changes in germ cells at risk for malignant transformation. Hamartomatous nodules, Leydig cell hyperplasia, decreased germ cells, tubular atrophy and stromal fibrosis were more pronounced as age increased ($p<0.001$). Expected residual AR activity acted as a positive predictor only for non-malignant germ cell survival in (post)pubertal patients ($p<0.05$). Immunohistochemical studies indicated that delayed maturation of germ cells was present in three patients, while intermediate changes that occurred between delayed maturation and intratubular germ cell neoplasia, designated pre-intratubular germ cell neoplasia, were identified in four cases. Intratubular germ cell neoplasia was observed in one patient. Neither POU5F1 nor KITLG expression was dependent on expected residual AR activity. An independent effect of inguinal *versus* abdominal position of the gonads was difficult to assess because inguinal gonads were present primarily in the youngest individuals. In conclusion, many histological changes occur increasingly with age. Expected residual AR activity contributes to better survival of the general germ cell population in (post)pubertal age; however, it did not seem to play an important role in the survival of the germ cells at risk for malignant transformation (defined by POU5F1 positivity and KITLG overexpression) in complete androgen insensitivity. Comparison of the high percentage of patients in our study that were carrying germ cells with delayed maturation or pre-intratubular germ cell neoplasia with previously reported cumulative risk of tumor development in adult patients indicates that not all such precursor lesions in complete androgen insensitivity will progress to invasive germ cell cancer.

Introduction

Complete androgen insensitivity syndrome is a clinical condition that is caused by an inactivating mutation in the androgen receptor (AR) gene, leading to a female phenotype in an individual with a male karyotype (46,XY) [1]. Typical changes in the gonadal histology of patients with complete androgen insensitivity have been described [2]. These changes develop with increasing age and may be influenced by two major factors: abnormal gonadal location and decreased AR activity [2,3]. Among others, germ cell anomalies are of crucial importance. Increased risk of germ cell cancer development has been noted in complete androgen insensitivity syndrome [4-6]. The occurrence of germ cell cancer has been reported to be up to 22% in adult patients [5]. Germ cell cancer is very rare in childhood and adolescence [7]; however, non-invasive precursor lesions characterized as intratubular germ cell neoplasia, also termed as carcinoma *in situ* of the testis have been repeatedly described in this age group [2,8]. Interestingly, the occurrence of intratubular germ cell neoplasia in pediatric patients, which is at a maximum of 6% of cases, does not reach the frequency of germ cell cancer in adulthood reported in most literature [2,8-11].

The prevalence of intratubular germ cell neoplasia appears to be remarkably higher (15%) in pediatric patients with partial androgen insensitivity than in patients with complete androgen insensitivity [4]. Hannema *et al* described that residual activity of AR has a positive effect on the development of Wolffian structures and the enlargement of seminiferous tubules during puberty in patients with complete androgen insensitivity syndrome [2,12]. Whether residual AR activity also has an impact on the survival of normal and/or atypical germ cells has not yet been reported.

The aim of the present study was to investigate the role of residual AR activity and abnormal gonadal location on the survival of atypical germ cells, as well as on the development of other histopathological changes in complete androgen insensitivity. We assessed 37 gonads of 19 patients with complete androgen insensitivity syndrome. The investigation included immunohistochemical detection of POU class 5 homeobox 1 (POU5F1, also known as OCT3/4) and KIT ligand (KITLG, also known as SCF) [13,14]. These two markers were only recently applied in the assessment of early changes in germ cells at risk for malignant transformation. To our knowledge, this is the first study exclusively dealing with patients with complete androgen insensitivity syndrome using both markers. In addition, the expression of Testis-

specific Y encoded protein 1 (TSPY1), a candidate for Gonadoblastoma locus on Y chromosome that is normally expressed in (pre-)spermatogonia, was investigated [15].

Material and Methods

Patients and histological material

We investigated 37 testes from 19 Czech and Dutch patients with the complete form of androgen insensitivity syndrome, i.e., patients with an unambiguously female phenotype who were diagnosed based on having AR gene mutation identified by direct sequencing in a diagnostic set up. Patients ranged in age from 3 months to 18.5 years (mean = 9.2 years). The samples were collected between 1992 and 2005 and were included only if the activity of AR could be inferred from the sequencing results or if a reference about the functional studies existed (see below). Based on the type of mutation (frame shift mutations, mutations leading to the introduction of a stop codon after internal initiation-of-translation sites) and a search of the literature (point mutations with zero activity in ligand-binding or transactivation studies), no residual activity of AR was expected in 11 patients [16-19]. In five patients, a point mutation in the ligand-binding domain resulted in a mutated AR with some residual activity in transactivation studies [12,20-22]. In the remaining three patients, the activity of AR was uncertain: mutation of an intronic region led to abnormal splicing of AR in one sibling pair [23], and AR truncated at the amino terminal side was detected as a result of an early introduced stop-codon in the third case [24]. One or both gonads were originally positioned in the inguinal region in six patients and were relocated into the abdominal cavity at hernioplasty during infancy or early childhood. Only a single gonad was situated in the labial region and was grouped with inguinal gonads for statistical analysis. The gonads were removed as a prophylactic measure in all cases (see Table 1).

This study was approved by the local Ethics Committee of the University Hospital Motol, Prague (EC 237/2009), and 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 (Federa, 2011).

Table 1: Patient information for age, mutation (Reference sequence: hg19, NM 000044) (Gotlieb et al. 2012), AR residual activity and gonadal location.

	Age (yrs)	Mutation nucleotide (HGVS)//amino acid level	Residual AR Activity, (method used)	Reference	Gon	Gonadal location
S1	16.50	c.1769-11T>A//p.?	???	Brüggenwirth et al., 1997	r	inguinal
		Sister of S5	TA (0% activity with 10 nM R1881)		l	inguinal
S2	0.25	c.2566C>T//p.Arg856Cys	No	Weidemann et al., 1996	r	inguinal
			LBA (0% ligand binding activity)		l	inguinal
S3	0.80	c.1721C>A//p.Ala574Asp	No	Brüggenwirth et al., 1998	r	inguinal
			TA (0% activity with 10 nM R1881)		l	inguinal
S4	1.20	c.1000insT//p.Gly334Valfs*7	No		r	inguinal
		Sister of S9			l	abdominal
S5	1.25	c.1769-11T>A//p.?	???	Brüggenwirth et al., 1997	r	inguinal
		Sister of S1	TA (0% activity with 10 nM R1881)		l	inguinal
S6	2.50	c.2343G>A//p.Met781Ile	Yes	Knoke et al., 1999	r	inguinal
			TA (100% activity with 3 nM R1881)		l	inguinal
S7	2.75	c.178C>T //p.Gln60*	???	Zoppi et al., 1993	r	NA
			TA (30% with 2 nM DHT)		l	inguinal
S9	3.00	c.2522G>A//p.Arg841His	Yes	Beitel et al., 1994	r	labial
			TA (60% activity with 2.2 nM mibolerone)		l	inguinal
S9	3.20	c.1000insT// p.Gly334Valfs*7	No		r	abdominal
		Sister of S4			l	abdominal
S10	6.00	c.2197G>A//Asp733Asn	Yes	Hannema et al., 2004	r	i/a (1 y)
		Sister of S17	TA (120% activity with 10 nM mibolerone)		l	i/a (1 y)
S11	9.50	c.832-833dupGC//p.Val279Leufs*18	No		r	i/a (6 y)
		Sister of S18			l	i/a (6 y)
S12	13.66	c.1847G>A//p.Arg616His	No	Mowszowitz et al., 1993	r	abdominal
			TA (0% activity with 10 nM mibolerone)		l	abdominal
S13	15.20	c.2567G>A//p. Arg856His	Yes	Marcelli et al., 1994	r	inguinal
			TA (80% activity with 2 nM mibolerone)		l	inguinal
S14	15.50	c.2546dupA//p.Asn849Lysfs*32	No		r	abdominal
		Sister of S15			l	i/a (0 y)
S15	15.66	c.2546dupA//p.Asn849Lysfs*32	No		r	i/a (0 y)
		Sister of S14			l	abdominal
S16	15.66	c.1774C>T//p. Gln592*	No		r	abdominal
					l	abdominal
S17	16.20	c.2197G>A//Asn733Asn	Yes	Hannema et al., 2004	r	i/a (1 y)
		Sister of S10	TA (120% activity with 10 nM mibolerone)		l	i/a (1 y)
S18	16.66	c.832-833dupGC//p.Val279Leufs*18	No		r	i/a (3 y)
		Sister of S11			l	i/a (3 y)
S19	18.50	c.159-171del13//p.Leu54Serfs*117	No		r	abdominal
					l	abdominal

TA - transactivation; LBA - ligand binding activity; ??? - unknown; Gon - gonad; l - left; r - right; i/a (xy) - original location inguinal, replaced to abdominal cavity at x years; NA - not available.

Gonadal histology assessment

All gonadal samples were fixed in 10% formalin and embedded in paraffin. At least one section from each gonad was stained with hematoxylin and eosin and assessed by a pathologist who was experienced in gonadal histology (JWO) for overall organization of the gonadal tissue, markers of pubertal maturation, and abnormal histological phenomena. The fraction of seminiferous tubules containing germ cells was calculated in at least 200 cross-sections of tubules in each gonad.

Definition of delayed maturation of germ cells, intratubular germ cell neoplasia and pre-intratubular germ cell neoplasia

Delayed maturation of germ cells is defined as the presence of POU5F1-positive germ cells with round nuclei located centrally in the seminiferous tubules of individuals over 6 months of age. Only occasionally are weakly POU5F1-positive cells at the basement membrane of the tubules accepted as indicators of delayed maturation. Such cells are considered to be in the process of turning off POU5F1 expression. KITLG is not expressed in gonads with delayed maturation germ cells [8,14].

For the diagnosis of intratubular germ cell neoplasia, the presence of at least one cross-section of a seminiferous tubule containing a homogeneous population of atypical germ cells with angulated nuclei is required. These cells must show either homogeneous double expression of POU5F1 (nuclear) and TSPY1 (cytoplasmic and membranous) or homogenous expression of POU5F1 (nuclear) in the absence of TSPY1. The involved tubule must show expression of KITLG, which usually presents as irregular spots associated with the cytoplasm of Sertoli cells.

A diagnosis of pre-intratubular germ cell neoplasia is made when the findings fall short of the criteria for intratubular germ cell neoplasia and are beyond those that are acceptable for the diagnosis of delayed maturation. KITLG may be expressed in testes containing pre-intratubular germ cell neoplasia [14,25].

Immunohistochemistry

Tissue samples from 36 gonads (22 gonads from patients with expected no AR activity; 9 gonads from patients with expected residual AR activity; 5 gonads from patients with uncertain AR activity) were available for immunohistochemical staining. After antigen retrieval (120°C, 0.9 Bar), 4-µm-thick sections were evaluated for the presence of POU5F1 (sc-5279, Santa Cruz Biotechnology, CA, USA, dilution 1:350, incubated 120' at room temperature) and TSPY1 (the antibody was kindly provided by Prof. Dr. C. Lau, Department of Medicine, VA Medical center, University of California, San Francisco, CA, USA; dilution 1:4000, incubated overnight at 4°C). POU5F1-positive samples were also stained for KITLG/SCF (sc-1302, Santa Cruz Biotechnology, CA, USA, dilution 1:250, incubated overnight at 4°C). Detection and visualization was conducted using biotinylated secondary antibodies and avidin-biotin-complex conjugated with horseradish peroxidase for POU5F1 (Vectastain ABC

kit Elite pk-6100 Standard) or alkaline phosphatase for TSPY1 and KITLG/SCF (Vectastain ABC kit pk-5000 AP). Diaminobenzidine/H_2O_2 (for POU5F1) and New Fuchsine/Naphtol ASMX phosphate (for TSPY1 and KITLG/SCF; N500 Sigma, Steinheim, Germany) were used as substrates. Adult testicular tissue with intratubular germ cell neoplasia was used as a positive control for all staining. The proportion of POU5F1 positive cells allocated to the basal lamina was assessed on 20 cross-sections of tubules, if available.

Double-staining

Samples with POU5F1-positive cells were investigated for co-expression of POU5F1 and TSPY1. Sections where pretreated with H_2O_2, pressure cooked and incubated with primary antibodies against TSPY1 (overnight, at 4°C) and POU5F1 (sc-8629, dilution 1:350, incubated 120' at room temperature). TSPY1 was detected using the avidin-biotin-complex conjugated with alkaline phosphatase complex and Fast Blue/Naphtol ASMX phosphate as a substrate. POU5F1 was detected with avidin-biotin-complex conjugated with horseradish peroxidase complex and 3-amino-9-ethyl-carbazole (Sigma, Steinheim, Germany)/H_2O_2 as a substrate. In between the two stainings, free biotin was blocked (Vector Laboratories, Burlingame, CA, USA). The adult testicular tissue containing intratubular germ cell neoplasia was used as positive control.

Statistics

Influence of age, gonadal location at the time of gonadectomy, and expected level of AR activity on particular histological features was analyzed. The results were assessed using Mann-Whitney and Chi-square tests, as well as logistic, multivariate and linear regressions. Correlation between original gonadal location and expected AR activity was evaluated by a Chi-square test. Correlations between expected AR activity and age and between gonadal location and age were analyzed by a Mann-Whitney test. A value of $p<0.05$ was considered significant. All analyses were performed using SYSTAT 10.0.

Results

The original anatomical gonadal location was significantly dependent on expected AR activity ($p<0.05$). While half of the gonads were originally situated in the abdominal cavity in patients with expected no residual activity, one gonad was labial, and the rest were inguinal in patients with expected residual activity of AR. Labial or inguinal location of the gonads possibly contributed to relatively earlier clinical diagnosis and therefore earlier gonadectomy ($p<0.01$) in our study; however, age of gonadectomy did not differ significantly ($p>0.05$) between patients with and without expected residual activity of AR.

Gonadal histology assessment

Intertubular stroma appeared edematous (Figure 1A) mostly in younger patients while in the older individuals it was rather fibrotic ($p<0.001$) (Figure 1B). Combination of both edematous and fibrotic stromal changes within a single gonad was also observed, mainly in prepubertal patients. Areas of ovarian-like stroma (Figure 1C) were present in the gonads of one sibling pair (S4 and S9). Remarkably dilated lymphatic vessels (Figure 1D) were observed in 67% of gonads throughout the entire cohort. Hamartomatous nodules (Figure 1E), i.e., well circumscribed nodules composed of Sertoli cell-only tubules and Leydig cells in between the tubules, were observed in all pubertal and postpubertal patients (13-years-old and older), and therefore, their development was significantly dependent on age ($p<0.001$). Tubular atrophy presented as fibrosis of the tubules (Figure 1F) was observed in 47% of cases of patients older than 6-years of age and was significantly dependent on higher age ($p<0.001$). Diffuse Leydig cell hyperplasia (Figure 1G), which was present in all pubertal and postpubertal patients, was also dependent on older age ($p<0.001$). Scattered Sertoli cells with eosinophilic granules in the cytoplasm (Figure 1H), so-called Hürtle cell-changes (a result of altered mitochondria), were identified in 14% of gonads, all in patients older than 9 years. Sertoli cell nodules, either with (Figure 1I) or without (Figure 1J) central hyaline deposits with partial calcification, were observed in almost 37% of patients, the youngest being 3 years old.

In regard to signs of pubertal development, tubular lumen formation was evaluated and was not consistently present in any of the cases, although sporadic lumen

(Figure 1K) were observed in the majority of the patients older than 9 years. Spermatogenesis was not encountered in any of the patients.

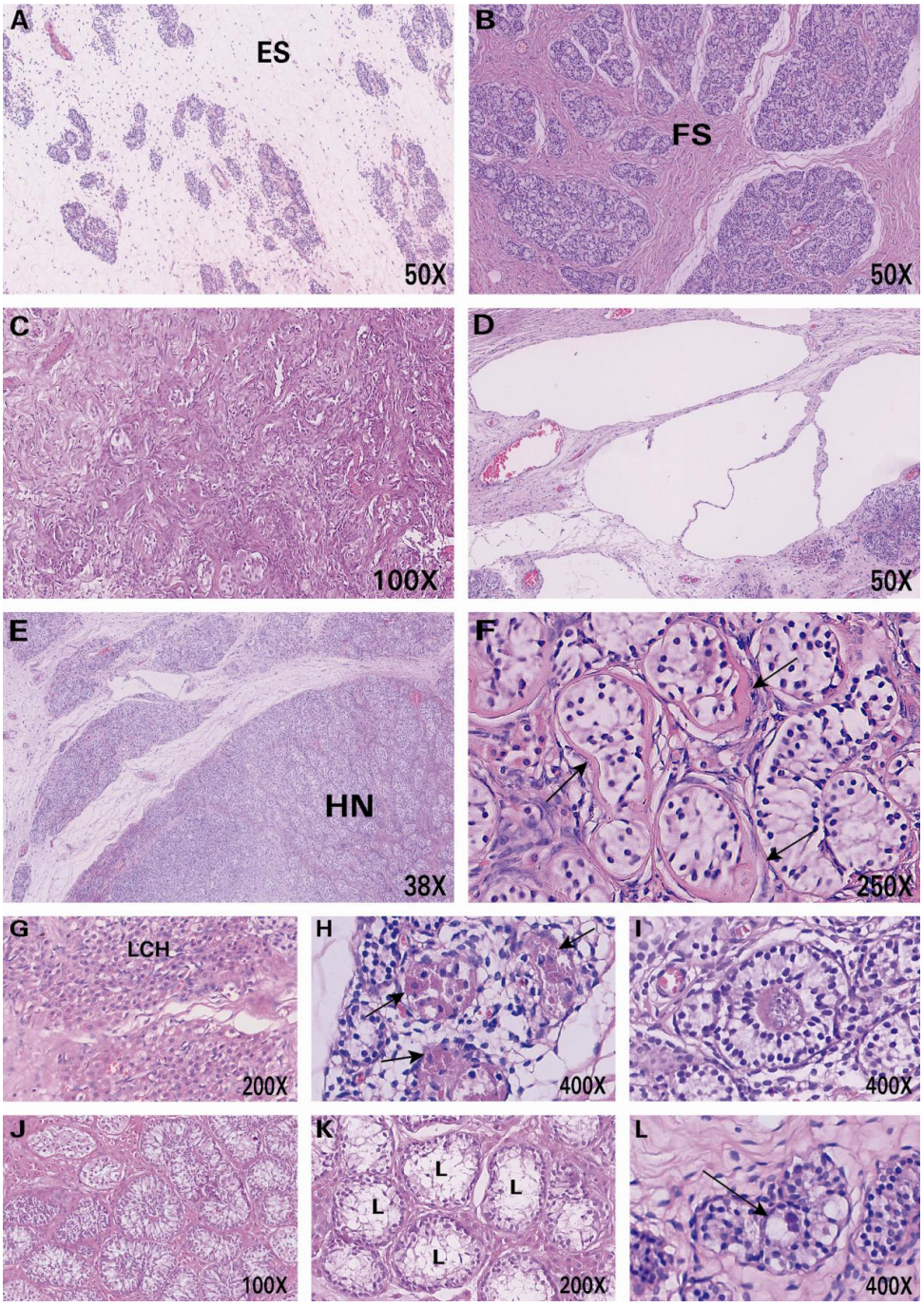

Figure 1: Different histopathological changes of the testis in complete androgen insensitivity syndrome, H&E staining. A - edematous stroma (ES); B - fibrotic stroma (FS); C - ovarian-like stroma; D - Dilation of lymphatic vessels; E - Hamartomatous nodule (HN); F - fibrotic atrophy of the seminiferous tubules (arrow); G - Leydig cell hyperplasia (LCH); H - eosinophilic granular changes of Sertoli cell cytoplasm (arrow); I - Sertoli cell nodule with central hyaline deposit; J - Sertoli cell nodules without hyaline deposit; K - sporadic lumen (L); L - multinucleate germ cell (arrow).

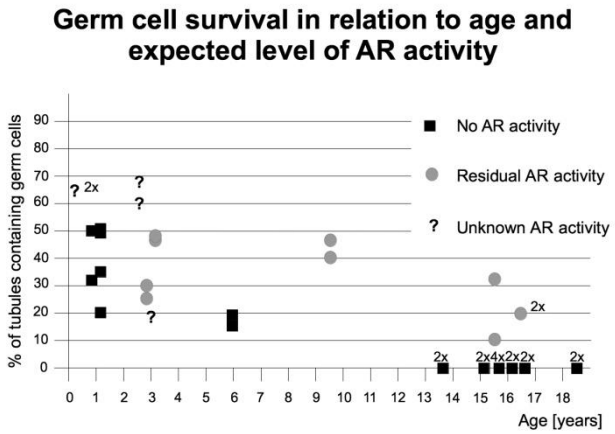

Figure 2: Germ cell survival in relation to age and expected level of AR activity. A significant difference was observed in patients older than 13 years; almost no tubules with germ cells were present in patients with zero residual activity whereas at least 11% of tubules containing germ cells were encountered in patients with expected residual AR activity.

At least one tubule with germ cells was found in 84 % of gonads. The number of tubules containing germ cells declined with age in the whole series ($p<0.001$). A significant difference in germ cell survival ($p<0.05$) between patients with and without expected residual AR activity in (post)pubertal age was observed. While the gonads of the patients with expected no residual activity lacked germ cells altogether or contained only solitary tubules with germ cells, a considerable number of tubules with germ cells was identified in patients with expected residual AR activity (Figure 2). Multinucleated germ cells (Figure 1L) were found in almost two-thirds of gonads in which some germ cells remained. Gonadal location (abdominal versus inguinal) was not an independent influential factor in any of the above-described features when corrected for age and expected AR activity.

Immunohistochemistry (Table 2)

Cells showing POU5F1-positive staining were detected in 13/36 (35%) gonads from 9/19 (47%) patients. A certain continuum in their distribution within the tubules was observed with increasing age. More than 85% of the positive cells were situated in the center of the tubules in 4 young patients (age 3 months to 3 years), bilaterally in 2 of them (Figure 3A). Positively stained cells were few in all cases, had round nuclei and were scattered throughout the whole gonad. They were TSPY1-negative or only slightly positive (Figure 3B). All the gonads were negative for KITLG (Figure 3C). Thus, the histochemical and morphological pattern resembled that of fetal gonads [14,26]. The findings were classified as delayed maturation in 3 patients who were older than 6 months.

The earliest atypical features were identified in both of the gonads of a 6-year-old patient (S9). Up to 33% of the POU5F1-positive cells were attached to the basal lamina. Distribution throughout the testis was patchy with regions of several adjacent positive tubules. The cells were both TSPY1-negative and positive regardless the location within the tubules. Additionally, the cells were morphologically atypical, made obvious by the shape of the nuclei. Some nuclei were round, and others were irregular. Some of the areas with POU5F1-positive cells were KITLG positive. However, there were also KITLG-negative tubules containing POU5F1-positive cells.

The changes were even more pronounced in the 4 oldest patients. POU5F1-positive cells occupied one well circumscribed lobule in a 9-year-old patient (S10). Two-thirds of the cells were in contact with basal lamina (Figure 3D). The cells were very heterogeneous in morphology and staining pattern, again TSPY1-positive or TSPY1-negative in all locations (Figure 3E). KITLG expression was very strong in this case (Figure 3F).

Several areas with POU5F1-positive cells were encountered in two 15-year-old twins (S14, S15), in both gonads in one of them. Interestingly, these were virtually the only regions with surviving germ cells (POU5F1-positive and negative) within the whole gonads. More than 60% of POU5F1-expressing cells were located at the periphery of the tubules, both positive and negative for TSPY1. KITLG expression was identified in most, but not all, regions.

Since the germ cells were very heterogeneous in morphology and expression within particular tubules and because normal spermatogonial cells (i.e., POU5F1-negative and TSPY1-positive) were often encountered in the same tubules as the atypical germ cells, a diagnosis of pre-intratubular germ cell neoplasia was made in patients S9, S10, S14 and S15.

According to the above criteria, intratubular germ cell neoplasia was present in one gonad of a 15-year-old patient (S13). More than 90% of the POU5F1-positive cells were attached to the basal lamina (Figure 3G). Clonal expansion of germ cells, which were uniform in POU5F1 and TSPY1 expression, was observed in several tubules (Figure 3H). The expression of TSPY1 in POU5F1-positive germ cells was heterogeneous in other regions. KITLG expression was present in some, but not all, tubules with POU5F1-positive germ cells (Figure 3I). Many tubules contained exclusively normal spermatogonia (POU5F1-negative and TSPY1-positive) in this patient.

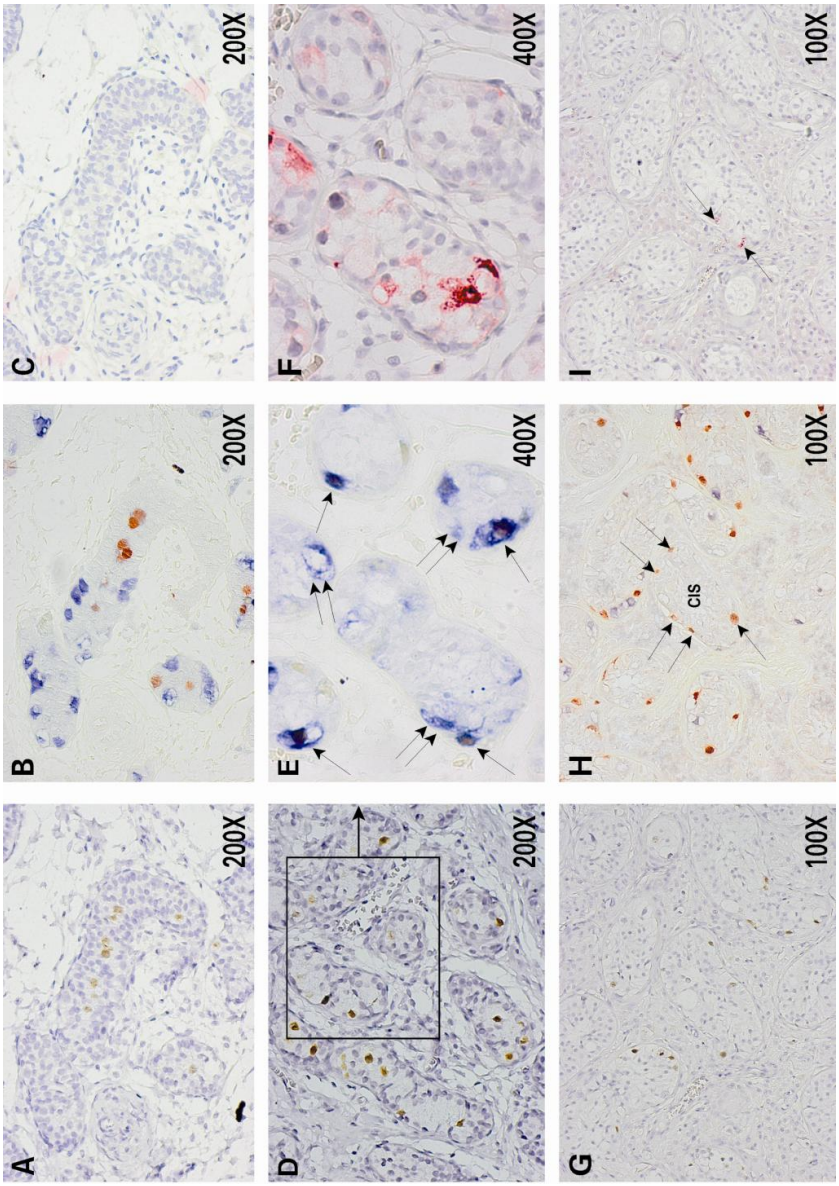

Figure 3: Immunohistochemical staining of gonads positive for POU5F1. A - delayed maturation - POU5F1-positive germ cells (brown) all in the center of the tubules in a gonad from 10-month-old individual (S2); B - same area as in A double-stained for POU5F1 (orange) and TSPY1 (blue), which are not co-expressed within the same cells; C - same area as in A and B negative for KITLG; D - pre-intratubular germ cell neoplasia - most of the POU5F1-positive germ cells are attached to the basal lamina in a gonad from 9-years-old individual (S10); E - detail of the same area as in D double-stained for POU5F1 and TSPY1, heterogeneity of the germ cells within tubules - cells are either positive for both POU5F1 and TSPY1 (arrow) or only for TSPY1 (double arrow); F - same area as in E strongly positive for KITLG (red); G - intratubular germ cell neoplasia - almost all POU5F1-positive germ cells are attached to the basal lamina in a gonad from 15-year-old individual (S13); H - same area as in G double-stained for POU5F1 and TSPY1 - seminiferous tubule (IGCNU) with a uniform population of germ cells which are positive for POU5F1 only (arrow); I - same area as in G and H stained for KITLG (positivity marked with arrow).

Table 2: Results of immunohistochemical staining in POU5F1-positive gonads.

	Age (yrs)	gon	% of POU5F1 (+) GC at the BL	KITLG	TSPY and POU5F1 double-staining	Pattern of double-staining	Overall evaluation
S1	0.25	r	0	(-)	Most GC only TSPY (+); POU5F1 (+) GC are mostly TSPY (-)	homogeneous	normal development
		l	3	(-)	Most GC only TSPY (+); POU5F1 (+) GC are mostly TSPY (-)	homogeneous	normal development
S2	0.8	r	8	(-)	Most GC only TSPY (+); POU5F1 (+) GC are mostly TSPY (-)	homogeneous	maturation delay
S4	1.25	l	6	(-)	Most GC only TSPY (+); POU5F1 (+) GC are mostly TSPY (-)	homogeneous	maturation delay
S8	3.2	r	13	(-)	Most GC only TSPY (+); POU5F1 (+) GC are mostly TSPY (-)	homogeneous	pre-CIS
		l	11	(-)	Most GC only TSPY (+); POU5F1 (+) GC are mostly TSPY (-)	homogeneous	pre-CIS
S9	6.0	r	33	(+)	Most GC only TSPY (+); POU5F1 (+) GC are both TSPY (+) and (-)	heterogeneous	pre-CIS
		l	19	(+)	Most GC only TSPY (+); POU5F1 (+) GC are both TSPY (+) and (-)	heterogeneous	CIS
S10	9.5	l	66	(+)	Most GC only TSPY (+); POU5F1 (+) GC are both TSPY (+) and (-)	heterogeneous	pre-CIS
S13	15.5	r	93	(+)	Many GC only TSPY (+); POU5F1(+) GC are both TSPY (+) and (-)	homogeneous in few tubules	pre-CIS
S14	15.66	r	61	(+)	GC only in POU5F1 (+) areas, some only TSPY (+); POU5F1 (+) GC are both TSPY (+) and (-)	heterogeneous	pre-CIS
S15	15.66	r	66	(+)	GC only in POU5F1 (+) areas, some only TSPY (+); POU5F1 (+) GC are both TSPY (+) and (-)	heterogeneous	
		l	69	(+)	Many GC only TSPY (+); POU5F1(+) GC are both TSPY (+) and (-)	heterogeneous	

gon - gonad; l – left; r - right; GC - germ cells; BL - basal lamina; (+) - positive; (-) - negative.

Discussion

According to current knowledge, two major factors influence postnatal testicular development: the location of the gonad and the androgen action [2,3]. Some histopathological changes, e.g. hamartomatous nodules or Leydig cell hyperplasia, develop during puberty as a consequence of the almost or entirely absent activity of androgens in complete androgen insensitivity syndrome. Other features, such as a decrease in germ cells, multinucleated germ cells, tubular atrophy, anomalous intertubular stroma, hyaline deposits, or lymphatic dilation, occur early in childhood. Therefore, their development is probably caused by abnormal (inguinal or abdominal) location of the gonads, which commonly occurs in patients with complete androgen insensitivity [27]. Differences in the effects of inguinal versus abdominal position of

the gonads are difficult to assess due to an unequal age distribution of the study group, with inguinal gonads being more frequent in younger patients.

A positive impact of expected residual AR activity on the development of Wolffian duct structures and on the enlargement of seminiferous tubules after the onset of puberty in complete androgen insensitivity was described by Hannema, *et al*. [2,12]. Additionally, we observed a significant positive effect on non-malignant germ cell survival after 13 years of age. Only single tubules containing germ cells were encountered in the gonads of patients with expected no residual activity of AR, whereas up to 34% of tubules of patients from the other group contained germ cells. No clear influence of expected residual AR activity was discovered on the other above-mentioned changes.

The survival of germ cells with fetal features (i.e., POU5F1-positive) and their progression towards an invasive tumor in patients with complete androgen insensitivity syndrome are separate and very important issues. The level of risk for germ cell cancer development in complete androgen insensitivity remains a matter of debate. Manuel *et al.* estimated the cumulative risk to be 3.6% at 25 years of age and 33% at 50 years of age [6]. According to Deans *et al.*, tumors occurred in 14% of adult patients based on historical literature [5]. The risk was estimated to be 0.8% in another meta-analysis, but this estimation was mainly based on a group of patients in which gonadectomy was carried out during childhood [4]. In fact, the risk is very difficult to predict because the approaches to manage patients with complete androgen insensitivity have continually changed over time. While older reports included many adult patients and mainly reported the presence of invasive germ cell tumors [6,28,29], more recent studies on pediatric patients refer to pre-invasive lesions, i.e., intratubular germ cell neoplasia, of the testis [2,8]. Intratubular germ cell neoplasia is encountered mostly in pubertal or adolescent patients [2,8]; however, the risk is calculated for entire patient groups that often include very young individuals in whom intratubular germ cell neoplasia has not had time to develop. Moreover, an intermediate stage between delayed maturation and adult type of intratubular germ cell neoplasia exists [8]. Whether this lesion is predetermined to evolve into invasive cancer in all cases is unknown. In the general population fully developed intratubular germ cell neoplasia is estimated to progress to an invasive tumor in 50% of cases during 5 years, and its prevalence is similar to that of germ cell tumors, suggesting a 100% progression to invasiveness over time [30].

We observed a developmental continuum between germ cells with delayed maturation and intratubular germ cell neoplasia in our study. POU5F1-positive, TSPY1-negative germ cells with round nuclei located in the center of the tubules, i.e., immature germ cells, were identified in at least one gonad in 4 patients whose age ranged between 3 months and 3.2 years. Cools *et al.* reported that some single POU5F1-positive germ cells are present in testes of normal individuals 6 months of age or younger [8]. Nevertheless, if the youngest patient from our series is disregarded, 3 out of 7 patients (43%) between 6 months and 3.2 years of age carried germ cells with delayed maturation. POU5F1-positive germ cells with irregular nuclei attached to the basal lamina of the tubules, which in some cases were TSPY1-positive and located within KITLG-positive areas, were identified in at least one gonad in 5 out of 11 patients (45%) older than 6 years of age. The lesion was classified as intratubular germ cell neoplasia in one case and as pre-intratubular germ cell neoplasia in the rest of the cases. Taken together, at least one gonad in 8 out of 18 (44%) patients older than 6 months contained POU5F1-positive germ cells, which may have given rise to an invasive tumor.

Regardless of whether we consider the proportion of the patients with (pre-) intratubular germ cell neoplasia among the older patients or the proportion of the patients older than 6 months with any kind of germ cell abnormality, the number of the gonads at risk is exceptionally high in comparison with other studies on pediatric patients. It is also high in comparison to cumulative risk for germ cell cancer in adult patients with complete androgen insensitivity (33% in 50 years) estimated by Manuel [2,6,8]. Such a high occurrence of atypical germ cells in our study may be incidental, but it may also indicate that not all abnormal germ cells will progress into an invasive tumor in patients with complete androgen insensitivity syndrome. We suspect two possible mechanisms that may explain the difference between the high prevalence of germ cell anomalies in this series of pediatric/adolescent cases and the much lower prevalence of invasive tumors in adults. The first is an absolute loss of the abnormal germ cells in adulthood when the gonads would have been retained. The second is a failure of progression of the pre-invasive lesions into an invasive cancer. Both mechanisms may be caused by a lack of androgen action in complete androgen insensitivity, resulting in a low-androgen environment. This lack-of-androgen theory would correlate with a significantly higher risk for tumor development in patients with partial androgen insensitivity syndrome (15% versus 0.8% in complete androgen

insensitivity syndrome according to Cools *et al*.) [4]. Because we did not observe any significant differences in the persistence of POU5F1-positive germ cells and KITLG expression in relation to the expected level of AR activity among patients with complete androgen insensitivity syndrome, the residual activity of AR in complete androgen insensitivity is likely not powerful enough to achieve a similar effect on survival of abnormal germ cells and their progression into neoplasia as in partial androgen insensitivity. The result may be partly influenced by the relatively small sample size.

In conclusion, many histopathological changes in testis develop with increasing age and are mostly influenced by abnormal gonadal location (inguinal or abdominal) and impaired androgen action in patients with complete androgen insensitivity. An independent effect of inguinal versus abdominal position of the gonads is difficult to assess because inguinal gonads were present primarily in the youngest individuals. Expected residual AR activity contributes to better survival of the general germ cell population in (post)pubertal age; however, it did not seem to play an important role in the survival of POU5F1-positive germ cells and KITLG overexpression and thus appears to be unrelated to a higher cancer risk in patients with complete androgen insensitivity syndrome. Moreover, expected residual AR activity did not prevent the development of lack-of-androgen phenomena such as hamartomatous nodules and Leydig cell hyperplasia. The high percentage of patients with germ cell abnormalities in our study suggests that most of the lesions do not progress to intratubular germ cell neoplasia and subsequent invasive germ cell tumors in complete androgen insensitivity. The level of risk in such cases remains to be elucidated. We observed one case with intratubular germ cell neoplasia and no invasive tumors in our study. This finding, together with other studies on pediatric patients, supports the current practice of postponing prophylactic gonadectomy to an adult age [1].

Acknowledgements

This work was supported by an ESPE Research Fellowship granted by Novo Nordisk A/S and by the project (Ministry of Health, Czech Republic) for conceptual development of research organization 00064203 (University Hospital Motol, Prague, Czech Republic). MC is supported by the Flanders Research Foundation (FWO). Special thanks in memoriam to Dr. Zuntova from Department of Pathology, University

Hospital Motol, Prague who collected the Czech samples and to Dr. Lanska for statistical analysis.

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Chapter 4

Gonadal pathology and tumor risk in relation to clinical characteristics in patients with 45,X/46,XY mosaicism

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J Clin Endocrinol Metab 2011;96(7):E1171-80.

Abstract

Context: Gonadectomy is avoided whenever possible in boys with 45,X/46,XY. However, no clinical markers are currently available to guide clinicians in predicting gonadal tumor risk or hormone production.

Objective: The objective of the study was to test the hypothesis that gonadal histology and risk for development of a malignant germ cell tumor are reflected by the clinical presentation of a 45,X/46,XY individual.

Design: The design of the study was the correlation of clinical data [external masculinization score (EMS), pubertal outcome] with pathology data (gonadal phenotype, tumor risk).

Setting: This was a multicenter study involving two multidisciplinary disorders of sex development teams.

Patients: Patients included genetically proven 45,X/46,XY (and variants) cases, of whom at least one gonadal biopsy or gonadectomy specimen was available, together with clinical details.

Interventions: Patients (n=48) were divided into three groups, based on the EMS. Gonadal histology and tumor risk were assessed on paraffin-embedded samples (n=87) by morphology and immunohistochemistry on the basis of established criteria.

Main outcome measures: Gonadal differentiation and tumor risk in the three clinical groups. Clinical outcome in patients with at least one preserved gonad.

Results: Tumor risk in the three groups was significantly related to the gonadal differentiation pattern ($p<0.001$). In boys, hormone production was sufficient and was not predicted by the EMS.

Conclusions: The EMS reflects gonadal differentiation and tumor risk in patients with 45,X/46,XY. In boys, testosterone production is often sufficient, but strict follow-up is warranted because of malignancy risk, which appears inversely related to EMS. In girls, tumor risk is limited but gonads are not functional, making gonadectomy the most reasonable option.

Introduction

Sex chromosome mosaicism (45,X/46,XY and variants) occurs with an estimated incidence of 1.5 per 10 000 [1] and may be due to loss of the Y chromosome through anaphase lag or to interchromosomal rearrangements with final loss of a structurally abnormal Y chromosome. The clinical spectrum is highly heterogeneous, with no obvious correlation between the phenotypic appearance and the respective cell line counts on routine peripheral blood karyotyping [1-3] or even on the basis of gonadal cell line counts [4]. Up to 95% of individuals may live undiagnosed as normal males [1]. However, ambiguous genitalia in a newborn, but also mild undervirilization (e.g. hypospadias) in boys or even typical Turner syndrome in girls may be associated with 45,X/46,XY mosaicism [3].

Individuals with 45,X/46,XY, as some other patients with a disorder of sex development (DSD), namely those who have (a specific part of) the Y chromosome in their karyotype (eventually only at the gonadal level) are at increased risk for the development of malignant germ cell tumors [5,6], referred to as Type II germ cell tumors [7], (see Ref. 8 for a review). This has been related to the presence and aberrant expression of the testis-specific protein on Y *(TSPY)* gene, proximal on Yp [9-13].

The recent change in attitude toward clinical management of DSD patients, with increased emphasis on a conservative approach, and the delay of irreversible surgery until adulthood [14-17] has created doubt concerning the optimal approach with regard to gonads at risk for malignant transformation, *e.g.* in individuals with 45,X/46,XY mosaicism and male gender. Gonadectomy is not the treatment of choice in these patients, but on the basis of a review of the relevant literature, tumor risk in 45,X/46,XY individuals has been reported to be around 15% [8]. However, clinical experience suggests a much lower incidence in 45,X/46,XY Turner syndrome girls. On the other hand, recent research has identified undifferentiated gonadal tissue (UGT), for which 45,X/46,XY is a known risk factor, as the precursor lesion for gonadoblastoma [18,19]. More generally, on the basis of pathological studies in gonadal samples from DSD patients, it has been demonstrated that a disturbed process of gonadal development, affecting Sertoli or granulosa cell differentiation and function, results in insufficient microenvironmental stimuli for the germ cells and hence in a delay or block in their maturation. Immature germ cells are

immunohistochemically characterized by increased TSPY expression and prolonged expression of embryonic germ cell markers, including the octamer binding transcription factor 3/4 (OCT3/4), encoded by the gene Pit-Oct-Unc domain class 5 transcription factor 1 *(POU5F1)* [18,20-23], a condition which has been linked to malignant transformation and proliferation [13]. The precise function of TSPY remains unknown. However, its aberrant expression has been related to increased proliferation of germ cells and oncogenic activity [10-12,24-26]. In embryonic stem cells, OCT3/4 is involved in the maintenance of pluripotency [27,28], but in primordial germ cells, experimental data rather suggest a role in their survival [29]. In this context, the position of the OCT3/4 positive germ cells within the testis tubule is of relevance: a luminal position corresponds to simple maturation delay, whereas a position on the basal lamina points at resistance to apoptosis of an aphysiological immature germ cell [20]. Immunohistochemical staining for the c-KIT ligand stem cell factor (SCF, also known as KITLG), which is of pathogenetic relevance in the development of germ cell tumors [30,31], constitutes an important additional marker supporting the differential diagnosis between maturation delay and neoplastic transformation of germ cells because SCF positivity is consistently detected in carcinoma *in situ* (CIS), gonadoblastoma and testicular germ cell tumors, but not in testes with maturation delay, [32,33]. Thus, recent research has provided us with tools to detect not only early stages of CIS and gonadoblastoma but also to identify premalignant lesions and germ cells at risk for neoplastic transformation, allowing to predict tumor development in gonadal biopsy samples and prophylactically removed gonads at a young age [18,19,33-35].

The present study was designed to refine our knowledge on tumor risk in the highly heterogeneous condition, which is 45,X/46,XY mosaicism. Specifically we examined whether a precise description of the clinical phenotype could be supportive in optimal patient management, including gonadal surgery and follow-up for tumor risk.

Materials and methods

Collection of gonadal samples and clinical data

Most samples (n=75, from 39 patients), obtained by biospy or gonadectomy, were retrieved from the archives of the pathology departments of the Erasmus Medical Center Rotterdam, the University Hospital Ghent, and the University Hospital Motol,

Prague. Samples were reviewed by M.C., J.P. and J.W.O., experienced in gonadal histology and germ cell tumor pathology. The 45,X/46,XY mosaicism was diagnosed on the basis of routine karyotyping, in case of doubt, additional investigations based on local protocols [fluorescence *in situ* hybridization (FISH) with centromere Y probe, buccal smear chromosome analysis, gonadal karyotyping by FISH] were used to confirm the diagnosis. Patients were excluded if sufficient or reliable clinical data were not available, or if the diagnosis 45,X/46XY (or variants) mosaicism was uncertain. Clinical data were recorded by the treating physicians (M.C., P.H., E.V.L., J.L., S.L.S.D., and K.P.W.) and reviewed by M.C. and J.P. Additional samples (n=12, from nine patients) were obtained from referring centers; corresponding clinical data were provided by the treating pediatric endocrinologists. Patients were classified into three groups, based on the external masculinization score (EMS), which represents a clinical scoring system (based on the position of the gonads, length of the phallus, presence of scrotal fusion and position of the urethral meatus) to quantitatively assess the degree of undervirilization in DSD patients [36]. The EMS was calculated from data of the first clinical presentation: Group 1, mild undervirilization, EMS 7-12; Group 2, ambiguous phenotype, EMS <7; and Group 3, female phenotype, representing in fact girls with Turner syndrome (without clitoromegaly).

Immunohistochemical staining

Tissue fixation was performed with 10% formalin for 24 h, followed by paraffin embedding and preparation of slices of 3 µm thickness. For immunohistochemistry, heat-induced antigen retrieval was applied in all stainings. OCT3/4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), dilution 1:350; for pretreatment: H_20_2 for 5' + biotin blocking was used; the incubation time was 2 h at room temperature; the secondary antibody was biotinylated rabbit-antimouse. TSPY (kindly provided by Prof C. Lau, Department of Medicine, VA Medical Center, University of California, San Francisco, CA): was used at a dilution of 1:3000; incubation time was overnight at 4°C; the secondary antibody: swine-antirabbit, biotin labeled. SCF (Santa Cruz Biotechnology) was used at a dilution of 1:350 to 1:500, with an incubation time of overnight at 4°C; the secondary antibody: horse-antigoat, biotin labeled. Detection was performed using diaminobenzidine/H_20_2 (OCT3/4) or New Fuchsin/Naphtol ASMX phosphate (Sigma, Steinheim, Germany) (TSPY, SCF), and counterstaining was with hematoxylin.

Microscopy and assessment of tumor risk

Based on the general morphology, as assessed on hematoxilin-eosin (HE) staining, the samples were categorized as (dysgenetic) testis, UGT, ovary, streak, or a combination of these. The sample was considered to be at risk for germ cell tumor development if either an *in situ* neoplastic lesion (gonadoblastoma or CIS) or one or more indices for premalignancy (UGT, OCT3/4 positive cells on the basal lamina of testis tubules, or positive SCF staining) were present.

Statistical analysis

Results were analyzed with the SPSS software (version 15.0; Chicago, IL), comparison of categorical variables was performed using a Fisher exact test.

Ethics

The study was approved by the medical ethical committees of the University Hospital Ghent (MEC 2008/098), the Erasmus Medical Center Rotterdam (MEC 02.981), and University Hospital Prague (EC 237/09). 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).

Results

An overview of the patients and samples is provided in Table 1. The group of individuals with female phenotype is overrepresented which probably relates to the finding that most 45,X/46,XY cases live undiagnosed (as normal males) [1]. Surgery is delayed in this group, due to later diagnosis (mostly because of short stature) than in groups with mild undervirilization and ambiguous phenotype.

In 16 of 87 samples (18.3%), no gonadal tissue was found; hence, for these samples (further referred to as vanished), the gonadal position could not be determined.

In total, 84 gonadal samples were considered for further analysis; one sample was excluded because it was a gonadectomy specimen of a previously biopsied gonad and revealed no new findings. In one patient who received a left biopsy and a right gonadectomy, the EMS could not be determined based on available clinical

information. For the different phenotypic groups, the gonadal position, as recorded during the biopsy/gonadectomy procedures, is represented in Figure 1A.

Table 1: Overview of the study population and available samples

	Mild undervirilization (EMS ≥7)	Ambiguous phenotype (EMS < 7)	Female phenotype (Turner syndrome)
Patients	10	14	23
Gonadal samples	15	24	46
Biopsy	9	6	
Gonadectomy	6	18	46
Sex of rearing			
Male	10	10	
Female	0	4	23
Mean age at surgery (yr)	4.0	2.2	12.2

EMS and sex of rearing unknown in one patient

Figure 1B shows the prevalence of the various gonadal differentiation patterns in our series, and Figure 1C represents the distribution of these patterns within each phenotypic group.

Scrotal gonads were all recognized as testes. Gonads in the inguinal position were mostly testes (72%), although UGT (18%) and streak (9%) were also encountered. Abdominal gonads mostly presented as streak tissue (68.5%), but interestingly, also testis (20.5%) or a combination of testis+UGT (3.7%) is also possible. An abdominal gonad with UGT differentiation was found in 5.6%, the only gonad with ovarian differentiation was in the abdominal position (data not shown).

Figure 2 shows representative examples of the gonadal differentiation patterns that were encountered in the 45,X/46,XY individuals included in this study.

An *in situ* neoplasia, but no invasive tumors, was found in four different patients (Table 2). One patient with an EMS of 7.5 of 12 received prophylactic gonadectomy of a right abdominal gonad, which on microscopic examination contained UGT with gonadoblastoma. On the left side, a scrotal testis was present. One patient with an EMS of only 1 of 12 received prophylactic surgery at the age of 1 yr and was found to have UGT with gonadoblastoma in the left abdominal gonad; the right abdominal testis displayed no neoplastic features. One patient (EMS 1.5 of 12) had a gonadoblastoma in a severely dysgenetic inguinal testis, the right abdominal gonad was a streak. Surgery was performed at the age of 1 yr. The last patient was diagnosed with Turner syndrome after work-up for delayed puberty. None of the treating physicians noticed any clitoral enlargement. However, prophylactic

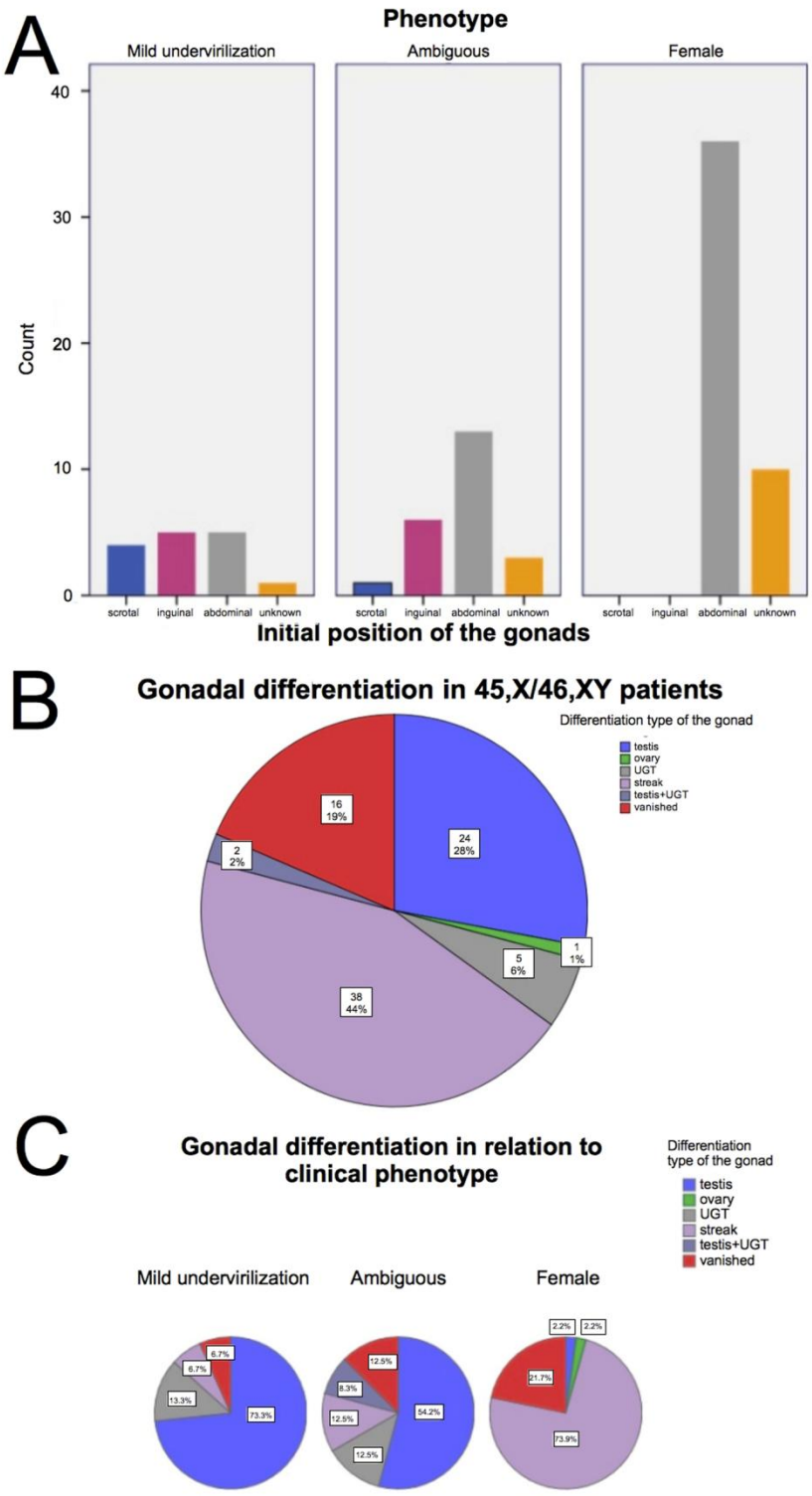

Figure 1: A - location of the gonads in the different phenotypic groups. Samples with an unknown position contained no gonadal tissue on microscopic evaluation; B - distribution of the encountered gonadal differentiation patterns; C - distribution of gonadal differentiation patterns in the different phenotypic groups.

gonadectomy performed at the age of 16 yr, revealed a testis containing CIS in the right abdominal gonad, whereas the left specimen contained no gonadal tissue.

All tumors in our series were *in situ* germ cell neoplastic lesions, discovered after prophylactic gonadectomy. There were no invasive tumors. Tumor risk was calculated from the presence of either *in situ* neoplasia or preneoplastic changes, as described in *Materials and Methods*. [a] $P < 0.001$.

Table 2: Summary of gonads containing tumors or preneoplastic lesions in patients with 45,X/46,XY mosaicism, taking to account the clinical phenotype.

	Mild undervirilization	Ambiguous phenotype	Female phenotype	Total
No risk	14	21	45	80
Tumor	1	2	1	4
Preneoplastic lesion	1	10	0	11
Risk[a]	2/15 (13%)	12/23 (52%)	1/46 (2.2%)	15/84 (18%)

Discussion

Tumor risk has been estimated at 15% in 45,X/46,XY individuals [8]. However, in clinical practice, histological examination of prophylactically removed gonads in Turner girls with 45,X/46,XY suggests a much lower incidence, whereas no data are available for boys with 45,X/46,XY. Specifically in this group, it is of interest to preserve gonads to allow endogenous hormone production, and therefore spontaneous puberty induction and maintenance. In previous years, tools have been developed by our group to recognize germ cells with premalignant characteristics, such as a maturation delay or block of germ cells, an immature environment, and an increased potential to proliferate and to resist apoptosis [13,18,20-23,32) (reviewed in Refs. 7,8,19,34,35,37-39). It was shown that a combination of these characteristics may lead to CIS or gonadoblastoma, depending on the context of the microenvironment [18,20,40,41]. For CIS, it has been demonstrated that all cases will become invasive over a lifetime period [42]; for gonadoblastoma this is less clear. This study was undertaken to examine in a large series of patients with 45,X/46,XY, clinically a very heterogeneous group, if the clinical phenotype reflects the gonadal phenotype and tumor risk, and whether this may provide a tool in clinical practice to

guide management with regard to gonadal biopsy or gonadectomy in this patient population.

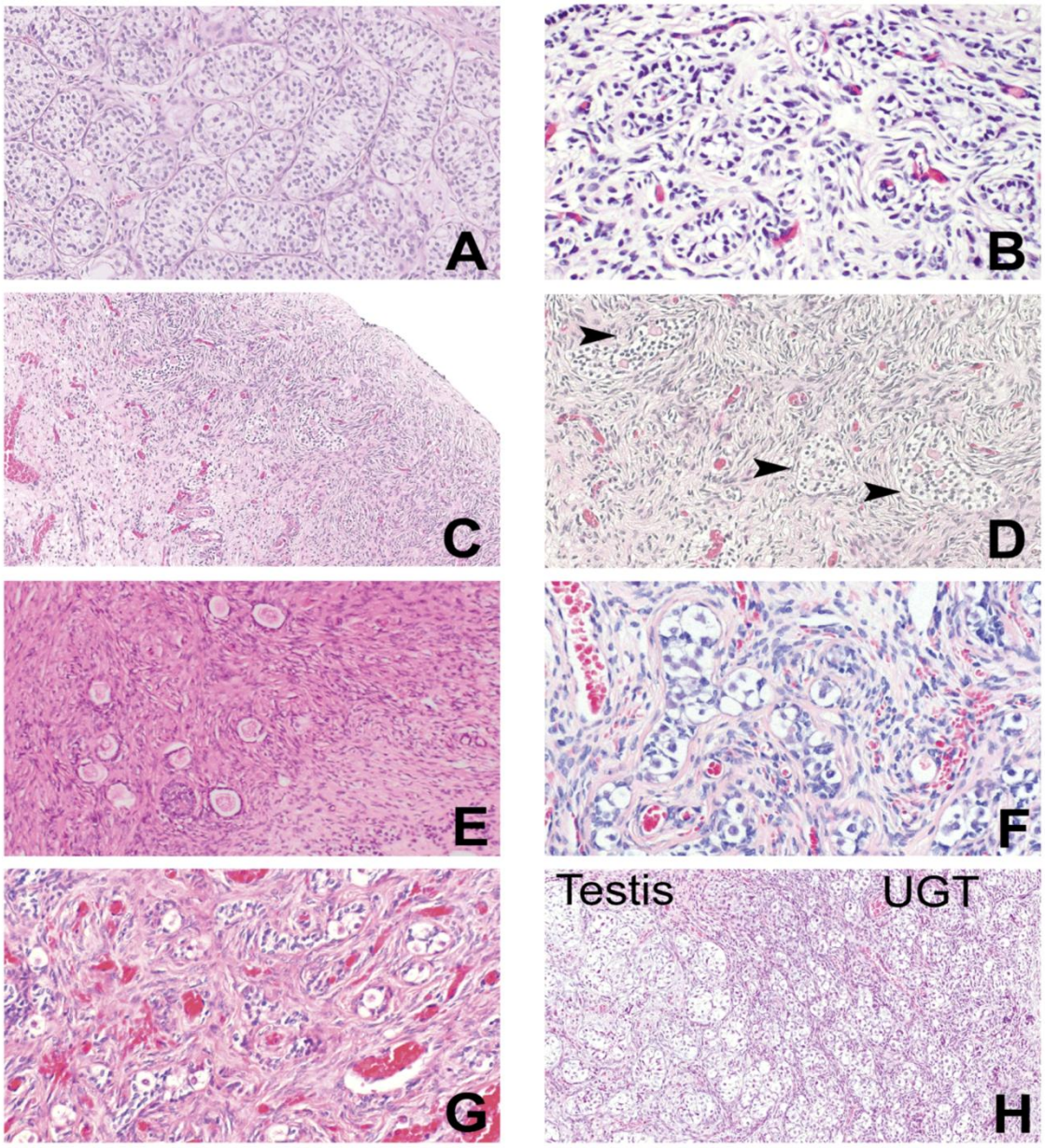

Figure 2: Representative examples of the gonadal differentiation patterns that were encountered in our series of patients with 45,X/46,XY mosacism. A - normal testis, HE, 200x; B - dysgenetic testis tubules, showing a thin basal lamina, an irregular tubular shape, and increased stromal background, HE, 200x; C - streak, HE, 100x; D - enlargement of C, clearly showing primitive testis cord-like structures (arrows), HE, 200x; E - ovarian follicles, encountered in only 1 gonad in our series, HE, 200x; F and G - UGT, HE, 200x; H - combined pattern with testis (left) and UGT (right), HE, 100x.

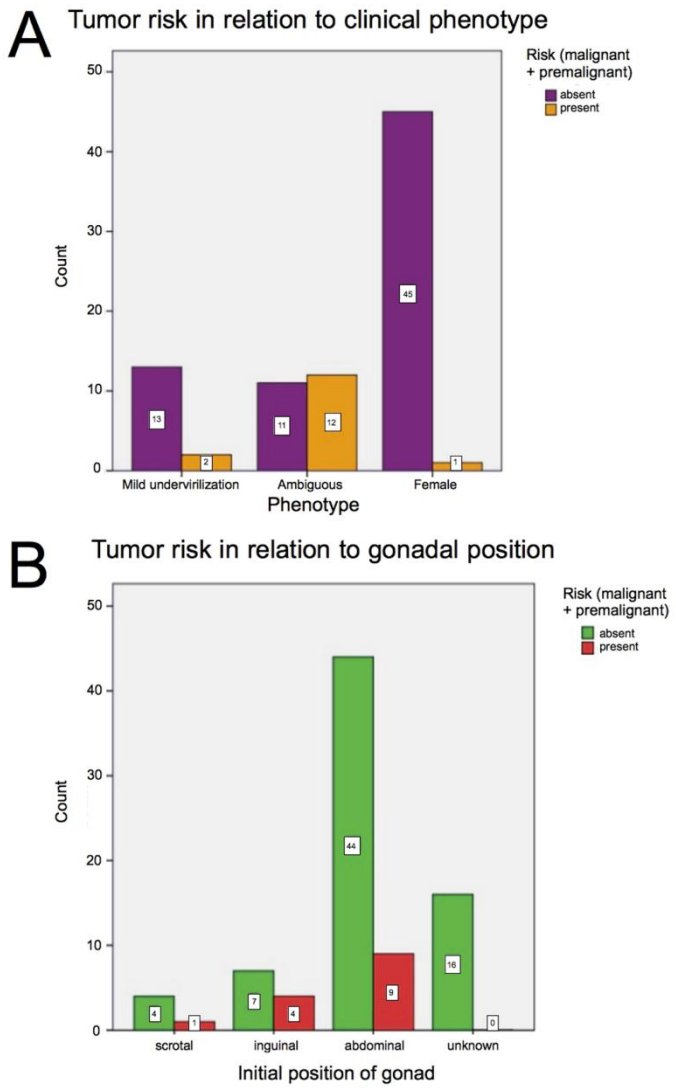

Figure 3: A - The presence of premalignant lesions or an *in situ* neoplasia in the gonads from patients with 45,X/46,XY mosaicism, categorized according to their clinical phenotype; B - presence of premalignant lesions or an *in situ* neoplasia in gonads from patients with 45,X/46,XY mosaicism, categorized according to the position of the gonad.

The distribution of respective cell lines as obtained from peripheral blood karyotyping was not taken into account for this study because previous observations revealed no correlation between peripheral blood karyotype and gonadal karyotype or gonadal differentiation patterns [4].

Morphological examination of the specimen revealed some interesting findings. First, the absence of gonadal tissue was observed frequently in our series (18%), as in other causes of gonadal dysgenesis (M. Cools, unpublished observation). Although theoretically it cannot be excluded that the surgeon missed the gonad while

performing the gonadectomy procedure, this is unlikely, taken into account the frequency of this finding, and the experience of the involved surgeons. Therefore, we hypothesize a mechanism in which the gonadal anlage, if unable to develop into a more mature stage, regresses by apoptosis. Second, categorization of gonadal differentiation patterns in 45,X/46,XY was difficult because they represent a continuum between two extremes (normal testis and normal ovary) rather than easily determinable separate entities, as is shown in Figure 2. Third, streak, defined as non-functional gonadal tissue, or even UGT was often referred to as ovarian-type stroma in official pathology reports, probably due to the background of stromal cells. However, the terminology of ovarian-type stroma was interpreted by some clinicians as ovarian tissue (defined by the presence of follicles including germ cells), eventually available for cryopreservation. In fact, irrespective of the clinical phenotype, the finding of ovarian follicles was rare in 45,X/46,XY mosaicism (one of 87 samples), even from tissue removed at a very young age. This is in contrast to observations in 45,X and 45,X/46,XX gonads [43]. Likewise, ovarian follicles in the context of an ovotestes (defined as the copresence of testis and ovarian tissue, including follicles, in one individual) were not encountered in our population, unlike in 46,XX/46,XY chimerism (M. Cools, unpublished observation). Streak tissue (in our series present in 44% of samples) by definition does not contain germ cells, but also in dysgenetic testes and UGT, germ cells were scarce. Increased apoptosis of germ cells has been attributed to a defective microenvironment and impaired meiosis of aneuploid germ cells [44].

Tumor risk was significantly reflected by the clinical phenotype in our series ($P < 0.001$) and revealed to be very high (52%) in cases with an ambiguous phenotype. This group has the highest prevalence of UGT (20.8%), which has been recognized as the precursor lesion for gonadoblastoma [18] (Figure 1C). Moreover, testes, if present, were severely dysgenetic in this group, and often contained immature OCT3/4-positive cells on the basal lamina, in contrast to patients with mild undervirilization, in whom UGT was less frequently observed (13.3%), and testes had attained a more mature stage, with less pronounced shape irregularity of the tubules and more frequent loss of OCT3/4 expression in germ cells that had reached the basal lamina. Moreover, testes were more often in the scrotal position in this group (Figure 1A). Cryptorchidism is known as an independent risk factor for the development of germ cell tumors [45], which has been related to maturation delay of

germ cells [46]. This risk is probably higher in inguinal than in abdominal gonads, due to early apoptosis of germ cells in the latter position [Ref. 47 and M. Cools, unpublished observations]. In our study, inguinal gonads revealed the highest tumor risk but this was not statistically significant, maybe due to small sample size (Figure 3B). Twenty percent of 45,X/46,XY testes with spontaneous scrotal descent revealed premalignant characteristics but this number represents in fact only one of five gonads, from an individual with an EMS of 5.5 and so belonging to ambiguous phenotype group. In interpreting these data, it has to be kept in mind that the differentiation patterns in these gonads and the clinical phenotypes of the patients were very heterogeneous, independently influencing tumor risk, in contrast to studies

Table 3: Summary of available clinical functional data in male patients with at least one preserved gonad or in females before gonadectomy.

Patient	EMS	Gonadal position[a]	Assessment	T	Remarks
Childhood					
3	8.5	1 inguinal testis 1 scrotal testis	HCG, 3yr HCG, 10yr	60 ng/dl (2.1 nmol/l)[b] 54 ng/dl (1.9 nmol/l)[b]	
8	7.5	1 scrotal testis	HCG, 1.5yr	109 ng/dl (3.8 nmol/liter)[b]	Test after unilateral gonadectomy
14	1	1 abdominal testis 1 abdominal UGT	HCG	Good	
28	4.5	1 abdominal testis 1 inguinal testis	HCG, 2wk	140 ng/dl (4.8 nmol/liter)[c]	
35	4	1 abdominal testis 1 vanished gonad	T, 3wk	135 ng/dl (4.7 nmol/liter)[c]	Bilat gonadectomy and raised female
37	5.5	1 inguinal testis 1 scrotal testis	T, 2wk	172 ng/dl (6.0 nmol/liter)[c]	
38	4.5	1 abdominal testis 1 inguinal testis	HCG, 9m	459 ng/dl (15.9 nmol/liter)[c]	Bilat gonadectomy and raised female
(Post)puberty					
2	11.5	1 inguinal testis 1 scrotal testis	PE	P3G3, 6/6 ml	No T suppletion
5	8	1 scrotal testis (originally inguinal)	PE, FSH, US, sperm count	Nl pubertal development, FSH↑, infertility, Nl US	Actually 30yr old, no T suppletion
10	4.5	1 scrotal testis (originally inguinal)	PE	G2, 4 ml	No T suppletion
12	1.5	1 scrotal testis	PE, FSH		Actually 15yr old, no T suppletion

Assay and age specific baseline references (reference values after HCG were not available): T - serum testosterone; HCG - human chorionic gonadotropin test, PE - physical examination; US - Ultrasound; Nl - normal.

[a] At the moment of assessment. [b] 8.6-14.4 ng/dl (0.3-0.5 nmol/liter). [c] 14-363 ng/dl (0.5-12.6 nmol/liter).

in patients with simple cryptorchidism. In the phenotypically female group, most gonads were streak or had vanished, resulting in a low tumor risk (Table 1 and Figure 1C). Only one of 46 gonads (2.2%), from a 16 years old girl with Turner syndrome and a vanished gonad on the contra-lateral side, displayed testis differentiation, notably with CIS. Remarkably, no virilization, not even clitoral enlargement was noticed in this girl. The reason for the discrepancy between this gonad and the 45 other samples from this group remains unexplained.

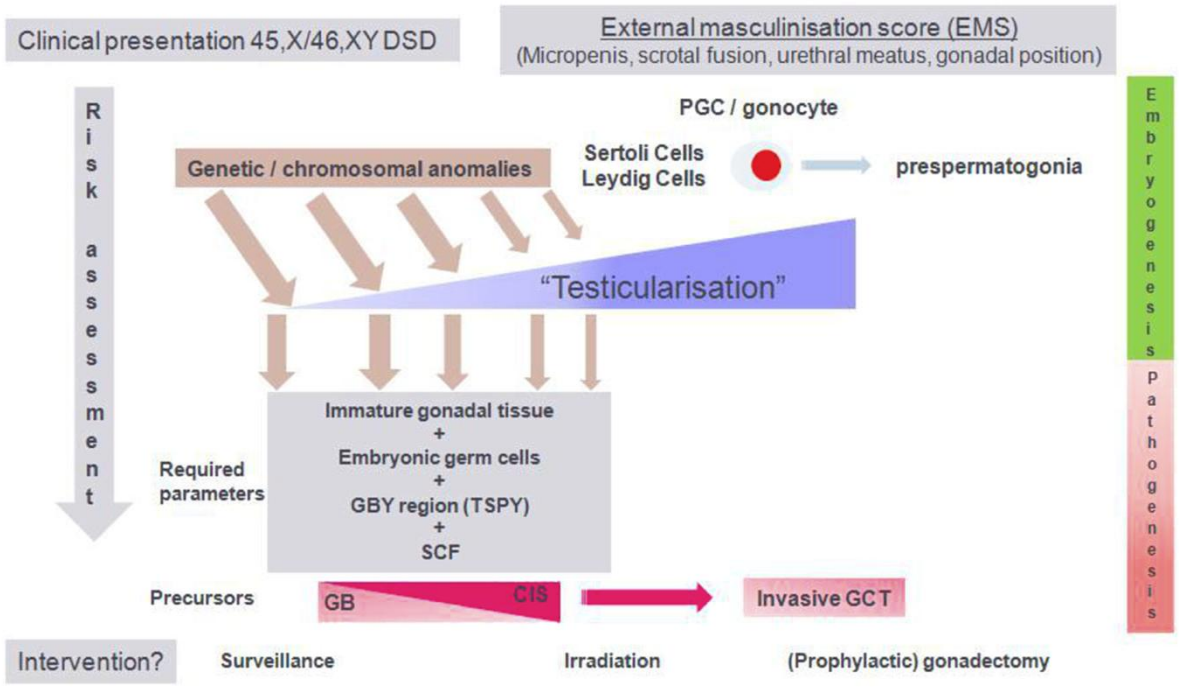

Figure 4: Suggested pathophysiology of germ cell tumor development in 45,X/46,XY gonads. The EMS score quantitatively describes the patient's phenotype. Higher EMS scores correlate with a more advanced process of testis formation at the gonadal level. Disturbed gonadal development, resulting from the abnormal karyotype, leads to impaired Sertoli/granulosa cell function. Subsequently, germ cells escape the normal control mechanisms exerted by the supportive cell lineage (i.e. differentiation and mitotic or meiotic arrest), causing a delay or block in their normal maturation process and leading to increased survival (prolonged OCT3/4) and proliferation (increased TSPY) chances. Especially in gonads with a low degree of testicularization, the four parameters required for malignant proliferation of germ cells are present, leading to a high risk for the development of gonadoblastoma or CIS, depending on the microenvironment, and subsequently to an invasive germ cell tumor. PGC - primordial germ cell; GB - gonadoblastoma; GBY - gonadoblastoma region on Y; GCT - germ cell tumor. [Adapted with permission from L.H. Looijenga et al.: Best Pract Res Clin Endocrinol Metab 2010;24:291-310 (19). © Elsevier.]

Functional outcome data in males with at least one preserved gonad were scarce but suggest a sufficiently conserved Leydig cell function to allow spontaneous puberty, with, as expected, high FSH levels, predicting impaired Sertoli cell function, and infertility [48]. However, these preliminary data emphasize the benefit for the male 45,X/46,XY individual if gonadectomy can be avoided. Guidelines for conservative follow-up of these patients, and for the timing and handling of testicular biopsies are awaiting.

In 45,X/46,XY Turner girls, without signs of virilization, tumor risk is low, but the gonadal tissue, if present, in most cases is a non functional streak, making the preservation of these gonads of no use. However, in cases where the girl is very reluctant to have surgery, or if surgery is contraindicated, gonadectomy can be postponed without great risk.

The question remains whether, in cases of phenotypical Turner syndrome and a diagnosis of 45,X monosomy after routine cytogenetic analysis, additional investigations are warranted to detect hidden mosaicism. FISH analysis with centromere X and Y probes on interphase nuclei, which is reported to be superior to PCR in this context due to a lower number of false positive results, reveals an additional 46,XX cell line in 30% and a 46,XY cell line in 10% of cases [49]. In view of this high number and in contrast to previous suggestions [8], we currently subscribe the American College of Medical Genetics guidelines, suggesting routine screening for hidden mosaicism in all 45,X women by additional FISH analysis on 200 interphase nuclei harvested from buccal smear [49]. However, to resolve this long-standing question, we believe it is mandatory to report on tumor incidence in larger series of 45,X/46,XY Turner women in whom even discrete signs of virilization, pointing at the presence of some testicular differentiation at the gonadal level, were explicitly sought and excluded.

To summarize, our data suggest that tumor risk in 45,X/46,XY patients is most pronounced in immature and/or poorly differentiated gonadal tissue and that the degree of testicularization of the gonad (defined as the process of testicular development in its broadest sense) is reflected by the clinical phenotype [19]. This hypothesis can modify our clinical approach to the 45,X/46,XY patient, resulting in an individualized management with regard to tumor risk and gonadectomy (Figure 4 and Table 4). Future research and long-term follow-up of these patients is necessary to demonstrate the safety and benefit of this approach.

Table 4: Guidelines for individualized management with regard to gonadectomy in 45,X/46,XY mosaicism

Mild undervirilization (EMS ≥ 7)
Orchidopexy
Regular self-examination (every 3 months) and ultrasound (annually) from puberty onward
One prepubertal biopsy (ideally between ages 1 and 9 yr or in combination with an orchidopexy procedure) and one post pubertal biopsy (*e.g*. at 17-25 yr of age) to assess tumor risk by specialized immunohistochemistry
In case of premalignant changes (OCT3/4 positive cells on the basal lamina/expression of SCF/presence of UGT) or *in situ* neoplasia: gonadectomy (or irradiation?)
Ambiguous genitalia (EMS < 7)
See guidelines for Mild Undervirilization
Low threshold to perform gonadectomy (e.g. insufficient hormone production necessitating hormone replacement therapy; impossibility to bring the gonad in a stable scrotal position; suspicion for malignancy on physical examination or ultrasound; immunohistochemical abnormalities related to pre-CIS lesions, such as OCT3/4-positive cells on the basal lamina or positive stem cell factor staining; or presence of UGT on the biopsy)
Female phenotype
Elective gonadectomy (if patient is reluctant to gonadectomy, consider leaving the gonads in place)
Cryopreservation not indicated

The approach to the 45,X/46,XY patient can be individually tailored, based on his/her phenotype, and varies from careful surveillence, (repeated) biopsy, irradiation of a CIS lesion, or prophylactic gonadectomy (see also Fig. 4)

Acknowledgement

We thank explicitly all referring clinicians (J.P. Bourguignon, M. Maes, G. Massa, G. Holmdahl, R. Gannaway, R. Goerse, O. Hiort, C. Clementson, J. Horejsi, M. Snajderova, J. Zapletalova, and D. Novotna) as well as the Pathology department of the University Hospital Ghent and the University Hospital Prague-Motol for their support in collecting tissue and clinical data. Members of the Mosaicism Collaborative Group include the following: J.P. Bourguignon, Department of Pediatrics, Sart Tilman University Hospital, Liège, Belgium; Kockum C. Clementson, Department of Pediatric Surgery, University Hospital Lund, Sweden; R. Gannaway, Department of Human and Molecular Genetics, Virginia Commonwealth University Medical Center, Richmond, VA; D. Gisselsson, Department of Clinical Genetics, University Hospital Lund, Sweden; R. Goerse, Department of Gynecology, University Hospital Regensburg, Germany; O. Hiort, Department of Pediatrics, University Hospital Schleswig-Holstein, Campus Lübeck, Germany; G. Holmdahl, Department of Pediatric Surgery, Queen Silvia Children's Hospital, Gothenburg, Sweden; M. Maes, Department of Pediatrics, University Hospital Saint-Luc, Brussels, Belgium; G. Massa, Department of Pediatrics, Virga Jesse Hospital, Hasselt, Belgium.

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Chapter 5

45,X/46,X,psu dic(Y) gonadal dysgenesis: Influence of the two cell lines on the clinical phenotype, including gonadal histology

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Sex Dev 2013;7(6):282-8.

Abstract

A child born with ambiguous genitalia (Prader III) was found to have a 45,X[92.2%]/46,Xpsu dic(Y)(p12)[7.8%] karyotype. Testosterone level was consistent with that of normal male, however, gonadotropins were elevated. Ultrasound and endoscopy of the urogenital sinus revealed well-developed Müllerian structures. At 3.5 months, the child was operated for right-sided incarcerated hernia, and the gonad situated at the inguinal region was biopsied and classified as primitive testis. Based on the presence of Müllerian structures, anatomy of external genitalia and wish of the parents the child was assigned female gender. She underwent removal of the left gonad at 4 months during another acute surgery; histology was similar to the right gonad. The rest of the right gonad was removed at 16 months, and feminizing genitoplasty took place at 3 years. The right and left gonad contained 28% and 22% of cells with Y chromosome, respectively. During further histological examination, dysgenetic features of the gonads were discovered. Some germ cells displayed abnormal development based on the specific expression of immunohistochemical markers (OCT3/4, TSPY, KITLG), indicating a possible risk for future malignant germ cell tumor development. Contribution of the 45,X cell line to the phenotype was also observed: the patient developed celiac disease, and her growth pattern resembled that of Turner syndrome responding to growth hormone treatment.

Introduction

A pseudodicentric Y chromosome is an aberrant Y chromosome with 2 centromeres that often appears in a mosaic karyotype with 45,X cell line. Regardless of the proportion of the 2 cell lines in peripheral blood, the clinical phenotype may vary widely between that of a normal infertile male and a female with a phenotype typical of Turner syndrome (TS) with gonadal failure and absent pubertal development [1-3]. Each of the cell lines may further contribute to the clinical presentation. This happens likely due to a varying distribution in the tissues [4]. The 45,X cell line is associated with typical issues including growth retardation, congenital disorders of the cardiovascular system and urinary tract, and a higher risk for autoimmune conditions [5,6]. The presence of a specific region on the Y chromosome in the karyotype, referred to as GBY (Gonadoblastoma locus on Y), increases the risk for development of a gonadal malignant germ cell tumor in the environment of a dysgenetic gonad [7-9]. The overall risk rate was estimated to be 15-40% in individuals with 45,X/46,XY karyotype [7]. A recent study correlating phenotype of external genitalia and risk for malignant germ cell tumor development in patients with mosaicism revealed that individuals with ambiguous genitalia are at significantly higher risk than those with female phenotype or mild hypovirlization [8].

We present a case report of a child with a 45,X/46,Xpsu dic(Y)(p12) karyotype and congenital ambiguous genitalia in whom the influence of both cell lines on the clinical phenotype was remarkably combined. To the best of our knowledge, we describe for the first time a detailed histological picture of the gonads of such an individual, including an immunohistochemical analysis of the expression of various markers of germ cell development (OCT3/4, TSPY, KITLG) [10-15] and supportive cell development (FOXL2, SOX9) [16].

Case report

Following an uneventful pregnancy, the child was born with ambiguous genitalia to unrelated healthy parents in the 39th week of gestation, with a birth weight of 2740 g (10th percentile) and length of 47 cm (10th percentile). The external genitalia were described as a 20-mm-long phallus with ventral chordae, a common orifice for the genital and urinary tract, a bifid scrotum and gonads bilaterally palpable in the groins

(Figure 1A), classified as Prader III. Hormonal levels were measured at day 10 for the first time. The testosteron level was within normal range for males (11.09 nmol/l), FSH and LH levels were elevated (11.1 U/l and 11.3 U/l, respectively) indicating dysgenetic nature of the gonads. The testosteron level increased (15.89 nmol/l) and the phallus temporarily elongated to 24 mm during hCG test. Two defined cell lines, 45,X [92.2%] and 46,X+Ymarker [7.8%], were identified in peripheral blood lymphocytes. Additional FISH analysis with probes targeted against centromeric and subtelomeric regions of the Y chromosome (Cytocell, Cambridge, UK) and SRY (Abbott-Vysis, Illinois, USA) identified the derivative chromosome as a pseudodicentric Y chromosome psu dic(Y)(pter→q11.2::q11.2→pter) carrying 2 copies of the *SRY* gene (Figure 1B,C).

A 20-mm-sized uterus was detected by ultrasonography, no asymmetry or hypoplasticity was described. Endoscopy of urogenital sinus revealed a common sinus ending with 2 openings: The first led to a vagina and further to a uterus with 1 prominent transverse mucosal fold; the second opening led into a short urethra. No additional investigations of the inner genitalia, such as MRI or laparoscopy, were performed. At the age of 3.5 months, the child underwent an urgent surgery due to a right-sided incarcerated inguinal hernia. The gonad located at the inguinal region was biopsied and fixed with adnexa (resembling tuba) in the abdominal cavity. The gonad was classified histologically as a primitive testis in the original report. Based on the presence of Müllerian duct structures (possibly allowing future fertility with donated oocyte) and better outcome from feminizing rather than virilizing genitoplasty, the multidisciplinary team recommended assigning the child female gender. A possible reduction of final height due to the 45,X line, generally better accepted in girls, was also considered. Last but not least, the wish of fully informed parents, who already had an older son, was respected. Two weeks after the first surgery, a contralateral hernia required a second surgical intervention. Because of the already assigned female gender, the gonad on the left side was removed during the operation (this did not prolong the urgent procedure) and was classified as a primitive testis as well. A gonadectomy of the residual right gonad was performed at 16 months (the detailed histology is given below) and a feminizing genitoplasty took place at 3 years of age after toilet mastering.

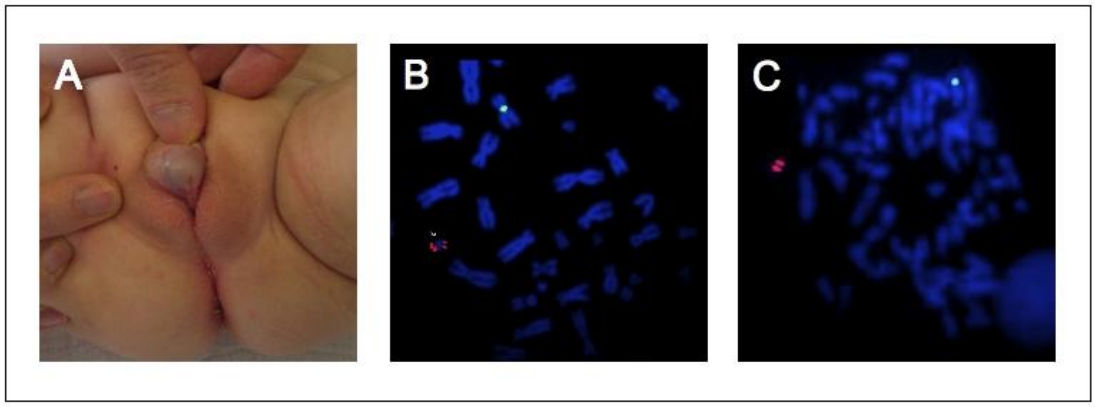

Figure 1: A – ambiguous genitalia; B – FISH detection of SRY region on Y chromosome (red) present in four copies and a single centromere of X chromosome (green) during mitotic metaphase; C – FISH detection of two centromeres of Y chromosome (red) and one centromere of X chromosome (green) during mitotic metaphase.

Despite the high percentage of 45,X cells in the peripheral blood, no typical external signs of Turner syndrome such as neck webbing, shield chest, low hair-line or lymphatic edemas were encountered. The patient developed celiac disease when she was 13 months old, and no other autoimmune disease has occurred to the current age of 5 years. Cardiac ultrasound revealed thickening of the wall of the ascending aorta with a mild pressure gradient. The girl was decribed to have relatively short lower limbs but no skeletal changes typical for TS such as Madelung deformity. Despite the midparental height at the 25th percentile, the girl had been growing at the 10th percentile until 9 months, but thereafter, her growth rate gradually declined. After the recognition of celiac disease and initiation of a gluten-free diet no catch-up growth had been observed during the subsequent 2.5 years, although the weight-for-height had improved during this period (Figure 2A). At the age of 3.3 years the bone age showed only minor delay of 4 months according to the Tanner-Whitehouse 3 method; IGF-I level was within the normal range at that time (80 IU/l). Therefore, celiac disease as a cause of growth retardation was considered unlikely, and growth hormone treatment at a daily dose recommended for patients with TS (0.05mg/kg) was started when the girl reached 3.5 years. The treatment led to an improved growth rate (Figure 2B).

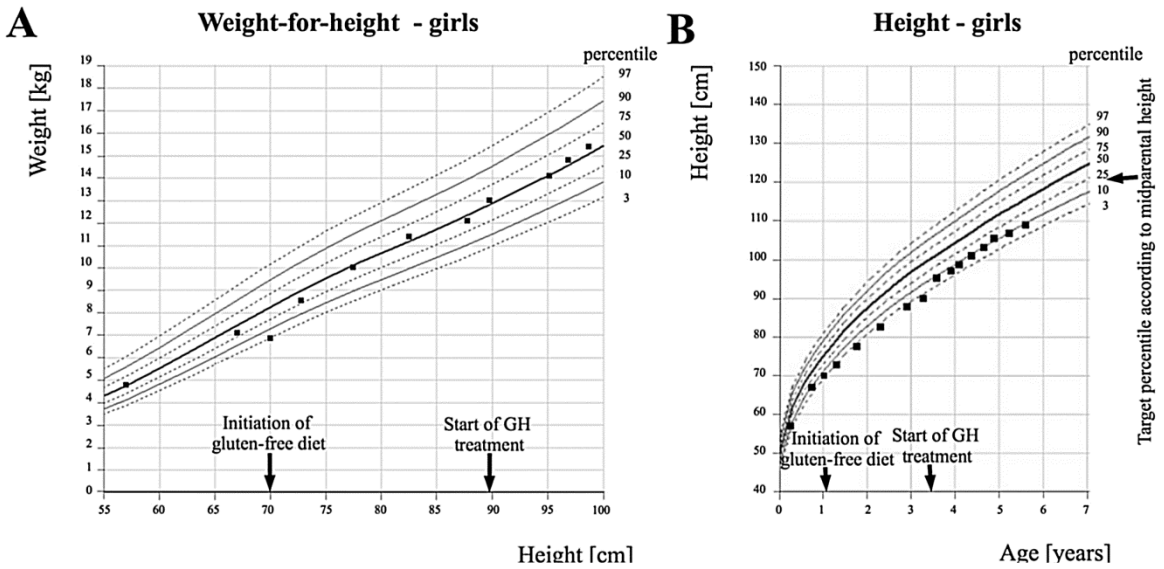

Figure 2: A - weight-for-height; B - growth chart.

Methods

The gonadal tissue was cultivated in Amniomax medium under standard conditions for 2-3 weeks. Cytogenetic samples were prepared according to standard protocols and assessed by the G-banding method. The gonadal histology was assessed on hematoxylin and eosin stained slides. Immunohistochemical detection of OCT3/4, TSPY, KITLG, FOXL2, and SOX9 (markers for gonocytes/carcinoma in situ of the testis, spermatogonia, early germ cell malignancy, granulosa cells, and Sertoli cells, respectively) (Table 1) [10-16] was performed on 4-µm-thick slides that had been pretreated in TRIS 0.01M/EGTA 0.001 and pressure-cooked (120°C, 0.9 Bar).

Results

Right gonad.

The cytogenetic analysis revealed that 28.7% of the cells in the right testis contained a pseudodicentric Y chromosome. The biopsy at age 3.5 months had the appearance of an immature testis with solid small seminiferous tubules. As expected at this age, no Leydig cells were observed. Several branching tubules indicated dysgenesis (Figure 3A). Germ cells occupied tubules in one region while only Sertoli cells were found in the tubules in the remainder of the specimen (Figure 3B). Many of the germ cells were positive for OCT3/4 and were situated not only in the center of the tubules,

as is typical for gonocytes (i.e. fetal germ cells), but also attached to the basal lamina, which suggests abnormal development. Some of the OCT3/4-positive cells were also positive for TSPY. KITLG (also known as SCF) was detectable only at very low levels. To determine the character of the supportive cells within the tubules, detection of SOX9 and FOXL2 was performed. Clusters of FOXL2-positive cells were detected in a small number of tubules, which contained SOX9-positive cells as well. The two markers were not found to be co-expressed in a single cell (Figure 3C). Germ cells were present in some but not all tubules with FOXL2-positive cells.

Table 1: Autoantibodies used for immunohistochemical analysis.

Antibody	Code/Source	Dilu-tion	Incubation	Secon-dary Ab	A B C	Chromogen
Primary antibody						
OCT3/4	sc-5279/Santa Cruz Biotechnology	1:350	2 hours; RT	RAM	HP	DAB
OCT3/4 for ds	sc-8629/Santa Cruz Biotechnology	1:150	1 hour; RT	HAG	HP	AEC
TSPY	Kindly provided by prof. C. Lau (Department of medicine, VA Medical center, University of California, San Francisco, USA)	1:4000	overnight; 4°C	SAR	AP	New Fuchsine
TSPY for ds		1:4000	overnight; 4°C	SAR	AP	Fast Blue BB
KITLG (SCF)	sc-1302/Santa Cruz Biotechnology	1:250	overnight; 4°C	HAG	AP	New Fuchsine
SOX9 for ds	AF3075/R&D Systems	1:200	2 hours; RT	HAG	AP	Fast Blue BB
FOXL2 for ds	Kindly provided by M. Fellous, Human Genetics, Cochin Institute, ICGM, Paris, France	1:500	2 hours; RT	SAR	HP	AEC
Secondary antibody						
RAM	E0413/Dako	1:150	30 minutes; RT			
SAR	E0431/Dako	1:150	30 minutes; RT			
HAG	BA-9500/Vector Laboratories	1:200	30 minutes; RT			

OCT3/4 – Octamer-binding factor 3/4; TSPY – Testis-specific protein Y-encoded; KITLG – c-KIT ligand; SCF - Stem cell factor; SOX9 – SRY-box 9; FOXL2 – Forkhead box protein L2; ds – double staining; RT – room temperature; RAM – Rabbit anti-mouse; SAR – Swine anti-rabbit; HAG – Horse anti-goat; ABC – avidin/biotin complex; HP – Horseradish peroxidase; AP – Alkaline phosphatase; DAB - 3,3´-diaminobenzidine; AEC – 3-amino-9-ethyl-carbazole.

The patient had a right-sided gonadectomy at age 16 months. Several differences were observed in the whole-gonad sample 12 months after the biopsy. A fibrotic scar with traces of haematin was visible in the middle of the cross-section after the biopsy. Multiple calcifications newly appeared in tubules throughout the entire gonad (Figure 3D). SOX9-positive supportive cells were identified, but there were no FOXL2-positive cells. The germ cells were numerous but present only in certain regions. Many of the germ cells were OCT3/4-positive and were located both in the center of the tubules and at the basal lamina. Germ cells that were double-positive for OCT3/4 and TSPY were observed. KITLG expression was more pronounced but was still relatively rare.

Left gonad. Cytogenetic analysis demonstrated that 22% of the cells contained a pseudodicentric Y chromosome in the left testis. The overall morphology was similar to the right gonad biopsy sample; the left gonad was an immature and slightly dysgenetic testis with several areas settled by germ cells. No calcifications were present. OCT3/4-positive germ cells were identified in the left gonad, and more of them were attached to the basal lamina and were double-positive for OCT3/4 and TSPY (Figure 3E) than in the right gonad. KITLG expression was much stronger in the matched areas (Figure 3F) in the left gonad. SOX9 and FOXL2-positive cells were present in approximately the same pattern as in the biopsy sample of the right gonad.

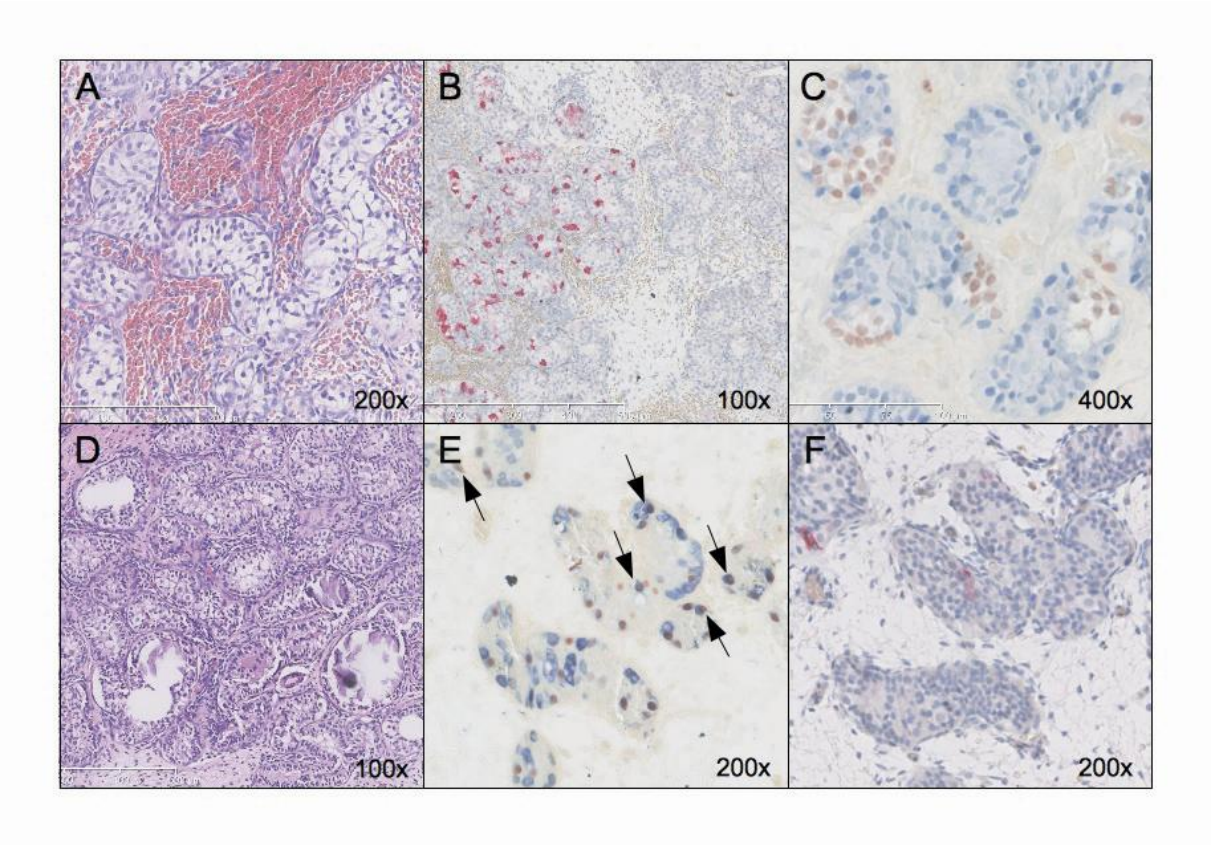

Figure 3: A – branching seminiferous tubule; B – region settled with germ cells (stained for TSPY, red) neighbouring with area of Sertoli cells only tubules; C – FOXL2 positive (granulosa) cells (orange) within seminiferous tubules settled by Sertoli cells positive for SOX9 (blue); D – multiple calcifications; E – double-staining for OCT3/4 (orange) and TSPY (blue) with double-positive cells attached to the basal lamina (arrow); F – same area with KITLG expression (red).

Discussion

Individuals with a mosaic karyotype including a 45,X line combined with a cell line containing a normal or derivative Y chromosome display a wide range of phenotypes. The clinical presentation is unpredictable on the basis of the peripheral blood karyotype and most likely depends on the representation of the 2 cell lines in particular tissues. While the line with a Y chromosome predominantly influences gonadal development and the genital phenotype, the 45,X cell lineage may affect multiple organs [5,8,17].

Both cell lines of our patient, 45,X and 46,X,psu dic(Y)(pter→q11.2::q11.2→pter), had a clear clinical effect. Although the phenotype of this patient did not display the characteristic external signs of TS, her growth pattern resembled that of patients with TS and responded well to growth hormone treatment. The patient developed celiac disease which is 11.6 times more prevalent in patients with TS than in the general population [18]. She had minor abnormal findings on a cardiac ultrasound.

The percentage of cells containing the derivative Y chromosome was 28% and 22% in the right and left gonad, respectively. This percentage and the presence of the *SRY* gene (2 copies) on the pseudodicentric Y chromosome contributed to bilateral testicular development. The incomplete virilisation of the external genitalia and the development of the Müllerian duct structures indicate impaired testicular function. Several signs of testicular dysgenesis could be observed. We identified FOXL2-positive cells in some seminiferous tubules. The expression of this marker is typical for granulosa cells, and it is not detectable in normal testicular tissue [16]. FOXL2-positive cells were present in samples from 3.5 and 4 months; however, they were not present in the sample from 16 months. This finding may indicate that these cells were unable to survive in testicular tissue. Formation of the tubules was most likely impaired to some extent, resulting in tubular branching. The microenvironment of the gonads was not favorable enough for the germ cell maintenance and led to a loss of germ cells in many of the seminiferous tubules.

Many of the persisting germ cells in the 3 histological samples retained expression of OCT3/4, a marker for fetal germ cells. This type of developmental delay has been reported in various forms of sexual development disorders. During normal development, unlike the case in our samples, germ cells switch off the OCT3/4 expression once they have reached the basal lamina. Sustained OCT3/4 expression,

in particular when co-expressed with TSPY (the most likely candidate for GBY, specific region of Y chromosome present in karyotype of DSD patients who developed malignant gonadal germ cell tumors) and combined with expression of KITLG, indicates abnormal germ cell development, which might have led to the development of carcinoma *in situ* (CIS) and an invasive germ cell tumor if the gonads had not been removed [7,9,14,15,19]. CIS cells are characterised by co-expression of OCT3/4 and TSPY, the staining is homogeneous in all germ cells within particular tubules suggesting the clonal growth; KITLG is always expressed in tubules containing CIS [14,19]. CIS is known to progress to an invasive tumor in 50% of cases within 5 years in postpubertal patients [20]. Since the samples lacked homogeneous pattern of double staining we cannot refer the germ cell lesion as a fully developed CIS in our case. Thus, it is actually unknown how high the real risk of further progression in this kind of abnormality is, and this needs to be investigated. The presence of testicular microlithiasis in the later sample of the right gonad further supports the argument that the germ cells in this case were at risk for malignant transformation because microlithiasis is known to be associated with testicular cancer development, especially in patients at increased risk (undervirilization, cryptorchidism) [21,22].

Gender assignment in individuals with ambiguous genitalia is currently a very important issue in pediatric endocrinology; however, there are no precise guidelies. During the decision-making process, one has to consider all circumstances of the individual case including parents' view. In our patient, both female and male gender could have been assigned. Presence of testicular tissue and male hormonal production, although allowing only partial virilization, spoke for male gender assignment; on the other hand, presence of Müllerian structures allowing IVF, high risk for development of gonadal germ cell tumor and possible reduction of final height were more favorable for female gender assignment. We finally respected parents' wish.

Because our patient was assigned female gender, she was recommended for removal of both gonads to prevent further virilization during the puberty and also due to the known high risk for malignant germ cell tumor development (15-40%) in patients with 45,X/46,XY mixed gonadal dysgenesis. Cancers can occur at relatively young age in these patients [7]. In case of male gender assignement orchidopexy and close follow-up of the gonads would be recommended [23].

Our case report confirms the need for a complex and multidisciplinary approach in patients with mosaic karyotype of a 45,X and a Y chromosome-bearing cell line in whom the effect of both may be combined. The early signs of the risk of the germ cells changes in our patient underscore the need for an early gonadectomy in the individuals raised as girls and cautious follow-up in boys. The presence of GBY confers a high risk for development of a malignant germ cell tumor.

Acknowledgement

This work was supported by the ESPE Research Fellowship granted by NovoNordisk A/S (JK-P) and by the project (Ministry of Health, Czech Republic) for conceptual development of research organization 00064203 (University Hospital Motol, Prague, Czech Republic) (MS, JL).

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Chapter 6

KITLG detection pattern correlates with number of abnormal germ cells in disorders of sex development

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Submitted

Abstract

The current approach to patients with Disorders of Sex Development (DSD) at increased risk of germ cell cancer tends to be more conservative. This is among others due to novel possibilities for histological diagnosis. Two immunohistochemical markers of early germ cell malignancy, POU5F1 and KITLG, have been identified and it was hypothesized that the level of KITLG positively correlates with number of abnormal, presumably (pre)malignant germ cells (POU5F1-positive cells in contact with basal lamina of seminiferous tubules; cells co-expressing POU5F1 and TSPY1). To test the hypothesis, 24 testicular DSD samples with POU5F1-positive cells, 9 samples of testis with intratubular germ cell neoplasia (IGCNU) from adult males and 17 samples of fetal testes were evaluated for KITLG positivity and for number of abnormal germ cells. The results were statistically analyzed. The number of (basal) POU5F1-positive cells decreased significantly with rising age in fetal gonads ($p<0.05$), in which up to 13.3% of POU5F1-positive cells were in contact with basal lamina, and 12.5% of the cells co-expressed TSPY1. In DSD and adult IGCNU samples the number of POU5F1-positive cells in contact with basal lamina of seminiferous tubules and/or the number of cells co-expressing POU5F1 and TSPY1 showed significant positive association with the pattern of KITLG positivity ($p<0.01$). DSD samples classified as normal or with delayed maturation were negative for KITLG and contained up to 18.5% of basal POU5F1-positive cells. All strongly KITLG-positive samples were diagnosed as pre-IGCNU or IGCNU and at least 31.7% of cells were located basally within the seminiferous tubules. In conclusion, association between KITLG staining pattern and number of germ cells with aberrant protein expression indicates an interconnection of the two phenomena in the malignant transformation of embryonic germ cells. In relation to the results, we propose criteria for delayed maturation and pre-IGCNU based on POU5F1 detection.

Introduction

Management of patients with Disorders of Sex Development (DSD) with an increased risk of germ cell cancer (GCC) has changed significantly during the past years. Especially the approach to individuals assigned to a male gender tends to be more conservative, among others due to the advances in diagnostics of early malignant characteristics of the germ cells [1].

Novel diagnostic tools include immunohistochemical detection of recently discovered markers of fetal/neoplastic germ cells (e.g., POU5F1, NANOG, AP2-gamma) and KITLG (c-KIT ligand; also known as Stem Cell Factor, SCF) [2-6]. KITLG is supposedly synthesized by Sertoli cells in physiological conditions and it has a unique position because it is, unlike the other markers, undetectable by common immunohistochemistry in testis with normal or delayed maturation of the germ cells, but consistently positive in the non-invasive progenitor of testicular GCC (intratubular germ cell neoplasia unclassified, IGCNU). It is usually detectable also in case of a IGCNU pre-stage, termed as pre-IGCNU [7].

Both POU5F1 (Pit-oct-unc domain 5 transcription factor 1, also known as Octamer-binding Transcription factor, OCT3/4) and KITLG are believed to be involved in the pathogenesis of GCC in DSD gonads. Whereas POU5F1 provides germ cells with capability of pluripotency and resistance to apoptosis, KITLG together with its receptor c-KIT controls migration of primordial germ cells towards the genital ridges and supports germ cell proliferation in the gonads [8-13]. Recently, van der Zwan and coworkers proposed a model of GCC development in which action of both proteins is interconnected (see also Discussion below) [14].

In line with this model, we hypothesized that the level of KITLG expression in DSD testes is positively associated with the number of abnormal germ cells, i.e., number of germ cells with persisting POU5F1 expression despite being in contact with the basal lamina, and/or number of germ cells which co-express POU5F1 and TSPY1 (Testis-Specific Y-encoded Protein 1, a spermatogonial marker, consistently found to be positive in pre-IGCNU and IGCNU). The two above described phenomena are rarely seen during normal testicular development and are believed to be related to GCC pathogenesis [15,16].

To test this hypothesis, we analysed 24 histological samples of testicular tissue with previously proven presence of POU5F1-positive cells obtained from patients with

various forms of DSD. It has to be emphasized here that we did not study DSD gonads containing more severely dysgenetic tissue, i.e., undifferentiated gonadal tissue, which is believed to give rise to a specific type of non-invasive GCC precursor, gonadoblastoma [17]. Because the germ cell changes met diagnostic criteria for the adult-type IGCNU only in one DSD testis, nine testicular samples with IGCNU from males without any signs of DSD were included to complete the spectrum of non-invasive germ cell lesions. Additionally, we analysed testicular samples from 17 fetuses of different gestational ages, supposedly with physiological germ cell development. We compared results from fetal gonads with findings in DSD.

Material and methods

Patients and tissue samples

We analysed 24 tissue samples of normally developed or dysgenetic testes from 19 patients with various forms of DSD. All included patients contain a specific part of the Y chromosome in their karyotype known as the GBY (GonadoBlastoma on the Y chromosome), for which *TSPY1* is the most likely candidate gene [18,19]. All the samples had been proven to contain POU5F1-positive germ cells during the diagnostic procedures. The tissue specimens were obtained from a biopsy or from prophylactic gonadectomy. The age of patients ranged from 4 weeks to 18 years (median age 1.5 years). The characteristics of the patients and gonads are summarized in Table 1. Five additional samples of testicular tissue from DSD patients (age range 9 months to 17 years, median 3.5 years) containing only POU5F1-negative germ cells were analysed for KITLG expression as negative controls.

The series was extended with nine samples of testicular tissue containing IGCNU which were obtained from adult males without any signs of DSD. The age at the time of biopsy or orchiectomy ranged between 18 and 49 years (median 30.0 years). IGCNU was adjacent to an invasive GCC front in 6 cases.

Finally, gonads from 17 male fetuses after spontaneous or induced abortions, or from preterm and term male neonates who died shortly after birth were assessed. Gestational age ranged from 15 to 40 weeks and was calculated based on the mother's last menstruation cycle and confirmed by measurement of foot lenght. Only fetuses without genital malformation and with well preserved testicular tissue were included.

Table 1: Overview of DSD gonads and patients involved in the study.

P	Diagnosis	Age	Gon	Histology	KITLG score	% of basal POU5F1 + cells	% of double-positive cells
1	SC-DSD	2.5y	right	DM	0	10.0	6.7
2	SC-DSD	4m	left	pre-IGCNU	2	54.7	66.3
		1.3y	right	pre-IGCNU	2	58.6	82.8
3	CAIS	9.5y	left	pre-IGCNU	2	70.6	52.9
4	CAIS	1.4y	left	DM	0	18.5	15.4
5	CAIS	6.0y	left	pre-IGCNU	1	23.3	43.3
		6.0y	right	pre-IGCNU	2	66.7	81.5
6	CAIS	15.7y	right	pre-IGCNU	1	55.6	72.2
7	CAIS	15.7y	right	pre-IGCNU	2	63.3	63.3
		15.7y	left	pre-IGCNU	2	64.2	45.3
8	PGD	2.5y	right	pre-IGCNU	2	51.0	77.6
9	PGD	1.5y	?	pre-IGCNU	1	26.8	44.3
10	UVS	1.3y	left	DM/pre-IGCNU	1	7.5	17.5
		1.3y	right	DM	0	7.4	29.6
11	CAIS	3m	?	N	0	7.1	3.4
		3m	?	N	0	6.1	4.1
12	CAIS	15.5y	right	IGCNU + pre-IGCNU	2	90.7	14.0
13	CAIS	10m	right	DM	0	9.8	7.3
14	17β-HSD	6m	right	pre-IGCNU	2	31.7	30.2
15	CAIS	18.0y	left	pre-IGCNU	2	71.4	38.8
16	SC-DSD	10.0y	?	DM	0	0	38.3
17	CAIS	4w	left	N/pre-IGCNU	1	18.9	8.6
18	UVS	1.5y	left	pre-IGCNU	2	64.4	46.6
19	CAIS	4m	right	N	0	4.7	7.8

CAIS - complete androgen insensitivity syndrome; IGCNU - intratubular germ cell neoplasia unclassified; DM - delayed maturation; Gon – gonad; N - normal development; P - patient; PGD - 46,XY partial gonadal dysgenesis; SC-DSD - sex chromosomal DSD (45,X/46,XY and variants); UVS - 46,XY undervirilization syndrome (impaired synthesis or action of androgens with unknown molecular cause); 17β-HSD - 17β-hydroxysteroid dehydrogenase deficiency; ? - not available.

Use of tissue samples remaining after diagnosis for scientific reasons was approved by Medical Ethical Committee (MEC) of the Erasmus MC Rotterdam (The Netherlands), permission 02.981. This included the permission to use the secondary tissue without further consent. Samples were used according to the “Code for Proper Secondary Use of Human Tissue in The Netherlands” developed by the Dutch Federation of Medical Scientific Societies (FMWV, http://www.federa.org/), Version 2002, update 2011.

Immunohistochemical detection of POU5F1 and KITLG

Formalin fixed paraffin embedded tissue samples were cut to 4µm-thick sections. The sections were deparaffinized, pretreated with 3% H_2O_2 (in case of POU5F1 detection), pressure cooked (120°C, 0.9 Bar) to retrieve the antigen, and blocked

with avidin and biotin kit. Subsequently, they were incubated with primary antibody against POU5F1 (sc-5279, Santa Cruz Biotechnology, CA, USA, dilution 1:350, for 120' at room temperature) or KITLG (sc-1302, Santa Cruz Biotechnology, CA, USA, dilution 1:250, overnight incubation at 4°C). Detection and visualization was done by biotinylated secondary antibodies and avidin/biotin complex conjugated with peroxidase (Vectastain ABC kit Elite pk-6100 Standard, USA) or alkaline phosphatase (Vectastain ABC kit pk-5000 AP, USA) in case of POU5F1 and KITLG, respectively. Diaminobenzidine/H_2O_2 or New Fuchsine/Naphtol ASMX phosphate (N500 Sigma, Steinheim, Germany) were used as substrate. Adult testicular tissue with IGCNU was used for all stainings as a positive control.

POU5F1 and TSPY1 double-staining

The sections were pretreated with 3% H_2O_2, pressure cooked, and blocked with avidin and biotin kit. They were then incubated with TSPY1 primary antibody (kindly provided by Prof. Dr. C. Lau, Department of Medicine, VA Medical center, University of California, San Francisco, CA, USA; dilution 1:4000, overnight incubation at 4°C) and subsequently with POU5F1 primary antibody (sc-8629, dilution 1:350, for 120' at room temperature). TSPY1 was detected using the avidin-biotin-complex conjugated with alkaline phosphatase complex and Fast Blue/Naphtol ASMX phosphate as a substrate. POU5F1 was detected with avidin-biotin-complex conjugated with horseradish peroxidase complex and 3-amino-9-ethyl-carbazole (Sigma, Steinheim, Germany)/H_2O_2 as a substrate. In between the two stainings, free biotin was blocked (Vector Laboratories, Burlingame, CA USA). The adult testicular tissue containing IGCNU was used as a positive control.

Assessment of germ cell abnormalities and KITLG expression pattern

Histological type of germ cell abnormalities in DSD gonads was evaluated according to the criteria summarized in Table 2 [20].

The following counting was performed in 20 tubules containing POU5F1-positive germ cells, if available per gonad: Regarding the numbers of abnormal germ cells, all POU5F1-positive germ cells were scored and a count of positive germ cells in contact with basal lamina of the seminiferous tubules in both single POU5F1 staining and double-staining was done. Moreover, all germ cells which co-expressed POU5F1 and TSPY1 were counted and as well as such cells attached to the basal lamina.

The samples were evaluated for the pattern of KITLG staining in areas which contained POU5F1-positive germ cells. They were divided into four categories as follows (representative images are shown in Figure 1): KITLG 0 - whole sample negative for KITLG (Figure 1A); KITLG 1 - only isolated spots (usually one or two per sample) were positive for KITLG (Figure 1B); KITLG 2 - KITLG positivity was not restricted to the isolated spots but was present in larger parts of the tubules which were usually numerous, not all tubules with POU5F1-positive germ cells were KITLG-positive (Figure 1C); KITLG 3 - all tubules containing POU5F1-positive germ cells were positive for KITLG (Figure 1D).

Table 2: Histological findings in testis with POU5F1-positive cells based on age and immunohistochemical analysis.

Finding	Age	Contact of POU5F1+ cells with basal lamina	TSPY1 in POU5F1+ cells	KITLG
Normal	< 6 months	occasional	occasionally +	negative
Delayed maturation	> 6 months	occasional	occasionally +	negative
Pre-IGCNU	any age	more than occasional	usually more than occasionally +	positive
IGCNU*	any age	(almost) all	usually + *	positive in all involved tubules

* Tubule with IGCNU is occupied by a uniform population of POU5F1-positive cells which replace the normal germ cell population and which are either all TSPY1-positive or less frequently all TSPY1-negative.

Statistics

Samples with less than 20 tubules counted were standardized to 20 tubules. Agreement between POU5F1 cell counts in the single-staining and the double-staining was assessed by Pearson's correlation coefficient (r) and linear regression modeling. The relation between embryonic age and cell counts was assessed using Spearman's correlation coefficient (ρ). In the DSD and/or IGCNU cases differences and correlation between the KITLG categories and cell counts were assessed using the Jonckheere–Terpstra test and Spearman's correlation coefficient (ρ), respectively. P values < 0.01 were considered significant until otherwise specified. Analyses were performed in SPSS 20.0.0 on a 64 bits Windows 7 system. Visualizations were created in SPSS and Microsoft Excel 2010.

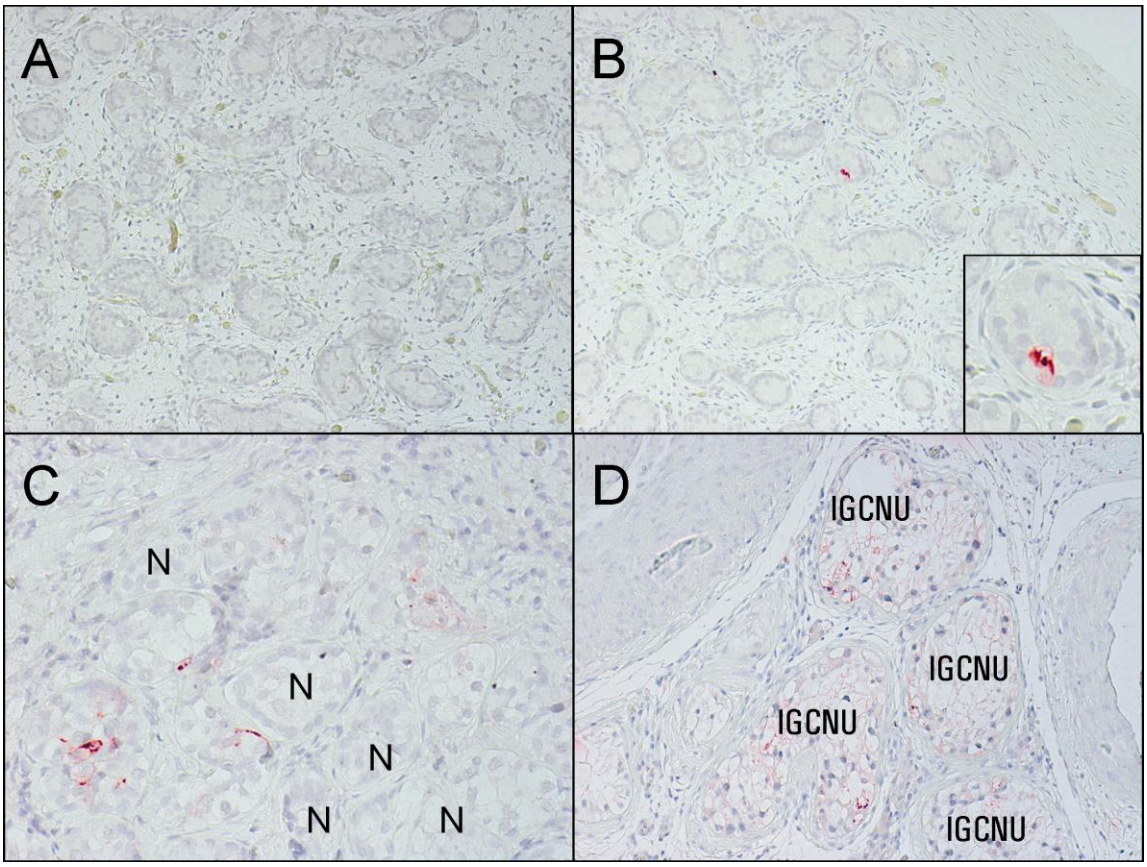

Figure 1: Examples of KITLG categories. A - gonad of 17-months old infant with an undervirilization syndrome (P10, right) negative for KITLG (magnification 100x); B - contralateral gonad of the same patient with a single spot of KITLG over-expression (red, magnification 100x), detail in the right lower corner; C - gonad of 15-years old patient with complete androgen insensitivity syndrome (P7, left) with consistent positivity of KITLG, some of the tubules remain KITLG-negative (N) (magnification 200x); D - adult IGCNU, all involved tubules (IGCNU) are positive (magnification 100x).

Results

Pattern of KITLG expression

According to the above set criteria, POU5F1-positive DSD samples were assessed for KITLG expression. Eight were KITLG-negative (KITLG 0), five contained few isolated spots of KITLG staining (KITLG 1), and in 11 of them KITLG was positive in numerous but not all tubules with POU5F1-positive cells (KITLG 2). None of the DSD samples reached KITLG 3 category unlike all the cases of adult testis with IGCNU. Both the group of fetal samples and POU5F1-negative DSD samples were KITLG-negative.

Agreement between single and double-staining

Upon visual inspection, POU5F1-positive cell counts in the single and double stainings with TSPY1 showed high levels of agreement. Correlation and regression analysis were applied to quantify systematical differences between the cell counts in both stainings. The results are summarized in Table 3 and Figure 2A and B. Correlation is close to perfect, but the regression analysis shows coefficients close to 0.8 indicating on average about 20% less POU5F1-positive cells in the double-staining for each sample. As expected, the intercept is not significantly different from 0. This discrepancy is possibly larger in the total POU5F1-positive counts, especially in samples with low counts (Table 3, Figure 2A). It might be due to the higher cell counts ("outliers") which dominate the slope of the regression curve. Subsequent analyses were performed on the cell counts from the double staining only.

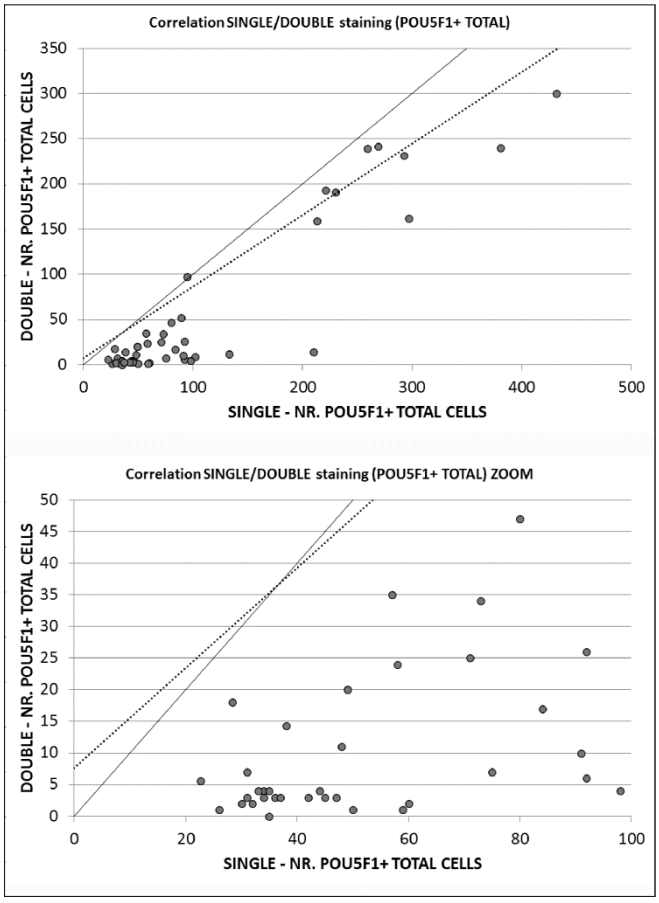

Figure 2: Scatter plot of POU5F1-positive cell counts in single and double staining. A - Total cell counts; B - Basal cell counts. Circles represent individual samples. The solid line represents the situation of perfect agreement (x=y) while the dotted line represents results of the regression analysis (Table 3).

Table 3: Correlation and regression analysis on POU5F1+ cell counts on single and double staining.

Sample set	POU5F1+ total			POU5F1+ basal		
	r	B_0±SE	B_1±SE	r	B_0±SE	B_1±SE
Embryo, DSD, IGCNU	0.964*	7.632±4.462	0.790±0.032*	0.987*	1.885±2.236	0.834±0.020*

If there would be perfect agreement between the immunohistochemical stainings and both sections are comparable, cell counts should show a perfect correlation (Pearson's r=1) and a regression coefficient of B_1=1 with intercept B_0=0. r=Pearson's correlation coefficient, B_0=intercept, B_1=regression coefficient of single staining predicting cell count in double staining, SE=standard error. *=p<0.01, 2-tailed. Constants were not significant.

Fetal gonads

The total number of POU5F1-positive cells as well as the number of positive cells in contact with basal lamina significantly decreased with gestational age (p=0.006 and p=0.021, respectively). In contrast, the total count of double-positive (POU5F1 and TSPY1) cells and count of double-positive cells attached to the basal lamina did not show a significant correlation with gestational age (Figure 3). Phenomena which are considered to be transitional during normal development, i.e., POU5F1-positive cells in contact with basal lamina and double-positive cells, were relatively rarely seen. In fact, a maximum of 13.3% of POU5F1-positive cells were in contact with the basal lamina (sample from 27-weeks old preterm neonate) and a maximum of 12.5% of cells co-synthesized POU5F1 and TSPY1 (sample from 35-weeks old preterm neonate).

Germ cell abnormalities present in DSD gonads

A continuum between no signs of malignancy and overt/adult-type IGCNU in our DSD samples with POU5F1-positive cells was observed. The findings were classified as normal in three gonads from individuals younger than 6 months. In these cases more than 90% of POU5F1-positive germ cells were located in the centre of the seminiferous tubules, these cells were mostly TSPY1-negative, and negative for KITLG (Figure 4A and B). In one gonad of the youngest patient (4 weeks of age) an isolated spot of KITLG positivity was detected. In addition, almost 20% of POU5F1-positive cells in this sample were in contact with basal lamina, thus this finding was on the threshold between normal and pre-IGCNU (Figure 4C and D). So called delayed maturation of germ cells (POU5F1-positive TSPY1-negative germ cells in the

center of the tubules without KITLG overexpression) was present in five cases of individuals older than 6 months, (Figure 4E and F). A borderline situation between delayed maturation and pre-IGCNU (due to a single spot of KITLG staining, was observed in a gonad of 17-months old patient. In this case, less than 10% of POU5F1-positive cells were in contact with basal lamina (Figure 4G and H). The germ cell lesion was described as pre-IGCNU in 13 cases (Figure 4I and J), the youngest being 4-months old. Finally, IGCNU (a uniform population of POU5F1-positive cells accompanied by KITLG overexpression), was detected in several tubules in a gonad of a 15-years old patient. These tubules were scattered within more numerous tubules with pre-IGCNU. Surprisingly, POU5F1-positive cells were predominantly TSPY1 negative in this case (Figure 4K and L). Germ cells in IGCNU and also cells of most advanced pre-IGCNU cases differed in shape from cells delayed in maturation. As is expected, IGCNU and pre-IGCNU cells had irregular angular nuclei where as nuclei of immature cells were of round shape.

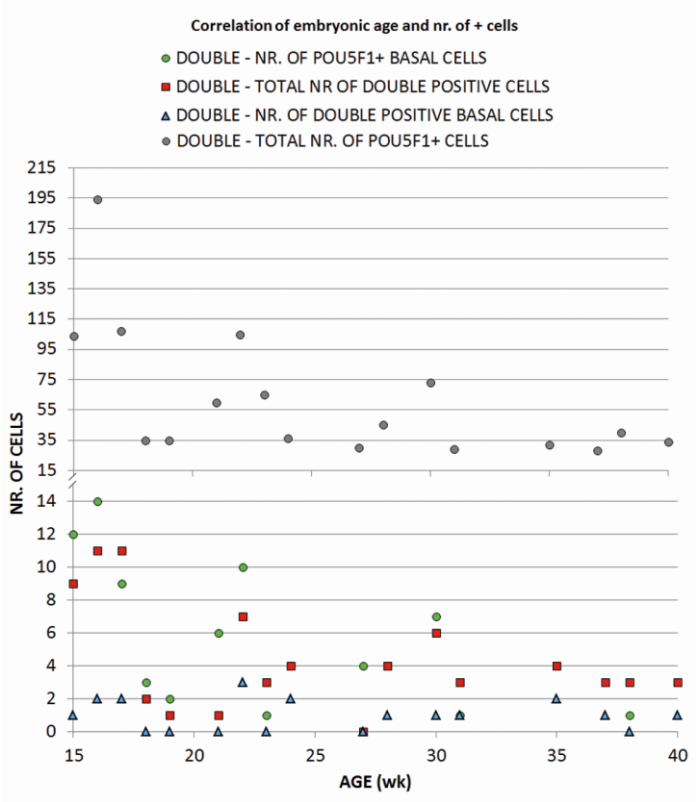

Figure 3: Cell count during embryonic development.

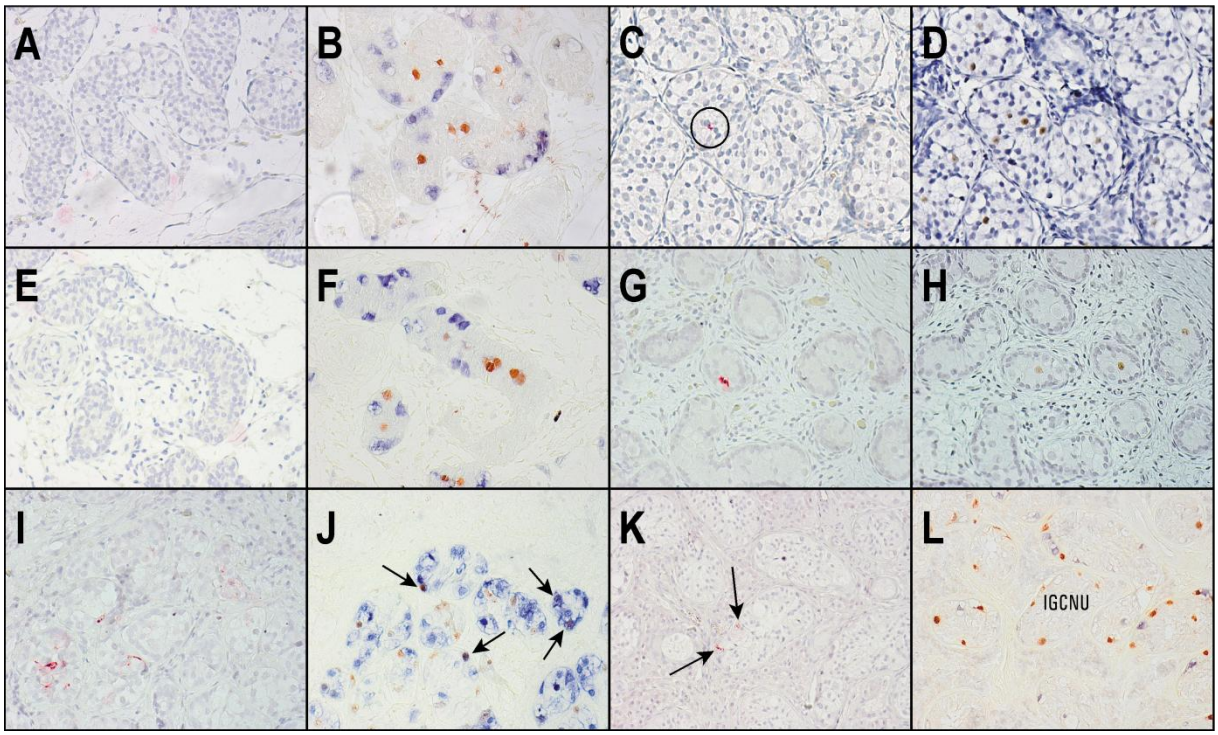

Figure 4: Examples of histological findings in DSD gonads. A - gonad of 4-months old infant with complete androgen insensitivity (P19) negative for KITLG (magnification 200x); B - same area as in A stained for POU5F1 (orange) and TSPY1 (blue), POU5F1-positive cells are TSPY1-negative and situated in the center of the tubules (magnification 200x); C - gonad of 4-weeks old infant with complete androgen insensitivity (P17) with a spot of KITLG positivity (red, in the circle)(magnification 300x); D - same area as in C stained for POU5F1 (brown), majority of the positive cells are situated in the center of the tubules (magnification 300x); E - gonad of 10-months old infant with complete androgen insensitivity (P13) negative for KITLG (magnification 200x); F - same area as in E stained for POU5F1 and TSPY1, POU5F1-positive cells are TSPY1 negative and situated in the center of the tubules (magnification 200x); G - gonad of 17-months old patient with undervirilization syndrome (P10, left) with a single spot of KITLG positivity (magnification 200x); H - same area as in G stained for POU5F1, positive cells are situated in the center of the tubules (magnification 200x); I - gonad of 15-years old patient with complete androgen insensitivity (P7, left) stained for KITLG, many but not all tubules are positive (magnification 200x); J - same area as in I stained for POU5F1 and TSPY1, majority of POU5F1-positive cells are attached to the basal lamina, some of them co-express TSPY1 (arrows) (magnification 200x); K - gonad of 15-years old patient with complete androgen insensitivity (P12) stained for KITLG which is positive (arrows) in a tubule with IGCNU when matched with double-stained sample (magnification 100x); L - same area as in K stained for POU5F1 and TSPY1, most of the POU5F1-positive cells are attached to the basal lamina and TSPY1 negative, tubule marked as IGCNU contains uniform population of germ cells positive for POU5F1 and negative for TSPY1 (magnification 100x).

Proportion of abnormal germ cells within particular KITLG categories

In samples within the KITLG 0 category 0 to 18.5% of all POU5F1-positive cells were attached to the basal lamina and 3.4 to 38.4% of all POU5F1-positive cells were TSPY1-positive (the data come from samples with minimal and maximal percentage, respectively). The proportion of POU5F1-positive basal cells ranged between 7.5 and 55.6% in samples categorized as KITLG 1, and between 31.7 and 90.7% in samples classified as KITLG 2. The percentage of double-positive cells varied between 8.7 and 72.2 in KITLG 1 and between 14.0 and 82.8 in KITLG 2 samples. In case of IGCNU in adult testis (KITLG 3) more than 82% cells were attached to the basal lamina and more than 73% were double-positive for POU5F1 and TSPY1. Notably, some tubules with IGCNU contained exclusively POU5F1-positive but TSPY1-negative cells. Results described in the two above paragraphs are summarized in Table 1.

Association between KITLG and POU5F1-TSPY1 protein expression

Initially, DSD and IGCNU samples were combined for this analysis. The association between KITLG category and positive cell count was evaluated, taking into account that a higher KITLG category is hypothesized to be associated with more POU5F1-positive and/or POU5F1 and TSPY1 double-positive cells. This association was significant and relevant for (basal) POU5F1-positive cell counts and (basal) POU5F1 and TSPY1 double-positive cell counts ($p<0.01$, Jonckheere-Terpstra Test & Spearman correlation coefficient >0.9, $p<0.01$, except for the total POU5F1-positive count ($\rho=0.725$), Figure 5A-D). A large difference between the highest (IGCNU samples) and all other (DSD samples) KITLG categories was observed with regards to all cell counts. This single observation could be the cause of the significant result of the Jonckheere-Terpstra test and high correlation without any actual relation present between the lowest three KITLG categories and the cell counts. Therefore the DSD set was also analysed separately, i.e. leaving out the highest KITLG category (adult IGCNU samples). In this subgroup the total number of POU5F1-positive cells is no longer significantly associated with the KITLG category ($p=0.178$, Spearman's correlation coefficient and $p=0.195$ Jonckheere-Terpstra Test). The total POU5F1-positive count already showed the weakest association in the DSD + IGCNU analysis above. The other cell counts continue to show significant differences

and strongly positive correlation between KITLG category and cell count ($p<0.01$ Jonckheere-Terpstra Test & Spearman's correlation coefficients >0.778, $p<0.01$).

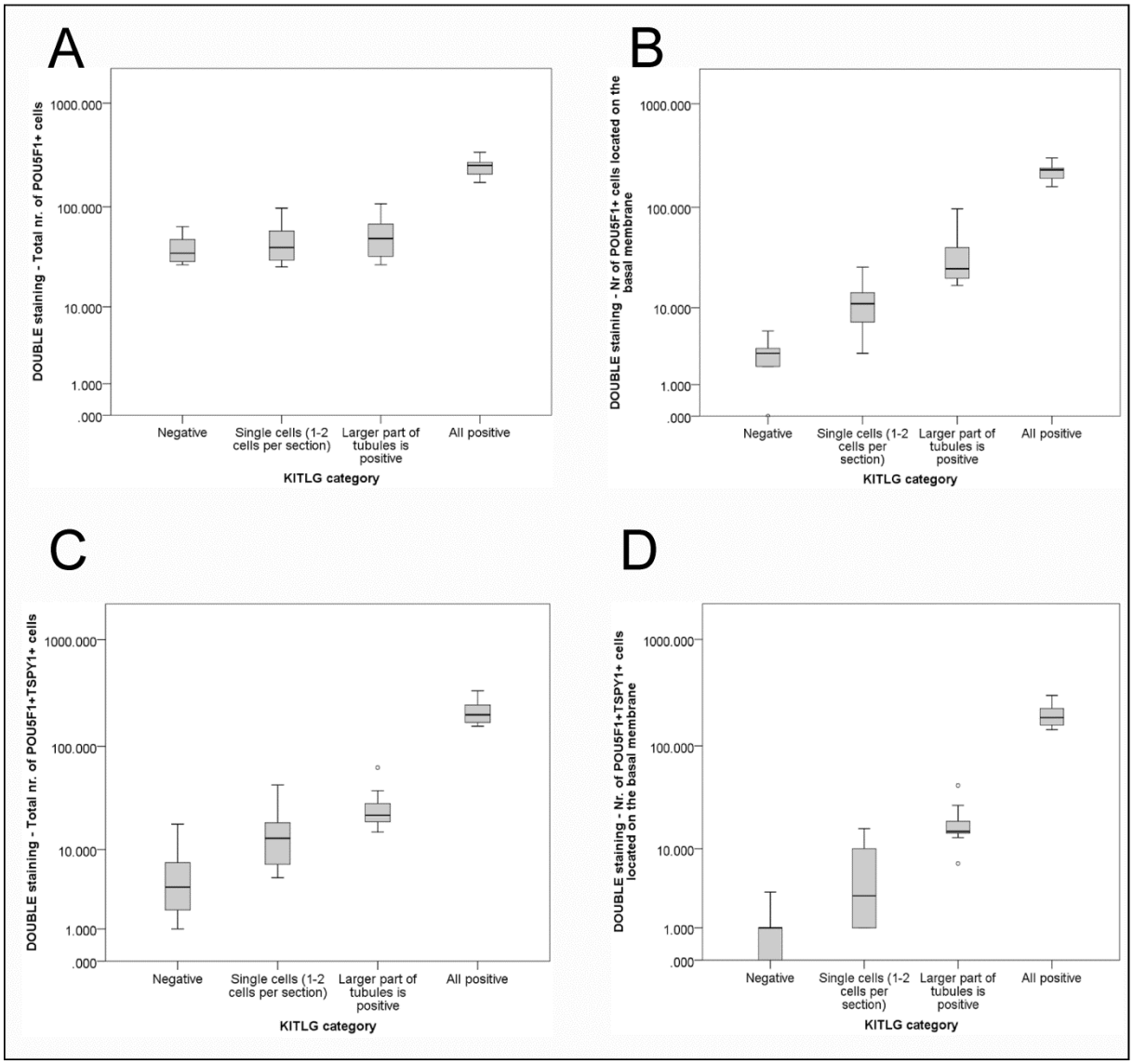

Figure 5: Distributions of cell counts between KITLG categories. A - total number of POU5F1-positive cells *versus* KITLG category, $\rho_{DSD+CIS}=0.725$, ρ_{DSD}=ns; B - basal POU5F1-positive cells *versus* KITLG category, $\rho_{DSD+CIS}=0.939$, $\rho_{DSD}=0.863$; C - total number of POU5F1-TSPY1 double-positive cells *versus* KITLG category, $\rho_{DSD+CIS}=0.907$, $\rho_{DSD}=0.778$; D - basal POU5F1-TSPY1 double-positive cells *versus* KITLG category, $\rho_{DSD+CIS}=0.929$, $\rho_{DSD}=0.835$; ρ indicates a significant ($p<0.01$) correlation coefficient (Spearman's ρ) and a significant Jonckheere-Terpstra Test between cell KITLG category and the respective cell count. ns indicates non-significant correlation.

Discussion

Role of the studied proteins in GCC development with regard to DSD

DSD patients who bear a GBY locus in their karyotype are at increased risk of gonadal GCC [19]. The risk varies between 0.8 and 60% among different disorders [16]. Development of GCC starts already in the prenatal period when further development of fetal germ cells (primordial germ cells/gonocytes) is blocked or retarded due to a more or less disturbed gonadal (testicular) microenvironment [21-23].

The arrested fetal germ cells keep synthesizing a typical spectrum of proteins (among others POU5F1) which provide the cells with unique stem-cell-like qualities, including capability of proliferation and resistance to apoptosis [9,10,15]. Persistence of the fetal pattern of expression regardless of the contact between germ cells and basal lamina of the seminiferous tubules is a sign of maldevelopment that may lead to IGCNU and subsequently also to an invasive cancer [11]. Perhaps with the purpose to maintain their population, the blocked germ cells often produce TSPY1 in combination with fetal proteins. *TSPY1* is the most likely candidate gene within the GBY region [18,19]. It is normally detectable in spermatogonia and its role is likely to enhance their proliferation activity [24,25].

KITLG together with its receptor c-KIT (with tyrosine-kinase activity) plays multiple roles in germ cell development. It is involved in migration of primordial germ cells towards the genital ridges, and most importantly, it supports early germ cell proliferation in the testis [8,12,13]. KITLG is physiologically synthesized by Sertoli cells at very low levels in the adult testis. It can be visualised by fluorescent immunodetection in frozen sections but not by non-fluorescent immunohistochemistry in formaline fixed and paraffin embedded tissue samples. However, it was shown that KITLG is abundantly expressed and thus detectable by non-fluorescent immunohistochemistry in testis containing abnormal germ cells, namely pre-IGCNU, IGCNU, and less consistently in invasive tumors [6]. Engagement of the c-KIT/KITLG pathway in GCC development is supported by association of GCC with a specific KITLG polymorphism and by an increased frequency of c-KIT mutations in specific subtypes of GCC, most notably in seminomas [8, 26-30].

Remarks on the observations

As described above, all the proteins which were analysed in our study supposedly play a role in GCC development in DSD gonads. A continuum between normal findings and IGCNU was observed in our DSD series. A gradually increasing number of POU5F1-positive cells attached to the basal lamina as well as an increasing number of (basal) POU5F1-TSPY1 double-positive cells was significantly associated with increasing levels of KITLG expression. This indicates that the two phenomena (i.e., abnormal protein synthesis in germ cells and KITLG expression) are interconnected in the process of germ cell malignant transformation. This is in line with GCC pathogenic model proposed recently by Van der Zwan and coworkers which merges results of two independent studies [14]. The first study demonstrated that a site of KITLG polymorphism associated with GCC is in fact a p53 response element [31]. The second study inferred that POU5F1 is stabilized through a specific phosphorylation regulated by AKT [32]. Based on these observations it was hypothesized that stress conditions (e.g. disturbed gonadal microenvironment) activate KITLG (with the specific polymorphism) through the up-regulation of p53 and this results in POU5F1 posphorylation and AKT activation. The described process finally leads to increased survival of fetal germ cells and a block in their pluripotent stage [14]. Possible links between the above described model and the role of TSPY1 in GCC development remain to be elucidated.

It is believed that presence of TSPY1 as the most likely candidate within the GBY region is required for GCC development in DSD individuals and that abundant expression of TSPY1 is one of the driving forces in GCC ontogeny in DSD patients [18,19]. Most of the cases which were classified as pre-IGCNU or IGCNU in our series indeed showed co-expression of POU5F1 and TSPY1 with higher frequency in comparison to the fetal samples where it was observed in 12.5% of the cells at maximum. However, we also noticed several exceptions. Tubules with POU5F1-positive but TSPY1-negative cells were observed in some cases of adult IGCNU (likely in line with the frequent loss during progression of IGCNU to invasive growth). This picture was also present in the only DSD testis with several tubules containing IGCNU (P12) where only 14% of cells co-expressed TSPY1. On the other hand, in case of a testis from another DSD patient (P16) none of the POU5F1-positive cells were attached to the basal lamina, KITLG was negative, but almost 40% of cells co-expressed TSPY1. From those findings we concluded that to judge the presence of

double-positive germ cells is less likely to be a reliable marker for pathological germ cell changes than evaluation of POU5F1-positive cells in contact with basal lamina. The percentage of POU5F1-positive germ cells at the basal lamina overlaped between the subsequent KITLG categories. This overlap was not present between KITLG 0 and KITLG 2 samples. The KITLG 0 category consisted of cases evaluated as normal or with delayed maturation if in line with age (see Table 2). The proportion of POU5F1-positive basal germ cells in this group was at maximum 18.5%. This was close to the situation in fetal gonads where at maximum 13.3% POU5F1-positive cells were in contact with the basal lamina. Of note, the percentage reached 18.8% in one fetal case in single POU5F1 staining.

The KITLG 2 category contained pre-IGCNU, in one case in combination with IGCNU. The lowest proportion of basal POU5F1-positive cells was 31.7% here. The group of gonads with only isolated and scarce KITLG positivity (KITLG 1) was the most heterogeneous. Proportion of basal cells ranged from 7.5 to 55.6%, and the overall diagnostic classification varied between close to normal and clear pre-IGCNU.

Clinical application of the findings

Kolesinska et al. reported on the current trend to assign individuals with remarkably masculinized external genitalia (median External Masculinization Score 6 out of 12) male gender [33]. To preserve the endogenous production of sex hormones and perhaps even to preserve fertility in such patients, physicians tend to favor a conservative approach instead of prophylactic gonadectomy. Of course, retained gonads need to be under close surveillance. Besides the self-monitoring and ultrasound controls, diagnostic biopsy with immunohistochemical search for germ cell abnormalities should be undertaken. Because of the limited representativeness of the biopsy for the whole gonad, it is recommended to repeat this procedure after some time [1,34].

The importance of immunohistochemical detection of IGCNU cells was demonstrated by Van Casteren et al. [35]. After the identification of abnormal germ cells (i.e., cells positive for fetal/neoplastic markers, specifically POU5F1), a proper evaluation is necessary especially in DSD gonads in which we may observe a wide range of abnormalities [7]. Our series of DSD gonads nicely shows that there does not exist any clear separation between delayed maturation and pre-IGCNU. Based on the above described differences between KITLG categories and using data obtained

from fetal samples, we propose to classify the findings with up to 10-15% POU5F1-positive cells in contact with basal lamina as a delayed maturation or normal development (if in line with age). Gonads with more than 30% of basal POU5F1-positive cells should be considered as pre-IGCNU. KITLG detection remains to be highly informative especially in borderline cases.

Delayed maturation is supposedly at a lower risk of progression towards malignancy than more advanced changes. However, the exact risk of particular lesions remains to be elucidated. It may possibly vary between different DSD types. Results of our previous study on gonads of individuals with complete androgen insensitivity syndrome indicated that not all pre-IGCNU would develop into cancer in that particular disorder [20]. On the other hand, the number of gonads containing non-invasive neoplastic precursor (IGCNU or gonadoblastoma) or its pre-stage (pre-IGCNU or undifferentiated gonadal tissue) was in accordance with a previously estimated GCC risk in a study on 45,X/46,XY mosaicism, so supposedly all would have progressed into cancer [36].

In conclusion, our study supports a hypothetic model of interconnection between KITLG and POU5F1 in GCC development based on the finding that the number of abnormal germ cells is positively associated with increased expression of KITLG in DSD testes. The position of POU5F1-positive germ cells in contact with the basal lamina seems to be a more reliable marker of malignant transformation than POU5F1 and TSPY1 co-expression. We proposed criteria for delayed maturation and pre-IGCNU based on POU5F1 staining. The role of KITLG as an additional important diagnostic tool, especially in borderline cases, was confirmed.

Acknowledgement

Special thanks belong in memoriam to Dr. Anna Zuntová from Department of Pathology and Molecular Medicine, Faculty Hospital Motol, Prague, Czech Republic, who collected an important part of the analysed DSD samples. This work was supported in part by ESPE Research Fellowship granted by NovoNordisk A/S (JK), Translational grant, Erasmus MC (MR), Project (Ministry of Health, Czech Republic) for conceptual development of research organization 00064203 (University Hospital Motol, Prague, Czech Republic) (JL, MS), TSPY KWF (Dutch Cancer Society 2006-3607) project, and The Flanders Research Foundation - FWO (MC).

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Chapter 7

General Discussion

Disorders of sex development: lessons from nature.

Gonadal differentiation and initiation of germ cell tumor development are the two entities that are nicely interconnected within the DSD. In fact, we have learned a lot about these issues from the DSD *in vivo* models, although we are still lacking some information.

Our knowledge of gonadal development is mostly obtained from research done in mice and other animals, nevertheless, it can in general be applied to humans as well, although exceptions exist [1-3]. Gonadal (testicular and ovarian) differentiation is a complex process orchestrated supposedly by tens of transcriptional factors and signaling molecules [4]. That is one of the main reasons why we are successful in uncovering genetic mechanisms of the disease only in a part of the disorders [5]. However, thanks to the novel genetic technologies, several new genes and pathways were recently confirmed to be engaged in disrupted gonadal development in humans when mutated or deleted [6-8].

As described above, the process of GCC development starts already *in utero* [9,10]. The underlying conditions are most apparent in gonadal dysgenesis, a consequence of sex chromosome aberrations or mutations and copy number changes of involved genes. In such cases, differentiation of several gonadal cell types (i.e., supportive cells, hormone producing cells, germ cells) is clearly disturbed. Indeed, maturation of germ cells is delayed or blocked, or the cell population may even decline under these circumstances [11].

Changes in gonadal histology are more subtle in case of 46,XY DSD undervirilization syndromes (i.e., disorders caused by impaired androgen synthesis or action). That is likely a reason why GCC manifests usually later in live in these syndromes than in gonadal dysgenesis. The disturbed microenvironment of close to normal gonads causes a delay of germ cell maturation; more apparent histological changes develop postnatally with rising age [12,13].

GCC is relatively frequent in the general young male Caucasian population [14]. It is most typically diagnosed in a stage of invasive tumor. Finding of isolated carcinoma *in situ* is less common and is usually secondary during a diagnostic process of infertility or in a biopsy due to the contralateral cancer [15,16]. Earlier stages of GCC development are likely present in childhood and therefore are difficult to diagnose in the current setup. The one exception may be a group of boys with cryptorchid testes

who are taken biopsy during the orchidopexy in some medical centers [17]. However, in these cases the boys are usually very young and so a delayed maturation of germ cells rather than further stages of malignization is likely to be observed.

In past years, a prophylactic gonadectomy was widely performed in DSD patients at risk for GCC and very often took place early after the diagnosis of the disorder had been made [18]. Therefore, gonads of different ages, from neonatal period to adulthood, are available. Thanks to this it is possible to trace gradual morphological and expressional changes of the germ cells that lead to the development of CIS/IGCNU in testis or gonadoblastoma in dysgenetic gonad as described hereunder. The role of proteins engaged in GCC development was reviewed in Chapter 2 and will not be discussed in detail here.

First step of the germ cell malignization in the testis is so called delayed maturation [12]. It resembles the fetal phase of germ cell development. Immature germ cells are located in the center of the seminiferous tubules and synthesize typical proteins (among others OCT3/4, NANOG, AP2-gamma, PLAP) [19-21]. Rarely, spermatogonial markers (e.g. TSPY) are co-expressed in such cells. Contact with basal lamina of these embryonic-like germ cells is also scarce. Both these phenomena are considered to be transitional [19]. KITLG is negative in delayed maturation when using non-fluorescent immunodetection [22].

It is believed that germ cells are able to complete the maturation in the above described stage. However, immature germ cells may escape normal development in yet unknown part of the cases. Subsequently, more and more cells that keep expressing fetal proteins are associated with the basal lamina; they also frequently co-synthesize a number of spermatogonial markers [12]. Moreover, KITLG becomes detectable [22]. The phase succeeding delayed maturation but not reaching criteria for CIS/IGCNU is termed as pre-CIS/pre-IGCNU.

To complete the list of testicular non-invasive germ cell lesions, CIS/IGCNU has to be characterized. Germ cells of the CIS/IGCNU display a clonal growth (i.e., they have identical morphological and expressional qualities), they are typically attached to the basal lamina and replace other germ cells (i.e., spermatogonia) in the seminiferous tubule. They do synthesize fetal markers and may produce TSPY in the same time, and KITLG is always detectable [23].

In fact, we may observe a continuum of changes between delayed maturation and invasive GCC in DSD testes. Importantly, we are not able to predict how high is the

risk of further malignization and of progress to an invasivness in particular non-invasive lesions in DSD. Is it 100% as is the case of CIS/IGCNU in general male population? [24] To solve this is a big task of the future research. What may complicate our effort is that differences between various disorders and even between different mutations of a single gene may exist.

The level of risk of further malignant progression is also not clear in changes of dysgenetic gonad. These are so called undifferentiated gonadal tissue (UGT), which resembles the developmental stage of sex cords, and gonadoblastoma, which is believed to arise from UGT. In both lesions OCT3/4-positive germ cells may co-express TSPY and KITLG is positive [11,25,26].

With a current view of GCC risk in particular types of DSD and possibilities of detection of an early germ cell malignancy, nowadays applied strategy represents a compromise between safety and avoidance of overtreatment. For instance, it is considered that pre-CIS/pre-IGCNU or UGT is an informative risk factor and a reason for gonadectomy, although it is not precisely known how high is the probability of their progression into an invasive tumor [27].

To further optimize the guidelines, several studies that aimed to search for features associated with tumor risk in particular DSD subtypes and to look for widely accessible tools for diagnostics of early germ cell malignancy were performed. The output will be discussed below.

Complete androgen insensitivity syndrome: factors influencing gonadal histology including germ cell pathology

Although CAIS belongs to the rare variants of DSD, it is one of the most frequent among 46,XY DSD [28]. Therefore, not only case reports, but also cohort studies and meta-analyses are available [29-35]. From these it was concluded that risk of development of invasive GCC and even presence of non-invasive neoplasia (CIS/IGCNU) before puberty is relatively low. The later was observed in 0.8% of the cases [29,35]. The risk rises in adolescence and adulthood and was recently estimated to be 14% [34]. Besides the analysis of overall histopathological changes of the gonads (regarding the topic of the thesis, details will not be discussed here), our study focused specifically on the relationship between tumor risk and two factors which influence testicular development, i.e., gonadal location and level of AR activity.

Cryptorchidism is known to be associated with GCC in the general population [36]. Unfortunately, it was not possible to evaluate the impact of normal versus abnormal location, because position of the gonads in our series was mostly pathological (abdominal or inguinal) in the context of the chromosomal constitution and expected physical development, in fact only a single gonad was situated in the labium. This is in accordance with previous findings in literature [37]. We also failed to compare independent influence of the abdominal and inguinal position because abdominal gonads were associated with both higher age at the time of gonadectomy (and so longer effect of the position) and zero activity of the AR.

No significant differences were observed in tumor risk between patients with residual AR activity and with no activity of the AR. This result was inconsistent with our hypothesis. Based on the importantly higher risk in individuals with partial form of androgen insensitivity syndrome than in individuals with CAIS, higher risk of tumor development in patients with residual activity of the AR might have been expected [35]. Moreover, importance of the AR function in GCC growth was demonstrated recently [38,39]. Likely, AR with residual activity in CAIS patients is not powerful enough to support tumor progression and growth significantly. Relatively small sample size might have also contributed to the result.

Interestingly, many of the samples in our series contained germ cell abnormalities (delayed maturation or pre-CIS/pre-IGCNU) that once may undergo malignization. The number of abnormalities was high not only in comparison with similar (pediatric) cohorts but also with estimated tumor risk in adulthood [30-35]. This probably means that not all the abnormalities would progress into GCC in CAIS. The phenomenon might again be explained by an impact of low-androgen environment. Importantly, it points on how little we know about the risk of further malignization of postnatally surviving cells with fetal characteristics in DSD gonads.

Gonadal pathology and tumor risk in relation to clinical characteristics in patients with 45,X/46,XY mosaicism

Estimated incidence of 45,X/46,XY mosaicism and its variants is 1.5 per 10,000 [40]. Clinical presentation of this chromosomal disorder is highly heterogeneous and varies from female phenotype with Turner syndrome (TS) characteristics through more or less undervirilized individuals to normal male [41,42]. Previously calculated

overall occurrence of GCC was about 15% in this disorder [35], however, clinical experience indicated important differences between individuals with different level of masculinization. To determine the risk more accurately is important especially in patients assigned male gender in whom we tend to spare gonads to avoid necessity of hormone replacement therapy.

The overall occurrence of gonads at risk was 18% in our series. This is in line with previous estimation [35]. As we hypothesized, clinical phenotype of the patients reflected gonadal histology and therefore also conditions for germ cells to either develop normally, decline, or escape normal development and show signs of (pre-) malignancy. Thus, it was possible to prove that there exists a significant difference in tumor risk between patients with female phenotype (2.2%), ambiguous genitalia (52%), and mild undervirilization (13%).

Gonadal dysgenesis was most pronounced in patients with female phenotype. The gonads were described as a streak or as vanished in vast majority of the cases, and so from definition lacked germ cells. Surprisingly, one of the gonads had a testicular appearance and contained CIS. Histology of gonads of patients with ambiguous genitalia was the most diverse. The tissue was severely dysgenetic, mostly described as a testis, UGT or combination of both. This kind of microenvironment supposedly led to a block of maturation of surviving germ cells and so we observed non-invasive neoplasia in two gonads, and pre-neoplastic changes in 10 additional cases. Gonads in patients with mild undervirilization were mainly testes with lower degree of dysgenesis in comparison to the previous group. Non-invasive neoplasia, a gonadoblastoma within associated UGT, was observed in one case.

Obtained results allowed us to propose guidelines for management of patients diagnosed with 45,X/46,XY mosaicism. The proposal respects current trend of sparing gonads in male individuals [42]. The benefit of this approach was demonstrated on several males included in the study, who experienced spontaneous puberty with normal onset and progression. Indeed, orchidopexy of the retained gonads and close follow-up including (properly evaluated) gonadal biopsy are mandatory due to the substantial cancer risk. Because of the limited representativeness of the biopsy sample, particularly in dysgenetic gonads, the procedure of biopsy should be repeated in time. The risk seems to be importantly lower in individuals with female phenotype, however it is still present and justifies gonadectomy of non-functional gonads.

45,X/46,X,psu dic(Y) gonadal dysgenesis: Influence of the two cell lines on the clinical phenotype, including gonadal histology

A case of 45,X/46,X,psu dic(Y) mixed gonadal dysgenesis offers a detailed view on the mosaicism disorder which was analyzed in previous chapter. An emphasis is put on a clinical impact of both cell lines. The 45,X cell line, which is typically related to the Turner syndrome, represents a pathophysiological background of growth retardation and development of celiac disease in this case [43,44]. The other cell line, 46,Xpsu dic(Y), is responsible for testicular differentiation of gonadal tissue and related ambiguous phenotype of external genitalia. Further histological studies revealed that left as well as right testis displayed dysgenetic features and contained abnormal germ cells (pre-CIS/pre-IGCNU). KITLG was detectable in both gonads. Thus, both gonads were at risk for future GCC development according to current belief [45]. The probability of future malignization was supported by presence of calcifications which developed during one year between biopsy and gonadectomy of the right gonad [46,47].

We observed an interesting phenomena related to supportive cells which demonstrated that differentiation of the gonads of 45,X/46,XY individuals oscillates between testis and ovarium. FOXL2-positive supportive cells were present in the tissue samples obtained in 3.5 and 4 months; these cells were no more present in the sample from 16 months. FOXL2 is characteristically synthesized by ovarian granulosa cells and is not present in the testicular tissue [48]. Absence of FOXL2-positive cells in later specimen indicates that the testicular environment was unfavorable for their survival.

The case also touched upon the issue of gender assignment in patients with ambiguous genitalia. It should be always strictly individual and take to account both future need of hormonal replacement, fertility possibilities, related disorders, and last but not least wish of well informed parents [49]. Indeed, potential gonadectomy is related to the assigned gender as it was indicated in the previous chapter.

KITLG detection pattern correlates with number of abnormal germ cells in disorders of sex development

Approach to DSD patients at risk of GCC nowadays tends to be more conservative especially in patients assigned male gender. This is besides others thanks to novel possibilities for histological diagnosis of early germ cell malignancy (pre-CIS/pre-IGCNU) which encompass for instance detection of OCT3/4 and KITLG [23]. The both proteins are believed to be involved in GCC ontogeny [20,22]. Van der Zwan and co-authors recently proposed a model of GCC pathogenesis which interconnects role of KITLG and OCT3/4 [50]. It is based on a known association between GCC risk in the general population and a certain KITLG polymorphism which resides in a site of p53 response element [51]. According to the model, stress conditions (e.g. impaired gonadal microenvironment) activate (susceptible polymorphic) KITLG via up-regulation of p53. Activation of KITLG supposedly results in a specific phosphorylation and stabilization of OCT3/4 [50]. Outcomes of our study support this hypothetic link. We proved that number of germ cell abnormalities, specifically presence of OCT3/4-positive cells attached to the basal lamina and presence of cells co-expressing OCT3/4 and TSPY, is positively associated with the increasing KITLG expression in DSD testes. However, whether the KITLG polymorphism is associated with greater risk of GCC also in DSD remains to be uncovered. Its influence is more likely in normally developed testes, i.e., in undervirilization syndromes, than in gonadal dysgenesis where extreme conditions may lead to expression of p53 in such extend that even non-polymorphic KITLG is highly activated.

Further results of the study revealed distinct differences in number of germ cell abnormalities between gonads with different level of KITLG expression. This together with observations on fetal gonads allowed us to propose criteria for germ cell maturation delay and pre-CIS/pre-ICGNU based on OCT3/4 detection. However, because germ cell changes in DSD gonad represent a continuum without a clear border, KITLG remains to be highly informative for the precise diagnosis [23].

Clinical impact of the results

We were not able to analyse the association between gonadal location and germ cell abnormalities in CAIS for reasons discussed above. We also failed in confirming any

correlation between germ cell abnormalities and level of AR activity in this particular disorder. However, we pointed on a likelihood that not all the premalignant germ cell lesions (delayed maturation as well as pre-CIS/pre-IGCNU) progress to a GCC in CAIS. In our series CIS/IGCNU was observed only in a single case of 15-years old patient, moreover only several seminiferous tubules were involved. Thus, this finding supports recent opinion on safety of postponement of gonadectomy to the adolescence or young adulthood in CAIS to let patients undergo spontaneous pubertal development [52].

The study targeted on 45,X/46,XY mosaicism revealed association between level of masculinization of external genitalia, gonadal histology and GCC risk. More detailed knowledge about the risk in different phenotypic groups allowed us to propose guidelines for management of the patients with mosaicism.

The 45,X/46,Xpsu dic(Y) case report underscored the complexity of the DSD and pointed on a link between phenotype, gender assignment and management regarding GCC risk.

The study correlating level of KITLG expression and number of abnormal germ cells in DSD testes proved the positive association of the two phenomena. The results support the recent model of interconnection between KITLG and OCT3/4 in GCC pathogenesis. Moreover, the results and their comparison with situation in fetal gonads allowed us to propose criteria for delayed maturation and pre-CIS/pre-IGCNU based on OCT3/4 detection. This may be helpful in situations when KITLG detection is not available. Nevertheless, KITLG analysis remains to be highly informative, especially in borderline cases.

Future prospects

Thanks to the growing interest in DSD in the last years, multiple advances have been achieved in various areas related to these disorders, including management of the patients at risk of GCC. However, low frequency of the disorders restricts our possibilities significantly. To surpass this limitation, broad international collaboration is mandatory and should be initiated by professional societies involved in care of DSD patients (e.g. societies of pediatric endocrinologists, urologists, gynecologists, etc.). The i-DSD registry was founded for this purpose several years ago and there are already several outcomes available from this act [53,54].

Regarding specifically the issue of GCC risk, there are still many questions waiting for the answer. Here are some of them: First, we are aware of the tumor risk in patients with 46,XY gonadal dysgenesis in general, but our knowledge about the risk for individual mutated genes is limited [35,55]. The same is true for the rare undervirilization syndromes.

Second, although we failed to correlate gonadal position and germ cell tumor risk in patients with CAIS, this correlation might be possible in patients with PAIS and other genetically well defined disorders if an enough large cohort of patients is available and all other influencing factors are considered.

Third, we are currently able to detect very early stages of malignancy, however, we do not know how many of these lesions would really progress into an invasive tumor in particular DSD. This issue may struggle with ethical problems. Who would dare to let the precursor germ cell lesions evolve into invasive tumor in a prospective study?

Fourth, several polymorphisms, among others in KITLG, are associated with GCC risk in the general population. Whether they play an important role in GCC development also in (some) DSD is currently unknown [56-58], however it may be studied retrospectively on an enough large series of DSD patients, e.g. patients with CAIS or with mutation in the *SRY* gene.

Last but not least, a promising non-invasive diagnostic method, detection of GCC specific microRNAs in the serum, is being under development in general male population; whether it will be possible to apply it also on DSD patients needs to be discovered [59]. A prospective study combining microRNA serum analysis prior to the gonadectomy/biopsy and histological assessment of gonadal tissue might be an option.

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Chapter 8

Summary/Samenvatting, acknowledgement and appendix

Summary

Disorders of sex development (DSD) constitute a heterogeneous group of conditions which were newly classified in 2005. DSD bring several important issues, among others increased risk of type II gonadal germ cell tumors (also germ cell cancer, GCC) in individuals with a defined part of the Y chromosome in the karyotype. Origin of GCC is believed to be in fetal germ cells which were arrested in their development. Both genetic and environmental background is suspected. During the last decade, important progress has been achieved in histological diagnostics (immunohistochemistry) of GCC.

Because of the well-known association between particular types of DSD and gonadal GCC risk, vast majority of the patients underwent prophylactic gonadectomy in the past. Current approach tends to be more conservative, especially in individuals assigned male gender. This is allowed thanks to several advances in our knowledge: 1) only a specific part of Y chromosome, so called GonadoBlastoma locus on Y, brings the risk; 2) the risk differs importantly between various forms of DSD; 3) novel possibilities in diagnostics of non-invasive germ cell neoplasia, so called carcinoma *in situ* of the testis (CIS; also intratubular germ cell neoplasia unclassified, IGCNU) and its pre-stage (pre-CIS/pre-IGCNU) have been established. Nevertheless, every additional information which might help us to better target on patients at a high risk of tumor would be appreciated. From this requirement arises the topic of this thesis.

Chapter 2 reviews the data about DSD in relation to GCC which had been available before the below listed studies were performed. It describes physiological germ cell development as well as the development of GCC. The suspected role of OCT3/4, TSPY and KITLG in pathogenesis of GCC is depicted with an emphasis on their employment in detection of germ cell abnormalities including delayed maturation of the germ cells and early germ cell malignancy (pre-CIS/pre-IGCNU). Finally, level of the tumor risk in relation to particular gonadal phenotype and to the type of DSD is provided.

A study described in **Chapter 3** aimed to investigate whether there exists any association between presence of histopathological changes of the gonadal tissue in patients with complete androgen insensitivity syndrome (CAIS) and the two factors

with a possible influence, gonadal location and level of androgen receptor (AR) activity. Special attention was put on germ cell abnormalities. Several observed features occurred already in very young individuals and thus were likely due to the abnormal location of the gonads which was present in all but one cases. Other phenomena developed only with the onset of puberty and are likely caused by lack of AR activity. Residual activity of AR was related to better survival of general germ cell population in (post)pubertal patients. We were not able to analyse an independent impact of gonadal location (inguinal *versus* abdominal) on germ cell pathology because of the unequal distribution in relation to age and AR activity. We did not prove any association between level of AR activity and germ cell abnormalities. Importantly, we detected a surprisingly high number of cases of germ cells maturation delay and of pre-IGCNU in comparison to the estimated tumor risk. This finding indicates that not all such lesions would progress into an invasive tumor in CAIS.

Study presented in **Chapter 4** proved our hypothesis that phenotype of external genitalia reflects gonadal histology and predisposition of tumor development in individuals with 45,X/46,XY mosaicism. We observed a significant difference in tumor risk between three groups of patients with different level of masculinization assessed by using the External Masculinization Score (EMS). The risk was the lowest in patients with female (Turner syndrome) phenotype (2.2%); patients with mild undervirilization displayed 13% risk; patients with ambiguous genitalia were the most highly at risk (52% risk). Based on the findings, we proposed guidelines for safe management concerning the tumor risk that allow to spare gonads in male individuals. The conservative approach was supported by the fact that in several patients from our study, who were raised as males, retained gonads produced sufficient amounts of androgens to allow spontaneous puberty.

Chapter 5 reports about the case of a child with 45,X/46,X,psu dic(Y) gonadal dysgenesis and ambiguous genitalia. Impact of the two cell lines on the phenotype, including gonadal histology, is described in detail and the complexity of the disorder is underlined. The case report also demonstrates how the management regarding cancer risk is dependent on the gender assignment in similar situations.

Study introduced in **Chapter 6** revealed that there exists an association between pattern of KITLG detection and number of abnormal germ cells (i.e., number of OCT3/4-positive germ cells attached to the basal lamina of seminiferous tubules and number of (basal) cells co-expressing OCT3/4 and TSPY; $p<0.01$) in testicular tissue of DSD patients. This result is in line with a hypothetic model of GCC pathogenesis proposed by van der Zwan et al., which interconnects role of KITLG and OCT3/4. Percentage of OCT3/4-positive germ cells in contact with basal lamina in gonads which were KITLG-negative was clearly distinct from percentage of such cells in gonads strongly positive for KITLG. This finding together with results obtained from series of fetal testes allowed us to propose criteria for delayed maturation and pre-CIS/pre-IGCNU based on OCT3/4 staining. However, this cannot fully replace detection of KITLG, which remains to be highly informative especially in borderline cases.

General discussion points out the contribution of the DSD cases to our knowledge on gonadal differenciation and GCC development. The major results of particular studies are discussed with an emphasis on the clinical output. Possible directions of further research in the field are outlined in the closing part.

Samenvatting

Disorders of sex development (DSD) omvatten een heterogene groep van condities welke in 2005 opnieuw geklassificeerd zijn. DSD verenigen verschillende belangrijke onderwerpen, waaronder de toename in het risico op het ontwikkelen van type II gonadale kiemceltumoren (ook wel kiemcelkanker genoemd, KCK) in individuen met een bepaald deel van het Y chromosoom in hun karyotype. De oorspong van KCK wordt verondersteld gelegen te zijn in foetale kiemcellen die geblokkeerd zijn in hun ontwikkeling. Zowel genetische als ook omgevings factoren worden verondersteld hierbij betrokken te zijn. Gedurende de laatste 10 jaar zijn belangrijke ontwikkelingen doorgemaakt ten aanzien van de histologische diagnose (immunohistochemie) van KCK.

Op grond van de bekende associatie tussen bepaalde vormen van DSD en gonadaal KCK risico, ondergingen de meerderheid van deze patiënten in het verleden profylactische gonadectomie. De huidige behandeling is meer conservatief, met name in individuen met het mannelijke geslacht (sekse). Dit is het gevolg van een aantal inzichten, en wel: 1) enkel één bepaald deel van het Y chromosoom, het zogenaamde GonadoBlastoma locus op Y (GBY), is gerelateerd met KCK risico; 2) het risico verschilt sterk tussen de verschillende varianten van DSD; 3) nieuwe mogelijkheden betreffende de diagnostiek van de niet-invasieve kiemcel neoplasie, i.e., carcinoma *in situ* van de testis/zaadbal (CIS; of Intratubular germ cell neoplasia unclassified, IGCNU) en het daarvoor liggende stadium (pre-CIS/pre-IGCNU) zijn beschikbaar gekomen. Desondanks zal alle additionele informatie behulpzaam kunnen zijn in het beter identificeren van de patiënten met het hoogste risico op KCK ontwikkeling. Op grond van deze randvoorwaarden zijn de onderwerpen beschreven in dit proefschrift tot stand gekomen.

Hoofdstuk 2 geeft een overzicht van de gegevens betreffende DSD in relatie tot KCK op het moment dat de in dit proefschritft opgenomen studies nog niet waren uitgevoerd. Het beschrijft de fysiologische kiemcel ontwikkeling, als ook de ontwikkeling van KCK. De veronderstelde rol van OCT3/4, TSPY en KITLG in de pathogenese van KCK is weergegeven met de nadruk op hun waarde voor het aantonen van kiemcel afwijkingen, inclusief vertraagde uitrijping van de kiemcellen

en de vroege KCK stadia (pre-CIS/pre-IGCNU). Ten slotte wordt het KCK risico in relatie tot bepaalde gonadale fenotypes en varianten van DSD besproken.

De studie beschreven in **Hoofdstuk 3** heeft als doel te bestuderen of er een relatie bestaat tussen de aanwezigheid van histopathologische afwijkingen van het gonadal weefsel in patiënten met de complete vorm van androgeen ongevoeligheid (CAIS) en twee factoren met een mogelijk efect: gonadale localisatie en het niveau van de androgeen receptor (AR) activiteit. Speciale aandacht is hierbij besteed aan kiemcel afwijkingen. Verschillende eigenschappen werden waargenomen, welke al aanwezig waren in hele jonge individuen en op grond daarvan waarschijnlijk het gevolg zijn van de afwijkende anatomische localisatie van de gonaden, zoals in alle, behalve één geval, geidentificeerd werd. Andere karakteristieken ontwikkelden zich pas bij de start van de puberteit en zijn waarschijnlijk het resultaat van de afwezigheid van AR activiteit. Rest AR activiteit is gerelateerd met een betere overleving van de algemene kiemcel populatie in (post)pubertale patienten. Het was niet mogelijk om te bestuderen of er een onafhankelijk effect was van de gonadale localisatie (inguinaal *versus* abdominaal) op de kiemcel pathologie op grond van de ongelijke verdeling in relatie met leeftijd en AR activiteit. Tevens was er geen bewijs bevonden voor een associatie tussen niveau van AR activiteit en kiemcel afwijkingen. Van belang is dat er een opvallend hoog percentage van gevallen van kiemcel maturatie vertraging en/of pre-CIS/IGCNU gevonden werd ten opzichte van het verwachte KCK risico. Deze gegevens impliceren dat niet alle lesies progressie zullen vertonen naar een invasive KCK in CAIS.

De studie gepresenteerd in **Hoofdstuk 4** ondersteunt de hypothese dat het fenotype van de uitwendige genitaliën een afspiegeling is van de gonadal histologie, en een weerspiegeling geeft van de predispositie van KCK ontwikkeling in individuen met 45,X/46,XY mosaicisme. Er werd een significant verschil gevonden betreffende het risico op een KCK tussen drie groepen van patiënten met verschillende niveaus van masculinisatie bepaald op grond van de zogenaamde External Masculinization Score (EMS): het risico was het laagste in patiënten met een vrouwelijk (Turner syndrome) fenotype (2.2%); patienten met een milde ondervirilisatie hadden 13% risico; patiënten met ambigue genitalia hadden het hoogste risico (52% risk). Op grond van deze bevindingen stellen wij richtlijnen voor ten aanzien van veilig management

betreffende KCK risico waarbij het mogelijk is om gonaden te sparen in mannelijke individuen. De conservatieve benadering wordt ondersteund door het feit dat verschillende patiënten in de studie, welke opgegroeid zijn als mannen, spontane puberteit ondergingen als gevolg van de androgeen productie in de behouden gonaden.

Hoofdstuk 5 beschrijft een casus van een kind met 45,X/46,X,psu dic(Y) gonadale dysgenesie en ambigue genitalia. The impact van de twee cellijnen op het fenotype, inclusief gonadale histologie, wordt in detail beschreven en de complexiteit van de aandoening wordt geïllustreerd. De casus demonstreert tevens hoe het management betreffende het KCK risico afhankelijk is van de geslachtstoewijzing in vergelijkbare situaties.

De studie beschreven in **Hoofdstuk 6** toont aan dat er een associatie bestaat tussen het patroon van KITLG aankleuring en het aantal afwijkende kiemcellen (i.e., aantal OCT3/4-positieve kiemcellen gelegen op de basale lamina van de tubuli seminiferi en het aantal (basaal gelegen) kiemcellen met co-expressie van OCT3/4 en TSPY; $p<0.01$) in testiculair weefsel van DSD patiënten. Deze bevinding is in lijn met het hypothetische model van KCK pathogenese zoals beschreven door Van der Zwan *et al*., waarbij een relatie verondersteld wordt tussen KITLG en OCT3/4. Het percentage van OCT3/4-positieve kiemcellen in contact met de basale lamina in gonaden zonder KITLG aankleuring was duidelijk anders dan het percentage van deze cellen in gonaden met een positive KITLG aankleuring. Deze bevinding tesamen met de resultaten verkregen uitgaande van een serie van foetale zaadballen, heeft het mogelijk gemaakt criteria te formuleren voor het onderscheiden van vertraagde uitrijping en pre-CIS/pre-IGCNU gebaseerd op OCT3/4 aankleuring. Ondanks dit resultaat, zijn de resultaten van de KITLG aankleuring van grote waarde, met name in bordeline gevallen.

De algemene discussie beschrijft de bijdragen verkregen uit het bestuderen van de DSD gevallen voor het verder ontwikkelen van onze kennis betreffende gonadale differentiatie en KCK ontwikkeling. De belangrijkste resultaten van de verschillende studies worden bediscussieerd met nadruk op de mogelijke klinische consequenties. Tot slot worden mogelijke ideeen betreffende toekomstig onderzoek bepresenteerd.

Acknowledgement

My way to this thesis was not straightforward. All started in Prague thanks to professor Jan Lebl (a head of the Department of Pediatrics of 2nd Faculty of Medicine, Charles University in Prague and University Hospital Motol) who honoured me with his confidence and guided me together with assisted professor Marta Šnajderová through my PhD studies in Prague. I would like to express many thanks to both of them and also to my colleagues from the Endocrine Unit for their support during the MD-PhD period, to all collaborating Czech clinicians and to statistician Dr. Lánská. My gratitude belongs in memoriam to Dr. Zuntová, who collected most of the gonadal tissue samples from Czech patiens, and to professor Kodet, the head of the Department of Pathology and Molecular Medicine, Charles University in Prague, 2nd Faculty of Medicine and University Hospital Motol, who granted the samples for the studies.

Professor Stenvert Drop, a former head of the Department of Endocrinology at Sophia Children´s Hospital, is the key person in my way to Rotterdam. It was him who organized the collaboration. Dear professor Drop, I am very grateful for the chance you gave me. Thank you for your hospitable care and for your passion for Antonín Dvořák which symbolized a piece of homeland for me when I was so far away from my closests.

My deepest gratitude belongs to my promotor professor Leendert Looijenga, the head of the Laboratory of Experimental Patho-Oncology (LEPO), Erasmus MC. Dear Leendert, I am afraid that I do not have enough words to express how much I have been appreciating your care and attitude with which you finally persuaded me to apply for doctorate at EUR. Thank you also for the possibility to spend marvellous moments with your family and thank you for your support in Prague in April 2014. And of course thank you, Katharina, for your hospitality and support during my days in Rotterdam.

My laboratory work was based on an immunohistochemical detection of selected proteins in gonadal tissue and I was very lucky to have the most skilled tutor, an IHC magician, Hans Stoop. Dear Hans, I consider you as being my lab father. Thank you so much for all you have taught me. Thank you for your patience and kind help. Thank you for introducing Bergen op Zoom and Antwerps to me. I am very lucky knowing such a Rennaisance man with broad interests as you are.

I had a great possibility to discuss the histological findings with professor J.Wolter Oosterhuis, a co-author of worldwide accepted germ cell tumor classification and a former head of the Department of Pathology at Erasmus MC. Dear professor Oosterhuis, although I likely never become a pathologist, every our session ment such an enriching experience for me! Thank you very much for that and also for all your valuable advices regarding the manuscripts.

I was highly honoured by a possibility to collaborate with associated professor Martine Cools, a head of the Department of Pediatric and Adolescent Endocrinology and Diabetology, University Hospital Ghent, Belgium, whose work in the field of DSD is so inspirative, influencing clinical approach to the patients at risk of germ cell cancer. Thank you very much, Martine.

I remember very well my first moments in LEPO. I was told that I surprised most of those who were there by my arrival. However, I felt nicely welcomed, especially thanks to you, Dr. Eikenboom. Wil, thank you for that, for many nice moments we have spent together, and of course for your kind help with information seeking. I have already told you that patients, who were treated by you, were so lucky in their misfortune, and I still think so.

Three former PhD students from LEPO helped me importantly (not only) with my research project. Martin, Yvonne and Remko, thank you all very much.

Many thanks belong also to Ad, Martine, Berdine, Ronak, Marcia and others who have been creating the nice atmosphere in LEPO. I would also like to thank Bestari Setyawati from Semarang, Indonesia with whom I explored both mysteries of laboratory work and beauties of the Netherlands.

I would like to thank Dr. Hennie Brüggenwirth from Department of Clinical Genetics, Erasmus MC for her help with CAIS manuscript and to Dr. Katja Wolffenbuttel from Department of Pediatric Urology, Sofia Children´s Hospital for her comments and suggestions on some of the manuscripts.

Dear all in LEPO, it was really an honour for me to be for some time a part of your team which I believe represents one of the leading centers of germ cell cancer research in the world.

Last but not least, I am very grateful to my family, because without them I would not be what I am and I would not be where I am.

Curriculum vitae

Jana Kaprová (Pleskačová) was born on 29th of March 1983 in Prague, in former Czechoslovak Socialistic Republic. After she had graduated at Gymnázium nad Alejí in 2002, she started her master program of General medicine at the 3rd Faculty of Medicine, Charles University in Prague. During her studies, she worked as a junior lecturer at the Department of Anatomy. She graduated cum laude in 2008. She was a laureat of Margaret Bertrand Foundation Award which is granted to the best student of the graduating year. After the graduation she gained a position of a resident in pediatrics at the Department of Pediatrics at the University Hospital Motol in Prague. At the same time she was enrolled in the PhD program at the 2nd Faculty of Medicine, Charles University in Prague under the supervision of prof. Jan Lebl and as. prof. Marta Šnajderová. Her laboratory work took place in Laboratory of Experimental Patho-Oncology at the Department of Pathology, Erasmus MC, Rotterdam, where she was guided by prof. Leendert L.H. Looijenga. The collaboration was supported by ESPE Research Fellowship. She finished her Czech PhD in 2014 defending the thesis *„Pathogenesis of germ cell tumor development: Application of current knowledge in early diagnostics in patients with disorders of sex development (DSD).“* Jana married Robert Kapr in 2012, together they have one son Antonín born in 2013.

List of publications

Articles published in Pubmed indexed journals:

- *Pleskačová J, Šnajderová M, Lebl J*: Disorders of sex differentiation: Genes responsible for development of genital system and final phenotype. Čes Gynekol 2009;74(3):193-197.
- *Pleskacova J, Rucklova K, Popelova J, Cerny S, Syrucek M, Snajderova M, Lebl J*.: Aortic dissection and rupture in a 16-year-old girl with Turner syndrome following previous progression of aortic dilation. Eur J Pediatr 2010;169(10):1283-6.
- *Pleskacova J, Hersmus R, Oosterhuis JW, Setyawati BA, Faradz SM, Cools M, Wolffenbuttel KP, Lebl J, Drop SL, Looijenga:* Tumor risk in disorders of sex development. Sex Dev 2010;4(4-5):259-269.
- *Cools M, Pleskacova J, Stoop H, Hoebeke P, Van Laecke E, Drop SL, Lebl J, Oosterhuis JW, Looijenga LH, Wolffenbuttel KP; on behalf of the Mosaicism Collaborative Group*. Gonadal Pathology and Tumor Risk in Relation to Clinical Characteristics in Patients with 45,X/46,XY Mosaicism. J Clin Endocrinol Metab 2011; 96(7):E1171-80.
- *Kaprova-Pleskacova J, Snajderova M, Stoop H, Koudova M, Kocarek E, Novotna D, Drop S, Obermannova B, Lebl J, Oosterhuis W, Looijenga L*: 45,X/46,X,psu dic (Y) gonadal dysgenesis: Influence of the two cell lines on the clinical phenotype, including gonadal histology. Sex Dev 2013;7(6):282-8.
- *Kaprova-Pleskacova J, Stoop H, Bruggenwirth H, Cools M, Wolffenbuttel KP, Drop SLS, Snajderova M, Lebl J, Oosterhuis JW, Looijenga LHJ*: Complete androgen insensitivity syndrome: factors influencing gonadal histology including germ cell pathology. Mod Pathol 2014;27(5):721-30.

Articles published in other peer-reviewed journals:

- *Pleskačová J, Šnajderová M, Lebl J*: Disorders of sex development: Currently recommended classification and new knowledge of gonadal maldevelopment. Čes-slov Pediatr 2010;65(2):79-85.
- *Pleskačová J, Šnajderová M, Lebl J.* Disorders of sex development and germ cell tumors: pathogenesis, prevalence, clinical attitude. DMEV 2011; 14(2):79-83.

List of abbreviations

AFP	Alpha-fetoprotein
AKT	V-akt murine thymoma viral oncogene homolog 1
AP2-gamma	Activating protein 2 - gamma
AR	Androgen receptor
CAIS	Complete androgen insensitivity syndrome
CIS	Carcinoma *in situ* (also IGCNU)
c-KIT	Tyrosine-protein kinase kit
DSD	Disorders of sex development
EMS	External masculinization score
ESC	Embryonic stem cells
ESPE	European Society for Pediatric Endocrinology
FISH	Fluorescent *in situ* hybridization
FOXL2	Forkhead box L2
FSH	Follicle stimulating hormone
GB	Gonadoblastoma
GBY	Gonadoblatoma locus on the Y chromosome
GCC	Germ cell cancer
GCT	Germ cell tumor
hCG	Human chorionic gonadotropin
HE	Hematoxylin and eosin
IGCNU	Intratubular germ cell neoplasia unclassified (also CIS)
IGF-1	Insulin-like growth factor 1
IVF	I*n vitro* fertilization
KITLG	KIT ligand (also SCF)
LH	Luteinizing hormone
LWPES	Lawson Wilkins Pediatric Endocrinology Society
miRNA	Micro ribonucleic acid
NANOG	Homeobox transcription factor NANOG
OCT3/4	Octamer-binding transcription factor 3/4 (also POU5F1)
PAIS	Partial androgen insensitivity syndrome
PGC	Primordial germ cells
PLAP	Placental alkaline phosphatase
POU5F1	Pit-oct-unc domain 5 transcription factor 1 (also OCT3/4)
RSPO1	R-spondin family member 1
SCF	Stem cell factor (also KITLG)
SNP	Single nucleotid polymorphism
SOX2	SRY-box 2
SOX9	SRY-box 9
SOX17	SRY-box 17
SRY	Sex determining region Y
TDF	Testis determining factor
TDS	Testicular dysgenesis syndrome
TS	Turner syndrome
TSPY(1)	Testis-specific Y-encoded protein (1)
UGT	Undifferentiated gonadal tissue
WNT4	Wingless-type MMTV integration site family member 4
WT1	Wilms tumor suppressor gene 1

Summary of Ph.D. training and teaching

Name of PhD student: Jana Kaprová — PhD period: 10/2009-5/2015

Erasmus MC Department: Pathology — Promotor: prof. Dr. L.H.J. Looijenga

Research school: Molecular Medicine

PhD training		
	Year	**Work load**
General courses		
- Course on the Fundamentals of the Research Work (ASCR[1])	2010	35 hours
In-depth courses		
- Advances in Molecular Biology and Genetics (ASCR)	2008	40 hours
- Course on Molecular Medicine	2010	0.75 ECTS
Presentations		
- Days of Pediatric Endocrinology (Hradec Králové, Czech Republic)	2009	1x
(Olomouc, Czech Republic)	2011	2x
(Mladá Boleslav, Czech Republic)	2013	1x
(Plzeň, Czech Republic)	2014	1x
- Days of Endocrinology 2009 (Český Krumlov, Czech Republic)	2009	1x
- LEPO[2] work group meeting (Rotterdam, The Netherlands)	2010	1x
- The 7th International Medical Postgraduate Conference (Hradec Králové, Czech Republic)	2010	1x
- 19th Annual Meeting of ES-PCR[3] (Bratislava, Slovak Republic)	2010	1x
- Students' Research Conference, 2nd Faculty of Medicine, Charles University (Prague, Czech Repubic)	2011	1x
- The 18th Annual Meeting of the MESPE[4] (Gozd Martuljek, Slovenia)	2012	1x
International conferences		
- The 15th Annual Meeting of the MESPE (Balatonfüred, Hungary)	2008	
- The 49th Annual Meeting of the ESPE[5] (Prague, Czech Republic)	2010	2x poster
- 20th Meeting of ES-PCR (Brno, Czech Republic)	2011	poster
- NICHe Conference[6] (Schloss Liebenberg, Berlin, Germany)	2011	
- The 51st Annual Meeting of the ESPE (Leipzig, Germany)	2012	poster
Seminars and workshops		
- ESPE Science School	2011	2.5 days
- ESPE Advanced Seminar on Developmental Endocrinology	2011	2 days
- IPOKRaTES Clinical Seminar (Diagnosis and management of adrenal and gonadal disorders)	2013	2 days
- ESPE Winter School	2013	5 days
Teaching activities		
	Year	**Work load**
- Junior lecturer (lectures and seminars for students of Master program), Dpt. of Anatomy, 3rd Faculty of Medicine, Charles University, Prague	2/2005 - 6/2008	0.25 part time
- Lectures within the Course on Molecular Pathogenesis of Endocrinological Diseases (Master), 2nd Faculty of Medicine, Charles University, Prague	2011 - 2014	6 hours
- Supervision on laboratory work of a Master student (BA Setyawati), Dpt. of Pathology, Erasmus MC	11/2009 - 3/2010	

[1] Academy of Sciences of the Czech Republic
[2] Laboratory of Experimental Patho-oncology
[3] European Society of Pediatric Clinical Research
[4] Middle European Society for Pediatric Endocrinilogy
[5] European Society for Pediatric Endocrinology
[6] New Inroads to Child Health