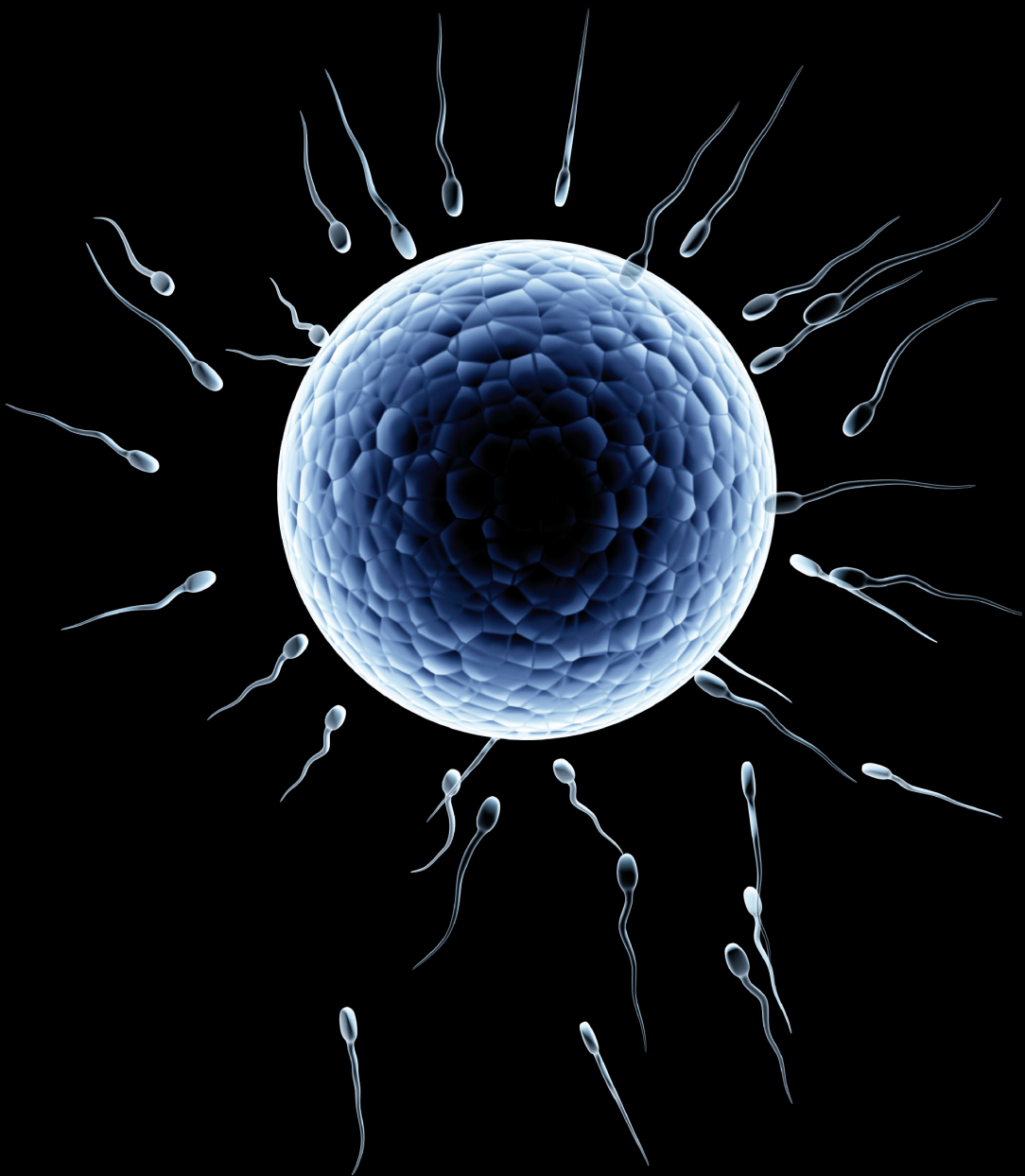
Disorders of Sex Development and Germ Cell Cancer; genetics and microenvironment



Remko Hersmus

Disorders of Sex Development and Germ Cell Cancer; genetics and microenvironment

Stoornissen in de sex ontwikkeling en kiemcelkanker;
genetica en micromilieu

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

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“It always seems impossible until it's done.”

Nelson Mandela

Voor mijn ouders: Ellie en Jan
Voor Elza

Contents

Abbreviations

AMH: anti-Müllerian hormone
AP: alkaline phosphatase
CIS: carcinoma *in situ*
CNV: copy number variation
c-KIT: stem cell factor receptor (CD117)
DAX1: DSS-AHC critical region on the X chromosome protein 1 (NR0B1)
DHT: dihydrotestosterone
DSD: disorders of sex development
DDS: Denys-drash syndrome
DMRT1: doublesex and mab-3 related transcription factor 1
FGF9: fibroblast growth factor 9
FOXL2: forkhead box L2
FS: Frasier syndrome
GATA4: GATA binding protein 4
GB: gonadoblastoma
GBY: gonadoblastoma region on the Y-chromosome
GCC: germ cell cancer
GCT: germ cell tumor
GIST: gastro-intestinal stromal tumors
INSL3: insulin-like 3
KITLG: c-KIT ligand (SCF)
MAP3K1: mitogen-activated protein kinase kinase kinase 1
NANOG: Nanog homeobox
NR0B1: nuclear receptor subfamily 0, group B, member 1 (DAX1)
NR5A1: nuclear receptor subfamily 5, group A, member 1 (SF1)
OCT3/4: octamer-binding transcription factor 3/4 (POU5F1)
PDGFRA: platelet-derived growth factor receptor alpha
PGC: primordial germ cell
POU5F1: POU class 5 homeobox 1 (OCT3/4)
RSPO1: R-spondin 1
SCF: stem cell factor (KITLG)
SF1: steroidogenic factor 1 (NR5A1)
SNP: single-nucleotide polymorphism
SOX9: SRY-related HMG-box, gene 9
SRY: sex-determining region on Y
TDF: testis determining factor
TDS: testicular dysgenesis syndrome
TS: Turner syndrome
TSPY: testis-specific protein, Y-encoded
WNT4: wingless-type MMTV integration site family, member 4
WWOX: WW domain containing oxidoreductase

Chapter 1

General Introduction

1.1 Male and female development

The ultimate purpose of sexual reproduction, which depends on specialized male and female anatomy and physiology, is to enable continuation of a species and introduction of genetic diversity. In mammals the developmental path towards a male or a female is in principle determined at the moment of fertilization, when either a Y- or an X-chromosome is inherited from the father. The subsequent chromosomal constitution, either XY (male) or XX (female) (referred to as chromosomal sex), will eventually drive formation of a testis or an ovary (the so called gonadal sex). This in turn will result in the next step in sex determination (the phenotypic sex), ultimately leading to a phenotypical male or female respectively. Because of the relevance of the general principles related to this phenomenon in understanding the various levels in which pathological gonadal processes can occur, the next paragraphs will explain these issues in more detail. These are schematically shown in Figure 1 and 2. Some of the items to be discussed are (partially) presented in Chapter 3.

1.1.1 Male gonadal development

Early in the process of embryogenesis, both in chromosomal males and females, the initially indifferent or bi-potential gonads are formed, under influence of factors like WT1, DMRT1, GATA4 and FGF9 [1]. In the embryo composed of XY cells, expression of the sex-determining region on the Y-chromosome (*SRY*) gene, also known as the testis determining factor (TDF), in the genital ridges is the pivotal and initiating event in the process of testis formation. SRY starts to be strongly expressed around week 6 of gestation in gonadal stromal cells, being the precursor of Sertoli cells [2]. When proper amounts of SRY are present at this time, amongst others depending on the stabilizing effect of a specific splice variant of the *WT1* gene (+KTS) [3], the down stream target *SOX9* will be upregulated through SRY binding to its testis specific enhancer. It has been shown in mice that a feed forward loop is initiated by Sox9 through upregulation of *Fgf9*, and expression of Sox9 is further stimulated by prostaglandin D2 [4-7]. SOX9 will subsequently induce a signal transduction cascade ultimately leading to differentiation of stromal cells into (pre-)Sertoli cells [8-9]. During this process of differentiation as well as proliferation, the size of the gonad increases, also through an influx of cells from the adjacent mesonephros. These cells will form, amongst others, peritubular myoid cells, endothelial cells and Leydig cells [10-13].

The germ cells, by that time having arrived from their migration from the epiblast (see below), become enclosed by these early Sertoli and peritubular

myoid cells and testis cords start to be formed, later becoming the seminiferous tubules. It must be noted that in contrast to the female situation (see below) presence of germ cells is not required for this process to occur. At the same time, in the interstitial space, steroidogenic Leydig cells start to form, producing testosterone and insulin-like factor 3 (INSL3). Together with, amongst others, Anti-Müllerian Hormone (AMH) formed by Sertoli cells, these factors are responsible for further development of the male internal and external genitalia (see below) [14-15].

1.1.2 Female gonadal development

In the absence of a functional Y-chromosome (i.e. SRY) the fate of the bi-potential gonad is directed toward the ovarian lineage, long believed to be the default pathway. In this process, the germ cells are surrounded by granulosa cells to form follicles, which are surrounded by interstitial cell types including the steroidogenic theca cells. However, ovarian development is not only depending on the presence and survival of germ cells (in contrast to testicular development), i.e. the absence of germ cells will eventually result in so-called streak gonads (non-functional stroma without germ cells). In contrast, it has been elegantly demonstrated that ovarian development requires expression of a specific set of genes. Instead of one single "master" gene, it appears that there are several proteins at play in parallel for specific cell lineages, including FOXL2, RSPO1, and WNT4. In the mouse, Foxl2 is necessary for the formation and maintenance of the granulosa cells and steroidogenic cells [16-21]. Disruption of RSPO1 leads to complete female-to-male sex reversal in humans [22], and in the mouse Rspo1 is found to positively regulate Wnt4 signaling [23]. Loss of both Wnt4 and Foxl2 leads to testis development and female-to-male sex reversal in mice with a 46,XX chromosomal constitution [24] and Wnt4 acts as an antagonistic signal to Fgf9 in regulating mammalian sex determination [25].

1.1.3 Male and female genital development and phenotype

During early embryogenesis the mesonephros and coelomic epithelium give rise to two separate systems, the Wolffian and Müllerian ducts, initially present in developing males and females alike.

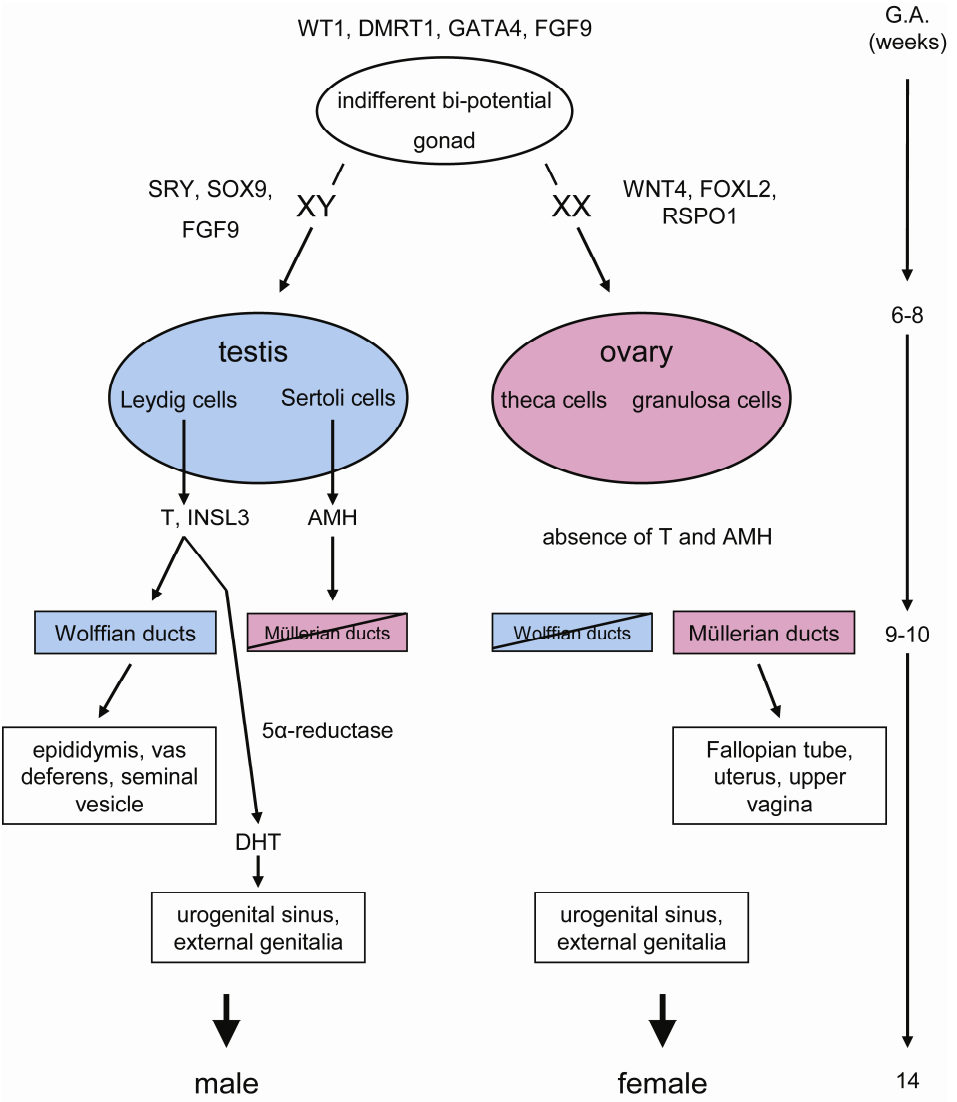

Figure 1. Schematic representation of male and female development. Both in chromosomal males and females, early in development the indifferent, bi-potential gonads are formed. In the XY embryo, expression of SRY (located on the Y-chromosome), is the pivotal and initiating event in the process of testis formation. SRY up regulates SOX9, which induces a signal cascade ultimately leading to formation of (pre-)Sertoli cells. During this time the size of the gonad increases and peritubular myoid cells, endothelial cells and the steroidogenic Leydig cells form. In the XX embryo, the absence of a functional Y-chromosome (i.e. SRY), directs the fate of the bipotential gonad toward the ovarian lineage. Instead of one single "master" gene, several proteins seem to be at play in parallel. Early in embryogenesis, two separate duct systems, the Wolffian and Müllerian ducts are formed in both males and females. In the male, Anti-Müllerian Hormone (AMH) produced by the Sertoli cells will cause the Müllerian ducts to regress. The Leydig cells will produce testosterone (T), stabilizing the Wolffian ducts, and INSL3, which together with dihydrotestosterone (DHT) will direct the formation of the male internal and external genitalia. In the female, independent of presence of functional ovaries, the combined absence of AMH and androgen action leads to development of the internal and external genitalia along the female pathway. G.A., gestational age. See text for further details.

If functional testes are formed (see above), under influence of levels of testosterone produced by the Leydig cells, the Wolffian ducts, expressing the androgen receptor (AR), will differentiate into epididymis, vas deferentia and seminal vesicles [26]. AMH produced by the Sertoli cells will cause the Müllerian ducts to regress [27]. In the absence of testosterone, the Wolffian ducts will degenerate. The generated testosterone will be converted by the enzyme 5α-reductase into dihydrotestosterone (DHT), which has a higher affinity to bind to the AR than testosterone. DHT is in fact the hormone responsible for virilization of the urogenital sinus and external genitalia, taking place between week 10 and 14 of gestation [28]. Testosterone production peaks at mid-gestation and then gradually decreases until term, driving penile enlargement and testicular descent. INSL3, like androgens, play a role in the first phase of testicular descent [29], Insl3 null mice have undescended testes, and mutations in this gene have been described in cryptorchid patients [15, 30]. In the female, independently of the presence of functional ovaries, the combined absence of AMH and androgen action leads to development of the internal and external genitalia along the female pathway. Müllerian ducts will give rise to the fallopian tubes, uterus and upper part of the vagina, and the Wolffian ducts will regress.

1.1.4 Germ cell development

The initially identified embryonic cells that will later form spermatozoa in males and oocytes in females are called primordial germ cells (PGCs). In the mouse, PGCs can be recognized (based on markers like alkaline phosphatase (AP) and OCT3/4) at day 6.5 post coitum (weeks 5-6 gestational age in humans) [31-32]. The PGCs arise in the proximal epiblast, and migrate through the hindgut towards the genital ridges, during this time the PGCs proliferate extensively [33]. This process of migration, proliferation and survival, is highly dependent on the stem cell factor (SCF, also known as KITLG)-c-KIT pathway [34-36]. After their arrival in the gonadal ridges they are called gonocytes, although they cannot be distinguished, either morphologically or based on marker profile, from PGCs, and will continue to proliferate. Depending on the presence or absence of SRY, the (at that point still bi-potential) gonad will form a testis or an ovary (see above). When a testis is formed the PGCs associate with the (pre)Sertoli cells, go into mitotic arrest and differentiate into (pre-) spermatogonia. During this process they lose expression of the embryonic markers (like OCT3/4, NANOG, AP and c-KIT), and will enhance expression of amongst others, TSPY and VASA [37-41].

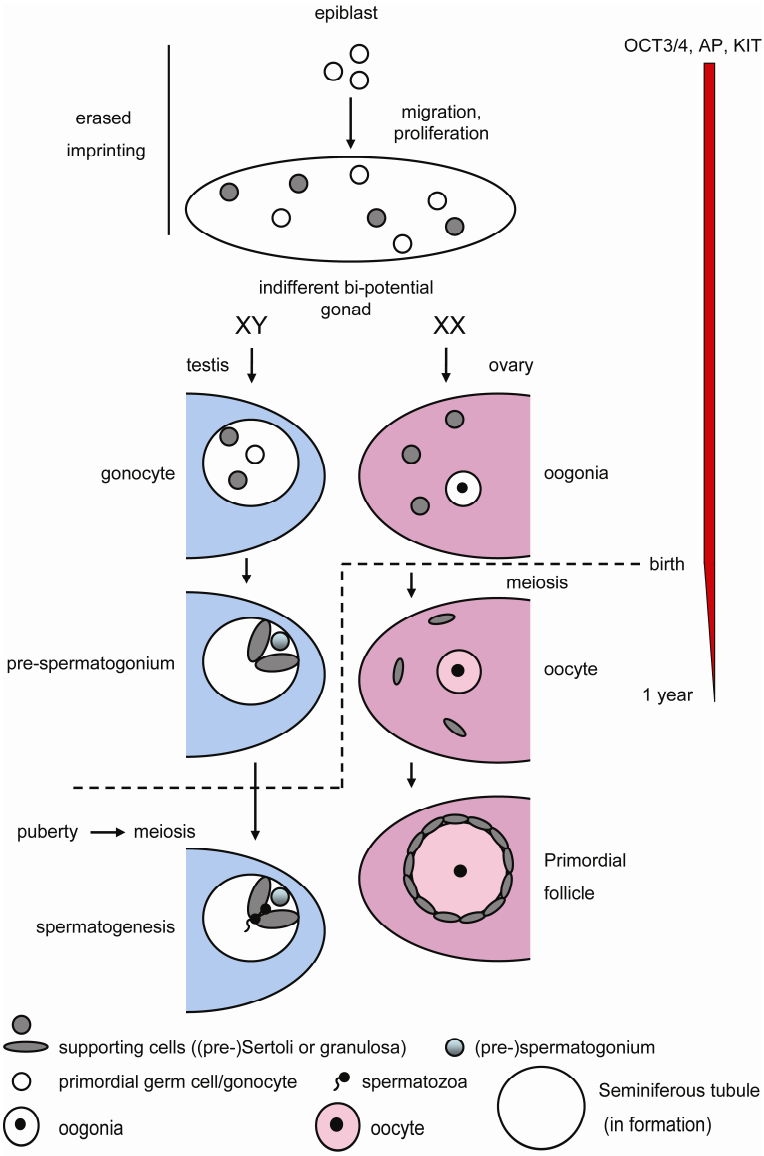

Figure 2. Schematic representation of germ cell development. In both males and females, the primordial germ cells (PGCs) arise early in embryonic development in the proximal epiblast, and migrate towards the genital ridges. The PGCs will undergo extensive epigenetic changes, and at the time they arrive in the gonadal ridges, genomic imprinting is completely erased. A paternal or maternal pattern of imprinting will be established depending on the presence of a testicular or ovarian environment respectively. Depending on the presence or absence of SRY, the at that point still bi-potential gonad will form a testis or an ovary (see Figure 1 and text). When a testis is formed the PGCs/gonocytes associate with (pre-)Sertoli cells, go into mitotic arrest and differentiate into (pre-)spermatogonia. During this process they lose expression of the embryonic markers (like OCT3/4, AP, and c-KIT) and will enhance expression of, amongst others, TSPY and VASA. Full spermatogenesis will only start after puberty, which is highly dependent on the hormone testosterone. In the female situation, the gonocytes will differentiate into oocytes, also losing expression of the before mentioned embryonic markers. In contrast to the male situation, in the female meiotic oocytes are present from post-partum onwards.

The expression of embryonic markers is fully lost during the first year of post-natal life, and if germ cells expressing these markers are identified after birth, they are delayed in maturation and at risk for malignant transformation [42-43]. Full spermatogenesis will only be initiated after puberty, for which the hormone testosterone is highly important [44]. This is in contrast to the female situation, in which the germ cells go into meiosis I arrest, characterized by the presence of meiotic oocytes from post-partum onwards. When the gonad forms an ovary, the gonocytes will differentiate into oocytes, also losing expression of the above mentioned embryonic markers [39, 45-46], and also delay in maturation can be found. Next to the changes described above, the PGCs also will undergo extensive epigenetic (i.e. chromosomal modifications and DNA methylation) changes. Expression of certain genes is determined by their parental origin, with some genes showing expression after paternal inheritance while others are expressed only when inherited from the maternal germ line. To accomplish this expression pattern, these genes are subject to germ line specific epigenetic modifications. To establish a uniparental paternal or maternal pattern of genomic imprinting, the originally bi-parental genomic imprinting present in the zygote has to be erased in the PGCs. At the time the PGCs arrive in the gonadal ridges genomic imprinting is completely erased, [47-50] and they will gain a paternal pattern in a testicular environment and a maternal pattern in an ovarian environment [51-52].

1.2 Disorders of sex development

Disorders of sex development (DSD) are defined as congenital conditions in which development of chromosomal, gonadal or anatomical sex is atypical, previously referred to as intersex conditions [53]. This new classification of patients with DSD is based on the chromosomal constitution, and consists of three main groups, namely; 46,XY-DSD, 46,XX-DSD, and sex chromosome-DSD, and although this classification has its limits, it prevents misunderstanding due to the different terminologies used (Table 1) [53]. As a whole, DSD includes relatively mild forms as hypospadias (1:500 live births), as well as more severe conditions such as ambiguous genitalia (1:4,500 live births) and complete sex reversal in 46,XY females and 46,XX males (1:20,000 live births) [54]. Some of the variants of DSD have an obvious explanation, for example the absence of a functional SRY due to deletion, or inactivating mutation of the gene, leading to complete sex reversal in 46,XY-DSD [55-57]. Others include: the translocation of SRY to one of the X-chromosomes in 46,XX-DSD [58]; duplications of *DAX1*, resulting in suppression of the SRY

pathway in 46,XY-DSD [59]; deletions of chromosome 9p, leading to haplo-insufficiency of *DMRT1* [60]; and the absence of the Y-chromosome in a subgroup of sex chromosome-DSD patients (45,X, Turner syndrome) [61]. However, sometimes in patients with a single genetic abnormality, and in patients with a mosaic constitution of the sex chromosomes the phenotype can be heterogeneous. The latter cannot be easily explained by the presence of Y-chromosome containing cells in the testicular areas [62], and it seems that in these patients a threshold level and sufficient density of the SRY is needed to activate SOX9 and the further downstream cascade [63-64]. Patients with hypovirilization, i.e. 46,XY-DSD with bilateral well differentiated testes (located in the abdomen, inguinal region or scrotal), can present with an ambiguous or female phenotype. Patients with specific forms of DSD have an increased risk to undergo malignant transformation of germ cells, resulting in a specific form of cancer, which will be discussed in the next paragraph.

Table 1. DSD classification

Sex chromosome DSD	46,XY-DSD	46,XX-DSD
A: 47,XXY (Klinefelter syndrome and variants)	A: Disorders of gonadal (testicular) development	A: Disorders of gonadal (ovarian) development
B: 45,X (Turner syndrome and variants)	1. Complete or partial gonadal dysgenesis	1. Gonadal dysgenesis
C: 45,X/46,XY (mixed gonadal dysgenesis)	2. Ovotesticular DSD	2. Ovotesticular DSD
D: 46,XX/46,XY (chimerism)	3. Gonadal regression	3. Testicular DSD
	B: Disorders in androgen synthesis or action	B: Androgen excess
	1. Disorders of androgen synthesis	1. Fetal
	a. LH receptor mutations	a. 3 β-hydroxysteroid dehydrogenase 2
	b. Smith-Lemli-Opitz syndrome	b. 21-hydroxylase
	c. Steroidogenic acute regulatory protein mutations	c. P450 oxidoreductase
	d. Cholesterol side-chain cleavage	d. 11 β-hydroxylase
	e. 3 β-hydroxysteroid dehydrogenase 2	e. Glucocorticoid receptor mutations
	f. 17 β-hydroxysteroid dehydrogenase	
	g. 5 α-reductase 2	2. Fetoplacental
		a. Aromatase deficiency
	2. Disorders of androgen action	b. Oxidoreductase deficiency
	a. Androgen insensitivity syndrome	
	b. Drugs and environmental modulators	3. Maternal
		a. Maternal virilizing tumours (e.g. luteomas)
		b. androgenic drugs
	C: Other	C: Other
	1. Syndromic associations of male genital development (e.g. cloacal anomalies, Robinow, Aarskog, Hand-Foot-Genital, popliteal pterygium)	1. Syndromic associations (e.g. cloacal anomalies)
	2. Persistent Müllerian duct syndrome	2. Müllerian agenesis/hypoplasia (e.g. MURCS)
	3. Vanishing testis syndrome	3. Uterine abnormalities (e.g. MODY5)
	4. Isolated hypospadias	4. Vaginal atresis (e.g. KcKusick-Kaufman)
	5. Congenital hypogonadotropic hypogonadism	5. Labial adhesions
	6. Cryptorchidism	
	7. Environmental influences	

Adapted from [53], without references to genes.

1.3 Disorders of sex development and germ cell cancer

Germ cell tumors (GCTs) are often considered a heterogeneous group of neoplasms, with the common characteristic that they all arise from the germ cell lineage. GCTs have traditionally been classified based on histological composition, which does not take into account the underlying differences in pathogenesis, thereby complicating clinical treatment, as well as identification of pathological mechanisms. Therefore an alternative classification system in which five types of GCTs are identified was proposed, which has been

recognized by the World Health organization and specialized pathologists in the field [65-67]. In the context of DSD only the so called type II GCTs, here referred to as Germ Cell Cancers (GCC) are of interest, and to be discussed in the next paragraph.

1.3.1 Type II germ cell tumors–germ cell cancers

Although the overall incidence in the general population of GCC is low, certain populations, like patients with specific forms of DSD (see below), have a high risk. The GCC of the testis, also referred to as testicular germ cell tumors or cancers (TGCTs-TGCC), are the most common diagnosed cancer in men aged between 20 and 40 years of age [68]. The incidence (6-11 per 100,000) has increased in the Caucasian populations in recent decades, and the incidence is still rising [69], and in the Netherlands approximately 750 new cases (6.6 per 100,000) are diagnosed each year [70]. Pure seminomas make up about 50% of TGCC (median age at diagnosis of 35 years of age), non-seminomas comprise about 35% (median age at diagnosis 25 years of age) and the rest are combined tumors (presenting at an intermediate age). Treatment of the disease by surgery and/or chemotherapy or irradiation is very effective, with a ten year survival of about 95% [71]. However, there are long term side effects related with systemic therapy in these patients [72-73]. It has been hypothesized by Skakkebæk and co-workers that the so-called Testicular Dysgenesis Syndrome (TDS) is the underlying entity in this cancer [74-75]. TDS links clinical observations like cryptorchism, subfertility/infertility and hypospadias with exposure to certain environmental factors [74, 76]. It must be noted however, that genetic factors are also recognized to play a role in the disease [77-78]. It can be argued that DSD is at the far spectrum of TDS, this is strengthened by the description of two independent families in which members with TDS and DSD were presented [79-80].

1.3.2 Precursor lesions: carcinoma *in situ* and gonadoblastoma

The precursor lesion of the TGCC is the so-called carcinoma *in situ* (CIS) [81], also referred to as Intratubular Germ Cell Neoplasia Unclassified (IGCNU) [66] and Testicular Intratubular Neoplasia (TIN) [82], and long term follow-up data implies that, if left untreated, CIS will always develop into an invasive TGCC [83]. CIS cells are located on the basement lamina of the seminiferous tubules, under the tight junctions of Sertoli cells in the adult testis, and they are often found adjacent to invasive TGCC [84]. CIS cells are the malignant counterpart of an embryonic germ cell, most likely the PGC/gonocyte, which they resemble

phenotypically and ultrastructurally and also regarding their expressed protein profile like AP, c-KIT, OCT3/4, TSPY and SCF. In addition, they show an erased pattern of genomic imprinting [38, 85-90]. In DSD patients not only CIS, but also gonadoblastoma (GB) can be found as precursor lesion, even within one gonad [91-92]. GB is composed of a mixture of embryonic germ cells, associated with supportive cells that resemble granulosa cells [66, 93]. GB shows expression of the same embryonic markers as CIS, and it is suggested that both CIS and GB are derived from fetal germ cells arrested in an early stage of development [94-96]. As is the case with CIS also GB is known to progress into an invasive GCC [91], resembling the invasive components which arise from CIS. In the context of testicular tissue CIS develops and GB is mainly seen in undifferentiated gonadal tissue [97]. Most likely the prolonged expression of OCT3/4 and increased expression of TSPY (see below) give germ cells residing in this unfavorable environment the tools to survive and proliferate [42, 93]. Important in this respect is to distinguish germ cells delayed in maturation from malignant germ cells, both express the same set of markers, which could lead to overdiagnosis [42]. SCF is a useful additional tool in this respect, as this marker is expressed in CIS and GB cells, but is negative in cells showing maturation delay [89].

1.3.3 Germ cell cancer in disorders of sex development patients

The most common gonadal tumors found in DSD patients are GCC, being the (non-)seminoma in the testis (see above) and its counterpart in the dysgenetic gonad, the (non-)dysgerminoma. Dysgerminomas histologically resemble seminomas and they cannot be distinguished from seminomas based on mRNA and miRNA profiling [98-99]. DSD patients with specific variants, i.e. gonadal dysgenesis and hypovirilization, have a high risk to develop GCC [100-101]. In gonadal dysgenesis there is an incomplete formation or malformation of the gonads, due to a disturbed migration of the germ cells, and/or their correct organization in the fetal gonadal ridge [102]. Hypovirilization can be caused by either errors in testosterone or DHT biosynthesis, testicular unresponsiveness to stimulation from the pituitary or by defects in androgen dependent target tissues [102-103]. Pivotal for the development of GCC in these patients is the presence of (part of) the Y-chromosome. In 1978 Page postulated that a gene on the Y-chromosome (gonadoblastoma on the Y-chromosome, GBY) may act as an oncogene in the context of a dysgenetic gonad [104]. The most likely candidate gene in this region is the *TSPY* gene. Expression of the protein is found in spermatogonia of the adult male, believed to be related to their mitotic

proliferation, and in the fetal gonad TSPY is expressed at a constant level, but the protein becomes more abundant in CIS, GB and sometimes seminoma [38, 105-106].

1.4 Concluding remarks

The GCC have their origin in embryonic germ cells, when these cells are retained in a testicular environment they can give rise to CIS and will subsequently progress into an invasive tumor. In DSD patients the spectrum in gonadal differentiation can vary from testicular to a more ovarian appearance, and these patients can, next to CIS, develop GB as precursor lesion. In normal development the decision if a testis or an ovary will be formed is made early in development by the presence of the Y-chromosome and the timely expression a specific set of genes. Specific subgroups of DSD patients can be explained in part by mutations in these genes, the absence of a Y-chromosome (45,X karyotype), or a mosaic sex chromosomal karyotype. However, for some of these patients the underlying defects only partly explain the disease and for a large group of patients the defects are not known. Insight into the genes/factors involved in the (mal)formation of the gonads, giving a better understanding of the micro-environment in which CIS and GB develop, can lead to an improved identification of patients at risk for developing a GCC. This will facilitate early diagnosis and treatment, preventing the development of an invasive and possibly metastatic cancer.

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Chapter 2

Aims and outlines of the thesis

Disorders of sex development (DSD) refer to a congenital condition in which there is an atypical development of chromosomal, gonadal or anatomical sex. DSD patients with hypovirilization and gonadal dysgenesis with part of the Y-chromosome have a high risk to develop a so called malignant type II germ cell tumor/cancer (GCC), related to the presence of the GBY region (i.e. TSPY). In gonadal dysgenesis migration of the germ cells and/or their organization in the gonad is disturbed, leading to incomplete formation of the gonads. Defects in androgen dependent target tissues, errors in testosterone biosynthesis and testicular unresponsiveness to stimulation of the pituitary, cause hypovirilization. The precursor lesions found in these patients can be both carcinoma *in-situ* (CIS) and gonadoblastoma (GB). The determinants for formation of either CIS or GB are largely unknown so far. To gain a better insight into the pathogenesis, especially the earliest developmental stages, of GCC, **Chapter 3** describes the presence of these precursor lesions (i.e. CIS and/or GB) related to GCC risk in a large series of gonads of DSD patients of whom the underlying molecular defect is known.

The development of the precursor lesions GB and/or CIS depends on the level of testicular differentiation of the gonad, directly linked to formation of either Sertoli cells or granulosa cells. CIS is found in a testicular context being associated with supportive Sertoli cells, while GB is found being associated, based on morphological criteria alone, with supportive cells having granulosa cell like characteristics. To further elucidate the gonadal context in which GB and CIS develop, **Chapter 4** describes a detailed histological analysis of a series of normal developed embryonal and adult, as well as DSD gonads with markers specific for Sertoli (SOX9) and granulosa cells (FOXL2). The study gives insight into the differentiation patterns in normal gonadal differentiation versus DSD and the histological context in which GB and CIS can be found.

GB is the *in situ* germ cell malignancy found in the dysgenetic gonad of DSD patients with gonadal dysgenesis or hypovirilization. In a substantial number of cases GB will develop into an invasive dysgerminoma or non-dysgerminoma, being histological and genetically the counterparts of the seminoma and non-seminoma of the testis, respectively. The precursor lesion for the latter is, as mentioned, CIS, and is found in well differentiated testicular tissue. It has been suggested that the so-called testicular dysgenesis syndrome (TDS) is the underlying entity for the formation of the testicular type II GCTs. It links various clinical observations with environmental factors, leading to an increase in GCT risk. However, it must be noted that a role for genetic factors is also recognized. **Chapter 5** demonstrates the possibility, importance and

relevance of early recognition of TDS and DSD for timely diagnosis of malignant GCC, thereby possibly preventing progression to metastasized disease, requiring systemic treatment for cure.

Early in embryonic development the bi-potential gonads have the ability to develop either into a testis or an ovary. A key player in this process is the *SRY* gene, encoding the SRY protein, expression of which will lead to the up-regulation of SOX9, in turn leading to a cascade in signal transduction leading to the development of (pre-)Sertoli cells and subsequent testis formation. Functional Sertoli cells produce, amongst others, AMH and direct the formation of testosterone producing Leydig cells, leading to the physiological formation of male internal and external genitalia. In DSD patients with gonadal dysgenesis the bi-potential gonads can fail to develop along the male pathway. Several genetic factors are known to play a role in DSD, although many are unclear so far. In **Chapters 6-8** the consequences of deactivating or activating mutations of genes involved in gonadal development are studied in greater detail, specifically in relation to GCC development.

In 10-15% of 46,XY DSD patients inactivating mutations in SRY are found, with most residing in the HMG domain. Additional identification and detailed description of patients will enhance our understanding of the biology related. In **Chapter 6** a novel *SRY* mutation is described, detected in a 46,XY DSD patient, of which the functional effects are investigated. Besides mutations in *SRY,* amongst others also specific mutations in the intron 9 splice-site of the Wilms' tumor 1 gene (*WT1*) can lead to a specific 46,XY DSD variant (old term: Frasier syndrome). Patients harboring this specific mutation show gonadal dysgenesis with a high risk for GB development and renal failure in early adulthood. In **Chapter 7** a novel mutation in *SRY*, found in a 46,XY DSD patient, is described, of which the functional analysis on trans-activational activity and nuclear import are described. Occurrence of delayed progressive kidney failure in the patient triggered analysis of the *WT1* gene, showing a pathogenic *WT1* intron 9 splice-site mutation next to the mutation in *SRY*. The consequences of the *SRY* and *WT1* mutations are discussed.

Chromosomal DSD consists, amongst others, of patients with a mosaic (45,X/46,XY and 46,XX/46,XY) chromosomal constitution, and these patients have an elevated GCT risk due to presence of Y-chromosomal material. As described, SRY plays a critical role in male development and mutations in the gene can cause gonadal dysgenesis. Interestingly, *SRY* mutations have also been reported in a minority of DSD patients with a mosaic karyotype. These have been identified by conventional methods. To further elucidate the

occurrence and possible role of *SRY* mutations in patients with mosaic chromosomal DSD, presence of variations within the gene was investigated by a sensitive next generation (deep) sequencing approach in a series of fourteen patients, described in **Chapter 8**.

Activating mutations in *c-KIT*, being the stem cell factor receptor, are found in a substantial percentage of testicular seminomas. It is known that in gastro-intestinal stromal tumors next to mutations in *c-KIT* also mutations in *PDGFRA* play a role, and that they are mutually exclusive. Malignant ovarian dysgerminomas morphologically and genetically resemble testicular seminomas and a similar pattern of activating *c-KIT* mutations is found. Although suggested, it is not clear if activating mutations in *c-KIT* play a substantial role in the development of GB and dysgerminoma in DSD patients. In **Chapter 9** a series of sixteen DSD patients showing GB and/or dysgerminoma and fifteen patients with ovarian dysgerminomas was investigated for activating *c-KIT* and *PDGFRA* mutations by meltingcurve analysis using specific probes, together with conventional sequence analysis. The results are linked with karyotype, histology of the gonads, expression of TSPY and c-KIT found in the tumors and precursor lesion.

In **Chapter 10** the results and observations made in the different chapters are discussed and integrated into current knowledge of DSD and GCC development.

Chapter 3

New insights into type II germ cell tumor pathogenesis based on studies of patients with various forms of disorders of sex development (DSD)

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Chapter 3

Abstract

Disorders of sex development (DSD), previously known as intersex, refer to congenital conditions in which development of chromosomal, gonadal, or anatomical sex is atypical. Patients with specific variants of this disorder have an elevated risk for the development of so-called type II germ cell cancers, i.e., the seminomatous and nonseminatous tumors, referred to as germ cell tumors (GCTs). Specifically DSD patients with gonadal dysgenesis or hypovirilization are at risk. A prerequisite for type II GCT formation is the presence of a specific part of the Y-chromosome (referred to as the GBY region), with the *TSPY* gene being the most likely candidate. Also the octamer binding transcription factor OCT3/4 is consistently expressed in all type II GCTs with pluripotent potential, as well as in the precursor lesions carcinoma *in situ* (CIS) in case of a testis and gonadoblastoma (GB) in the DSD gonad. The actual risk for malignant transformation in individual DSD patients is hard to predict, because of confusing terminology referring to the different forms of DSD, and unclear criteria for identification of the presence of malignant germ cells, especially in young patients. This is specifically due to the phenomenon of delay of germ cell maturation, which might result in over diagnosis. This review will give novel insight into the pathogenesis of the type II GCTs through the study of patients with various forms of DSD for which the underlying molecular defect is known. To allow optimal understanding of the pathogenesis of this type of cancers, first normal gonadal development, especially regarding the germ cell lineage, will be discussed, after which type II GCTs will be introduced. Subsequently, the relationship between type II GCTs and DSD will be described, resulting in a number of new insights into the development of the precursor lesions of these tumors.

Introduction

Developmental processes are a key to the complexity of multi-cellular organisms, including mammals. Studies showing how mistakes in developmental programming can lead to diseases, including tumors (benign and malignant), may be instrumental to understand the fundamental basis of the pathogenetic process(es) involved. Based on this knowledge, new approaches can be developed to prevent initiation of the mal-development and/or deal with the disease once it is formed. Human germ cell tumors (GCTs, see Table 1 for complete list of abbreviations), especially those we refer to as type II GCTs (i.e., the seminomatous and nonseminomatous tumors), are unique, because they hold the capacity to develop into any cell type of the body, as well as extra-embryonic lineages [1-2]. In addition, it was recently demonstrated that they can also generate the germ cell lineage [3]. In other words, type II GCTs are really totipotent, which is likely related to their cell of origin, the primordial germ cell (PGC)/gonocyte. Therefore, in our view, investigation of these malignancies will shed light on regulation of processes like proliferation and differentiation, leading to normal or aberrant embryogenesis. A comprehensive study of these tumors, and specifically a detailed comparison with normal germ cell development as well as embryogenesis will allow elucidation of the relevant pathogenetic mechanisms leading to malignancy. Vice versa, such an experimental set up will lead to new insight into

Table 1. List of abbreviations

AMH	Anti-Mullerian Hormone
AP	Alkaline phosphatase
AR	Androgen receptor
CAIS	Complete androgen insensitivity syndrome
CIS	Carcinoma *in situ*
c-KIT	Stem cell factor receptor
DAX1	Dosage-sensitive sex reversal/adrenal hypoplasia congenita critical region on the X chromosome
DMRT1	Double sex and mab-3 related transcription factor 1
DSD	Disorders of sex development
EC	Embryonal carcinoma
ES cells	Embryonic stem cells
FOXL2	Forkhead box L2
GB	Gonadoblastoma
GBY	Gonadoblastoma locus on the Y chromosome
GCT	Germ cell tumor
OCT3/4	Octamer binding transcription factor 3/4
PAIS	Partial androgen insensitivity syndrome
PGC	Primordial germ cell
PLAP	Placental/germ cell alkaline phosphatase
RA	Retinoic acid
SCF	Stem cell factor
SOX9	SRY (sex determining region Y)-box 9 (campomelic dysplasia, autosomal sex reversal)
SRY	Sex determining region on Y
TDS	Testicular dysgenesis syndrome
TGCT	Testicular germ cell tumor
TGF-beta1	Transforming growth factor beta 1
TSPY	Testis specific protein-Y encoded
UGT	Undifferentiated gonadal tissue
WNT4	Wingless-type MMTV integration site family, member 4
WT1	Wilms tumor 1

normal development. A selected number of these aspects will be discussed in this review, particularly regarding information obtained from multidisciplinary studies of patients with disorders of sex development (DSD), previously referred to as intersex. Moreover, a number of likely informative lines for future studies in this context will be proposed.

Normal germ cell development before entrance into the gonad

The embryonic stem cells that will form the germ cell lineage (i.e., resulting in mature germ cells, spermatozoa in males and oocytes in females), also called primordial germ cells, are first recognised as a separate entity (based on various markers like alkaline phosphatase (AP) and OCT3/4 (also known as POU5F1)) in the mouse at day 6.5 post coitum (weeks 5-6 gestational age in humans) [4]. At this time, these cells separate from the proximal epiblast and start to migrate through the hindgut to the genital ridge. During migration these cells undergo extensive rounds of proliferation. Amongst others, the stem cell factor (SCF) – c-KIT pathway is crucial in this process [5]. PGCs are positive for the receptor, and the SCF will function as a chemo-attractant, leading the cells to the genital ridge, their final destination. This signalling pathway has various functions, including survival by preventing induction of apoptosis [6]. Interestingly, disturbances in this signalling pathway result in various anomalies, including abnormal migration of PGCs, possibly leading to infertility. The absence of SCF in extragonadal sites induces apoptosis of germ cells that have migrated to non-gonadal sites [7]. Besides migration, PGCs undergo a process highly specific for the germ cell lineage, called epigenetic reprogramming [8]. This is a requirement to allow these cells to transfer capacity of pluripotency to the next generation. Important to remember in this context is the fact that these cells are themselves not pluripotent, but they are able to transfer pluripotency to the next generation, which is crucial for the continuation of the species. Pluripotency is, amongst others, related to specific activity of a number of genes depending on their parental origin, known as genomic imprinting [9]. The status of genomic imprinting, e.g. biparental, erased and finally uniparental, is reflecting the germ cell maturation status as well as sex lineage (paternal or maternal). PGCs that arrive at the genital ridge are called gonocytes, although they can not be distinguished from PGCs based on morphology or on marker expression. Gonocytes have a status of erased genomic imprinting, i.e. they have lost their parental specificity of imprinted genes, related to a demethylated genome [8].

Normal male versus female development

In the process of male or female sex formation, different levels can be distinguished, which are consecutive and determinant in the final outcome. The process starts with the chromosomal sex, in mammals due to either the constitution of XY (male) or XX (female). In non-mammalian organisms various other sex-determining systems are functional, which will not be discussed here, because they have been extensively presented elsewhere [10]. The chromosomal sex normally determines gonadal sex, i.e., either testis or ovary. The decision about the final sex determination of an individual (male or female) is primarily made at the gonadal ridge when the germ cells have entered after their migration from the proximal epiblast. In normal developing males, this is due to induction of expression of a specific gene, *SRY* (also known as testis determining factor), expressed in the stromal cells of the gonad [11], for review. This gene is encoded on the pseudo-autosomal region of the short arm of the Y chromosome (Yp). If sufficient amounts of SRY are formed in the proper cells at the right time, amongst others depending on the stabilizing effect of a specific splice variant of the *WT1* gene (+KTS) [12], this results in induction of expression of the transcription factor *SOX9*. Stable SOX9 expression in turn leads to a cascade of signal transduction ending in differentiation of the stromal cells into Sertoli cells [13]. Subsequently, the next step in the process of sex differentiation, i.e. the phenotypic sex [14]), for review, is initiated, depending on the development of functional Sertoli cells, and later on Leydig cells, in males, and their counterparts granulosa – and theca cells, in females. Processes beyond sex determination will not be discussed in this review, although a number of relevant mechanisms have been identified which are involved in male development. These include the delay of meiosis initiation by retinoic acid (RA) involving pathways [15-16], resolution of Mullerian structures after expression of anti-Mullerian hormone (AMH, also called Mullerian inhibiting substance: MIS) by Sertoli cells [17], and the descent of the testicles into the scrotum [18] for instance. In the absence of proper SRY expression, either by absence of the gene (in case of a deletion), or by inactivating mutations, the initial cascade does not occur. Therefore, stromal cells will not differentiate into Sertoli cells, but will embark on the female pathway. In other words, these stromal cells are bipotential in origin, and are able to generate either Sertoli cells (during initiation of male development) or granulosa cells (during initiation of female development). In the absence of Sertoli cell formation, the female differentiation pathway will be followed and a phenotypic female will eventually develop. However, it has become clear that the female developmental pathway is

not a default system in itself, as was originally thought. It requires the activation of a number of genes, including *FOXL2* and *WNT4*. Double knockout mice for these two genes result in testis development in spite of a XX sex chromosomal constitution [19]. These insights into normal sex determination must be kept in mind when searching for explanations for the various forms of DSD (see below).

Based on normal male and female development, the pattern of genomic imprinting of the germ cells will be determined; the erased PGCs will gain a fully maternal pattern of genomic imprinting in an ovarian environment, and a paternal pattern in a testicular environment [20]. In contrast to the female situation, which is characterized by the presence of meiotic oocytes from the post-partum time onwards, full spermatogenesis starting from spermatogonia is only initiated after puberty. For this process, the hormone testosterone is of crucial importance [21]. It is formed by Leydig cells in the testis, and acts via the androgen receptor (AR), which is present on Sertoli cells, but most likely not on cells belonging to the germ cell lineage [22]. It is important to realize that both oocytes and spermatogonia, during normal development lose their embryonic characteristics, including expression of AP, OCT3/4 and c-KIT.

Classification of human GCTs

Traditionally, GCTs are classified based on their histological composition. This results in the different variants of teratoma (somatic differentiation), yolk sac tumor and choriocarcinoma (extra-embryonic differentiation), and embryonal carcinoma (stem cell component), as well as seminomatous tumors (subdivided amongst others into classic and spermatocytic seminoma). The classical seminomas of the ovary are called dysgerminomas (as are germinomas of the brain, which will not be discussed here). However, this classification system does not take into account the underlying differences in the pathogenesis of these tumors, which complicates clinical treatment as well as identification of pathogenetic mechanisms. Therefore, we proposed an alternative classification system, in which five types of GCTs are identified, referred to as I-V [1-2]. These are summarized in Table 2. One of the parameters on which this classification is based is the pattern of genomic imprinting. For the purpose of this review, only the type II GCTs are of interest, and will be discussed in more detail below.

Testicular type II GCTs

Epidemiology and histology

The type II GCTs of the testis, named testicular germ cell tumors (TGCTs), account for up to 60% of all malignancies diagnosed in men between 20 to 40

years of age [23]. An annual increase in incidence of 3-6% in Caucasian populations has been observed in recent decades. About 50% of TGCTs are seminomas and 40% consist of nonseminomas, the rest are those containing both components, referred to as combined tumors. The median age of patients with seminoma is 10 years higher than that of patients with nonseminoma (35 versus 25 years), the combined tumors present at an intermediate age. Seminomas are homogenous tumors in which the cells resemble PGCs/gonocytes. The nonseminomas can be composed of different elements: embryonal carcinoma (stem cell component), the somatically differentiated teratoma component, yolk sac tumor and choriocarcinoma which represent the extra-embryonic differentiation component. Embryonal carcinoma cells can differentiate into the other histologies and are in fact the pluripotent stem cells of nonseminomas [24].

Table 2. The five types of germ cell tumors

Type	Anatomical site	Phenotype	Age	Originating cell	Genomic imprinting	Genotype	Animal model
I	Testis/ovary/sacral region/retroperitoneum/ mediastinum/neck/midline brain/other rare sites	(Immature) teratoma/yolk sac tumor	Neonates and children	Early PGC/ gonocyte	Biparental partially erased	Diploid (teratoma) Aneuploid (yolk-sac tumor): gain of 1q, 12(p13), and 20q, loss of 1p, 4, and 6q	Mouse
II	Testis	Seminoma/ non-seminoma	> 15 y (median:35 and 25 y)	PGC/ gonocyte	Erased	Aneuploid (+/- triploid: gain of X, 7, 8, 12p and 21, loss of Y, 1p, 11, 13 and 18	Not available
	Ovary	Dysgerminoma/ non-seminoma	> 4 y	PGC/ gonocyte	Erased	Aneuploid/tetraploid	Not available
	Dysgenetic gonad	Dysgerminoma/ non-seminoma	Congenital	PGC/ gonocyte	Erased	Diploid/tetraploid	Not available
	Anterior mediastinum (thymus)	Germinoma/ non-seminoma	Adolescents	PGC/ gonocyte	Erased	Diploid/tri-tetraploid	Not available
	Midline brain (pineal gland/hypothalamus)	Germinoma/ non-seminoma	Children (median age 13 y)	PGC/ gonocyte	Erased	Diploid/tri-tetraploid	Not available
III	Testis	Spermatocytic seminoma	> 50 y	Spermatogonium/ spermatocyte	Partially complete paternal	Aneuploid: gain of 9	Canine seminoma
IV	Ovary	Dermoid cyst	Children/ adults	Oogonia/ oocyte	Partially complete maternal	(Near) diploid, diploid/tetraploid, peritriploid (gain of X, 7, 12 and 15)	Mouse gynogenote
V	Placenta/uterus	Hydatiform mole	Fertile period	Empty ovum/ spermatozoa	Completely paternal	Diploid (XX and XY)	Mouse androgenote

y: years; PGC: primordial germ cell

Precursor lesion of the testis

The precursor lesion for all TGCTs is the so called carcinoma *in situ* (CIS) [25], sometimes referred to as intratubular germ cell neoplasia unclassified [26] and testicular intraepithelial neoplasia [27]. CIS cells are located at the basement membrane of the seminiferous tubules in close connection with the Sertoli cells in adult testis, and are often present in the adjacent parenchyma of invasive TGCTs [28-29]. In the male Caucasian population the incidence of CIS is similar to the lifetime risk of developing a TGCT and all patients with this lesion will eventually develop an invasive TGCT. CIS, as seminoma (see above), represents

the malignant counterpart of an embryonic germ cell, most likely a PGC/gonocyte. This is supported by immunohistochemical characteristics like expression of PLAP, c-KIT and OCT3/4 [30-31], CIS cells also phenotypically and ultrastructurally resemble PGCs [32], and epidemiological data suggest that the initiating event of TGCTs occurs during the fetal period [33]. Moreover, both CIS cells and PGCs show an erased pattern of genomic imprinting [34], and telomerase activity is present [35]. The nature of the risk factors for TGCTs, familial predisposition, a previous TGCT, cryptorchidism, infertility and various forms of DSD [36], further support the model that the initiating step in the pathogenesis of this cancer occurs during embryonal development. CIS, present in the adjacent parenchyma of invasive TGCTs, has the same ploidy as seminoma [37]. The chromosomal constitution of CIS cells shows an overall similar pattern of gains and losses as are present in the invasive tumors, although in CIS loss of parts of chromosomes 4 and 13, and gain of 2p are more frequently found. Most importantly gain of 12p is not consistently found in CIS, which indicates that over-representation of 12p is established during progression from pre-invasive to invasive behaviour [38-39]. The idea that genes on 12p are involved in Sertoli cell independent/invasive growth of the tumor cells is tempting. Of interest in this context is the finding that genes on 12p can be up regulated by TSPY (see below) [40]. Moreover, human embryonic stem (ES) cell lines show gain of chromosome 12 (and 12p) during continuous (semi-solid) *in vitro* culturing [41], which underlines the survival/growth advantage of pluripotent cells with over-representation of this genomic fragment. Interestingly, this could be related to the idea that gain of 12p is supportive for seminoma-like cells to survive outside their niche created by the Sertoli cells.

Testicular dysgenesis syndrome

The testicular dysgenesis syndrome (TDS) hypothesis [42], proposes that germ cells not properly nourished by Sertoli cells during fetal gonadogenesis undergo a delay in there maturation, resulting in an increased risk for subfertility and GCT formation. This model suggests that in various apparently unrelated conditions, such as certain conditions caused by chromosomal aberrations (*e.g.*, trisomy 21), the exposure of the male fetus to xeno-estrogens and anti-androgens, and DSD, the developmental delay of germ cells is the common underlying mechanism.

OCT3/4, biology and diagnostic marker

The octamer binding transcription factor OCT3/4 is consistently and specifically expressed in all GCTs with pluripotent potential, as well as in the neoplastic precursor lesions CIS in testis and gonadoblastoma (GB) in the DSD gonad [43-45]. OCT3/4 is highly expressed during the earliest stages of embryogenesis and in ES cells. In the early embryo after the blastocyst stage OCT3/4 is quickly repressed and becomes confined to the germ cell lineage [46-47]. Loss of OCT3/4 expression in PGCs leads to apoptosis [48], hence it is hypothesized that OCT3/4 is required for survival of PGCs. The level of expression of OCT3/4 in ES cell-derived tumors in mice is highly correlated with the formation and aggressive properties of these tumors [49]. These data suggests that aberrant expression of OCT3/4 in GCTs might be relevant to the development and oncogenic potential of these tumors. Besides the proven expression of OCT3/4 in ES cells, embryonal carcinoma (EC) cells and PGCs, an increasing number of articles have recently been published reporting OCT3/4 expression in normal adult tissues, various tumors and cell lines. This interest in OCT3/4 has been triggered by the concept of adult stem cells. However, multiple pseudogenes for OCT3/4 exist and all studies so far have used non-specific primers that detect transcripts from both the OCT3/4 gene as well as from pseudogenes [50-52]. Recently a specific primer set for OCT3/4 was developed [53]. Claims of OCT3/4 expression in somatic cells have to be interpreted with care, since from studies of murine embryogenesis it is known that at 8.5 dpc only the migrating PGCs are positive for OCT3/4, and PGCs that fail to reach the genital ridge go into apoptosis. There are no indications that there remain OCT3/4 positive cells in differentiating tissues during human embryogenesis. Moreover, recently it was shown that OCT3/4 does not play a role in mouse somatic stem cell renewal [54-55].

Type II GCTs and DSD

GB arises in the dysgenetic gonads of DSD patients with a part of the Y chromosome. In 1987 Page postulated the hypothesis that a gene on the Y chromosome (gonadoblastoma locus on the Y chromosome, GBY) may act as an oncogene in the context of a dysgenetic gonad [56]. Later the GBY susceptibility region was sub-localized to a region around the centromere of the Y chromosome. The most likely candidate gene in this region is TSPY. The TSPY protein is expressed in spermatogonia of the adult male, and is thought to be related to their mitotic proliferation [57]. In the fetal gonad TSPY protein is expressed at a constant level [31]. However TSPY protein becomes more

abundant compared to both normal embryonic and adult testis, in CIS, GB and sporadically in seminoma [58]. TSPY is homologous to SET/NAP proteins, which play a role in nucleosome assembly and chromatin remodelling [59]. Next to some pseudogenes, also a functional X chromosomal homolog of TSPY exists in humans (called TSPY-like 2 or TSPX, DENTT, hCINAP, CDA1, se20-4), which, in contrast to TSPY, is widely expressed and plays a role in growth arrest, TGFbeta1 signalling and regulates growth and differentiation of the intracellular parasite toxoplasma gondii [60-62]. One of the TSPY pseudogenes has been found to be mutated in a family with (among others) male to female sex reversal [63]. TSPY has been shown to influence expression of genes on 12p (see above) [40], and genes associated to cell cycling when transfected into HELA cells or NIH3T3 [64]. It can be hypothesized that germ cells residing in an unfavourable environment, like in the case of TDS and DSD, can survive and proliferate due to prolonged expression of OCT3/4 and increased expression of TSPY.

Disturbances in male versus female development, DSD

The physiological process of male and female development can be mis-regulated at different levels. A summary of the normal process is summarized in Figure 1A. Figure 1B, shows an overview of the currently characterized mutations and chromosomal abnormalities that are known to effect GCT risk and are which associated with DSD. This figure is not meant to represent the full spectrum of disturbances related to gonadal development, but offers some well defined examples. Previously DSD disorders were referred to as intersex. As indicated, DSD is defined as congenital conditions in which development of chromosomal, gonadal, or anatomical sex is atypical. Many different causes may lead to DSD, varying from mutations in genes encoding proteins that play a role in the different developmental programs and cascades (like SRY, but also the AR for instance), chromosomal imbalances (of sex chromosomes in Turner syndrome (45,X0), and various forms of mosaicisms) and, in other organisms than man at least, environmental influences. A consensus meeting was held in Chicago in 2005, in which it was decided that all patients with DSD will be classified based on the chromosomal constitution (i.e., karyotyping) of peripheral leukocytes. The classification system is summarized in Table 3. Although this system, like every other system, has limitations, it at least gives clarity regarding a number of issues, and prevents misunderstanding due to different terminologies used.

Table 3. Proposed new nomenclature

Previous: Intersex	Proposed: disorders of sex development (DSD)
Male pseudohermaphrodite Undervirilization of an XY male Undermasculinization of an XY male	46, XY DSD
Female pseudohermaphrodite Overvirilization of an XX female Masculinization of an XX female	46, XX DSD
Turner syndrome (45,X and variants) Klinefelter syndrome (47,XXY and variants) Mixed gonadal dysgenesis (45,X/46,XY)	Sex chromosome DSD
True hermaphrodite	Ovotesticular DSD
XX male or XX sex reversal	46, XX testicular DSD
XY sex reversal	46, XY complete gonadal dysgenesis

For some of the disorders, like 46,XX males, in which the *SRY* gene is translocated to one of the X chromosomes, the mechanism of disturbance is easily explained. Another example is 46,XY females, in which no functional SRY is present due to a deletion or an inactivating mutation [65]. Other examples are: 9p-deletions (haplo-insufficiency of *DMRT1*) [66] and *DAX-1* duplication in 46,XY females, which results in suppression of the SRY pathway [67]. However, the mechanistic basis for most of the variants of DSD remains unexplained. This is further complicated by the fact that a single genetic abnormality can give a heterogeneous phenotype. This is for example found in Frasier syndrome patients. These patients have the characteristic splice mutation in intron 9 of the *WT1* gene, which results in less formation of the +KTS form [68], leading to less SRY protein, and a diminished induction of SOX9 [12]. Most of these patients are 46,XY sex reversed females (with GB, see below), however phenotypical males with testis (and CIS, see below) have been reported as well.

A heterogeneous pattern (see Figure 1B) is also found in patients with a mosaic constitution of their sex chromosomes, which theoretically might be easily explained. However, recent findings indicate that the exact basis for this heterogeneity of either ovarian or testis development is not elucidated, and is not simply due to the predominant presence of the Y chromosome containing cells in the testicular area. A correlation between testicular differentiation and the percentage of Y chromosome positive cells was suggested in XX <-> XY chimeric mouse models, in which it was found that if the gonad contains less than 30% Y-positive cells, it develops as an ovary [69]. At first this model

seemed to be confirmed in humans [70]. However a study on a larger sample series done by us reveals that there is no direct correlation between the gonadal differentiation pattern and the presence of the Y chromosome [71].

It seems that in mosaic patients the timing, presence of a threshold level, and sufficient density of the SRY signal to activate the downstream cascade (especially the expression of SOX9) is important in determining the fate of the bipotential gonad [11, 72]. The possible role of prostaglandin in this process is under investigation.

Germ cell tumor development in patients with various forms of DSD

Besides cryptorchidism, familial predisposition, in(sub)fertility and birth weight, DSD is also a significant risk factor for type II GCTs. This is relevant for a number of reasons. For sure, it allows identification of selected groups of individuals for early diagnosis, but moreover, it also might be helpful to elucidate the underlying pathogenetic mechanisms. Specific variants of DSD (especially in patients with gonadal dysgenesis and hypovirilization) are related to an increased risk for type II GCTs (reviewed in [73]. Gonadal dysgenesis can be defined as an incomplete or defective formation of the gonads, as a result of a disturbed process of the migration of the germ cells and/or their correct organization in the fetal gonadal ridge. Hypovirilization is caused by errors in testosterone biosynthesis, by testicular unresponsiveness to stimulation by the pituitary, or by defects in androgen dependent target tissues [74]. Based on careful literature searches, a number of conclusions can be drawn regarding the risk of type II GCTs in DSD patients. These include the significantly lower risk for GCTs in patients with complete androgen insensitivity syndrome (CAIS) compared to partial androgen insensitivity syndrome (PAIS). This is likely due to apoptotic cell death of germ cells in CAIS patients [75]. The risk of cancer in the PAIS patients is influenced by the anatomical localization of the gonad, being the highest in abdominal sites, and the lowest in scrotal localization. Patients with ovo-testicular DSD, of which the gonads mostly consist of well-differentiated ovarian and testicular tissue, have a low risk for type II GCTs. Data regarding the prevalence of GCTs in patients with 17β-HSD deficiency, Leydig cell hypoplasia, and specific gene mutations is very limited or absent. The presence of the GBY region, possibly to be substituted by the *TSPY* gene and/or protein (see above), in the gonads should always be ruled out in the presence of dysgenetic testes in 46,XX males or patients with 45,X/46,XYp- or Yq mosaicism, this in light of its suspected role in the development of GCTs (see above).

The use of a uniform classification system of the various forms of DSD will hopefully shed light on the actual risk for malignant transformation of germ cells in the different DSD subgroups, which might result in a more conservative approach of gonadectomy in some patients. The benefits may include physiological induction of puberty and even fertility. To allow such a change in clinical decision-making, it is needed to characterize the germ cells in DSD patients in detail, to determine their stage of maturation and characteristics of malignant transformation. Such a screen requires a large series of well-described patients with the various forms of DSD. Because of the rareness of DSD in the general population, international collaborative studies, including pathology review of the gonads if available, using well described criteria, will be needed to acquire the numbers of patients allowing investigations of sufficient power.

Possible overdiagnosis of malignant germ cells due to delay of maturation

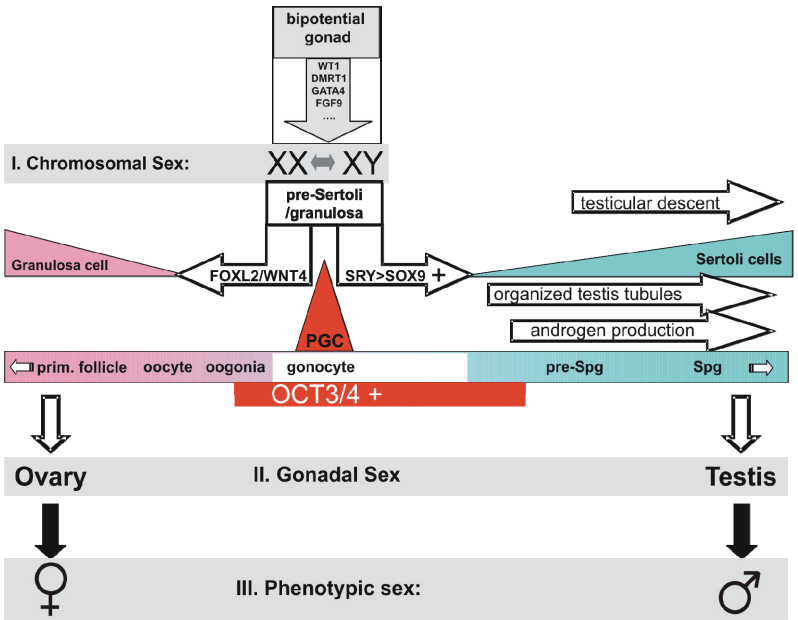
One of the difficulties in the diagnosis of malignant germ cells is that the diagnostic markers used are normally present in embryonic germ cells. This would not have been problematic if germ cells would normally mature in the gonads of patients with DSD. However, this is not always the case, especially not in patients with hypovirilization and gonadal dysgenesis. In these patients, germ cells may undergo delayed maturation, which results in a more prolonged expression of embryonic markers. This may result in overdiagnosis of malignant cells.

Overall this is a problem in young children. This notion was supported by studies of embryonic gonads of patients with trisomy 21, amongst others [76]. Specifically delay of germ cell maturation was found in very young male patients, whereas in older patients, germ cells had lost embryonic marker expression. Careful analysis of undervirilization patients identified a number of criteria that can be used to define malignant germ cells more precisely. These include the location of the OCT3/4 positive germ cells, either at the membrane or central in the seminiferous tubule, and the distribution through the testis [77]. However, identification of a marker that is absolutely specific for malignant germ cells would be very helpful for diagnostic purposes

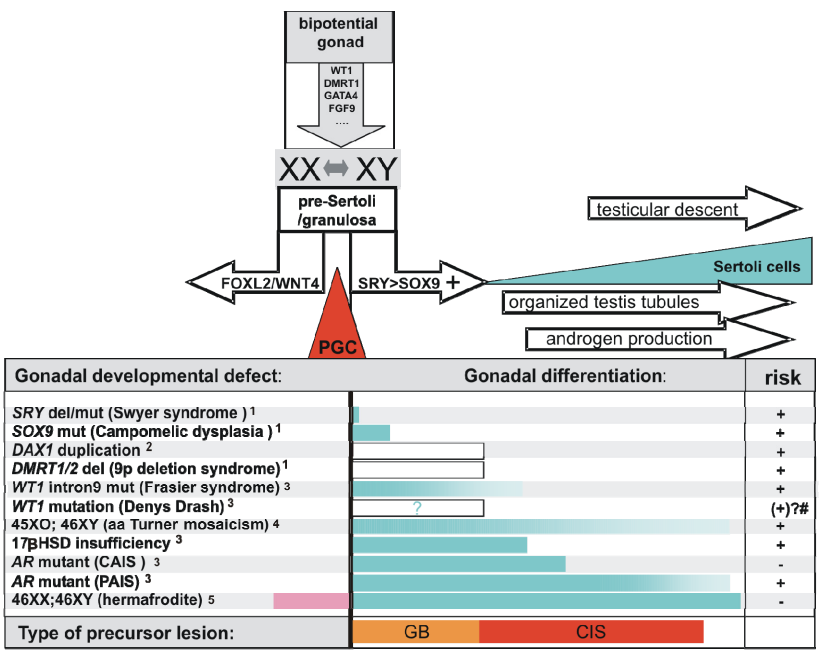
New insight into the precursor of type II GCTs in DSD patients

Although information on the occurrence of type II GCTs in patients with DSD is still limited, careful analysis of published results is revealing novel insights.

A



B



= no Denys Drash *WT1* mutant patient found in literature with GB, although this has been reported for non-specified Denys Drash patients

- = 0.5-5%, + >10%
Average risk normal: 0.01%

DSD classification; 1: 46, XY (complete) gonadal dysgenesis, 2: 46, XX testicular DSD, 3: 46, XY DSD, 4: sex chromosome DSD, 5: ovotesticular DSD

male female unknown; GB = gonadoblastoma; CIS = carcinoma *in situ*

Figure 1. Overview of normal development, level of virilization in DSD patients, precursor lesions and germ cell tumor (GCT) risk. (A) Overview of normal male and female development. The chromosomal sex (XY or XX) determines whether the bipotential gonad (formed under the influence of products of different genes, e.g. WT1, DMRT1) will eventually develop into either a testis (via the SRY/SOX9 pathway) or an ovary (via FOXL2/WNT4). This is predominantly determined by the formation of Sertoli – and granulosa cells, respectively. Initially the earliest developmental stages of the germ cells (PGCs/gonocytes) are positive for OCT3/4. Note that upon physiological maturation of the germ cells, both in the male and female direction, expression of OCT3/4 is lost. The final development of the gonadal sex (testis or ovary) determines the phenotypic male or female characteristics. (B) Relationship between gonadal developmental defect, level of virilization, risk of gonadoblastoma (GB) and/or carcinoma *in situ* (CIS) formation and GCT risk. The upper panel again represents normal gonadal development (especially in the male direction). The lower panel summarizes different gonadal developmental defects (with an identified genetic basis) related to gonadal differentiation, i.e. level of virilization. In addition, the risk for development of a type II GCT is indicated in the most left column. Note that depending on the level of virilization the precursor lesion presents itself as either GB or CIS

Such a screen of published data has been performed, of which the most important findings are represented in Figure 1B. The extent to which normal male developmental characteristics have been found in DSD patient gonads is based on patients for whom the exact mutation is known. The corresponding clinical syndromes of such patients are indicated to allow a link to previous publications using previous classification. However, when possible, this information was translated to the new DSD classification system. Again, Figure 1B is not representing a complete picture, but is meant to unravel a number of intriguing correlations between the underlying genetic defect and the risk for the development and type of precursor of type II GCTs. Moreover, because of the incompleteness of understanding the mechanism of sex determination, the presented scheme is, by definition, a simplification. The heterogeneity of the histological composition of the gonads in the various forms of DSD is visualized by bars.

When Figure 1B is analyzed in detail, a number of relevant conclusions can be drawn regarding the precursor lesion. These include: (I) a high risk of GB is found when sex determination is disrupted in an early stage of Sertoli cell differentiation (due to abnormalities in *SRY*, *SOX9*, *WT1* intron 9). It must be remembered that GB can only be formed in the presence of the GBY region of the Y chromosome. Early Sertoli cell development is also disturbed patients with 45,XO;46,XY mosaicism, who also carry a high risk to develop GB as precursor lesion The same is true for patients with 9p deletions, likely related to the loss of *DMRT1*. In conclusion, GB is found in patients that lack a certain level of Sertoli cell development. Careful histological analysis of gonadal tissue of DSD patients revealed that undifferentiated gonadal tissue (UGT) is the most likely precursor stage of GB [78]. Identification of this stage allows better diagnosis and further elucidation of the pathogenetic pathway(s); (II) defects

occurring later in gonadal development, like for instance 17β-HSD insufficiency and AR mutants (predominantly PAIS), results in enhanced risk of CIS as precursor, as can be found in males without any form of DSD as defined in this review (e.g. in simple cryptorchidism), albeit with a much lower incidence.

Continuum between testicular CIS-undifferentiated gonadal tissue-GB-invasive type II GCTs

It has been indicated that GB and CIS are fundamentally different [79-80]. We will summarize a number of arguments to support the model that this is not the case, and that GB and CIS are in fact a continuum, of which the phenotypic presentation is determined by the micro-environment, i.e., the level of virilization.

The similar types of invasive GCTs that can progress from it demonstrate the pathogenetic link between GB and CIS. In fact, all variants of invasive type II GCTs can (next to CIS) also be found associated with GB. That dysgerminomas (associated with GB) and seminomas (associated with CIS) are highly similar was recently shown using mRNA and microRNA expression profiling [81-82]. This is in line with previous observations that these tumors are similar with regard to the expression of immunohistochemical markers, e.g. OCT3/4 (POU5F1), c-KIT, and alkaline phosphatase, as well as chromosomal constitution (i.e., gain of 12p). These observations support the hypothesis that these tumors are in fact the same, which might be better represented by a modified nomenclature. The fact that DSD gonads predominantly contain dysgerminoma instead of the various nonseminomas is likely related to the anatomical localization of the gonad, and not due to a difference between GB and CIS. This is supported by the observation that intra-abdominal testes also more frequently develop seminoma than the various forms of nonseminoma [83] .

An interesting question is why a GB is formed in some patients and CIS in others. We hypothesize that this is due to the specific micro-environment, especially the absence of functional Sertoli cells, leading to a female development. In other words, CIS can only be formed at a certain level of testicular development. Thus, GB and CIS are simply two variants of the same defect. If this is true, it is likely that a pathologist because of the absence of obvious Sertoli cell differentiation will classify a lesion as a GB, and that the same lesion will be classified as CIS in a context where Sertoli cells are identified. This is indeed the case. The criteria used by a pathologist to classify a

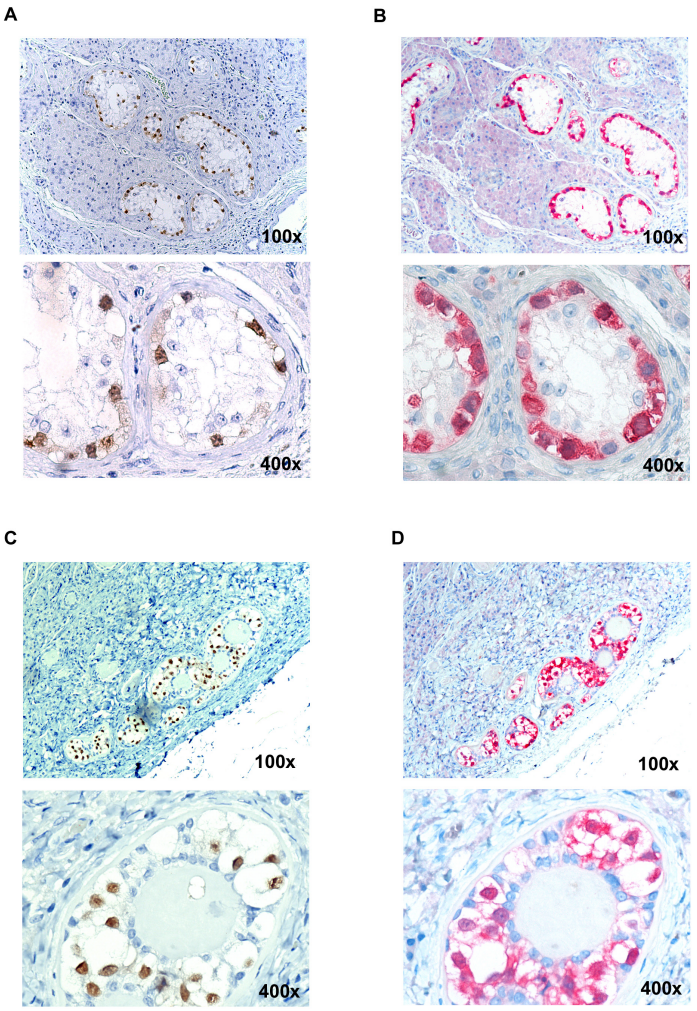


Figure 2. OCT3/4 and TSPY staining in CIS and GB occurring within a single DSD gonad. 46, XY DSD (Frasier syndrome) patient presenting with both CIS and GB. (A) OCT3/4 staining in CIS. (B) TSPY staining in same CIS tubule. (C) OCT3/4 staining in GB. (D) TSPY staining in same GB region. Magnification, 100x upper and 400x lower panel, for all (OCT3/4 staining = brown; TSPY staining = red). Slides are counterstained with hematoxilin.

GB are based on phenotypical environmental characteristics. A mixture of immature germ cells and stromal cells is diagnosed as GB, when found in the context of ovarian stroma. This is nicely illustrated in a Frasier syndrome patient diagnosed in our institute. Most Frasier patients (characterized by an intron 9 *WT1* mutation), as discussed above, are sex reversed 46,XY females with streak gonads eventually developing GB [84]. However, they can also present as males with ambiguous genitalia in which a CIS lesion has developed

[85]. In addition, we found that even CIS and GB can occur within a single gonad of such a patient (see Figure 2). Based on these and similar observations, and independently reported in literature [86], we conclude that indeed a continuum exists between GB and CIS.

However, there may be differences in DNA ploidy of GB and dysgerminoma versus CIS and seminoma (the latter two being consistently polyploid) [87]. This needs further investigation. Hence, it would be of interest to investigate the genetic defects and phenotypical characteristics of the gonad in which both CIS and GB is identified.

Future implications and studies to be initiated

An important question that remains to be answered is which forces direct the evolution from developmental delay towards tumor development? Which factors determine the survival of germ cells with embryonic characteristics in the DSD gonad? If CIS and GB represent indeed the same entity, but in a different environment, than what do they have in common that may explain the high tumor risk? How does the lack of Sertoli cell development play a role in the malignant transformation of the germ cells? Prevention of meiosis is likely essential for type II GCT development in the context of both female and male development. What are the crucial factors involved in maintaining the germ cells in an undifferentiated and possibly proliferative state and (how) can we manipulate them? Are there similarities between the effects of endocrine disruptors in the TDS that Skakkebaek proposed and in DSD in such a way that it could have implications for a much larger population than the patients with gonadal dysgenesis?

Take home message

In conclusion it can be said that the comparison of normal and aberrant development associated with type II GCTs leads to new insights in the mechanisms involved in tumor formation. The aberrant expression of normal developmental markers in these tumors and in DSD gonads, provides an insight into arrested development, which otherwise would be difficult to gain. However, it should be kept in mind that tumors may differ from normal development and such hypotheses need to be checked in the normal development of the gonads.

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Chapter 4

FOXL2 and SOX9 as parameters of female and male gonadal differentiation in patients with various forms of disorders of sex development (DSD)

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Abstract

The transcription factors SOX9 and FOXL2 are required for male and female mammalian gonadal development. We have used specific antibodies to investigate the role of these key proteins in disorders of sex development (DSD), specifically intersex states. In normal gonads SOX9 was found to be restricted to presence of (pre-)Sertoli cells, while FOXL2 was found in granulosa cells, and in stromal cells interpreted as early ovarian stroma. Both proteins were found within a single patient, when testicular and ovarian development was present; and within the same gonad, when both differentiation lineages were identified, like in ovotesticular-DSD (ie hermaphrodite). Especially SOX9 was informative to support the presence of early testicular development (ie seminiferous tubules), expected based on morphological criteria only. In a limited number of DSD cases, FOXL2 was found within reasonably well-developed seminiferous tubules, but double staining demonstrated that it was never strongly co-expressed with SOX9 in the same cell. All seminiferous tubules containing carcinoma *in situ* (CIS), the malignant counterpart of a primordial germ cell, ie the precursor of the type II germ cell tumours of the testis, seminomas and nonseminomas, showed the presence of SOX9 and not FOXL2. In contrast, gonadoblastomas (GB), the precursor of the same type of cancer, in a dysgenetic gonad, showed expression of FOXL2 and no, or only very low SOX9 expression. These findings indicate that gonadal differentiation, ie testicular or ovarian, determines the morphology of the precursor of type II germ cell tumours, CIS or GB respectively. We show that in DSD patients, the formation of either ovarian and/or testicular development can be visualized using FOXL2 and SOX9, expression, respectively. In addition, it initiates a novel way to study the role of the supportive cells in the development of either CIS or GB.

Introduction

The mechanism of female versus male development has been an intriguing subject for years. For a long period of time, it was dominated by the search for responsible genes. In 1959, the initial step of sex determination in mammals was elucidated by identification of the Y chromosome [1-2]. In fact, the chromosomal sex, as determined at the time of fertilization (XX or XY) was completely associated with final development of a female or male individual (ie phenotypic sex), respectively. Although the actual mechanism was unresolved, it was obvious that gonadal sex (development of either ovary or testis) was a crucial step in this process. The field changed significantly in 1990, when the SRY gene was identified as the testis determining factor [3]. Inactivation of SRY results, both in mice and men, in sex reversal [4-5]. In spite of a XY constitution, gonadal development and subsequent phenotype will be female. Later on, studies were initiated to further elucidate the SRY pathway, resulting in identification of SOX9 [6]. This transcription factor is an intermediate downstream target of SRY, and absolutely required for testis development. This is due to its function in formation and maintenance of (pre-) Sertoli cells, a critical step in further testis formation, and subsequent generation of the male phenotype [7-8]. Absence of SOX9 results in the same gonadal phenotype as is seen in the absence of functional SRY, but in addition leads to congenital abnormalities recognized as campomelic dysplasia [6].

In contrast to the male gonadal differentiation, it was initially believed that female development occurred by default, being simply the result of the absence of male development (ie SRY, SOX9 etc) [9-10]. However, it has been demonstrated recently that this is an oversimplification and that it is at least not a passive process. In fact, it requires activation of a number of genes, including WNT4 and FOXL2. This latter protein, like SOX9, is also a transcription factor, required for formation of granulosa cells, the female counterparts of Sertoli cells [11-12]. In other words, SOX9 and FOXL2 are the two earliest counteracting players in the differentiation of the bipotential precursor into either the male (Sertoli cell) or female (granulosa cell) pathway [13-15].

Under normal physiological conditions, the development of gonadal sex, ie formation of testicular or ovarian tissue, is highly separated in mammals and determined by the constitution of the sex chromosomes. Therefore, the phenotypical sex is unambiguous [16]. However, there are a number of pathological exceptions to this rule. Sometimes, both testicular and ovarian tissue can be formed, either in a single gonad, or in two different gonads [17-18]. This pathological condition has been referred to previously as hermaphroditism,

while according the current nomenclature it is classified as ovo-testicular disorder of sex development (DSD) [19]. In addition, a number of other variants of DSD are recognized, summarized in Table 1. DSD patients carry an increased risk to develop type II germ cell tumours. Most recently, a number of markers of the different maturation stages of the germ cells have been identified, which allows a straightforward identification of their maturity [20]. This has been highly informative for the diagnosis of both carcinoma *in situ* (CIS) and gonadoblastoma (GB) - the precursors of the type II malignant germ cell tumours, ie seminoma (dysgerminoma) and nonseminoma, in the testis and dysgenetic gonad, respectively. These include the transcription factor OCT3/4, also known as POU5F1, which is an absolute marker for CIS of the adult testis [21-23]. However, overdiagnosis must be excluded in dysgenetic gonads and in gonads at young age, because OCT3/4 is intrinsically positive in primordial germ cells and gonocytes. Delayed maturation, which is frequently found in DSD patients, will result in a positive staining of germ cells for OCT3/4 in most cases [24]. Up to now the maturation state of the surrounding tissue could only be determined based on morphological criteria, which makes it to some extent subjective.

Table 1. Proposed new nomenclature

Previous: Intersex	Proposed: disorders of sex development (DSD)
Male pseudohermaphrodite Undervirilization of an XY male Undermasculinization of an XY male	46, XY DSD
Female pseudohermaphrodite Overvirilization of an XX female Masculinization of an XX female	46, XX DSD
Turner syndrome (45,X and variants) Klinefelter syndrome (47,XXY and variants) Mixed gonadal dysgenesis (45,X/46,XY)	Sex chromosome DSD
True hermaphrodite	Ovotesticular DSD
XX male or XX sex reversal	46, XX testicular DSD
XY sex reversal	46, XY complete gonadal dysgenesis

The exact pathological relationship between CIS of the adult testis and GB of the dysgenetic gonad is unresolved so far. It is accepted that CIS exists in the testicular context, being in association with Sertoli cells [25]. However, for GB this is much less clear. Based on merely morphological criteria it has been suggested that the germ cells in GB are associated with supportive cells that show characteristics of granulosa cells [26]. Here we have investigated the presence of both SOX9 and FOXL2 in normally developed embryonic and adult gonads, as well as in gonads of patients with various forms of DSD. Both cases with and without CIS and GB are represented. We show that SOX9 is a highly informative marker for testicular development and FOXL2 for ovarian development, present in either an isolated or a mixed constitution. Moreover,

CIS was consistently found to be associated with Sertoli cells, being positive for SOX9, and GB with granulosa cells, being positive for FOXL2. Although SOX9 and FOXL2 could be present within a single histological context, it was never found to be expressed at high levels within the same cell. These observations demonstrate for the first time the additional value of immunohistochemistry for SOX9 and FOXL2, compared with morphology alone, in diagnosing the presence of either ovarian or testicular differentiation, or both, in patients with DSD. This allows an unbiased study of the role of the histological context in the formation of CIS and GB.

Materials and methods

Tissue samples

Thirteen male and female embryonic gonads and 31 adult testis, CIS, and ovary samples were tested. In total, 31 DSD patients were retrieved from our archives (16 DSD samples from Rotterdam and 15 DSD samples from Paris): ten sex chromosome DSD patients, 13 patients with 46,XY gonadal dysgenesis, four 46,XX testicular DSD and four ovotesticular DSD patients (Table 2). Use of tissues for scientific reasons was approved by an institutional review board. The samples were used according to the *Code for Proper Secondary Use of Human Tissue in The Netherlands*, as developed by the Dutch Federation of Medical Scientific Societies (version 2002).

Immunohistochemical staining

Immunohistochemistry was performed on paraffin-embedded tissue sections of 3 μm thickness. After deparaffinization and 5 min. incubation in 3% H_2O_2 to inactivate endogenous peroxidase, antigen retrieval was carried out by heating under pressure up to 1.2 bar in either 0.01M sodium citrate (pH6) in case of SOX9 staining or in 0.01M EGTA, 0.01M Tris (pH9) in case of FOXL2 staining. After blocking endogenous biotin using the avidin/biotin blocking kit (SP-2001, Vector Laboratories, Burlingame, CA USA), the sections were incubated for 2 h at room temperature, with either a polyclonal goat anti-human SOX9 antibody (AF3075; R&D Systems, Wiesbaden, Germany), diluted 1:250, or a polyclonal rabbit anti-human FOXL2 antibody [27] diluted 1:500. Subsequently a biotinylated horse-anti goat or a biotinylated swine-anti rabbit secondary antibody respectively was applied to the sections, and the bound antibody complex was visualized using horseradish peroxidase avidine biotin complex method, with 3,3'-Diaminobenzidine used as chromogene.

In the case of double staining, antigen retrieval was performed by heating up to 1.2 bar in 0.01M EGTA, 0.01M Tris (pH9) and detection of the first antibody (1:200 anti-SOX9, 2 h at room temperature) was carried out using a biotinylated horse-anti goat 1:200 antibody (BA-9500, Vector Laboratories), Avidin-alkaline-phosphatase complex (D0396; DAKO, Glostrup, Denmark) and subsequently Fast Blue BB / Naphtol ASMX phosphate staining. Before incubation with the second primary antibody (1:500 anti-FOXL2, 2 h at room temperature) previous avidin/biotin was blocked using the avidin/biotin blocking kit (Vector Laboratories). Subsequent detection of the second primary antibody was performed using a biotinylated swine-anti-rabbit antibody (E0431; Dako) 1:200, Avidin-horseradish-peroxidase complex (P0397, Dako) and staining using 3-amino-9-ethyl-carbazole.

Table 2. Patient characteristics and staining results

		T		O		M		GB		CIS	
Patient	**Morphology of the gonad**	**S**	**F**	**S**	**F**	**S**	**F**	**S**	**F**	**S**	**F**
Embryonal											
17/23/37 weeks GA		+ (n=3)	- (n=2)								
18/40 weeks GA											
22/24/37 weeks GA				- (n=3)	+ (n=5)						
20/22/24/36/41 weeks GA											
Adult											
Testis and CIS		+	-							+ (n=25)	- (n=25)
Ovary				-	+ (n=3)						
					- (n=1)						
DSD											
Sex chromosome DSD											
1	streak gonad					-	+				
2	testis + sex-cords	ND	ND			+/-	+/-				
3	testis + sex cords	+	- (+)*			+/-	+				
4	streak + sex cords					+/-	+				
5	testis	+	-								
6	testis	+	-								
7 A	testis + sex cords/ovarian stroma	+	- (+)*			+/-	+/-				
7 B	ovarian stroma					+	+				
8	Testis + streak	+	-			-	+				
9	Testis +streak	+	- (+)*			ND	ND				
10	Testis + streak	+	- (+)*			ND	ND				
Ovotesticular DSD											
11	ovarian tissue					+/-	+				
12	ovary			-	+						
13	ovary + testis	+	-	-	-						
14	ovary + testis	+	-	-	+						
46,XX testicular DSD											
15	Testis	+	-								
16	Testis	+	-								
17	Testis	-	-								
18	Testis	+	-								
46,XY gonadal dysgenesis											
19	GB + streak/sex cords					-	+	-	+		
20 A	testis + streak	+	- (+)*			-	+				
20 B	streak					+	+				
21	testis + sex cords	+	-			-	+				
22	CIS + intratubular seminoma + seminoma	+/-	-					-	+	+	-
23 A	GB + dysgerminoma + YST + imm. Teratoma							-	+		
23 B	GB					-(+)#	+	ND	+		
24	CIS + GB + dysgerminoma							-	+	+	-
25	Testis + streak	+	- (+)*			-	+				
26	GB + dysgerminoma							-	+		
27	Testis	+	- (+)*								
28	Testis + streak + GB	+	-			ND	ND	-	+		
29	Testis	+	- (+)*								
30	Testis + streak + GB	+	-			-	+	-	+		
31	Testis + streak + GB	+	- (+)*			-	+	ND	ND		

T=testicular differentiation, O=ovarian differentiation, M=mixed (ovarian stroma, UGT, sex cords,streak), GB=Gonadoblastoma, CIS=Carcinoma *in situ*, S=SOX9, F=FOXL2, GA=gestational age, YST=yolk sac tumor. ND=not done. DSD=disorders of sex development

one SOX9 positive tubule found

* occasional clusters or single cells positive for FOXL2 in testis tubules

Results

*SOX9 and FOXL2 protein expression in normal embryonic and adult gonads, both ovary and testis*To investigate the presence of SOX9 and FOXL2 during human embryonic gonadal development, a series of 18 normal gonadal tissues, all formalin-fixed, paraffin-embedded, were studied using immunohistochemical techniques. The antibodies and conditions used have been proven to be specific in gonadal context in earlier studies [27]. The samples included in this series are summarized in Table 2, in which also the results are indicated. None of the normal testicular tissues showed presence of FOXL2, at any time during development, and vice versa, none of the normal ovarian tissues showed SOX9 expression. Examples of the staining patterns found in the earliest developmental stages [23 and 37 weeks' gestational age (GA) male and 24 and 41 weeks' GA female] are shown in Figure 1. SOX9 but not FOXL2 was present in Sertoli cells, whereas FOXL2 and not SOX9 was positive in granulosa cells (see Figure 1).

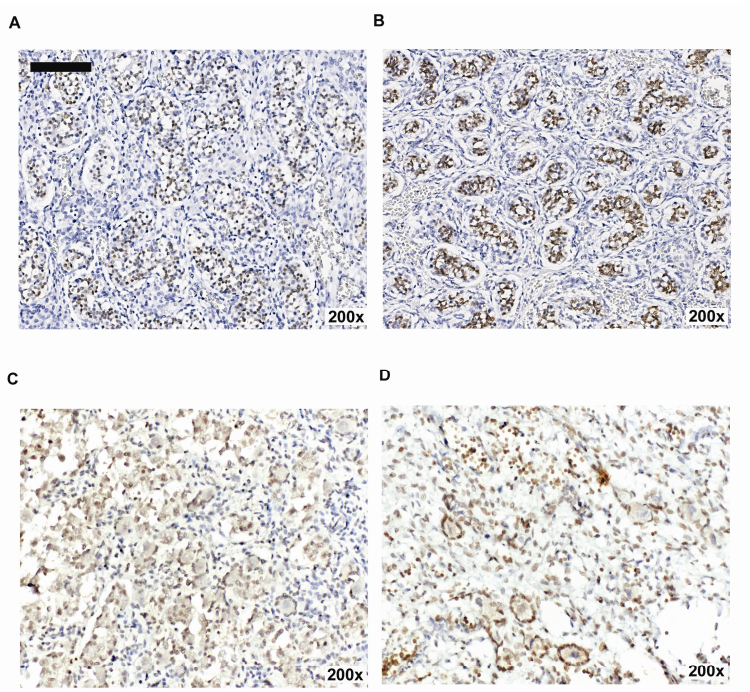

Figure 1. Immunohistochemical staining of male and female embryonic gonads. SOX9 staining (brown) of (A) a 23-week-old and (B) a 37-week-old male embryonic gonad. FOXL2 staining (brown) of (C) a 24-week and (D) a 41-week-old female embryonic gonad. Original magnification: x200. Bar = 100 μm. Counterstained with haematoxylin.

The staining was consistently nuclear, as expected for transcription factors. Because of the absence of FOXL2 in testicular tissue, and SOX9 in ovarian tissue, no double staining was performed in normal tissues.

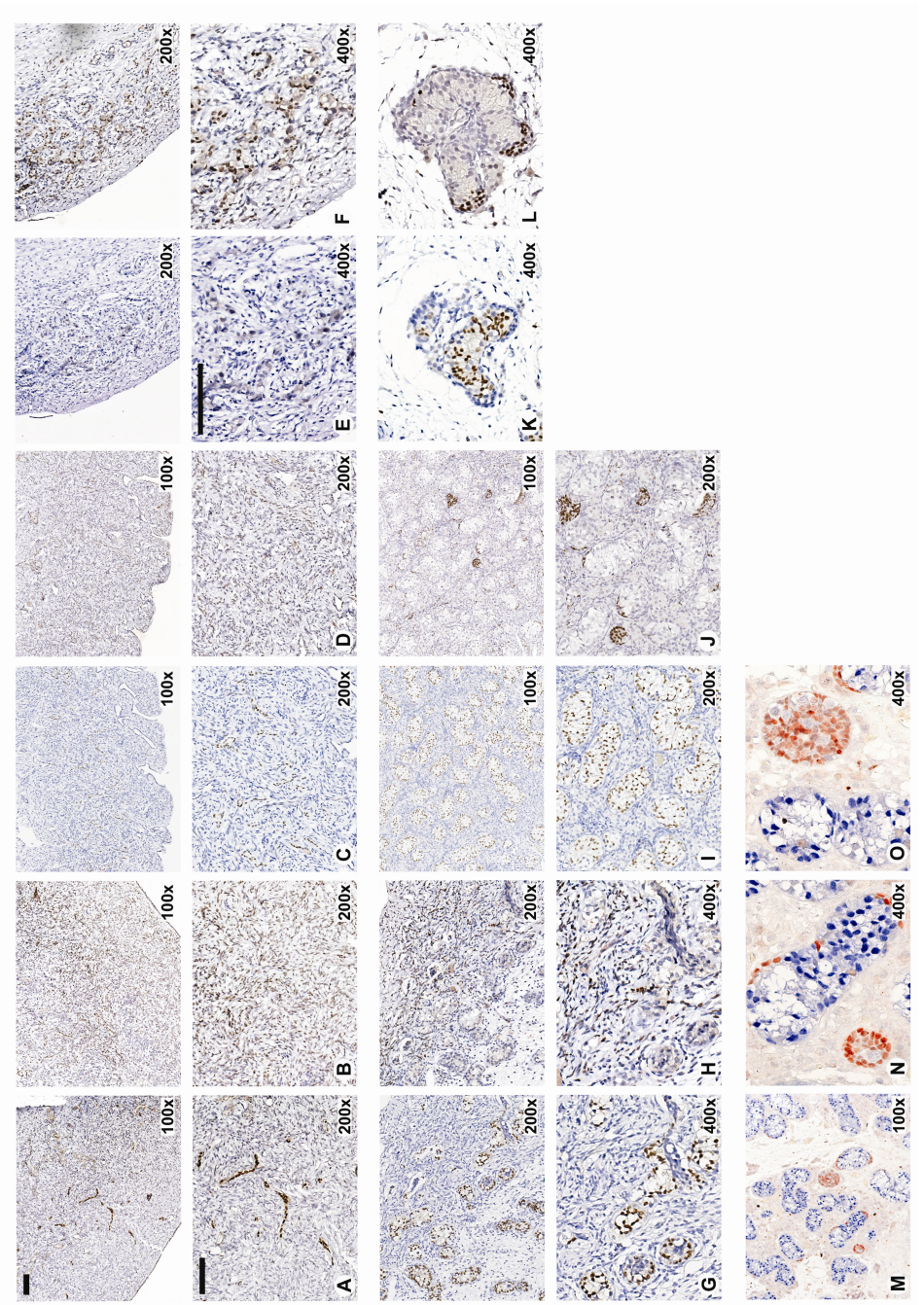

Figure 2. Patterns of SOX9 and FOXL2 protein expression in DSD gonads. (A, B) Staining of streak gonad of patient 20B. (A) Positive SOX9 staining (brown) in some single cells and strands of cells. (B) FOXL2 expression (brown): overall more cells express FOXL2 than SOX9. Original magnification: x100 upper and x200 lower panels. (C, D) Staining of ovarian stroma in gonad of patient 7B. (C) Expression of SOX9 (brown); most of the cells positive are arranged in strands. (D) An overall more scattered pattern of cells positive for FOXL2 (brown). Original magnification: x100 upper and x200 lower panels. (E, F) Staining in sex-cord region of patient 21**.** (E) Staining for SOX9 (brown) is very weak to absent. (F) Expression of FOXL2 (brown) is present in the initializing sex-cord region. Original magnification: x200 upper and x400 lower panels. (G, H) Transition from more testicular to undifferentiated tissue in gonad of patient 21. (G) SOX9 expression (brown) is strong in the Sertoli cells of the tubules in the testicular part of the gonad. (H) FOXL2 (brown) on the other hand is absent from the tubules, but gives positive staining in cells which are located in the more undifferentiated part of the gonad. Original magnification: x200 upper and x400 lower panels. (I-O) FOXL2 positive cells in testis tubules in testicular tissue of patient 3. (I) SOX9 expression (brown) is present in the Sertoli cells of the seminiferous tubules. (J) In the same region of the gonad FOXL2 (brown) positive staining can be seen in some of the tubules, and also some clusters of positive cells are present in the stroma. Original magnification: x100 upper and x200 lower panels. (K, L) Positive cells for SOX9 (K, brown) and FOXL2 (L, brown) are present in the same testis tubule shown; note that there seems to be no co-expression of the two proteins within cells (original magnification: x400). Alls slides are counterstained with haematoxylin. (M-O) Double staining of SOX9 (blue) and FOXL2 (red) in seminiferous tubules. (M, N) Some of the tubules show SOX9- and FOXL2-positive cells, but co-expression of both proteins within the cells was never seen. (M, O) Other tubules stain positive for SOX9 or FOXL2 alone. Original magnification: x100 and x400, respectively. Indicated bars = 100 μm

SOX9 and FOXL2 in gonads of patients with DSD

To study whether in pathological samples SOX9 and FOXL2 showed the same pattern as found during normal embryonic development, a series of 31 gonads of DSD patients were investigated. In Table 2, a summary is given of the staining patterns found for the different patients, together with their gonadal characteristics and DSD classification. Note that next to mature testicular and ovarian tissue, also immature structures could be identified, including what we previously classified as undifferentiated gonadal tissue (UGT) [28]. As expected, the pattern of SOX9 and FOXL2 staining found in the DSD samples clearly correlated with fully differentiated testicular and ovarian development, respectively. The pattern of expression was not influenced by the anatomical localization of the gonad, either scrotal or abdominal, or whether the testicular and ovarian tissue as found within a single patient was present in one or the two gonads. Representative examples of the different staining patterns are shown in Figure 2. In most of the streak gonads, previously generally interpreted as female, the majority of cells were FOXL2-positive. However, often isolated cells, strands of cells or structures suggesting initiation of testis tube formation clearly staining for SOX9 were found (Figure 2A and 2B). This strongly supported the

hypothesis of the pathologist that indeed some testicular development had occurred in these gonads. Also in gonads classified as ovarian stroma, the same pattern of SOX9 staining was found (Figure 2C and 2D). An example of a region of early sex-cord formation in which no expression of SOX9 could be seen, but FOXL2 was clearly present is shown in Figure 2E and 2F. However, sometimes faint SOX9 staining was observed in such regions. Another pattern we observed was the situation in which a transition from a streak gonad to a clearer testicular differentiation is seen (Figure 2G and 2H). The testis-like tubules stained positive for SOX9 but not for FOXL2, while in the undifferentiated tissue FOXL2-positive cells were present and SOX9 staining was absent.

A limited number of cases (indicated in Table 2) showed presence of both SOX9 and FOXL2, all in testicular tissue, of which representative examples are shown in Figure 2I-2O. Note that the majority of seminiferous tubules contain cells, often clearly recognized as Sertoli cells, which are positive for SOX9 (Figure 2I). However, intermixed, predominantly in clumps, FOXL2-positive cells could be identified both within and in between the tubular structures (Figure 2J). If both SOX9- and FOXL2-positive cells were present within one tubule, SOX9 and FOXL2 appeared not to be co-expressed within the same cells (Figure 2K and 2L, respectively, adjacent slides). To exclude if there was co-expression of both factors within cells, a double staining for SOX9 (shown in blue) and FOXL2 (shown in red) was performed (Figure 2M-2O). In tubules with both SOX9- and FOXL2-positive cells, expression is mutually exclusive (Figure 2M and 2N). Also tubule like structures with only FOXL2-positive cells could be found (Figure 2M and 2O).

SOX9 and FOXL2 in CIS of the testis and GB of the dysgenetic gonad

In the case of CIS the pre-malignant germ cells are always associated with Sertoli cells. The histological constitution of CIS is within the seminiferous tubules, under the tight junctions formed by Sertoli cells. Indeed, all cases of testicular CIS showed expression of SOX9 in the Sertoli cells, while no staining was found for FOXL2 (see Figure 3A). In contrast, in GB, where the male versus female development of the stromal cells has always been a matter of debate, no clear SOX9 staining was identified, while FOXL2 was consistently present (see Figure 3B).

Discussion

The presence of both testicular and ovarian gonadal tissue in a single individual is not a physiological condition in mammals, although it is found in other

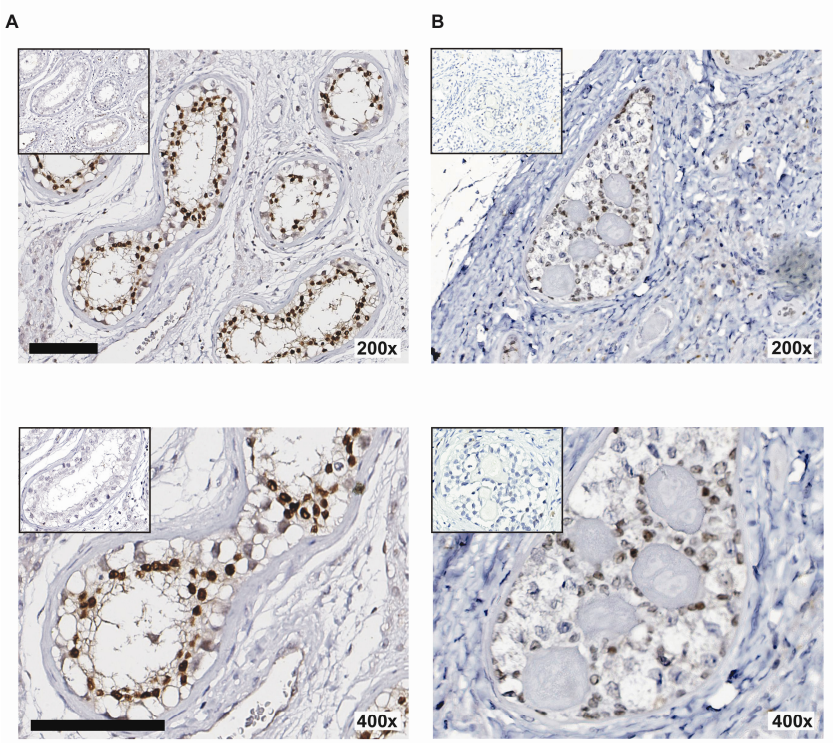

Figure 3. SOX9 and FOXL2 in carcinoma *in situ* (CIS) and gonadoblastoma (GB). (A) SOX9 positive Sertoli cells in CIS. Inset: no positive staining for FOXL2 in the same CIS tubules. (B) In contrast, in GB, only FOXL2-positive cells can be found. Inset: no SOX9-positive cells can be found. Both SOX9 and FOXL2 shown in brown. Original magnification: x200 upper and x400 lower panels. Bars = 100 µm. Slides were counterstained with haematoxylin.

species; for example, in nematodes [29], snails etc. [30]. Under specific pathological conditions in mammals, both mouse and human, these tissues can be identified within a single individual, and sometimes even within a single gonad. Patients with such gonads are currently classified as having DSD, previously referred to as intersex [19].

Different variants of DSD were investigated in this study for the presence of both SOX9 and FOXL2. These transcription factors are known for their necessity in testicular (Sertoli cell) and ovarian (granulosa cell) development respectively, and were used in this study as functional readouts of those processes. For comparison also normal embryonic and adult gonads of both males and females were included. Overall, the patterns of expression of SOX9 and FOXL2 in the gonads of DSD patients were in accordance with testicular and ovarian differentiation, respectively, as expected based on published data, and in line with the normal gonads included in this study. SOX9

was detected in (pre-)Sertoli cells and FOXL2 in (pre-)granulosa cells. However, some FOXL2 positive cells were identified in seminiferous tubule-like structures in a number of DSD patients, but never in the case of normal development. Strong co-expression of SOX9 and FOXL2 protein was never observed, which suggests mutual exclusiveness of expression of these genes. This might be due to direct or indirect (mutual) repression at the transcriptional level. However, regulation at other levels, like protein or mRNA stability, cannot be excluded.

Testicular CIS was consistently associated with SOX9 expression in the Sertoli cells, while GB clearly showed the presence of FOXL2 and not SOX9. It is known that both CIS and GB can progress to invasive germ cell tumours [31-33], referred to as type II germ cell tumours, the seminomatous tumours and nonseminomas. Also the fact that dysgerminomas (associated with GB) and seminomas (associated with CIS) are highly similar, as shown by mRNA and microRNA expression profiling [34-35], indicated that in fact CIS and GB are highly comparable, although the histological appearance is different. We propose a model in which the level of virilization, ie the maturation of the Sertoli cell, due to the SRY-SOX9 pathway is determining whether the malignant counterpart of primordial germ cells, will manifest itself as CIS or GB. The presence of FOXL2 in this context is supportive for this model. Classically, GB is described as a mix of immature germ cell and stromal cells, in which the stromal cells show characteristics of immature Sertoli or granulosa cells [26]. The presence of FOXL2 in these supportive cells is the first unbiased demonstration that, in contrast to the supportive cells associated with CIS, they are in fact granulosa-like cells. It will, therefore, be interesting to see whether the accompanying Leydig cells reported in cases of GB are in fact Leydig cells or whether they might be more related to their female counterpart: theca cells. It would be challenging to elucidate mechanistically why granulosa cell characteristics are found even when Leydig cells are formed. Possibly, the granulosa cells formed in GB are not completely functional, suggested by the observation that the germ cells in GB show more maturation along the male pathway, as suggested based on the presence of E-cadherin [36]. This needs further investigation.

In conclusion, this study for the first time demonstrates that SOX9 and FOXL2 can be used as informative markers to identify testicular and ovarian development in patients with various forms of DSD. The expression patterns of both transcription factors are highly restricted to the histological context of either testis (SOX9) or ovary (FOXL2). The pattern of expression is clearly different in CIS compared to GB. The data presented lead to the model that the

presentation of malignant germ cells in DSD patients can be either CIS or GB, or even a combination of both, depending on the level of virilization, ie the functionality of the SRY-SOX9 pathway. This model opens novel areas for further investigation.

Acknowledgements

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Chapter 5

Delayed Recognition of Disorders of Sex Development (DSD): A Missed Opportunity for Early Diagnosis of Malignant Germ Cell Tumors

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Chapter 5

Abstract

Disorders of sex development (DSD) are defined as a congenital condition in which development of chromosomal, gonadal or anatomical sex is atypical. DSD patients with gonadal dysgenesis or hypovirilization, containing part of the Y-chromosome (GBY), have an increased risk for malignant type II germ cell tumors (GCTs: seminomas and non-seminomas). DSD may be diagnosed in newborns (e.g. ambiguous genitalia), or later in life, even at or after puberty. Here we describe three independent male patients with a GCT; two were retrospectively recognized as DSD, based on the histological identification of both carcinoma *in situ* and gonadoblastoma in a single gonad as the cancer precursor. Hypospadias and cryptorchidism in their history are consistent with this conclusion. The power of recognition of these parameters is demonstrated by the third patient, in which the precursor lesion was diagnosed before progression to invasiveness. Early recognition based on these clinical parameters could have prevented development of (metastatic) cancer, to be treated by systemic therapy. All three patients showed a normal male 46,XY karyotype, without obvious genetic rearrangements by high resolution whole genome copy number analysis. These cases demonstrate overlap between DSD and the so-called Testicular Dysgenesis Syndrome (TDS), of significant relevance for identification of individuals at increased risk for development of a malignant GCT.

Introduction

Congenital conditions in which development of chromosomal, gonadal, or anatomical sex is atypical are termed "Disorders of Sex Development" (DSD) [1], and have replaced the formerly used "intersex" term. It is estimated that DSD affects 1 in 4,500 to 5,000 live births in the general population, although with variability regarding the various DSD subtypes [1]. DSD patients are subdivided into different entities; 46,XY DSD; 46,XX DSD and sex chromosomal DSD. Within these subgroups, patients with gonadal dysgenesis (GD) and hypovirilization with presence of part of the Y chromosome (i.e. GBY), are known to have an increased risk to develop carcinoma *in situ* (CIS) or gonadoblastoma (GB), the precursor lesions of seminoma(SE)/dysgerminoma(DG) and non-seminoma, referred to as malignant type II germ cell tumors (GCTs) ([2-4], for review). In GD migration of the germ cells and/or their organization in the gonads is disturbed, leading to incomplete formation of the gonads. Hypovirilization is caused by defects in androgen dependent target tissues, errors in testosterone biosynthesis and testicular unresponsiveness to stimulation from the pituitary [5], leading to underdevelopment of the male differentiation lineage.

GB is the *in situ* germ cell malignancy of the ovary and dysgenetic gonad which, in a significant number of cases, will develop into an invasive dysgerminoma or, less often, non-dysgerminoma, being histologically and genetically counterparts of testicular seminoma and non-seminoma [6]. GB is composed of a mixture of embryonic germ cells (OCT3/4 and SCF (official term: KITLG) positive, amongst others) and supportive cells, with characteristics of granulosa cells (FOXL2 positive) [7]. GB can be found in undifferentiated gonadal tissue and in gonadal tissue with immature testis differentiation [8], overall related to a low level of testicularization (i.e., level of testis formation). CIS (cells also positive for OCT3/4 and SCF, amongst others), on the other hand, being the precursor of the similar types of cancer (SE and non-seminoma) of the testis, is associated with SOX9 positive Sertoli cells [7] and is found in well-differentiated testicular tissue [9].

For malignant transformation of embryonic germ cells in the context of type II GCTs, presence of part of the Y chromosome is crucial, referred to as GonadoBlastoma on the Y chromosome (GBY) region by Page in 1987 [2]. *TSPY* is currently considered to be the most likely candidate gene for this genomic region [10-11], and of diagnostic value, because both CIS and GB show co-expression of OCT3/4, SCF and TSPY.

In spite of the overall low incidence in the general population, type II testicular GCTs are the most common malignancy in Caucasian males aged between 15 and 45 years, the incidence of which is still rising [12]. It has been suggested that the so-called Testicular Dysgenesis Syndrome (TDS) is the underlying reason [13], estimated to affect 1 in 500 live births. However, existence of TDS is also questioned [14]. TDS links various clinical observations like cryptorchidism, subfertility/infertility and hypospadias with exposure to certain environmental factors, with either a xeno-estrogen or anti-androgen function. However, genetic factors, especially a limited number of Single Nucleotide Polymorphisms (SNPs) are also recognized to play a role in development of this type of cancer [15-16]. Most likely, the pathogenesis is a close and subtle interplay between both genetic- and environmental factors, referred by us to as "Genvironment".

Here three unique unrelated male patients are presented demonstrating the relevance of TDS- and DSD recognition for early diagnosis of malignant type II GCTs, possibly preventing progression to metastasized disease.

Materials and methods

Patients

Three unrelated male patients, all with hypospadias and cryptorchidism in their clinical history are described. All patients underwent hypospadias corrections and two patients had orchidopexy early in life. Two of the patients were only retrospectively recognized as having DSD based on the presence in a single gonad of GB next to CIS as precursor lesions. The third patient described, having been recognized early in life as having DSD/TDS (i.e. hypospadias and cryptorchidism), shows that early identification of the condition can lead to early detection of the cancer precursor lesion before progression to invasiveness occurs. Detailed description is presented in the Results section.

Tissue samples

Collected tissue samples were diagnosed according to WHO standards [17] by an experienced pathologist in gonadal pathology, including GCTs (JWO). Use of tissue samples for scientific reasons was approved by an institutional review board (MEC 02.981 and CCR2041). 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 (FMWV, Version 2002, updated 2011). Fresh tissue material was fixed in 10% buffered formaline for 24 hrs and paraffin embedded according to standard protocols.

Immunohistochemical staining

Immunohistochemistry was performed on paraffin-embedded tissue sections of 3-µm thickness. Hematoxylin (Klinipath, Duiven, The Netherlands) and eosin (Klinipath) counterstaining was performed according to standard procedures. After deparaffinization and 5 min. incubation in 3% H_2O_2 to inactivate endogenous peroxidase activity, antigen retrieval was carried out by heating under pressure of up to 1.2 bar in an appropriate buffer; 0.01M sodium citrate (pH 6) or 0.01M EGTA, 0.01 M TRIS (pH 9). After blocking endogenous biotin using the avidin/biotin blocking kit (SP-2001, Vector Laboratories, Burlingame, CA, USA), the sections were incubated for either 2 hrs at room-temperature (OCT3/4, SOX9) or overnight at 4˚C (TSPY, FOXL2, SCF). Appropriate biotinylated secondary antibodies were used for detection and were visualized using the avidin-biotin detection and substrate kits (Vector Laboratories). The antibodies used directed against OCT3/4, TSPY, SCF, SOX9 and FOXL2 have been described before [7, 18-20].

Fluorescent in situ hybridization

Slides of 5 µm thickness were deparaffinized and heated under pressure of up to 1.2 bar in appropriate buffer; 0.01M sodium citrate (pH 6). Slides were digested using 0.01% pepsin (Sigma Aldrich, St. Louis, MO USA) in 0.02 M HCl at 37°C, with an optimal digestion time of 2.5 min. Slides were rinsed, dehydrated and the probes dissolved in hybridization mixture were applied. Probes for centromere X (BamHI) and centromere Y (DYZ3) were used, labeled with digoxigenin-11-dUTP and biotin-16-dUTP (Roche Diagnostics, Mannheim, Germany) using a nick-translation kit (Gibco BRL, Paisley, UK). After denaturizing (80°C for 10 min), hybridization overnight (37°C) and washing steps, probes were visualized using Cy3-conjugated avidin (1 : 100, Jackson ImmunoResearch, West Grove, PA USA) and Sheep-anti-dig FITC (1 : 50, Roche Diagnostics) and analyzed using a fluorescent microscope (Leica Microsystems, Rijswijk, The Netherlands).

Copy number analysis

Genomic DNA was isolated from peripheral blood (patient 1 and 3) and frozen gonadal tissue without presence of malignant cells (patient 2) using standard procedures. For each sample 200 ng of DNA was labelled and hybridized onto the Human OmniExpress microarray (Illumina, San Diego, CA USA) at the Australian Genome Research Facility (Melbourne, Australia) following

manufacturer's instructions. Data was analyzed with Genome Studio (Illumina) and cnvPartition, using default settings.

Results

Clinical history, hormonal and genetic data, and immunohistochemical analyses

Patient 1: Review of the existing clinical data, was prompted by the histological evaluation of the right testis at the age of 26 years (showing dysgenetic characteristics, see below). It was found that the patient had multiple surgical corrections of proximal hypospadias between his second and tenth year of age, because of the severity of this anomaly. Orchidopexy of the left testis by an inguinal approach was performed at three years of age, while no right gonad was found during inguinal exploration on the right side at that time. At 26 years of age the patient underwent surgery for a left sided inguinal hernia. During the procedure the right testis (inguinal position) was identified at the left hand side (i.e. crossed testicular ectopia), and removed because of a macroscopically abnormal/tumor-like appearance.

Histological examination of this gonad showed dysgenetic characteristics, containing CIS, GB, DG and SE (representative hematoxylin & eosin (H&E) staining shown in Figure 1A). The CIS- and GB-germ cells showed a positive staining for OCT3/4 (Figure 1B, brown), TSPY (Figure 1C, red) and SCF (Figure 1D, brown). The supportive cells in context of CIS stained predominantly positive for SOX9 (Figure 1E, brown), while those in the context of GB stained predominantly for FOXL2 (Figure 1F, brown). Co-expression is however observed, suggesting an issue of balance. In line with current treatment options, the patient received prophylactic radiotherapy according to standard guidelines. During close follow-up (3 years), the patient showed no relapse of the disease.

Genetic analysis by karyotyping of peripheral blood lymphocytes, and FISH using X and Y centromeric probes on gonadal tissue (representative FISH shown in Figure 1G) indicated a normal male 46,XY constitution.

Hormonal data analysis at the age of 24 years indicated a sub-optimal testicular function (hypergonadotrophic hypogonadism): FSH 12 and 17.5 U/L (normal 2.0-7.0 U/L), LH 5.6 and 8.4 U/L (normal 1.5-8.0 U/L), testosterone 13.2 and 16.2 (normal 10-30 nmol/L), Inhibin B 119 and 74 ng/L (normal 150-400 ng/L). Tumor markers measured after removal of the affected gonad with the cancer showed a slightly elevated level of AFP 15-19 µg/L (normal <10-15

µg/L), normal levels of ß-HCG <0.5 IU/L (normal <0.5 IU/L) and LDH 152-314 U/L (normal <450 U/L).

Taken together; the histological observations, clinical history, karyotyping and hormonal data support the diagnosis of the patient as a 46,XY DSD, type A: disorder of testicular development, 1: partial gonadal dysgenesis [1]. A summary of the various actions and observations are schematically shown in Figure 1H.

Patient 2: Review of the (limited) clinical history was provoked by the histological evaluation of the left testis at the age of 21 years, showing dysgenetic characteristics (see below). It revealed presence of bilateral intra-abdominal testes, while the male patient also showed hypospadias, as well as presence of a uterus. At 20 years of age the patient was diagnosed with a right intra-abdominal testicular SE (of which no material or further information could be retrieved). During surgical removal of the affected gonad, the left sided intra-abdominal testis was positioned at an inguinal site. This remaining testis was biopsied six months later because of unexplained enlargement, and was subsequently removed because of presence of CIS and GB (see below).

Histological evaluation of the left biopsy showed the presence of GB and CIS, which was followed by orchidectomy. Further histological examination indicated the presence of dysgenetic characteristics, CIS and GB (representative H&E shown in Figure 2A), supported by staining for OCT3/4 (Figure 2B, brown), TSPY (Figure 2C, red) and SCF (Figure 2D, brown), next to SE and DG. The supportive cells in GB stained again positive for FOXL2 (Figure 2E, brown) and for SOX9 in CIS (Figure 2F, brown). Because of proven metastasized disease the patient received chemotherapy following standard procedures. No follow up information is available.

Genetic analysis by karyotyping of peripheral blood lymphocytes, and FISH using X and Y centromeric probes on gonadal tissue indicated a normal male 46,XY karyotype (data not shown). No hormonal or tumor marker data was available.

In summary, histological evaluation, review of clinical history, and karyotyping indicate that the patient must be diagnosed as a 46,XY DSD, type A: disorder of testicular development, 1: partial gonadal dysgenesis [1]. A summary of the various actions and observations are schematically shown in Figure 2G.

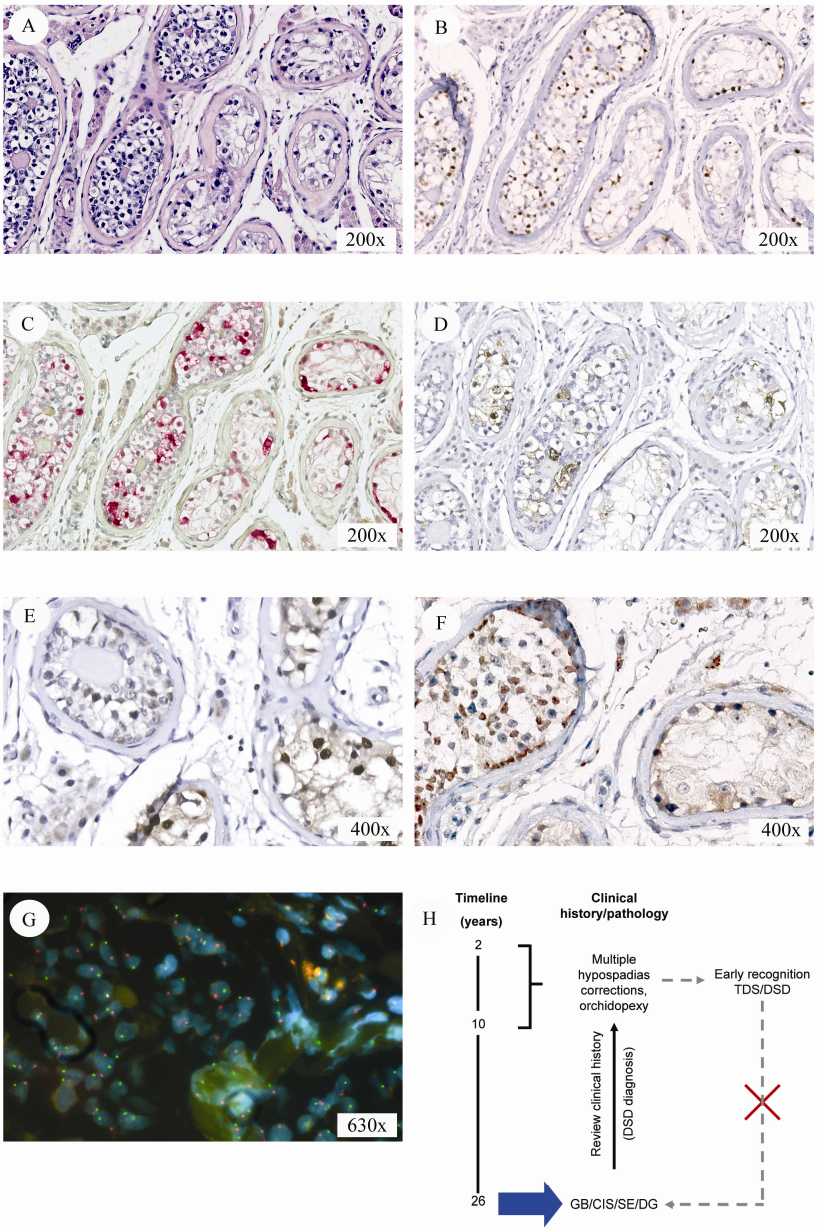

Figure 1. Immunohistochemical staining and fluorescent *in situ* hybridization (FISH) of the gonadoblastoma and carcinoma *in situ* lesions of patient 1. (A) Representative hematoxylin and eosin staining. The germ cells present in the GB and CIS stain positive for (B) OCT3/4 (brown), (C) TSPY (red) and (D) SCF (brown). (E) The supportive cells in the CIS lesion are SOX9 positive (brown staining) and are negative for FOXL2. (F) In the GB the supportive cells stain positive for FOXL2 (brown staining) and are negative for SOX9. (A-F) In every image the GB lesion is shown on the left side (embryonic germ cells intermixed with granulosa like supportive cells), CIS containing seminiferous tubules on the right side (CIS cells associated with Sertoli cells on the basal lamina). Magnification 200x and 400x for all. Slides (B–F) are counterstained with hematoxylin. (G)

Representative FISH with Y centromere-specific probe (shown in red) and X centomere-specific probe (shown in green). Magnification 630x. (H) Schematic representation of the different moments in time of clinical intervention: Blue arrow; identification of a malignant type II germ cell tumor, together with GB and CIS as precursor lesions at the age of 26 years. Review of the clinical history showed hypospadias and cryptorchid testes, signs of TDS/DSD which were not recognized at an early age. Grey dashed arrows; early recognition of TDS/DSD could have allowed early detection and treatment of the malignancy, thereby preventing the need for additional systemic treatment.

Patient 3: The male patient showed multiple congenital anomalies at birth, amongst others penoscrotal hypospadias and bilaterally cryptorchid testes. He underwent multiple hypospadias corrections at one and two years of age. Orchidopexy of the right testis to a high scrotal position was performed at two years of age, and a herniotomy and orchidopexy, to a high scrotal position, of the left testis was carried out at 3 years of age. Overall appearance of the left testis together with total dissociation of epididymis and testis prompted a biopsy to be taken at that time (representative H&E shown in Figure 3A). It was diagnosed as pre-pubertal testicular parenchyma with seminiferous tubules containing Sertoli cells and germ cells, without indication for malignancy. The patient was lost to follow-up until 12 years of age at which time he was examined because of incontinence problems, and came under attention of the initial clinician treating the hypospadias by coincidence. Physical examination showed a pubertal boy (Tanner stage P4G3) with a scrotal localization of the right testis, while the left testis was not palpable. Further examination using ultrasound showed an inguinal position of the left testis (ascending testis), and bilateral testicular microcalcifications (microlithiasis). Because of the inability to position the left testis in the scrotum, and the knowledge about the increased risk for development of a malignant GCT based on the clinical characteristics, the left testicle was removed and the right testis was biopsied.

Histological examination of the left testis (representative H&E staining shown in Figure 3D), showed seminiferous tubules containing CIS, supported by staining for OCT3/4 (Figure 3E, brown), TSPY (Figure 3F, red) and SCF (Figure 3G, brown). Co-staining of these markers in single CIS cells was identified indicated by the arrow). The presence of CIS initiated re-examination of the biopsy taken at the age of three years. Because of limited material available, only staining for OCT3/4 (Figure 3B, brown), and TSPY (Figure 3C, red) could be done, showing the presence of pre-malignant germ cells, referred to as pre-CIS. This conclusion was not made at the time of original sampling because of lack of appropriate markers. The biopsy taken from the right testis showed normal testicular parenchyma without malignancy (negative OCT3/4 staining, data not shown).

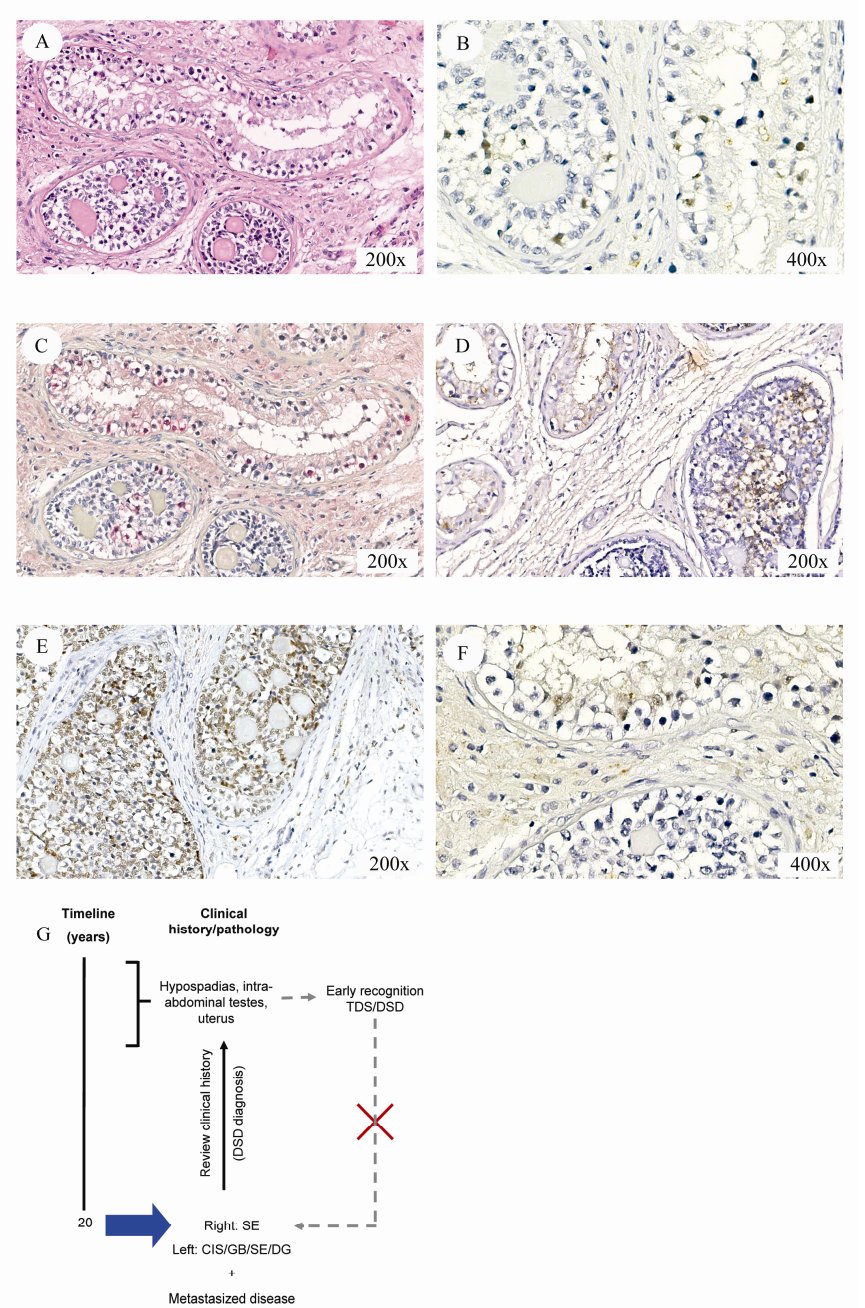

Figure 2. Immunohistochemical staining of the gonadoblastoma and carcinoma *in situ* lesions of patient 2. **(**A) Representative hematoxylin and eosin staining. Positive staining for (B) OCT3/4 (brown), (C) TSPY (red) and (D) SCF (brown) of the germ cells present in the GB and CIS. (E) In the GB the supportive cells stain positive for FOXL2 (brown). (F) The supportive cells in the CIS lesion are SOX9 positive (brown staining) and are negative for FOXL2. (A-D, F) Again, both GB (embryonic germ cells intermixed with granulosa like supportive cells) and CIS (associated with

Sertoli cells on the basal membrane of the tubules) are shown. Magnification 200x and 400x for all. Slides (B–F) are counterstained with hematoxylin. (G) Timeline showing the clinical history, histology and actions taken.

Genetic analysis by karyotyping of peripheral blood lymphocytes, and FISH using X and Y centromeric probes on gonadal tissue indicated a normal male 46,XY karyotype (data not shown). Hormonal data at the age of 12 years were as follows: FSH 1.8 and 3.1 U/L (normal <6.0 U/L), LH 0.4 and 1.0 U/L (normal <2.5 U/L), testosterone 1.6 and 6.7 nmol/L (normal 3.0-6.5 nmol/L), and AMH 18.8 μg/L (normal 30-200 μg/L). The tumor markers tested were within the normal range: AFP <1 μg/L (normal <10 μg/L) and ß-HCG 0.1 IU/L (normal <0.5 IU/L).

Taken together, histological evaluation, review of clinical history, and karyotyping indicate that the patient must be diagnosed as 46,XY DSD, type A: disorder of testicular development, 1: partial gonadal dysgenesis [1]. A summary of the various actions and observations in time are schematically shown in Figure 3H.

Copy number analysis of known DSD genes

A peripheral blood DNA sample from patient 1 and 3, and a DNA sample isolated from frozen gonadal tissue from patient 2 (as no peripheral blood was available) were checked for copy number changes by genome-wide SNP analysis using Illumina OmniExpress Beadchips. This supported the 46,XY karyotype, and showed no aberrations affecting known DSD genes (data not shown).

Discussion

Here, two unrelated male patients are presented, both diagnosed with an invasive malignant type II GCT. One was prophylactically treated with irradiation for a stage I seminoma, and the other received chemotherapy for proven metastasized disease. These treatment protocols have been found to increase the risk for long term sequelae [21]. Presence of GB, known to be associated with DSD [8], besides CIS, as precursor in these patients, triggered review of their clinical history. Both cases showed severe hypospadias and cryptorchidism. These are identifiers of DSD, as well as TDS, both conditions known for their increased risk of malignant type II GCTs [3, 13]. In addition, patient 1 had crossed testicular ectopia, a very rare anomaly, reported to be associated with TDS and DSD [22]. No genetic confirmation of an underlying DSD was found in any of the patients, even using high resolution genome wide analysis. In spite of this lack of identification of the molecular basis of the

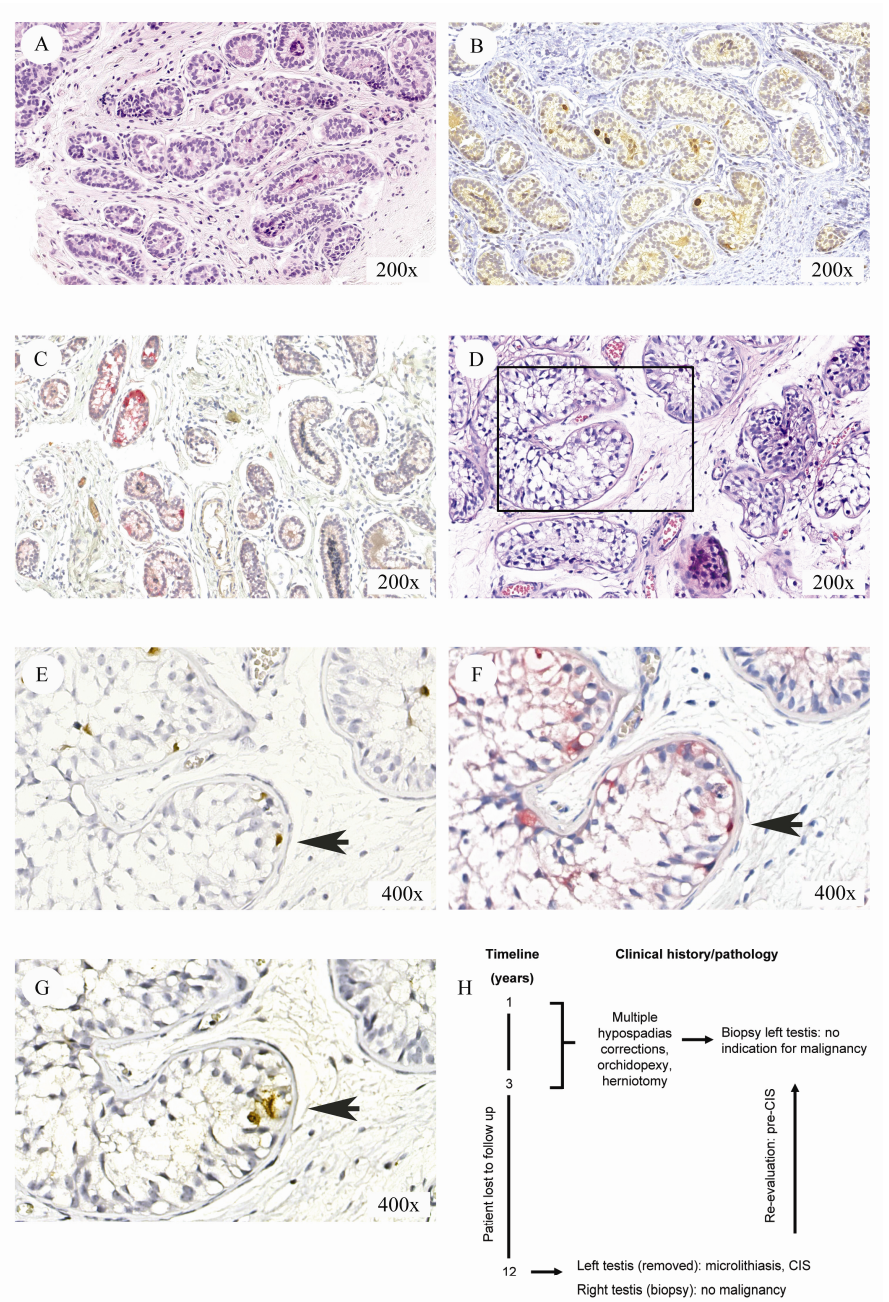

Figure 3. Immunohistochemical staining of the carcinoma *in situ* lesion of patient 3 at three and twelve years of age. **(**A) Representative hematoxylin and eosin staining. Positive staining for (B) OCT3/4 (brown), (C) TSPY (red) of the germ cells present in the CIS. (A-C) Biopsy tissue at 3 years of age. (D) Representative hematoxylin and eosin staining. Positive staining for (E) OCT3/4 (brown), (F) TSPY (red) and (G) SCF (brown) of the CIS cells. (D-G) Gonadal tissue at 12 years of age. (E-G) Region indicated with a square in (D) is shown. Note the expression of OCT3/4, TSPY

and SCF in the CIS cell indicated by the arrow. Magnification 200x and 400x for all. Slides (B–G) are counterstained with hematoxylin. (H) Timeline showing the clinical history, histology and actions taken.

underlying disorder, the observations have significant implications regarding development of strategies for early diagnosis of type II GCTs, as well as understanding the biology of the disease.

DSD patients can be diagnosed early in life based on various characteristics, including sexual ambiguity; family history; discordant karyotype and genital appearance; aberrant male and female genitalia. In children and young adults however, DSD can present as an inguinal hernia in a girl, incomplete or delayed puberty, virilization in a girl, primary amenorrhea, breast development in a boy and a previously unrecognized genital ambiguity [23].

When sex determination is disrupted in an early stage of Sertoli cell differentiation a high risk for GB is found [9]. The GB lesion is composed of immature germ cells intermixed with supportive cells classified as granulosa [24]. The GB lesions found in the two presented patients showed these characteristics as well, based on immunohistochemical finding using OCT3/4, TSPY, SCF, SOX9 and FOXL2. Next to the GB component, CIS was also present in both. This in fact triggered the search for additional clinical arguments in line with the diagnose of these patients as DSD. The findings presented indicate that by proper application of the current knowledge of risk factors for type II GCTs, these patients could have been diagnosed earlier, thereby possibly preventing the use of irradiation and chemotherapy. That this is in fact a feasible option is demonstrated by the third patient presented. It demonstrates the power of applying the current markers for diagnosis of the pre-malignant lesions of type II GCTs. In fact, re-evaluation of the biopsy of this patient, taken at three years of age, showed co-expression of OCT3/4 and TSPY in germ cells located on the basal lamina. These cells are referred to as pre-CIS, from which CIS will develop. Proper identification of the risk factors for type II GCTs, in particular related to DSD and TDS will increase the possibility to identify patients at risk for malignancy at an early age, allowing application of limited-harmful treatment protocols.

OCT3/4 expression is most likely related to the survival of the germ cells [25], while the role of TSPY is less clear. It has been suggested to be related to cell cycle regulation [26-28]. In addition, SCF is informative to diagnose CIS and GB, especially to distinguish CIS from germ cells delayed in their maturation [29]. Of interest in this context is the linkage of specific single nucleotide polymorphisms with development of type II GCTs in the general

Caucasian population, including involvement of SCF [15-16]. However, the impact of these risk alleles in the DSD populations remains to be investigated.

The left testis of patient 1 and the right testis of patient 3 are still *in situ* at a scrotal localization. For the first patient available hormonal data indicated sub-optimal testicular function (high FSH, low inhibin, testosterone low normal range). In spite of treatment by prophylactic irradiation, and absence of metastasis (based on routine examinations), the patient is under close surveillance because of a minor elevated AFP level. No hormonal indications for testicular dysfunction could be observed in patient 3, while no data were available for patient 2.

Recently two families were independently reported showing an overlap between DSD and TDS. One family showed two sisters with XY sex reversal, gonadal dysgenesis and GB, and the other family included one daughter with a mosaic karyotype and GB. All patients showed a *SRY* mutation, inherited from the father, being mosaic. The fathers of both families presented with TDS, one with oligoasthenozoospermia and a testicular SE, the other with hypospadias, cryptorchidism, oligoasthenozoospermia and a testicular SE as well [30-31]. In other words, TDS and DSD form a continuum, which is informative to identify individuals at risk for type II GCTs.

As indicated, the two patients demonstrated here with a type II GCT, and proven metastasized cancer in one, at time of diagnosis, also show the value of identification of parameters known to be related to TDS and DSD, including cryptorchidism, hypospadias and the presence of GB (in the latter). Based on these characteristics, these two patients would have been diagnosed as 46,XY DSD, Disorder of testicular development, 1: partial gonadal dysgenesis [1], being at increased risk for development of a malignant type II GCT. Feasibility of early diagnosis, leading to prevention of development of an invasive, and possibly even a metastatic cancer, is clearly demonstrated by the third patient.

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Chapter 6

A novel SRY missense mutation affecting nuclear import in a 46,XY female patient with bilateral gonadoblastoma

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Abstract

Patients with disorders of sex development (DSD), especially those with gonadal dysgenesis and hypovirilization, are at risk to develop so-called type II germ cell tumors (GCTs). Both carcinoma *in situ* and gonadoblastoma (GB) can be the precursor lesion, resulting in a seminomatous or nonseminomatous invasive cancer. SRY mutations residing in the HMG domain are found in 10-15% of 46,XY gonadal dysgenesis cases. This domain contains two nuclear localization signals (NLSs). Here we report a unique case of a phenotypical normal woman, diagnosed as a patient with 46,XY gonadal dysgenesis, with an NLS missense mutation, on the basis of the histological diagnosis of a unilateral GB. The normal role of SRY in gonadal development is the upregulation of SOX9 expression. The premalignant lesion of the initially removed gonad was positive for OCT3/4, TSPY and stem cell factor in germ cells and for FOXL2 in the stromal component (ie, granulosa cells), but not for SOX9. On the basis of these findings, prophylactical gonadectomy of the other gonad was performed, also showing a GB lesion positive for both FOXL2 (ovary) and SOX9 (testis). The identified W70L mutation in the *SRY* gene resulted in a 50% reduction in nuclear accumulation of the mutant protein compared with wild type. This likely explains the diminished SOX9 expression, and therefore the lack of proper Sertoli cell differentiation during development. This case shows the value of the proper diagnosis of human GCTs in identification of patients with DSD, which allows subsequent early diagnosis and prevention of development of an invasive cancer, likely to be treated by chemotherapy at young age.

Introduction

Congenital conditions in which development of chromosomal, gonadal, or anatomical sex is atypical are refered to as disorders of sex development (DSD), previously known as intersex [1]. DSD patients with 46,XY complete gonadal dysgenesis (previously referred to as Swyer syndrome) have a high risk of developing a type II malignant germ cell tumor (GCT) with gonadoblastoma (GB), often bilateral, as the precursor lesion (see Cools *et al* [2] for a review). What these patients have in common is that during early embryonic development, the initially immature bi-potential gonads fail to differentiate along the male (testicular) pathway. Pivotal to this process is the sex-determining region on the Y chromosome (*SRY*) gene. Typically during male development, expression of SRY in the gonadal stromal cells will upregulate the transcription of another key DSD gene and transcription factor SOX9 through DNA binding to the testis enhancer of *SOX9* [3, 4 , 5]. This will subsequently lead to a cascade of signal transduction with differentiation of the stromal cells into (pre-)Sertoli cells. The development of functional Sertoli cells produces anti-Müllerian Hormone (AMH) and directs the formation of testosterone-producing Leydig cells, ultimately giving rise to male internal and external genitalia [6]. In 10-15% of XY patients with male-to-female sex reversal (46,XY DSD) inactivating mutations in *SRY* have been identified, with the majority residing in the HMG domain, and affecting the binding and bending of DNA [7-8]. At the N- and C-terminal ends of this domain two nuclear localization signals (NLSs) are present. The biological effect of a number of the reported mutations can be explained by the fact that they reside in one of these NLSs, thereby disrupting nuclear import. In general, mutations in *SRY* can lead to failure of the bi-potential gonads to develop into testes with the consequence that testosterone and AMH will not be produced. Without testosterone, the external genitalia will not virilize, and the Wolffian ducts fail to develop into epididymis, vas deferens and seminal vesicles. Without AMH the Müllerian ducts will not regress, and subsequently develop into female internal reproductive organs, namely the fallopian tubes, uterus and upper portion of the vagina [6]. At birth, these patients can show a phenotypical female appearance, but they may present at puberty with absence of secondary sexual characteristics and primary amenorrhoea. This is due to complete gonadal dysgenesis and lack of ovarian function. It is therefore unusual to observe a GB in a presumed normal female after puberty.

GB consists of a mixture of embryonic germ cells (primordial germ cells/gonocytes) and supportive cells that resemble immature Sertoli/granulosa

cells [9]. It is recognized as the *in situ* germ cell malignancy that in a substantial number of cases will develop into an invasive tumor (dysgerminoma or, less frequently, non-dysgerminoma). Carcinoma *in-situ* (CIS), the precursor of the seminoma and non-seminomatous tumors in the testis, is the GB counterpart of the male gonad. It exists in close association with Sertoli cells [10]. The supportive cells in GB stain positive for the granulosa cell marker, FOXL2, and are (almost) always negative for the Sertoli cell marker, SOX9 [11]. This reflects a blockage in testicular differentiation. CIS, GB and seminoma, dysgerminoma as well as embryonal carcinoma are positive for OCT3/4, which is a reliable marker for type II GCTs with pluripotent potential [12-14]. OCT3/4 is also known as POU5F1, and is one of the transcription factors involved in regulation of pluripotency [14-16]. In normal development OCT3/4 is specifically found in primordial germ cells/gonocytes, but is absent at later developmental stages. In cases of maturation delay, as can be observed in DSD patients with hypovirilization and gonadal dysgenesis, this can lead to overdiagnosis [17]. Recently it was shown that stem cell factor (SCF) is an additional valuable marker, which distinguishes germ cells delayed in their maturation from malignant germ cells, being negative and positive, respectively [18]. Development of a GB is dependent on presence of part of the Y chromosome, known as the GBY region [19]. One of the putative candidate genes for the involvement of this region is *TSPY*, which denotes Testis Specific Protein on the Y chromosome [20]. The encoded protein is found to be highly expressed in CIS and GB [21].

In this study, we report a unique case of GB in a phenotypical adult woman of 26 years of age. In the DNA extracted from peripheral blood cells from this patient a novel mutation was identified in the N-terminal NLS of SRY, resulting in reduced nuclear import, leading to gonadal dysgenesis. This was identified on the basis of the diagnosis of a unilateral GB. Subsequent prophylactic removal of the remaining gonad indeed demonstrated the presence of another GB. This intervention prevented development of an invasive type II germ cell tumor, and the need of chemotherapy for treatment.

Materials and methods

Immunohistochemical staining

Immunohistochemistry was performed on paraffin-embedded slides of 3-µm thickness. The antibodies used directed against OCT3/4, SCF, TSPY, SOX9 and FOXL2, have been described before [11, 13, 18, 22-23]. Briefly, after deparaffinization and 5 min incubation in 3% H_2O_2 to inactivate endogenous

peroxidase activity, antigen retrieval was carried out by heating under pressure of up to 1.2 bar in appropriate buffer. After blocking endogenous biotin using the avidin/biotin blocking kit (SP-2001; Vector Laboratories, Burlingame, CA USA), sections were incubated either overnight at 4 °C (SCF, TSPY) or for 2 h at room temperature (OCT3/4, SOX9, FOXL2) and detected using the appropriate biotinylated secondary antibodies and visualized via avidin-biotin detection and substrate kits (Vector Laboratories). Double staining for FOXL2 and SOX9 was carried out as described before [11].

Fluorescent in situ hybridization

Slides of 5 μm thickness were deparaffinized and heated under pressure as described above. Slides were digested using 0.01% pepsin (Sigma Aldrich, St. Louis, MO USA) in 0.02 M HCl at 37 °C, with an optimal digestion time of 2.5 min. Slides were rinsed, dehydrated and the probes dissolved in hybridization mixture were applied. Probes for centromere X (BamHI) and centromere Y (DYZ3) were used, labeled with digoxigenin-11-dUTP and biotin-16-dUTP (Roche Diagnostics, Mannheim, Germany) using a nick-translation kit (Gibco BRL, Paisley, UK). After denaturation (80 °C for 10 min), hybridization overnight (37 °C) and washing steps, probes were visualized using Cy3-conjugated avidin (1 : 100, Jackson ImmunoResearch, West Grove, PA USA) and Sheep-anti-dig FITC (1 : 50, Roche Diagnostics) and analyzed using a fluorescent microscope (Leica Microsystems, Rijswijk, The Netherlands).

SRY functional study and plasmids

Direct sequencing of the SRY gene on peripheral blood DNA from the patient was done at the Department of Clinical Genetics. pcDNA3-FLAG-SRY and pcDNA3-FLAG-R75N plasmids have been described previously [24]. pcDNA3-FLAG-W70L was created by PCR with primers 5'-CATCATGGATCCGCCACCATGGACTACAAAGACGATGACGACAAGATGCAATC TTAT-3' containing a BamHI restriction site, KOZAK translational start site, FLAG-tag epitope, and SRY ATG site, and 5'-ATGATGAATTCTACAGCTTTGTCCAG-3' containing a *Eco*RI restriction site and the SRY TAG site using standard conditions. The PCR product was subsequently cloned into the pcDNA3 vector using the introduced restriction sites. All constructs were verified by sequencing.

NT2/D1 cells seeded in six-well plates were transfected with 2 μg per well of either pcDNA3-FLAG-SRY wild-type, pcDNA3-FLAG-R75N mutant or

pcDNA3-FLAG-W70L mutant using Fugene 6 (Roche Diagnostics). After transfection, immunohistochemistry was carried out using mouse monoclonal antibody against FLAG tag (1:400). The secondary antibody used was Alexa 488-conjugated donkey antimouse IgG (1:500, Molecular Probes, Eugene, OR USA). DNA was stained with 0.1 μg/ml of 4',6-diamidino-2-phenylindole (Molecular Probes). Image analysis was performed using NIH ImageJ (public domain software). SRY fluorescence was quantitated as described before [25]. Briefly, measurements (average of two independent transfections, n=50) were taken of the density of fluorescence from the cytoplasm and the nucleus with the background fluorescence subtracted from the equation: Fn/c = (n–bkgdn)/(cp–bkgdcp), where n is the nucleus and bkgdn is the background in the nucleus, cp the cytoplasm and bkgdcp the background in the cytoplasm.

Results

Clinical history

A phenotypical female patient aged 26 years presented at the clinic with abdominal pain on the right side, which had previously been diagnosed as irritable bowel syndrome. She had a history of treatment-resistant irregular menstrual cycles after menarche at the age of 14 years. Echographic examination revealed an enlarged ovary of 4.8 x 3 cm on the right side, suspected to be a dermoid cyst. A right-sided salpingoophorectomy was performed by laparoscopy.

Histological and immunohistochemical analyses

Histological examination revealed that the removed lesion thought to be a dermoid cyst was in fact an exceptionally large GB, of which a representative image of the hematoxylin and eosin staining is shown in Figure 1A. In agreement with this diagnosis, the germ cells showed positive staining for OCT3/4 (Figure 1B), TSPY (Figure 1C) and SCF (Figure 1D). Furthermore, in the supportiving cells, positive staining was observed for the granulosa cell marker FOXL2 (Figure 1E), while SOX9 was absent (Figure 1F). Within the lesion a small invasive component was identified by H&E staining and immunohistochemistry, being a mixture of dysgerminoma, immature teratoma and yolk-sac tumor (data not shown). Tumor markers collected 26 days after removal of the tumor showed the following: AFP 5 (normal range: 0-9 μg/l), hCG + beta hCG 2.0 (normal range: 0-6.9 IU/L), LDH 391 (normal range: 0-449 U/L). Although the time delay between surgery and serum sampling for

hormone level determination is considerable, it suggests that the tumor produced AFP as well as hCG. Based on the decline of the markers, as well as the (relative small) invasive component, situated in the middle of the tumor, the decision was taken not to treat the patient with chemotherapy, but surveillance was decided on. After 14 months, the patient is still relapse free, based on tumor marker status, namely AFP 1, hCG + beta hCG <0.1 and LDH 219. The presence of a GB, staining positive for TSPY, initiated discussion about the karyotype of the patient.

In situ hybridization and karyotyping

Fluorescent in situ hybridization was performed on the gonadal tissue, (see Figure 1G) and in parallel, karyotyping of peripheral lymphocytes was performed. Both approaches demonstrated the presence of both a single X and a Y chromosome per nucleus.

Prophylactically removed gonad

After intensive discussion with the patient, the remaining left gonad was surgically removed, which on laparoscopical analysis presented as a streak gonad. This was consistent with the non-functionality of the in situ gonad, based on the patients' requirement for hormonal support to prevent menopause after the initial surgery. Histological examination revealed predominantly a streak appearance, with a small GB lesion, confirmed by a positive staining for OCT3/4, TSPY, SCF (data not shown) and FOXL2 (Figure 2A). In contrast to the GB lesion in the right gonad, also SOX9-positive supportive cells were also identified (Figure 2B). Double staining for both FOXL2 and SOX9 showed that in the majority of cells expression was mutually exclusive; however a small subset of cells seemed to stain positive for both markers (Figure 2C and D).

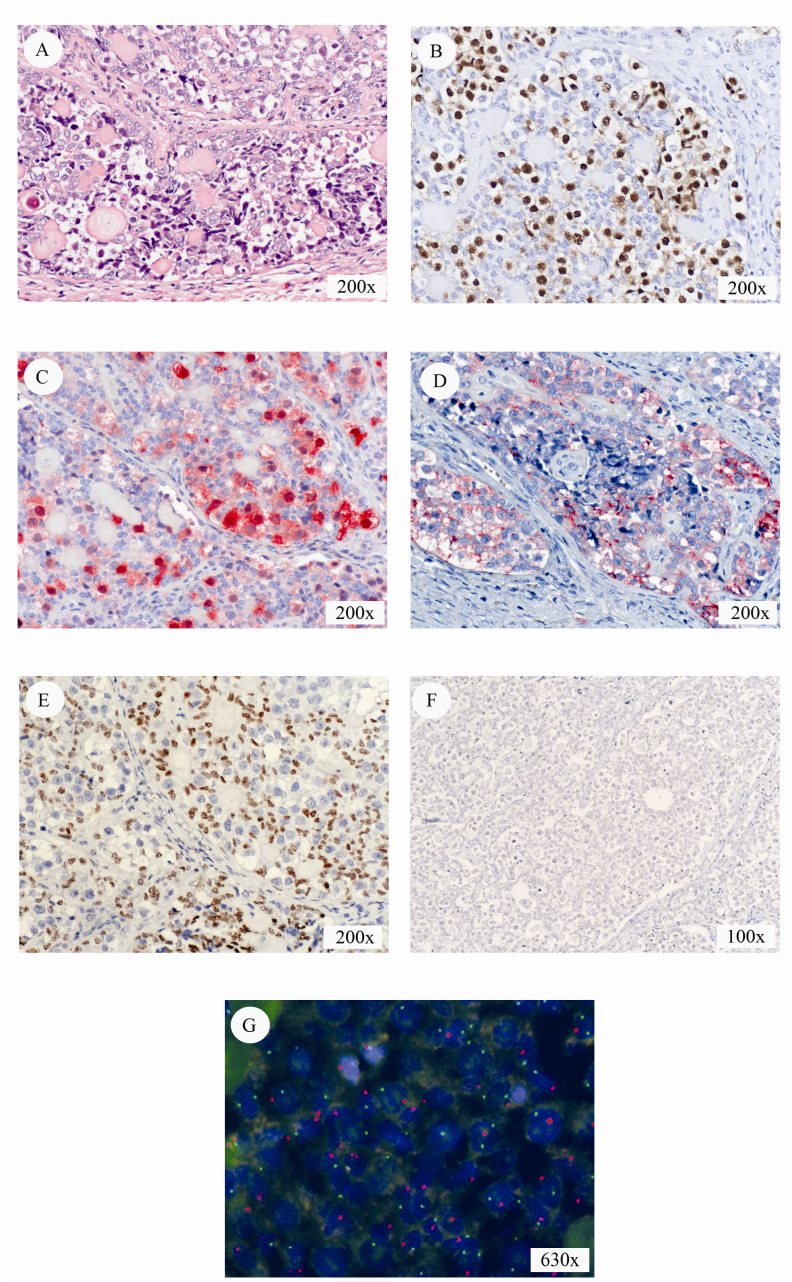

Figure 1. Immunohistochemical staining and fluorescent *in situ* hybridization of the right gonadoblastoma lesion. (A) Representative hematoxylin and eosin staining. The germ cells present in the GB stain positive for (B) OCT3/4, (C) TSPY and (D) SCF. (E) The supportive cells in the GB lesion are FOXL2 positive, and (F) SOX9 negative. Magnification 100x and 200x for all. Slides (B-F) are counterstained with hematoxilin. (G) Representative FISH with Y centromere shown in red and X centomere shown in green. Magnification 630x.

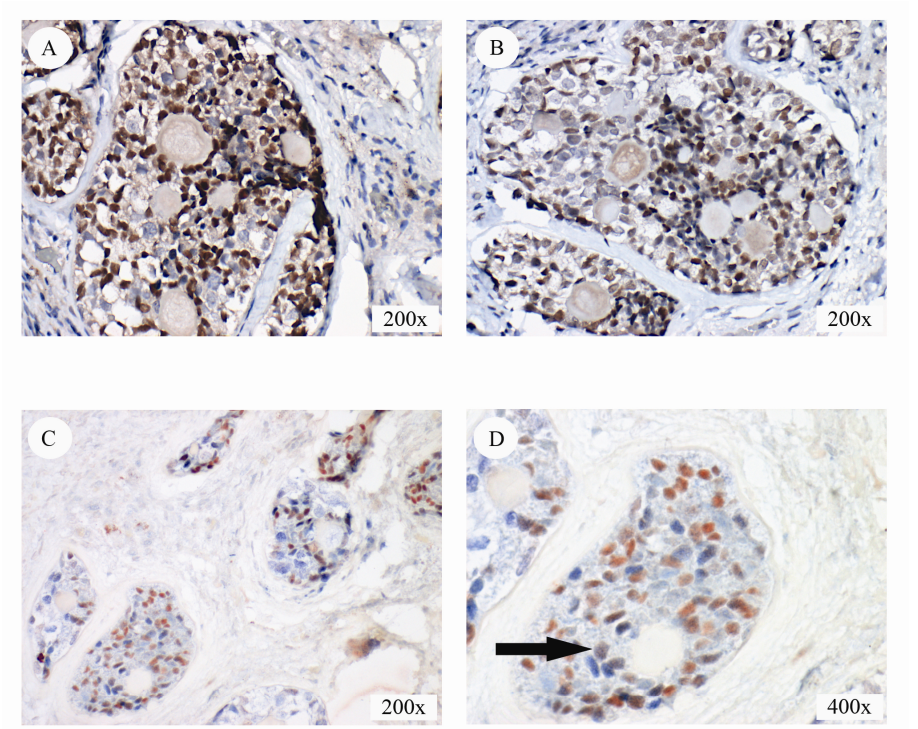

Figure 2. Immunohistochemical staining of the left gonadoblastoma lesion. (A) Supportive cells in the GB staining positive for FOXL2. (B) Also SOX9 positive supportive cells can be found. All slides are counterstained with hematoxylin. (C and D) Double staining for SOX9 (red) and FOXL2 (blue) of the left GB lesion. Note that although most cells stain positive for either SOX9 or FOXL2, a few cells seem to have co-expression of both markers, indicated by an arrow. Magnification, 200x and 400x for all.

Mutational and functional analysis of SRY

One of the first genes implicated in 46,XY sex reversal is *SRY*. Direct sequence analysis of the *SRY* gene revealed the presence of a single nucleotide change at position 209 (G to T, see Figure 3A), resulting in a missense mutation (tryptophan (W) to leucine (L) amino acid change) at position 70 in the SRY protein. A W70L missense mutation in SRY has not been reported to date.

The W70L sequence variant is located within the N-terminal NLS sequence of SRY (Figure 3B). Therefore the nuclear import activity of this SRY mutant was investigated in a cell-based transfection assay using expression plasmids encoding wild type and mutant full-length SRY. The subcellular localization of SRY was determined using indirect immunofluorescence. Wild-type SRY efficiently accumulated in the nucleus (Figure 3C, upper panel). As a positive control of a nuclear accumulation defect, the mutant R75N was included, showing a strong reduction of nuclear accumulation (28% of wild-type) consistent with a previous observation [24] (Figure 3C, middle panel and

Figure 3D). The mutant W70L also showed a significant reduction (P<0.0001) of nuclear accumulation to approximately 50% of wild type SRY activity (Figure 3C lower panel and Figure 3D).

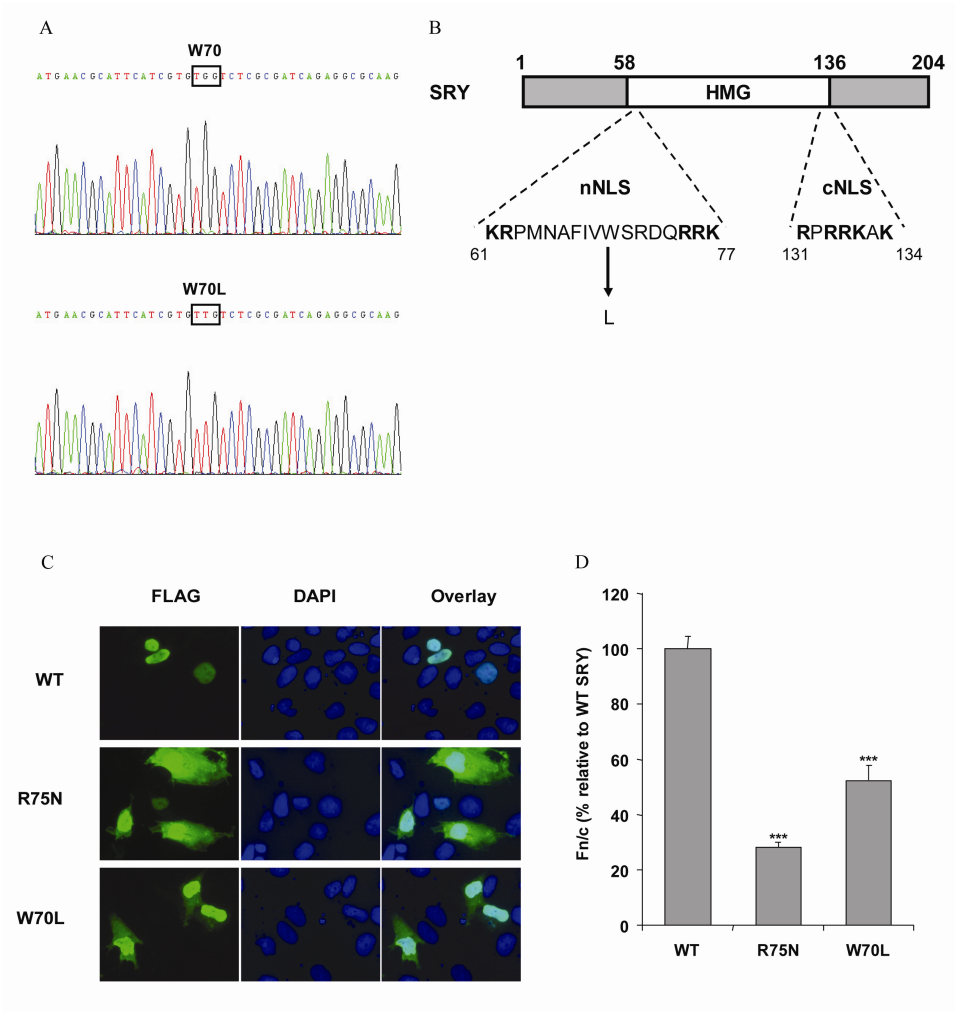

Figure 3. Mutational and functional analysis of SRY. (A) Wild-type (WT, upper panel, control) and mutated sequence (lower panel, patient) of SRY. The G209T mutation results in a tryptophan (W) to leucine (L) amino acid change at position 70 of the SRY protein. (B) Schematic representation of the SRY protein; HMG domain and both the N- and C-terminal NLS are indicated. The W70L mutation resides in the N-terminal NLS. (C) Immunofluorescent analysis of WT and mutant FLAG tagged full-length SRY constructs (upper panel; nuclear accumulation of WT SRY, middle panel; nuclear accumulation of SRY R75N, and lower panel; nuclear accumulation of SRY W70L). Note the reduction in nuclear accumulation for both mutants compared to WT. (D) SRY fluorescence was quantitated as described before [25]. The SRY R75N control shows a strong reduction of nuclear accumulation (28% of WT), in line with previous observation [24]. The mutant W70L SRY shows a significant reduction of nuclear accumulation of approximately 50%. Error bars represent the standard error of mean values. Two-tailed T-test of unpaired sample means was performed, p < 0.0001 for both.

Discussion

GB is specifically found as a premalignant lesion in DSD patients with, amongst others, 46,XY gonadal dysgenesis (Swyer's syndrome) (see Cools *et al* [2] for a review). The failure of the indifferent gonads to develop during embryogenesis into testes ultimately leads to a phenotypical female, who will present with delayed puberty and amenorrhoea. It is therefore unusual to find GB in a presumed normal female after puberty, as is observed in the patient described in this study.

The GB in the right gonad was unusually large, and on sonographic investigation suspected for a dermoid cyst. The tumor cells were positive for the pluripotency marker OCT3/4, which is specifically and consistently expressed in the neoplastic precursors of type II GCTs, ie, CIS in testis and GB in the dysgenetic gonad [14, 16, 26]. Presence of this protein is essential for pluripotency of embryonic stem cells; and early in development, expression becomes confined to the germ cell lineage. Loss of OCT3/4 expression in mouse primordial germ cells leads to apoptosis [27], and thus is required for their survival. Upon differentiation of primordial germ cells/gonocytes to either pre-spermatogonia or oogonia, OCT3/4 is lost. Therefore, it is informative to identify embryonic germ cells during normal development, as well as malignant germ cells.

As OCT3/4 cannot distinguish between malignant germ cells and germ cells with maturation delay, staining for SCF was performed. This marker has most recently been shown to specifically stain CIS and GB, whereas germ cells with proven maturation delay were negative [18]. As expected, positive SCF staining was observed.

The presence of, part of, the Y chromosome is necessary for development of a GB in a dysgenetic gonad. This is the so-called gonadoblastoma locus on the Y-chromosome (GBY) region as originally postulated by Page [19]. This genomic region contains *TSPY* as one of the likely candidate genes. This is supported by the strong expression of TSPY in CIS and the germ cells of GB [28], as is also the case in this patient. Normally, TSPY is expressed in spermatogonia of the adult testis and is thought to be related to mitotic proliferation [29]. In fact, it influences cell-cycle associated genes when transfected into NIH3T3 and HELA cells [30], as well as genes on chromosome 12p, including *KRAS2* and *NANOG* [31]. This is an intruiging finding because the chromosomal constitution of CIS cells is overall similar to that of invasive type II GCTs, although gain of 12p is established during progression to invasiveness [32-33].

The SOX9 and FOXL2 staining patterns seen in the right GB lesion of this patient can be explained by the differentiation state of the gonad. In a XY individual, after SRY, the expression of SOX9 [34] is necessary for testis development. SOX9 recruits a subset of cells in the indifferent gonad to the Sertoli cell fate and these orchestrate further testis formation and subsequent formation of the male phenotype [4, 35]. Activation of female gonadal development, which initially was believed to be the simple result of the absence of male development, requires activation of amongst others FOXL2. This transcription factor is required for the development of granulosa cells [36-37]. The presence of SOX9 and FOXL2, can be used as a readout of testicular and ovarian differentiation respectively. Moreover, stromal cells associated with GB are more granulosa like cells, in contrast to CIS, which is associated with Sertoli cells [11]. The absence of SOX9 expression in the right gonad of this patient has led to the development in a more female direction, ie, less male, as shown by the presence of FOXL2, which has led to the environment in which the GB has been able to develop. The expression pattern seen in the GB of the left gonad differs from our previous observations [11]. Besides FOXL2 positive cells, also some SOX9 staining, and even cells which seem to stain positive for both was observed. In this context it is of interest that XY Sox9$^{-/-}$ mice (in the presence of Sry) show expression of two ovarian genes (*Wnt4* and *Foxl2*) [38]. This indicates that Sox9 can downregulate Wnt4 and/or Foxl2 in a XY gonad. In addition, evidence that Sertoli and granulosa cells have a common precursor exists [39]. The recent report of a transcriptional (nuclear) function for SRY in the direct binding and regulation of the *SOX9* gene [5] raises the possibility that in this patient reduced SOX9 levels can be an initial consequence of SRY. Overall 50% of wild-type activity might place SRY at a threshold of activity, whereby in some cells sufficient levels of SOX9 are reached and so FOXL2 is downregulated, while in other cells this threshold is not reached, as shown by the FOXL2 positive and SOX9/FOXL2 double positive cells. In mice, transfer of Y chromosomes (SRY alleles) from certain strains onto the C57BL/6J mouse strain causes an abnormal gonadal development due to aberrant interaction with autosomal genes, leading to ovaries, ovotestis or delayed testis cord development in a XY background [40]. Our study increases the possibility that other gonadal dysgenesis patients with GB and a similar FOXL2 and SOX9 staining pattern may be due to the *SRY* mutation causing partial loss of function.

The presence of a GB staining positive for TSPY warranted further investigation into the karyotype of this patient, which was shown to be 46,XY by

FISH and blood karyotyping. Subsequent laparoscopic examination and removal of the left gonad showed it to be a streak gonad containing a small GB lesion, staining positive for OCT3/4, TSPY, c-KIT and SCF as expected, which, if left in place, would eventually have developed in an invasive malignant tumor.

The presence of SOX9 in the GB of the left gonad shows that although nuclear import of SRY is impaired, it has been able, in a subset of cells, to induce expression of downstream targets and initiate the male pathway. Intriguing in this respect is the fact that, in contrast to most patients with Swyer's syndrome, the patient indicated that she had always been attracted to male activities, and she considers herself to be bi-sexual. Sex-specific differences in brain morphology are driven by the action of steroid hormones produced by the gonads. Recent data suggest that also genetic differences and even SRY itself have a role [41-43]. Possibly reduced penetrance of the mutation in brain sex development, either by some expression of steroid hormones by the gonads or directly due to SRY expression in the brain, has led to a more male development in this patient. If the SRY mutation has arisen *de novo* or is in the germ line cannot be determined as DNA from relevant family members is not available, although the first hypothesis is most likely.

Taken together, the reduced ability of the novel W70L mutant SRY, as identified in this patient, to induce proper testis differentiation, reflected by the FOXL2 staining in the supportive cell lineages, created an environment in which germ cells were delayed in maturation. Prolonged expression of OCT3/4 and presence of TSPY are suggested to allow subsequent survival and proliferation of these germ cells, which over time developed into a GB and invasive components. Knowledge about these issues will allow early (molecular) diagnosis of patients with a high risk for development of a malignant germ cell tumor, leading to prophylactic removal of the gonad and prevention of formation of an invasive cancer.

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Chapter 7

A 46,XY female DSD patient with bilateral gonadoblastoma, a novel SRY missense - combined with a WT1 KTS splice-site mutation

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Chapter 7

Abstract

Patients with Disorders of Sex Development (DSD), especially those with gonadal dysgenesis and hypovirilization are at risk of developing malignant type II germ cell tumors/cancer (GCC) (seminoma/dysgerminoma and nonseminoma), with either carcinoma *in situ* (CIS) or gonadoblastoma (GB) as precursor lesion. In 10 -15% of 46,XY gonadal dysgenesis cases (i.e. Swyer syndrome), *SRY* mutations, residing in the HMG (High Mobility Group) domain, are found to affect nuclear transport or binding to and bending of DNA. Frasier syndrome (FS) is characterized by gonadal dysgenesis with a high risk for development of GB as well as chronic renal failure in early adulthood, and is known to arise from a splice site mutation in intron 9 of the Wilms' tumor 1 gene (*WT1*). Mutations in *SRY* as well as *WT1* can lead to diminished expression and function of SRY, resulting in sub-optimal *SOX9* expression, Sertoli cell formation and subsequent lack of proper testicular development. Embryonic germ cells residing in this unfavourable micro-environment have an increased risk for malignant transformation. Here a unique case of a phenotypically normal female (age 22 years) is reported, presenting with primary amenorrhoea, later diagnosed as hypergonadotropic hypogonadism on the basis of 46,XY gonadal dygenesis with a novel missense mutation in *SRY*. Functional *in vitro* studies showed no convincing protein malfunctioning. Laparoscopic examination revealed streak ovaries and a normal, but small, uterus. Pathological examination demonstrated bilateral GB and dysgerminoma, confirmed by immunohistochemistry. Occurrence of a delayed progressive kidney failure (focal segmental glomerular sclerosis) triggered analysis of *WT1*, revealing a pathogenic splice–site mutation in intron 9. Analysis of the *SRY* gene in an additional five FS cases did not reveal any mutations. The case presented shows the importance of multi-gene based diagnosis of DSD patients, allowing early diagnosis and treatment, thus preventing putative development of an invasive cancer.

Introduction

Disorders of Sex development (DSD) are congenital conditions of incomplete or disordered gonadal development leading to discordance between genetic sex, gonadal sex, and phenotypic sex [1]. DSD occurs with an estimated incidence of 1:5000 [1]. Individuals with an underlying DSD, especially those with specific Y chromosomal material in their karyotype, have an increased risk for developing a type II germ cell tumor/cancer (GCC) [2]. GCCs arise from primordial germ cells (PGC) or gonocytes and can be subdivided into seminomas/dysgerminomas and non-seminomas with carcinoma *in situ* (CIS) or gonadoblastoma (GB) as precursor lesions [3-4]. GCC risk varies, but is estimated to be over 30% in patients with complete gonadal dysgenesis and is often bilateral [2].

Frasier syndrome (FS), currently classified as 46,XY DSD, complete gonadal dysgenesis, is characterized by gonadal dysgenesis, a high risk for development of a GCC and chronic renal failure in early adulthood. Usually patients with complete gonadal dysgenesis are not diagnosed at birth because of their normal female appearance of external genitalia. However, these patients will not develop secondary sex characteristics at pubertal age, and will generally attend the clinic because of primary amenorrhea, with hormonal analysis showing hypergonadotropic hypogonadism because of lack of gonadal function.

Wilm's Tumor 1 (WT1) is an important regulator of early gonadal and kidney development [5]. It is expressed earlier in time than SRY in the urogenital ridge, from which the gonads and kidneys are derived. All known WT1 isoforms share four C-terminal zinc fingers which are necessary for DNA/RNA binding. The two major WT1 isoforms are produced by alternative splicing, resulting in an insertion (+KTS) or exclusion (-KTS) of lysine, threonine and serine between zinc fingers three and four. The –KTS isoform mainly plays a role in transcription, and *AMH* transcriptional activation in Sertoli cells [6]. The +KTS isoform is involved in RNA processing, and in the mouse plays a role in *Sry* regulation *in vivo* [7].

Essential in the process of sex determination is the presence of the sex determining region on the Y chromosome (SRY) gene. If sufficient levels of SRY are present, expression of the transcription factor SOX9 is induced, resulting in formation of Sertoli cells and subsequent testis development [8]. If no functional SRY is present, specific stromal cells will follow the female pathway, become granulosa cells, and the gonad will develop as an ovary [9]. In the absence of the hormones produced by the testis there will be no sex differentiation in the male direction; in the absence of testosterone the Wolffian

ducts will not develop into epididymis, vas deferens and seminal vesicles. Without Anti-Müllerian Hormone (AMH) there will be no regression of the Müllerian ducts, and these will develop into normal female internal organs, i.e. fallopian tubes, uterus and upper part of the vagina [10].

SRY mutations residing in the HMG (High Mobility Group) domains are found in 10 -15% of the 46,XY gonadal dysgenesis cases and affect binding to and bending of DNA or nuclear transport [11-14]. As a consequence these mutations can lead to an early error in the process of sex determination preventing proper formation of a testis. Specific intron 9 splice site mutations in *WT1* resulting in a decreased WT1+KTS isoform are typically found in FS patients, leading to a diminished expression of SRY and subsequently SOX9, thereby disturbing testicular development [15]. Furthermore, knockout mice for the +KTS isoform showed sex reversal in males [16]. Thus both *SRY* and *WT1* mutations can cause (complete) sex reversal.

A highly informative marker for the presence of type II GCCs (i.e. GB, CIS and their invasive counterparts dysgerminoma and seminoma as well as embryonal carcinoma) is the transcription factor OCT3/4, also known as POU5F1 [17]. OCT3/4 is involved in the regulation of pluripotency, is expressed in PGCs and gonocytes during normal gonadal development, is required for PGC survival, and is lost after maturation to pre-spermatogonia in males and oogonia in females [17-20]. In DSD patients OCT3/4 positivity of the germ cells might be due to maturation delay and not due to malignant transformation. To distinguish between these, Stem Cell Factor (SCF, also known as KITLG) has been shown to be informative [21]. GB arises in the context of granulosa cells, staining positive for FOXL2 and negative for SOX9 (a Sertoli cell marker), this in contrast to the precursor lesion arising in a testicular environment, being CIS, in which the supportive (Sertoli) cells are negative for FOXL2 and stain positive for SOX9 [22].

Here, we present a unique case with bilateral GB and dysgerminoma in an adult woman presenting with primary amenorrhea at the age of 22 years, who was initially diagnosed with 46,XY gonadal dysgenesis. Mutation analysis identified a novel missense mutation (c.383A>G, p.Lys128Arg) in the HMG domain of the *SRY*, which did not have a significant effect on transcriptional activation and nuclear import *in vitro*. Laparoscopy revealed streak ovaries with GB and dysgerminoma on both sides. During follow-up the patient developed progressive renal failure based on focal glomerulosclerosis. Subsequent analysis of the *WT1* gene revealed a splice site exon 9 mutation (IVS9 +5 G>A) resulting in the final diagnosis FS. Sequence analysis of DNA from five additional FS

patients with a proven *WT1* mutation for *SRY* mutations did not reveal any variants, indicating that the presence of mutations in both genes in FS patients is rare. To our knowledge this is the first case describing a patient with a mutation in both *WT1* and *SRY*, and underlines the importance of proper diagnosis, especially in patients with an increased risk for GCC, allowing early diagnosis and treatment, thus preventing the development of invasive cancer.

Materials and methods

Tissue samples

Collected tissue samples were diagnosed according to WHO standards [23] by an experienced pathologist in gonadal pathology, including GCC (JWO). Use of tissue samples for scientific reasons was approved by an institutional review board (MEC 02.981 and CCR2041). 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 (FMWV (Version 2002, updated 2011).

Immunohistochemical staining

Immunohistochemical staining was performed on formalin fixed paraffin embedded samples of 3 µm thickness. The antibodies directed against OCT3/4, c-KIT (CD117), Stem Cell Factor (SCF), Testis Specific Protein on the Y chromosome (TSPY), SOX9 and FOXL2 have been described before [21-22]. Briefly, after deparaffinization and 5 min incubation in 3% H_2O_2 for inactivating endogenous peroxidase activity, antigen retrieval was carried out by heating under pressure of up to 0.9 bar in an appropriate buffer. After blocking endogenous biotin using the Avidin/Biotin Blocking Kit (SP-2001; Vector Laboratories, Burlingame, CA, USA), sections were incubated either overnight at 4°C (SCF, c-KIT, TSPY) or for 2h at room temperature (OCT3/4, SOX9, FOXL2) and detected using the appropriate biotinylated secondary antibodies and visualized using the avidin–biotin detection and substrate kits (Vector Laboratories).

SRY sequencing

Direct sequencing of the *SRY* gene on peripheral blood DNA from the patient was performed at the department of clinical genetics (reference sequence: NM_003140.1). For the additional samples DNA was isolated from either peripheral blood lymphocytes (4 patients) or from formalin fixed paraffin embedded material (from two independent blocks, 1 patient) according to

standard procedures. SRY was PCR amplified, analyzed on a 1% agarose gel, purified using the Agencourt AMPure XP kit (Beckman Coulter genomics, Danvers, MA, USA) and Sanger sequencing was done according to standard procedures.

SRY transactivation assay

DNA encoding wild type SRY, mutant SRY and SF1 were cloned into the pcDNA3 mammalian expression plasmid (Clontech, Mountain View, CA, USA), and sequence verified. To test for SRY activation of TESCO, *in vitro* luciferase assays were performed on a human embryonic kidney carcinoma cell line (HEK293T). Cells were cultured in DMEM, High Glucose, GlutaMAX media (Invitrogen, Life Technologies, Paisley, UK) containing 10% Fetal Bovine Serum, 1% sodium pyruvate and 1% penicillin-streptomycin. Cultures were grown at 37°C with 5% CO2. Cells were seeded in serum-free media 24 hours prior to transfection in 96-well tissue culture plates at a density of 30,000 cells per well.

Cells in each well were co-transfected with the reporter constructs TESCO-E1b-*Luc* (10 ng) or the empty vector E1b-*Luc* (8 ng), together with 40 ng of each of the expression constructs pcDNA3-SF1 and either pcDNA3-hSRY (wild-type) or pcDNA3-SRY-K128R (mutant). The reporter constructs contained the minimal E1b promoter driving a luciferase gene. pRL-TK-Renilla (Promega, Madison, WI, USA; 1 ng) was added to each well as an internal control. pcDNA3 and pUC DNA were added to make up a total of 100 ng DNA per well, and transfection was performed with 0.38 µl of FuGENE6 Transfection Reagent (Roche, Basel, Switzerland) following manufacturer's instructions. Cells were lysed 48h after transfection and firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega).

Six independent assays were performed, each in triplicate. Firefly luciferase activity (Luc) was normalized against that of Renilla luciferase (Ren). Luc/Ren readings for TESCO-E1b-*Luc* were further normalized against that of E1b-*Luc* to obtain the fold change of TESCO activity over that of the empty vector. Fold change of the mutant SRY-K128R construct was then normalized against that of wild-type SRY. Data are therefore represented in the form of mean percentage of wild-type SRY fold change. Statistical analysis was performed by conducting an unpaired t-test.

SRY nuclear import assay

pcDNA3-FLAG-SRY plasmid has been described previously [13]. pcDNA3-FLAG-K128R was created using site-directed mutagenesis. All constructs were verified by sequencing.

HEK293T cells seeded in 6-well plates were transfected with 2 µg/well of either pcDNA3-FLAG-SRY wild-type or pcDNA3-FLAG-K128R mutant using Fugene 6 (Roche). After transfection, immunohistochemistry was carried out using mouse monoclonal antibody against FLAG tag (1:400). The secondary antibody used was Alexa 488-conjugated donkey antimouse IgG (1:500, Molecular Probes, Life technologies). DNA was stained with 0.1 µg/ml of 4',6-diamidino-2-phenylindole (Molecular Probes, Life technologies). Image analysis was performed by using NIH ImageJ (public domain software). Briefly, measurements were taken of the density of fluorescence from the cytoplasm and the nucleus with the background fluorescence subtracted from the equation: Fn/c = (n – bkgdn)/(cp – bkgdcp), where n = nucleus and bkgdn = background in the nucleus, cp = cytoplasm and bkgdcp = background in the cytoplasm.

WT1 mutation analysis

Mutation analysis was performed at the department of clinical genetics of the Amsterdam Medical Center. Briefly: Exon 9 of the WT1 gene (NM_024426, but with the translation initiation codon starting at c.395), including flanking intronic sequences, was amplified by PCR followed by direct sequencing using Bigdye v1.1 chemistry and an ABI3100 sequencer (Life Technologies, Carlsbad, CA, USA). Sequences were analyzed using Codoncode Aligner (CodonCode Corporation, Dedham, MA, USA).

Results

Patient clinical history

A phenotypically normal female presented at the outpatient clinic with primary amenorrhoea at the age of 22. She reported to have had some vaginal bleeding at the age of 13 and 14 years which she thought was the start of menarche. This together with the fact that she grew up in different families was the reason of her late clinical presentation. Patient history mentioned migraine and severe asthma for which she was treated with corticosteroids. Physical examination showed normal female external genitalia, with Tanner stage III breast development and stage II pubic hair development. She had a scoliosis and 2.5 cm difference in length of her legs. Hormonal analyses at the age of 22 and 23 years revealed low oestradiol: 11 and <10 pmol/L (normal 100-1000 pmol/L),

testosterone: 1 nmol/L (normal 0.5-3 nmol/L), high FSH: 215 and 219 IU/L (normal 1-8 IU/L), and high LH: 78 and 75 IU/L (normal 2-8 IU/L) levels, indicating hypergonadotropic hypogonadism. Furthermore an increase in serum creatinine levels 111-217 umol/L (normal 90 umol/L) was found over the course of ten months suggestive of impaired kidney function, although not diagnosed at the time of presentation. Chromosome analysis on peripheral blood lymphocytes showed the presence of a 46,XY karyotype, and mutational analysis of the *SRY* gene revealed an, at that time, unclassified variant K128R (c.383 A>G, p.Lys128Arg). Based on these results the patient was diagnosed with 46,XY gonadal dysgenesis. Laparoscopic examination showed streak ovaries and a normal, but small, uterus. Because of the known tumor risk in these patients, both ovaries were removed during this intervention (for histology, see below).

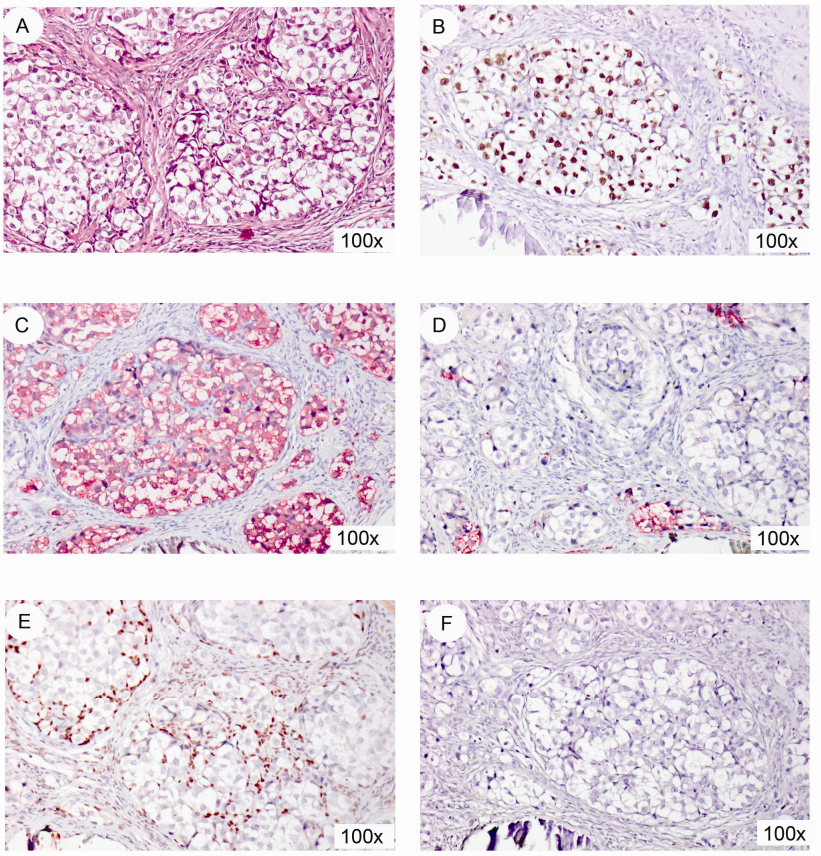

Figure 1: Immunohistochemical staining of the left GB lesion. (A) representative hematoxylin and eosin (HE) staining. The germ cells present in the GB stain positive for OCT3/4 (B), TSPY (C), and SCF (D). Supportive cells in the GB stain positive for FOXL2 (E), while SOX9 (F) is negative. All slides are counterstained with hematoxylin. Magnification 100x for all.

Two months after gonadectomy the patient visited the emergency room with complaints of agonizing headache, which were caused by severe hypertension; her blood pressure was 200/127 mmHg, with a good response to treatment with Amlodipine. In addition, blood analyses showed severe renal failure and additional examinations showed that the progressive renal failure was due to primary focal glomerulosclerosis. The rapid progression of kidney failure together with the diagnosis of 46XY gonadal dysgenesis and bilateral GB and dysgerminoma (for histology, see below) triggered investigation for a *WT1* mutation. The patient is currently on haemodialysis and awaits kidney transplantation, which has to be postponed for five years (until 2014) due to the treatment of the GCC.

Histological and Immunohistochemical analysis

Histological examination of both gonads showed that GB and dysgerminoma was present in a dysgenetic histological context. The lesions on both sides were restricted to the gonad. A representative image of the hematoxylin and eosin (H&E) staining is shown in Figure 1A. In agreement with this diagnosis, the germ cells showed positive staining (shown only for the left GB lesion) for OCT3/4 (Figure 1B), TSPY (Figure 1C) and SCF (Figure 1D). In addition the supportive cells stained positive for FOXL2 (granulosa cell marker, Figure 1E) and were negative for SOX9 (Sertoli cell marker, Figure 1F). The GB removed from the other side showed a similar staining pattern for all markers investigated (data not shown). Both gonads showed multiple micro-calcifications (microlithiasis), represented in the images of Figure 1.

Mutation analysis and functional analysis of SRY

Direct sequencing of the *SRY* gene showed the presence of a single nucleotide change at position 383 (A to G, see Figure 2A), resulting in a missense substitution (Lysine (K) to Arginine (R) amino acid change) at position 128 in the SRY protein (hemizygous pattern). A K128R missense mutation in SRY has not been reported to date. The K128R sequence variant is located within the HMG domain of SRY next to the C-terminal nuclear import signal (cNLS) (Figure 2B).

SRY activates *SOX9* expression together with SF1 via a testis-specific enhancer called TESCO, which is located approximately 13 kb upstream of *SOX9* [24]. The ability of the SRY K128R mutant form of SRY to activate *SOX9* via TESCO was analyzed. Results show that the K128R mutation of *SRY* did not

significantly affect TESCO activity *in vitro* compared to wild-type SRY (Figure 2C), although a reduction of about 20% was observed.

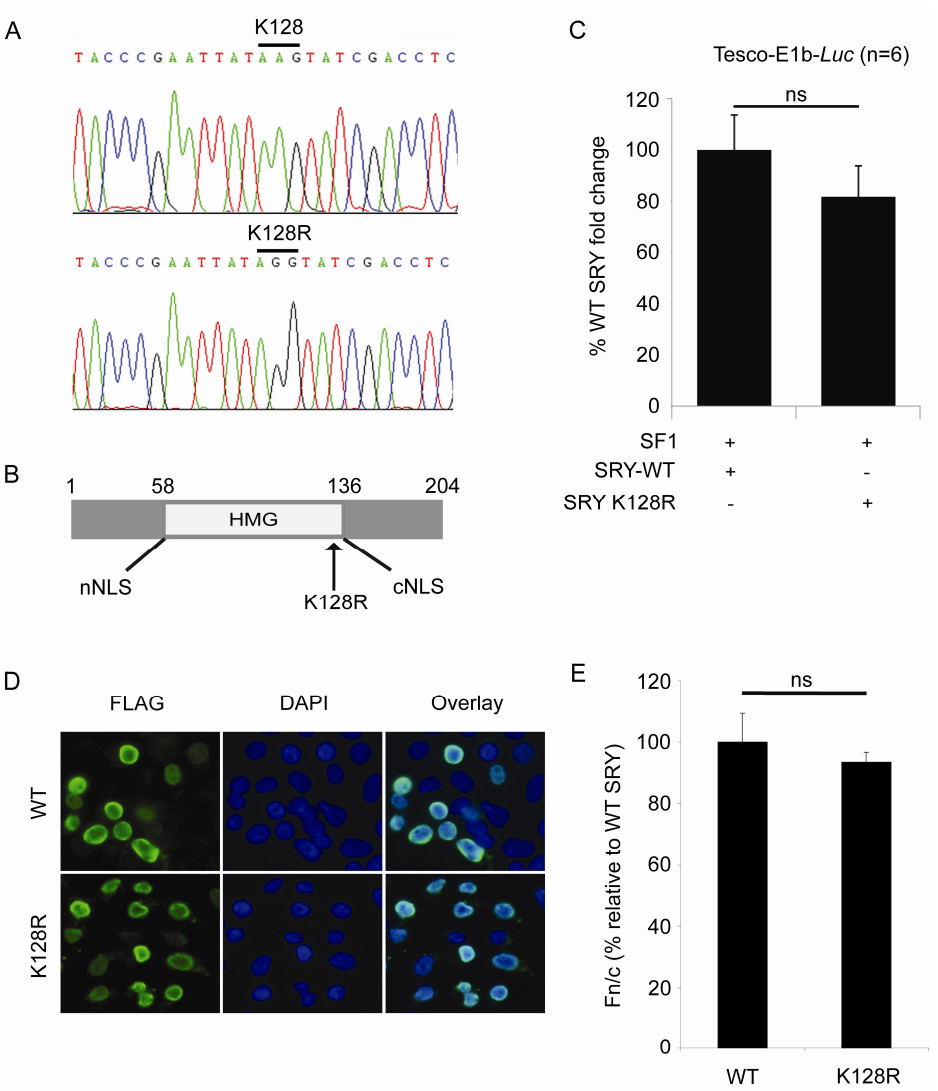

Figure 2: Mutational analysis of SRY. (A) wild type (upper panel, control) and mutated sequence (lower panel, patient) of SRY. (B) schematic representation of the SRY protein. The K128R mutation resides in the HMG domain, just before the cNLS. (C) *In vitro* luciferase assays of SRY-WT (wild-type) and SRY-K128R (mutant) in HEK293T cell line. Cells were co-transfected with TESCO-E1b-*Luc*, SF1 and WT or mutant SRY to assess for activation of TESCO. The mean percentages of fold change of luciferase activity of TESCO-E1b-*luc* over the empty vector, relative to WT SRY levels from six independent assays (each performed in triplicate) are shown. Error bars represent standard error of the mean (SEM). (D) pcDNA3-FLAG-SRY wild-type (WT, 2 µg) or pcDNA3-FLAG-SRY mutant (K128R, 2 µg) were transiently transfected into HEK293T cells using Fugene 6. Exogenous

SRY (WT or K128R) expression was detected using a FLAG antibody and a green fluorescent Alexa-488 dye coupled secondary antibody. Nuclei were stained with 4',6-diamino-2-phenylindole (DAPI). Both wild type and mutant SRY show strong nuclear staining. (E) SRY fluorescence was quantified as previously described [35]. Nuclear accumulation of SRY (WT or K128R) expressed as fluorescence in the nucleus over that in the cytoplasm (Fn/c) were background fluorescence has been subtracted. Measurements represent the average of 3 independent transfections. Results are relative to WT transfected cells (Fn/c given value of 100%). The number of cells analysed is n=111 (WT) and n=121 (K128R). Error bars represent the standard error of mean values. Two-tail t-Test of unpaired sample means was performed between WT transfected cells and mutant transfected cells and showed no significant differences. P=0.49

As the K128R substitution is located next to the cNLS, the effect on nuclear import was also investigated using expression plasmids encoding wild-type and mutants full-length SRY transfected in HEK293T cells. The subcellular localization of SRY was determined 48 h after transfection using indirect immunofluorescence and quantified using image analysis (Figure 2D and E). Wild type SRY efficiently accumulated in the nucleus. The mutant K128R also showed a slight reduced but non-significant difference in nuclear accumulation compared to the wild type protein, indicating that the K128R mutation does not affect the nuclear import function of SRY.

Mutation analysis WT1 and additional FS samples analyzed

As the patient had 46,XY gonadal dysgenesis together with renal failure (focal segmental glomerulosclerosis), and GB with dysgerminoma, without Wilm's tumor, all pointing to FS, the *WT1* gene was analyzed. Direct sequencing of the *WT1* gene showed a single nucleotide change at the start of intron 9 at the position +5 (IVS9 + 5G > A) in a heterozygous state (data not shown), characteristic for FS.

To determine if *SRY* mutations together with *WT1* mutations were present in other DSD cases with the same clinical characteristics a review of the literature was done (supplementary Table 1 and 2), showing that this has not been investigated to date. Therefore an additional five DNA samples from FS patients with a proven *WT1* mutation were analyzed for *SRY*, showing no aberrations in *SRY* in addition to the *WT1* mutation.

Discussion

Sex determination and specifically testis differentiation in males is critically dependent on transcriptional regulation of a selective number of genes including *WT1, SRY*, and *SOX9* [25-26]. Expression of the Y-chromosome located *SRY*, above a threshold and in a critical time window, is crucial in

triggering testis formation. SRY will upregulate SOX9 which will orchestrate the formation of the pre-Sertoli cells and further regulates testis development. *WT1* is expressed in the gonadal ridges before the onset of SRY, and plays an important role in testicular as well as kidney formation. It has been suggested that the WT1+KTS isoform functions in terminal Sertoli cell differentiation and homeostasis through the maintenance of a critical level of *SRY* and *SOX9* expression [15].

SRY mutations play a role in 46,XY sex reversal (46,XY DSD) and in about 15% of 46,XY gonadal dysgenesis cases mutations are found [27]. The majority of mutations reside in the HMG domain, which is involved in the binding and bending of DNA. Besides these, mutations located in one of the NLSs have been reported, resulting in a reduced nuclear import of SRY. The K128R mutation described here does not lead to a statistically significant reduction in transactivational activity as ascertained by an *in-vitro* assay, although a minor reduction (about 20%) was observed. In addition, although located adjacent to the cNLS of SRY, the mutation does not result in a significant reduction in nuclear import of the protein. This suggests that the phenotype of the patient is not due to a nuclear import defect as has been observed in other cases [13-14, 28]. Although the lysine on position 128 is conserved between man and mouse, mutation of lysine on position 128 to arginine does not affect regulation of SRY subcellular distribution by (de-)acetylation via p300 [29]. Taken together, the results show that the mutation has little effect on the *in vitro* transactivation and nuclear import assays available. Therefore it is unlikely that the SRY K128R mutation has a significant effect on the (gonadal) phenotype of the patient has, although a more dramatic effect of the mutation in an *in vivo* situation cannot be ruled out.

Reviewing the literature shows that almost all gonadal dysgenesis cases with a proven *SRY* mutation (86 cases in total, supplementary Table 1) show a female phenotype (n=81, 94%). Only a few cases show ambiguous genitalia (n=4), and one patient has a male phenotype with ambiguous genitalia (respectively 5% and 1%). In a total of 61 cases gonadal histology was analyzed: 18 showed a GB (30%), one a dysgerminoma (1%) and two GB along with dysgerminoma (3%). This strongly shows the known increased GCC risk in these patients (34% in this cohort). Only a limited number of papers describe the functionality of *SRY* mutations (20 in total, 23%), and the effects range from completely abolished DNA binding to no differences in DNA binding when compared to wild type SRY. Based on these data, no genotype-phenotype correlation can be gathered (supplementary Table 1). In some cases the

mutations described are also present (in mosaic form) in male family members, with one showing hypospadias and cryptorchidism, one diagnosed with a testicular seminoma, and one without GCC and a normal male phenotype [30-32] (refs 14, 15 and 54 in supplementary Table 1). Whether this is also the case in the patient described here, or the mutation arose *de novo,* cannot be investigated because family members are not available for analysis (see above).

The patient described here was initially diagnosed as a 46,XY DSD complete gonadal dysgenesis and a (until now unclassified) mutation in *SRY* was found (i.e. Swyer syndrome), associated with GB and dysgerminoma. However, upon follow-up the diagnosis of progressive renal failure based on focal segmental glomerulosclerosis, prompted analysis of the *WT1* gene. Initially the mild renal impairment found at presentation was not considered to be indicative to screen *WT1* for mutations.

Mutations in *WT1* play a role in 46,XY DSD (i.e. FS, Denys-Drash syndrome, and WAGR-syndrome), and those found in FS consist of *WT1* intron 9 splice-site mutations. These patients have complete 46,XY sex reversal, late onset kidney failure (between 10-20 years), focal segmental glomerulosclerosis, streak gonads, and a high risk for GB, but not Wilm's tumors [33]. Sequence analysis of the *WT1* gene in the patient described here revealed a classic FS mutation in the intron 9 splice-site (IVS9 +5 G>A). This ultimately results in the decrease of the +KTS isoform and it is known that the subsequent reversion in +KTS/-KTS ratio causes defects in the development of glomerular podocytes and male sex-determination, ultimately leading to nephrotic syndrome and male-to-female sex reversal, respectively [33-34]. Careful review of the literature revealed that this is the first patient described having both a *WT1* as well as a *SRY* mutation; however in almost all cases described a mutation screen of both *SRY* and *WT1* was not performed. Analysis of five additional FS patient samples with a proven *WT1* mutation by conventional Sanger sequencing of the *SRY* gene did not reveal any mutations. The majority of FS patients described in literature are phenotypically females (n=48, 96%) and only two phenotypically males are presented (4%, supplementary Table 2). It also underlines the high incidence of GB and/or dysgerminoma in this patient group; 18 out of 39 patients with described gonadal histology showed GB (46%), in one patient carcinoma *in-situ* (CIS) is described, the precursor lesion of GCC in the testis, and in one patient GB next to CIS is described. Five patients had an invasive dysgerminoma next to the GB, one patient is described as having GB and a metastatic tumor, and one patient is mentioned as having dysgerminoma. In the other patients with described gonadal histology, the majority show streak

gonads (n=17, 44%), in one it is described as a dysgenetic gonad and in one no gonadal tissue could be found. For the other patients no gonadal histology was analyzed (n=11).

It has been described that *SRY* and *SOX9* expression can be diminished in FS [15] and one could speculate that in the case presented here the effects from reduced *SRY* expression by a mutated *WT1* were exacerbated by the presence of the SRY K128R mutation, although a reduced SRY function could not be shown conclusively *in vitro*. This situation may have contributed to the maldevelopment of the gonads, thereby creating the micro-environment in which embryonic germ cells can survive, and are prone to become malignant. However, screening an additional five FS patients with a proven *WT1* mutation did not reveal any sequence variants in *SRY*. Although this is a limited series of these unique cases, it indicates that presence of *SRY* mutations in FS is rare.

To our knowledge this is the first patient described with a mutation in *SRY* together with a classical FS *WT1* mutation, and thus seems to be a rare condition. Nonetheless, in this patient an optimal diagnosis could have been made, if a screening for *WT1* mutation was performed at an earlier time point. The patient is currently on haemodialysis and awaits kidney transplantation, which has to be postponed for five years (until 2014) due to the GCC in her history. This case clearly demonstrates the significant role of proper diagnosis of the variants of DSD, especially in those with an increased risk for GCC, allowing early diagnosis and treatment, thus preventing the development of invasive cancer. The presence and type of *WT1* mutation has major consequences for the patient. We therefore suggest that *WT1* mutation screening should be performed in all patients with 46,XY gonadal dysgenesis, especially in case of an unclassified SRY variant, and not vice versa. In addition, careful evaluation of kidney function at early stage is recommended in these patients.

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Chapter 8

SRY mutation analysis by next generation (deep) sequencing in a cohort of chromosomal Disorders of Sex Development (DSD) patients with a mosaic karyotype

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Chapter 8

Abstract

The presence of the Y-chromosome or Y chromosome-derived material is seen in 4-60% of Turner syndrome patients (Chromosomal Disorders of Sex Development (DSD)). DSD patients with specific Y-chromosomal material in their karyotype, the GonadoBlastoma on the Y-chromosome (GBY) region, have an increased risk of developing type II germ cell tumors/cancer (GCC), most likely related to TSPY. The Sex determining Region on the Y gene (*SRY*) is located on the short arm of the Y-chromosome and is the crucial switch that initiates testis determination and subsequent male development. Mutations in this gene are responsible for sex reversal in approximately 10-15% of 46,XY pure gonadal dysgenesis (46,XY DSD) cases. The majority of the mutations described are located in the central HMG domain, which is involved in the binding and bending of the DNA and harbors two nuclear localization signals. *SRY* mutations have also been found in a small number of patients with a 45,X/46,XY karyotype and might play a role in the maldevelopment of the gonads. To thoroughly investigate the presence of possible *SRY* gene mutations in mosaic DSD patients, we performed next generation (deep) sequencing on the genomic DNA of fourteen independent patients (twelve 45,X/46,XY, one 45,X/46,XX/46,XY, and one 46,XX/46,XY). The results demonstrate that aberrations in *SRY* are rare in mosaic DSD patients and therefore do not play a significant role in the etiology of the disease.

Background

The development of a mammalian embryo into either female or male is primarily dependent on the sex chromosomal constitution, being XX and XY respectively. Normal male (46,XY) sex determination relies on the presence of the Y-chromosome, specifically on expression of *SRY* at the appropriate time and place during gonad development. Timely expression of this gene above a critical threshold is necessary to trigger testis formation [1-2]. If sufficient SRY is present, SOX9 will be up-regulated, leading to the formation of pre-Sertoli cells [3]. This will further orchestrate the formation of a functional testis, ultimately leading to the development of male primary and secondary sex characteristics [2]. In a 46,XX constitution, (i.e. the absence of the Y-chromosome and *SRY*) supportive cells in the gonad will, under the influence of FOXL2, WNT4, and RSPO1 amongst others, develop as granulosa and theca cells, leading to the formation of a functional ovary and female primary and secondary sex characteristics [2].

Turner syndrome (TS) is characterized by gonadal dysgenesis, short stature, and dysmorphic features (neck webbing amongst others). In 6 to 11 % of cases a cell line with a normal or abnormal Y-chromosome is identified by standard cytogenetic techniques [4]. Patients with chromosomal DSD as a result of a 45,X/46,XY karyotype (mixed gonadal dysgenesis) may present with a wide spectrum of phenotypes ranging from normal male through ambiguous genitalia to female with a TS phenotype [5]. They are characterized by the presence of dysgenetic testis and/or streak gonads, with persistence of the Müllerian ducts and inadequate virilization, and classically have a 45,X/46,XY karyotype. Y-chromosome mosaicism may lead to virilization and modifications in the female phenotype of TS patients, although a direct correlation between presence of the Y-chromosome and gonadal differentiation pattern has not been found [6-7]. The presence of a specific region of the Y-chromosome in TS patients is correlated with an increased risk of developing a GCC, namely the GonadoBlastoma on Y region (GBY, i.e. TSPY) [8-9].

Mutations in the *SRY* gene are known to be involved in 46,XY sex reversal and are found in approximately 15% of 46,XY gonadal dysgenesis cases [10]. Most of the mutations detected are located in the HMG domain, responsible for the binding and bending of DNA, but several mutations outside of this domain have been reported. Several reports have also described mutations in the *SRY* gene in individuals with a 45,X/46,XY karyotype [11-14], suggesting an additional effect of mutant SRY in the gonadal development of these patients.

Until recently the detection of genetic variants present in <50% of cells was technically challenging, as conventional Sanger sequencing does not routinely reveal such changes. The development of next-generation sequencing technology has greatly simplified this type of analysis, as the potential to generate millions of sequence reads allows the detection and precise quantitation of low frequency variants. This approach has been used for identifying mosaic changes in a range of different samples types [15-16]. Here we describe the analysis of the *SRY* gene using the 454 GS/FLX sequencer in fourteen mosaic patients, including twelve patients with 45,X/46,XY, one patient with a 45,X/46,XX/46,XY, and one patient with a 46,XY/46,XX karyotype, to evaluate the potential role of SRY mutations in these patients.

Results

In total fourteen chromosomal DSD patients with a mosaic karyotype were included in the study: twelve patients with a 45,X/46,XY, one patient with a 45,X/46,XX/46,XY, and one patient with a 46,XY/46,XX sex chromosomal DSD (Table 1). Age at biopsy or gonadectomy ranged from 6 months to 17 years of age (median age 3 years, Table 1). From seven patients the karyotype in peripheral blood lymphocytes was determined (cases 1 - 3, 5, 8, 12 and 14), and of five patients the gonadal karyotype was known (case 1 - 4, and 12). Eight patients (57%) had a male, and six patients (43%) had a female gender. Histology of the gonads showed streak gonads, undifferentiated gonadal tissue, ovotesticular and testicular differentiation patterns. In one case no gonadal tissue was found (case 7), only adnexal structures (fallopian tubes, epididymis and an underdeveloped/dysplastic uterus). In one patient (case 3) a gonadoblastoma was described, being the precursor lesion of the type II germ Cell Tumor/Cancer (GCC) in the dysgenetic gonad [9].

Sequencing of the pooled PCR products generated a total of 102,646 matched reads, an average of 3,666 reads per product (Supplemental Table 2). A variant in >2% of reads was identified in only one case (Sample 14, Table 1). This was a deletion of T on nucleotide position 197 in the SRY gene (c.49delT in reference sequence NM_003140.1) which was identified in 21% of sequence reads of the 45,X/46,XX/46,XY patient. Subsequent analysis of sample 14 by subcloning PCR product and analyzing 30 samples by conventional Sanger sequencing, could not confirm the deletion originally found by deep sequencing (data not shown).

Table 1. *SRY* mutations in mosaic DSD patients

				Histology of the gonads								Age biopsy/gonadectomy
Case No	**Sex**	**Karyotype (%) [%]**	**SRY Variants**	**T**	**O**	**S** [#]	**UGT**	**OT**	**NGT**	**Other**	**NA**	**Years**
1	F	45X/46XY (10%:90%) [58%/42%]				+ (L+R)						17
2	M	45,X/46,X,der(Y)(pter-q11.2::q11.2-pter) (44%:56%) [T:71%/29%;		+ (R)			+ (L)					6 months
3	F	45X/46XY (56%:44%) [Y present[§]]					+ (GB) (L)					17
4	M	45X/46X iso Y (NA) [96%/4%]				+ (L)						6 months
5	F	45X/46XY (50%:50%) [NA]				+ (R)						16
6	F	45X/46X iso Y (NA) [NA]				+ (L+R)						1
7	M	45X/46XY (NA) [NA]							+ (R)			1
8	M	45X/46XY (50%:50%) [NA]		+ (R) [&]								9 months
9	M	45X/46XY (NA) [NA]									+	NA
10	F	45X/46XY (NA) [NA]				+ (L+R)						15
11	M	45X/46XY (NA) [NA]									+	3
12	F	46XX/46XY (94%:6%) [T:16%/84%; O: 68%/32%]						+ (NA)				1
13	M	45X/46XY (NA) [NA]									+	5
14	M	45X/46XX/46XY (39%:49%:12%) [NA]	c.49delT 21%	+ (L)								3

				Histology of the gonads								Age biopsy/gonadectomy
Reference	**Sex**	**Karyotype (%) [%}**	**SRY Variants**	**T**	**O**	**S**	**UGT**	**OT**	**NGT**	**Other**	**NA**	**Years**
[22]	F	45X/46XXY (65%/35%) [NA[@]]	p. Y3X		+ (L+R) [‡]							NA
[11]	F	45X/46XY (82%/18%) [L:94%/6% R:98%/2% [€]]	p.S18N			+ (L+R) [†]						17
[11]	F	45X/46X mar (Y) (95.5%/0.5%) [100%/0% [€]]	p.S18N			+ (L+R) [†]						14
[20]	F	45X/46X psu dic (Y)(pter-q11::q11-pter) (40%/60%) [NA[@]]	p.R59G			+ (L+R)						24
[12]	F	45X/46XY (80%/20%) [NA]	p.N82X			+ (NA) *						24
[12]	F	45X/46XY (86%/14%) [NA]	p.L159TfsX167			+ (NA) *						20
[12]	F	45X/46XY (89%/11%) [NA]	p.Q74H			+ (L+R)						22
[14]	F	45X/46XY (15%/85%) [NA]	rs11575897							+ (GB) (L+R) [$]		13.5

(%): Karyotype in blood, [%]: Gonadal karyotype, GB: Gonadoblastoma, NA: Not Available, T: Testis, O: Ovary, S: Streak, Tissue, OT: OvoTestis, Ov St: Ovarian Stroma, UGT: Urdifferentiated Gonadal, NGT: No Gonadal Tissue, L: Lef: side, R: Right side

[#] Including ovarian stroma

[§] Not further specified

[@] Blood karyotype confirmed on gonadal tissue, not further specified

[&] Contains germ cells positive for OCT3/4, TSPY, SCF: at risk for malignant transformation (pre-CIS)

[‡] Gonads contained primordial follicles, not further specified

[†] Macroscopically streak

[€] Karyotype assessed in fibroblasts cultured from gonads

* Stromal tissue with similarities to testicular histopathology present, not further specified

[$] Described as Left dysgenetic testis with GB, Right dysgenetic gonad

Discussion

SRY is the founding member of the SRY-like HMG box (SOX) family of transcription factors, characterized by a HMG domain [17]. It is involved in the binding and bending of DNA and contains two nuclear localization signals. Mutations in *SRY* are present in 10-15% of 46,XY DSD patients [10], and these patients have an increased risk of developing GCC, related to the presence of the GBY region (with TSPY as the most likely candidate gene), and the prolonged expression of OCT3/4 (POU5F1) in the germ cells [8, 18-19]. Several authors have described mutations in *SRY* in rare cases with a mosaic sex chromosome constitution [11-13, 20-22], indicating a potential involvement of SRY in abnormal gonadal development of 45,X/46,X,der(Y) patients.

However, in this study no confirmed mutations in *SRY* were identified in any of the fourteen cases analyzed. In case no. 14 with a 45,X/46,XX/46,XY karyotype, a deletion of T on position 197 of *SRY* (ref. seq. NM_003140.1) was found by deep sequencing in 21% of the sequence reads. However, subsequent analysis by sequencing subcloned PCR products only produced wild type *SRY* sequences, indicating that the original deep sequencing result was most likely a false positive. The results presented here are in agreement with, and extend the data reported by (and others summarized in) Nishi *et al.* [14], who found only one SRY polymorphism (c.561C→T) in a group of 27 patients (fourteen TS and thirteen mixed gonadal dysgenesis patients. In Table 1, next to the cases analyzed here, an overview of *SRY* mutations that have been reported in chromosomal DSD cases is shown. The results published until now, showing a *SRY* mutation in approximately 8% of cases, have all been obtained using conventional Sanger sequencing; the findings presented here show that, although analyzed with a highly sensitive sequencing technique, variations in *SRY* are not common in patients with a mosaic sex chromosomal constitution.

Shahid *et al.* [13] describes a mosaic TS patient, with gonadoblastoma, having a frameshift mutation (L94fsX180) in *SRY* which was inherited from the father. He was found to be mosaic for the *SRY* mutation and had oligoasthenozoospermia and a testicular GCC (seminoma), which are signs of mild Testicular Dysgenesis Syndrome (TDS), the underlying entity proposed by Skakkebæk *et. al.* [23]. They suggest that the presence of the mutated *SRY* gene might play a role in the development of gonadoblastoma and seminoma, being the precursor lesion and the invasive component of GCC respectively. However, in the series of samples analyzed here and published by others, no clear link between presence of *SRY* mutations and development of a gonadoblastoma in these patients can be made (Table 1 and references therein). Domenice *et al.*

[21] describe a patient with partial gonadal dysgenesis and a *SRY* missense (S18N) mutation whose unaffected male relatives also harbored the mutation, showing no link between *SRY* mutation and TDS. However, a family with two sisters with 46,XY DSD, pure gonadal dysgenesis and a phenotypically normal brother has been described, in which a *SRY* frameshift mutation was found in the two sisters and in a mosaic constitution in their father. He showed signs associated with TDS; hypospadias, cryptorchidism, a testicular GCC (seminoma) and oligoasthenozoospermia, suggesting that mutations in SRY may be associated with TDS [24]. If variations in *SRY* play a significant role in TDS and the development of a testicular GCC remains unresolved, and may warrant further investigation.

It has been found in chimeric XX-XY mouse models that if the gonad contains less than 30% Y-positive cells, the gonad will develop as an ovary, suggesting a correlation between percentage of Y-containing cells and the gonadal differentiation pattern [25]. This seemed at first to be confirmed in humans [26-27], however two subsequent case reports and analysis of a larger series of samples show no correlation between the degree of gonadal mosaicism and differentiation pattern [6, 28-29]. The study by Cools *et al.* [6] revealed no clear correlation between peripheral blood karyotype and gonadal karyotype, or between the gonadal karyotype and differentiation pattern found in the gonads. The inconsistency between gonadal karyotype and gonadal differentiation pattern cannot be explained by the presence of *SRY* mutations, as they are found only in rare cases, and do not seem to correlate with the differentiation pattern reported [11-12, 14, 20, 22], even when ascertained by a highly sensitive next generation sequencing approach, as shown in this study.

This is, to our knowledge, the first study using next generation sequencing to detect mutations in the *SRY* gene in chromosomal DSD patients with a mosaic karyotype. Although a highly sensitive method, no aberrations in *SRY* were detected. Including the present study, a total of 91 patients with a mosaic sex chromosomal constitution have been screened for *SRY* mutations, of which only seven (8%) showed a variation. This indicates that mutations in *SRY* are rare in chromosomal DSD patients with a mosaic karyotype and only play a role in a minority of cases.

Material and methods

Tissue and DNA samples

Anonymized tissue samples were collected from our diagnostic archives and diagnosed according to WHO standards [30] by an experienced pathologist

(JWO). Use of tissue samples for scientific reasons was approved by the Medical Ethical Committee ErasmusMC (MEC 02.981 and CCR2041). 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 (FMWV (Version 2002, update 2011). Genomic DNA was isolated from peripheral blood lymphocytes following standard protocols.

Primer design and PCR amplification

SRY specific priming sequences were designed using reference sequence NG_011751. The complete coding sequence was covered in two overlapping PCR products, generating products of 383 bp and 372 bp. To facilitate analysis on the 454 GS/FLX sequencer (454 Life Sciences, Branford, CT, USA) the SRY-specific sequences were modified by adding a) the forward or reverse Titanium Primer and b) a 10 nucleotide multiplex identifier sequence, allowing all samples to be combined into a single reaction. All sequences are outlined in supplementary Table 1 (SRY mosaic amplification primers). PCR amplification was carried out in 25 µl volumes, using 1.25 U Pfusion High Fidelity Enzyme per reaction. Cycle conditions were: 1 cycle of 94 ^{o}C for 1 min; 35 cycles of 94 ^{o}C for 30 sec, 62 ^{o}C for 30 sec, 72 ^{o}C for 1 min; 1 cycle of 72 ^{o}C for 10 min. Samples were analyzed on a 1% agarose gel, then purified using the Agencourt AMPure XP kit (Beckman Coulter Genomics, Danvers, MA, USA) following the manufacturer's protocol.

Sequencing and data analysis

PCR products were pooled in equimolar concentrations and sequenced on the 454 GS/FLX sequencer (454 Life Sciences) at the Australian Genome Research Facility (Melbourne, Australia) following manufacturer's instructions. The reads were de-multi-plexed based on the unique 10 nt MID sequence. Variant detection was performed with NextGene (SoftGenetics, State College PA, USA), using NG_011751 as the reference sequence for alignment. Only variants present in >2% of reads for a given sample were chosen for further analysis.

PCR amplification and sequencing of sample 14

DNA was amplified using *SRY* specific primers SRY-up 5'-TTCAATTTTGTCGCAACTCTCC-3' and SRY-rev 5'-GATCGAATGCGTTCATGGGTC-3', generating a product of 237 bp. PCR amplification was performed using the BD Advantage 2 kit (BD Biosciences, Palo Alto, CA, USA). Cycle conditions were: 1 cycle of 95 °C for 1 min; 45 cycles of 95 °C for 45 sec, 57 °C for 45 sec, 68 °C for 1 min; 1 cycle of 68 °C for 3 min.

PCR product was analyzed on 1% agarose gel. Subsequently PCR product was cloned, transformed, plated and positive clones were analyzed using the TOPO TA Cloning Kit For Sequencing, following manufacturers instructions (Invitrogen, Life Technologies, Carlsbad, CA, USA). Sequences reactions were done with standard T3 and T7 primers, using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and run on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Life Techologies, Carlsbad, CA, USA) following manufacturer's instructions. Sequences were analyzed with MutationSurveyor software (Softgenetics, State College, PA, USA) using reference sequence NG_011751.

Acknowledgements

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Chapter 9

Prevalence of *c-KIT* mutations in gonadoblastoma and dysgerminomas in patients with Disorders of Sex Development (DSD) and ovarian dysgerminomas

Submitted

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Abstract

Background: Activating *c-KIT* mutations in exons 11 and 17 are found in 10-40% of testicular seminomas, two thirds of these being missense point mutations in codon 816. Malignant ovarian dysgerminomas represent approximately 3% of all ovarian cancers in Western countries, morphologically resembling testicular seminomas. A similar pattern of chromosomal aberrations and activating *c-KIT* mutations are found. DSD patients harboring specific Y-chromosomal material in their karyotype have an increased risk of developing a Type II Germ Cell Tumor, with gonadoblastoma as precursor lesion and mainly dysgerminoma as the invasive component. **Methods:** *c-KIT* and *PDGFRA* were analyzed in a series of 16 DSD patients presenting with gonadoblastoma and dysgerminoma and 15 patients presenting with pure ovarian dysgerminomas by conventional sequencing together with mutational analysis of *c-KIT* codon 816 by a sensitive and specific LightCycler melting curve analysis. These data were combined with results on TSPY and OCT3/4 expression. **Results:** Five *c-KIT* codon 816 mutations and three N822K mutations were detected in the group of pure ovarian dysgerminomas. Interestingly, in the group of DSD cases, a N505I and D820E mutation was found in a single tumor of a patient with gonadoblastoma and dysgerminoma. No mutations in *PDGFRA* were found. All gonadoblastomas and dysgerminomas investigated stained positive for OCT3/4, and TSPY expression was only seen in the gonadoblastoma/dysgerminoma lesions of the DSD patients. **Conclusions:** This data supports the existence of two distinct but parallel pathways in the development of dysgerminoma, in which mutational status of *c-KIT* might parallel the presence of TSPY.

Introduction

c-KIT belongs to the Type III tyrosine kinase receptor family, which also includes the platelet-derived growth factor receptor (PDGFR) and macrophage-colony stimulating receptor (M-CSFR). It contains a 5-repeat extra-cellular immunoglobin-like, a transmembrane, a juxtamembrane and two tyrosine kinase domains. The ligand for c-KIT is the stem cell factor (SCF, KITLG) and the SCF-KIT pathway regulates the differentiation of melanocytes, red blood cells, mast cells, interstitial cells of Cajal, and germ cells [1-3]. Moreover, this pathway also plays an important role in the survival of primordial germ cells (PGCs) [4-5]. Expression of c-KIT and gain-of-function mutations in *c-KIT* has been found in mastocytosis, leukemia and gastro-intestinal stromal tumors (GIST) [6-8]. In GIST activating mutations in *c-KIT* exons 8, 9, 11, 13 and 17 are found in 75 – 80% of cases, mutations in *PDGFRA* exons 12, 14 and 18 in 5 - 8%, and they are mutually exclusive [9] (for review).

Activating *c-KIT* mutations have also been found in human germ cell tumors/cancers (GCC), and approximately 10-40% of testicular seminomas harbor activating mutations in exons 11 and 17. About two thirds of these are missense point mutations at codon 816 in exon 17 [2, 10-12], which are also found in almost all mast cell tumors [13]. Noteworthy is the fact that in a subset of tumors which show the same histology of testicular seminoma, namely; mediastinal seminomas, intracranial germinomas and ovarian dysgerminomas, activating mutations of *c-KIT* have been found [14-16]. Next to gain-of-function mutations in c-KIT, also amplification of chromosome 4q12, harboring the *c-KIT* gene, has been described in testicular GCC, most likely related to the progression to the seminoma subtype [10]. Malignant ovarian dysgerminomas represent approximately 3% of all ovarian cancers in Western countries, and not only share a morphological resemblance, but also show a similar pattern of chromosomal aberrations [17] with testicular GCC. Families with both ovarian and testicular GCC have been reported, suggestive of a common etiology [18]. Bilateral disease is found in about 1-3% of patients with a GCC, showing a similar incidence in Europe, the United States and Japan. In the German studies on nontesticular germ cell tumors (MAKEI), up to 5% of patients with ovarian GCC showed bilateral disease, either as synchronous or metachronous manifestation. Several studies have shown a very high incidence (64 to 93%) of *c-KIT* mutations in this population of patients [19-20], although other reports have not reproduced these results. If the detection of *c-KIT* mutations in unilateral testicular GCC can be used as molecular screening for bilateral disease remains therefore controversial.

Disorders of Sex Development (DSD), previously referred to as intersex, are a congenital condition in which there is an atypical development of the chromosomal, gonadal or anatomical sex [21]. DSD is divided in three main groups; 46,XY DSD, 46,XX DSD and chromosomal DSD, and certain subgroups of these patients (i.e. those with gonadal dysgenesis and hypovirilization) harboring Y-chromosomal material in their karyotype have an increased risk of developing GCC [22-23] (for review). The precursor lesion which arises in the dysgenetic gonads of these patients is the gonadoblastoma (GB), or carcinoma *in situ* (CIS), depending on the level of testicularization of the gonad [24], and the invasive component is the dysgerminoma in most cases (being genetically the counterpart of the seminoma of the testis). For the development of GB the presence of the GonadoBlastoma locus on the Y-chromosome (GBY) is imperative, with the *TSPY* gene being the most likely candidate in this region. TSPY expression is linked to the proliferation and survival of germ cells, and expression has been shown to be increased in CIS, GB and sometimes seminoma [25]. OCT3/4 (POU5F1) is specifically expressed in all GCC with pluripotent potential, as well as in the neoplastic precursor lesions CIS and GB [26-27]. Germ cells residing in an unfavorable environment, as is the case in DSD, might escape cell death by prolonged expression of both OCT3/4 and TSPY. If mutations in *c-KIT* or *PDGFRA* play a significant role in the development of GB and the development of dysgerminoma in DSD patients is not clear so far because of the lack of multiple studies.

Here we report the analysis of activating mutations in codon 816 of *c-KIT* in 31 patients with a GB and/or dysgerminoma by LightCycler analysis, together with conventional sequence analysis of *c-KIT* exons 8, 9, 11, 13 and 17, and *PDGFRA* exons 12, 14 and 18, mutations in which are frequently found in GIST. These results are linked with karyotype, histology of the gonads, expression of TSPY in the tumors and putative role of the mutations found in the etiology of the disease.

Materials and methods

Tissue samples and immunohistochemistry

In total 31 cases, consisting of eleven cases of GB, fifteen cases of DG and eight cases of GB with DG were retrieved from the archives (Table 1). Collected tissue samples were diagnosed according to WHO standards [28] by an experienced pathologist (JWO). Use of tissue samples for scientific reasons was approved by an institutional review board (MEC 02.981 and CCR2041). 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 (FMWV: Version 2002, update 2011).

Immunohistochemistry was performed on paraffin-embedded tissue sections of 3-µm thickness. After deparaffinization and 5 min. incubation in 3% H_2O_2 to inactivate endogenous peroxidase activity, antigen retrieval was carried out by heating under pressure of up to 1.2 bar in an appropriate buffer; 0.01M sodium citrate (pH 6) or 0.01M EGTA, 0.01 M TRIS (pH 9). After blocking endogenous biotin using the avidin/biotin blocking kit (SP-2001, Vector Laboratories, Burlingame, CA, USA), the sections were incubated for either 2 hrs at room-temperature (OCT3/4, c-KIT (CD117) or overnight at 4˚C (TSPY). Appropriate biotinylated secondary antibodies were used for detection and were visualized using the avidin-biotin detection and substrate kits (Vector Laboratories). The antibodies used directed against OCT3/4, TSPY and c-KIT have been described before [29-31].

DNA isolation and c-KIT codon 816 mutational screen

DNA was isolated from formalin-fixed-paraffin-embedded material using a standard protocol, percentage of tumor present in each sample was over 50% unless indicated otherwise (Table 1). In brief, 10 slices of 10-µm thickness were cut and incubated three times with xylene for at least 30 min at RT, after which the pellet was washed each time with ethanol. Lysisbuffer consisting of 10 mM TRIS, 100 mM NaCl, 5 mM EDTA, 1% SDS and 1 mM $CaCl_2$ together with 10mg/ml proteinase-K was added, and the sample was incubated for 16 hrs at 50°C, while shaking at 1200 rpm. DNA was subsequently extracted by standard phenol/chloroform extraction and ethanol precipitation. DNA was dissolved in 10 mM TRIS with 1 mM EDTA. DNA quality and concentration was checked on the Nanodrop 1000 (ThermoScientific, Wilmington, DE, USA)

50 ng of DNA from each sample was screened for c-KIT D816V, D816H, D816Y mutations using a melting-curve based LightCycler assay (Roche Diagnostics, Mannheim, Germany) with forward primer KIT816For, CAGCCAGAAATATCCTCCTTACT; or KIT816 ForA, CTTTTCTCCTCCAACCTAATAG; reverse primer KIT816Rev, TTGCAGGACTGTCAAGCAGAG; and hybridization probes c-KIT-anchor, LC640-ATGTGGTTAAAGGAAACGTGAGTACCCA—PH; c-KIT-sensor VAL, AGCCAGAGTCATCAAGAATGATTCTA—FL; c-KIT-sensor TYR, AGCCAGACACATCAAGAATGATTCTA—FL; c-KIT-sensor HIS, AGCCAGATACATCAAGAATGATTCTA. To suppress wild type sequences, all reactions were performed with and without addition of a locked nucleic acid

(LNA), c-KIT probe GCCAGAGACATCAAGAATG (all primers produced by TIB molbiol, Berlin, Germany). Mixing experiments showed that with the addition of LNA to block wild type sequence, the lower limit of detection was 20 fg of mutant DNA in 50 ng of wild type DNA, and routinely 20 pg of mutant DNA could be detected (data not shown). As a control, samples containing the c.816 mutation under investigation were included in each experiment and were analyzed with and without LNA, together with the experimental samples. The PCR reaction was carried out in a 20 µL volume with 0.5 µM each of forward, reverse, anchor and appropriate sensor probe, 0.01 µM of LNA, 3 mM $MgCl_2$ and 2 µL LightCycler Fast-Start DNA Master HybProbe mix. Reactions were run on a LightCycler Instrument (Roche Diagnostics, Almere, The Netherlands). Amplification was performed with 45 cycles using 60°C annealing temperature. Final melting curve analysis was started at 40°C up to 95°C with a slope of 0.2°C/second and continuous detection with channel F2/F1. Lightcycler data was analyzed using the LightCycler 3.0 software (Roche Diagnostics). Samples showing an aberrant melting curve were run at least in duplicate.

Sequence analysis

All cases found to be positive in the c-KIT c.816 screen were confirmed by sequence analysis. Approximately 100ng of PCR product was treated with ExoSAP-IT (GE Healthcare Life Sciences, Piscataway, NJ, USA) following manufacturers instructions, and directly sequenced with 3.3 pmol of each forward and reverse primer using the Big Dye terminator Cycle Sequencing Kit (Applera, Darmstadt, Germany). After initial denaturation at 95°C for 5 min, 25 cycles at 94°C for 15 seconds and 60°C for 4 minutes were performed. Sequence analysis was performed on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

In addition to screening for activating mutations of c.816, in all samples *c-KIT* exon 8, 9, 11, 13, en 17 and *PDGFRA* exon 12, 14 and 18 were analyzed by conventional bidirectional cycle sequencing of PCR-amplified fragments. Amplification of 50 ng genomic DNA of each sample was performed with M13-tailed primers (Supplementary Table 1). After initial denaturation at 95°C for 3 min, 35 cycles of 95°C for 30 seconds, 60°C for 45 seconds, and 72°C for 45 seconds were performed, followed by 10 min at 72°C. Subsequent sequence analyses of the PCR products was carried out with M13 forward and reverse primers, essentially as described above.

Results

The mean age of diagnosis of the GB and/or DG was 15 years (range, 3 months-36 years, see Table 1). The mean age of diagnosis between the group of patients with DSD (cases 1-16), being 16 (3 months-36 years) and the group with ovarian dysgerminoma (cases 17-31), being 14 (6-19 years) did not differ significantly. Within the group of DSD patients the mean age of diagnosis did differ between patients showing GB, being 13 and patients who had a dysgerminoma with GB, being 21 years. In total, twenty-two cases showed a dysgerminoma component; thirteen patients had pure dysgerminoma, three patients had non-dysgerminoma components (yolk sac tumor and (immature) teratoma) next to the dysgerminoma component, and six patients showed GB next to dysgerminoma. One patient showed teratoma and yolk sac tumor next to GB. Eight patients did not have an invasive component; seven showed GB (one bilaterally) and one patient had GB next to CIS and intratubular seminoma. Five cases presented with bilateral disease; one case showing GB in both gonads, one patient having GB in one gonad and GB together with dysgerminoma in the other, one case with GB in one gonad and GB next to dysgerminoma, yolk sac tumor and immature teratoma in the other, and from two cases only material from one of the gonads was available, showing GB, CIS, and dysgerminoma in one patient and GB, teratoma and yolk sac tumor in the other patient (cases 1-5, Table 1). LightCycler analysis detected variants in exon 17 of *c-KIT* in five out of the total group of 31 patients (19%). Four were found in the group of ovarian dysgerminomas (27%: four out of fifteen cases), consisting of two D816V, one D816H and one D816Y mutation (cases 17, 19, 22 and 25, Table 1). All mutations at codon 816 were detected in the LightCycler assay, in analyses with and without LNA added, showing a shift in melting curves which were compared with control samples (Supplementary Figure 1). One variant in *c-KIT* exon 17 was found in the group of DSD patients, which changed the codon 178 sequence from ATC to ATT (I798I), which encodes a known synonymous SNP (rs. 55789615) (case 4, Table 1). This variation shifted the melting curve to a position different from that of any of the control mutation samples included (data not shown). Two other samples produced an aberrant melting curve (case 1 and 2, Table 1), but no mutation was detected in subsequent sequencing, despite analyzing the samples in triplicate for all three c.816 variants on two independent DNA isolations (data not shown). All mutations found were verified by sequencing the LightCycler products from reactions with and without LNA (Supplementary Figure 1). All other samples tested showed melting curves identical to non-mutated Asp 816 (data not shown).

						c- KIT LightCycler		Mutation detection								PDGFRA sequencing				Immunohistochemistry gonad		
								c-KIT sequencing														
Case No	Age	Sex	Malignancy	Karyotype	c-KIT LC	Seq. LC Products	Exon 8	Exon 9a	Exon 9b	Exon 11	Exon 13	Exon 17	Exon 12a	Exon 12b	Exon 14	Exon 18	c-KIT (CD117)	OCT3/4	TSPY
1	16	F	GB	46XY	shift		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+
1[§]	16	F	GB	46XY	shift				ND				P567P	P567P			+	+	+
2[§]	19	F	GB	46XY	shift				ND				ND	ND			+	+	+
2[§]	19	F	GB/DG	46XY	shift		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+
3[§]	22	F	GB	46XY		ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	+
3	22	F	GB/DG/YST/imTE	46XY		ND			ND	ND			ND	ND	ND	ND	+	+	+
4	21	M	GB/DG/CIS	46XY	shift	I798I			ND				ND				+/-	+	+
5[§]	9	F	GB/TE/YST	46XY		ND			ND			ND	P567P	P567P			+ (few cells)	ND	+
6[§]	14	F	GB	46XY		ND							P567P	P567P			-	+ (few cells)	+ (few cells)
7[§]	2	F	GB	46XY		ND							P567P	P567P	ND	ND	+	-	-
8	26	M	GB/CIS/ItSE	46XY		ND							P567P	P567P			+	+	+
9	14	F	GB/DG	46XY		ND			ND				P567P	P567P			+	+	+
10	18	F	GB/DG	46XY		ND							P567P	P567P			+	+	+
11	22	F	GB/DG	46XY		ND							P567P	P567P			+	+	+
12[§]	1	M	GB	45X/46XY		ND						ND	ND	ND	ND	ND	+	+	+
13[§]	17	F	GB	45X/46XY		ND							P567P	P567P			-	+ (few cells)	-
14	20	F	GB	45X/46XY		ND			ND				P567P	P567P			+	+	+
15[§]	3m	F	GB	46XX		ND							P567P	P567P			+	+	+ (few cells)
16	36	M	GB/DG	46XY[‡]		ND			**N505I**			**D820E**	P567P	P567P			+	+	+
17	14	NA	DG	NA	**His**	**D816H**						**D816H**	P567P	P567P			+	+	-
18	19	NA	DG	NA		ND							P567P	P567P			+	+	-
19	17	NA	DG	NA	**Val**	**D816V**						**D816V**	P567P	P567P			+	+	-
20	NA	NA	DG	NA		ND							P567P	P567P			+	+	-
21	19	NA	DG	NA		ND						**N822K**	P567P	P567P			+	+	-
22	15	F	DG	NA	**Val**	**D816V**						**D816V**	P567P	P567P			+	+	-
23	15	F	DG	46XX		ND			ND	ND		ND	ND	ND	ND	ND	ND	+	ND
24	12	F	DG	46XX		ND						**N822K**	P567P	P567P			+	+	-
25	7	F	DG	46XX	**Tyr**	**D816Y**						**D816Y**	P567P	P567P			ND	+	-
26	16	F	DG	46XX		ND							P567P	P567P			+/-	+	-
27	14	F	DG	46XX		ND			ND			**N822K**	P567P	P567P			+	+	-
28	14	F	DG	46XX		ND						I798I	P567P	P567P			+/-	+	-
29	16	NA	DG	46XX		ND							P567P	P567P			ND	+	-
30	6	F	DG/TE	46XX		ND							P567P	P567P			+	+	-
31	10	F	DG/YST	46XX		ND						**D816V**	P567P	P567P			ND	+	-

ND, not done; NA, not available; mutations indicated in bold, other samples wild type unless indicated otherwise; DG, dysgerminoma; GB, gonadoblastoma; CIS, carcinoma in situ; TE, teratoma (im: immature); YST yolk sac tumor; itSE, intratubular seminoma; seq, sequence; LC, LightCycler; c-KIT LC, LightCycler melting curve results; case numbers in bold are bilateral cases

§, tumor/precursor percentage below 50%

‡, as determined by FISH on gonadal tissue

I798I: heterozygous synonymous SNP, rs.5578615

P567P: homozygous synonymous SNP, rs.1873778

Next to the c.816 LightCycler analysis, conventional Sanger sequencing of *c-KIT* exons 8, 9, 11, 13, and 17 was performed on the DNA samples in a diagnostic

setting. This confirmed the presence of the four c.816 mutations found by the LightCycler analysis (cases 17, 19, 22 and 25, Table 1), but also revealed an additional D816V mutation (case 31, Table 1). Furthermore, in three ovarian dysgerminoma cases a N822K mutation was found (cases 21, 24 and 27 Table 1). In total, in eight out of fifteen ovarian dysgerminoma cases (53%) an exon 17 mutation was found. One patient (case 28, Table 1), showed a heterozygous synonymous SNP (rs. 55789615). In case 16 a D820E mutation in exon 17, next to a N505I mutation in exon 9 was found, being the only DSD case showing mutations in *c-KIT* (6%, 1 out of 16). No mutations in any of the other exons analyzed were found. Sequence analysis of *PDGFRA* exon 12, 14 and 18 did not reveal any mutations, only a homozygous synonymous SNP in exon 12 (rs. 1873778) was detected in all samples analyzed.

Immunohistochemical analysis of c-KIT showed no correlation between the presence of *c-KIT* activating mutations and protein expression in the tumor. In four cases c-KIT immunohistochemistry was not investigated (case 23, 25, 29 and 31), as no additional material was available (Table 1). Staining for c-KIT was variable in the whole series analyzed, ranging from absent through intermediate to strong staining and no clear difference between the DSD and ovarian dysgerminoma subgroups could be seen. As expected, staining for OCT3/4 was positive in the GB, and dysgerminoma components in all cases analyzed, with the exception of case 7. TSPY staining correlated with the two subgroups of patients analyzed, being positive in the DSD group (cases 1-16), with the exception of cases 7 and 13, which showed no staining, and negative in the ovarian dysgerminomas (cases 17-31)(p-value 3.6×10^{-8}).

Discussion

c-KIT expression has been demonstrated in a wide variety of human tumors, although in most types expression is variable. The highest percentages are seen in gastro-intestinal tumors, seminomas, adenoid-cystic carcinomas and malignant melanomas, and amplification and enhanced expression is associated with seminoma progression [10, 32]. The presence of activating mutations of *c-KIT* in testicular seminomas is well known. Although ovarian dysgerminomas resemble seminomas in morphology and chromosomal aberrations [33], expression of c-KIT is not extensively explored. Here we analyzed fifteen cases of pure ovarian dysgerminomas and found that c-KIT is expressed, although variable, in all but two of the cases analyzed. Mutations in *c-KIT* codon 816 were found in 5 (33%) and mutations in codon 822 in 3 (20%) out of the 15 pure ovarian dysgerminoma cases (case 17, 19, 22, 25, 31 and 21, 24, 27 respectively,

Table 1), accounting for 53% of cases analyzed. No mutations were detected in c-KIT exon 8, 9, 11, and 13. Although most ovarian dysgerminomas expressed c-KIT, we could not find a correlation between expression and *c-KIT* exon 17 mutations. It is known that in GIST in addition to mutations in *c-KIT*, also mutations in *PDGFRA* exon 12, 14 and 18 play a role and that these are mutually exclusive [9]. Sequencing *PDGFRA* did not reveal mutations in any of the dysgerminoma DNA samples analyzed, only a variation in exon 12 was found in almost all cases (homozygous synonymous SNP, rs. 1873778, Table 1). This indicates that mutations in *PDGFRA* do not play a major role in the development of (ovarian) dysgerminomas or GB. The results shown here extend those of Cheng *et al.* and Hoei-Hansen *et al.* [16, 34]. Cheng *et al.* [34] analyzed 22 cases of dysgerminoma and found a *c-KIT* codon 816 mutation in 27% of cases, and KIT expression in 87%. Hoei-Hansen *et al.* found c-KIT codon 816 mutations in five out of seventeen dysgerminoma cases (29%) with 80% expressing c-KIT [16]. Furthermore, also in gastro-intestinal tumors *KIT* mutation rate is lower than the expression rate of KIT [35-36]. The results presented here suggest that in about half of ovarian dysgerminomas activating mutations in *c-KIT* play a role, with about a third consisting of codon 816 mutations, as has been reported by others [16, 34], while the remaining 20% consisted of N822K mutations. Indeed, *c-KIT* N822K mutations have also been found in testicular GCC [10, 20, 37], indicating a role for this mutation in the development of GCC, independent of the origin in the testis or ovary. Next to these mutations a known synonymous SNP (rs55789615) was detected in exon 17 of case 30, which has been described before in a patient having a N822K mutation in the GCC of the contralateral testis [20]. Besides these, no other aberrations in the exons analyzed could be found in the group of ovarian dysgerminomas. Patients showing expression of c-KIT might benefit from targeted therapy with imatinib mesylate, as has been shown for patients with GIST [38], and also in a patient with metastatic seminoma [39]. This might therefore also be of interest to treat ovarian dysgerminoma.

DSD patients with gonadal dysgenesis or hypovirilization have an increased risk of developing GCC, with GB as the precursor lesion, linked to the presence of (part of) the Y-chromosome. Y-chromosomal material is detected in 90% of patients with dysgenetic gonads, with the *TSPY* gene being seen as the candidate gene in the GonadoBlastoma on the Y-chromosome (GBY) region [40]. Here we show that in 89% of cases (17 out of 19 analyzed) where GB was present, either with or without dysgerminoma, positive staining of the TSPY protein could be seen in the neoplastic cells. It is possible that in the two TSPY

negative cases (7 and 13) the staining was sub-optimal due to poor tissue fixation, as other markers tested showed unexpected (negative) results (data not shown). In contrast, all cases with ovarian dysgerminoma in a 46,XX (normal female) genetic background were negative for TSPY. The results underline the importance of presence of (part of) the Y-chromosome in the development of GB and point to the fact that in the case of DSD and ovarian dysgerminomas the pathways leading to the tumors are distinct. This is in line with, and extends the results reported by Hoei-Hansen and co-workers [16], who showed TSPY in five out of seven cases with GB, the precursor lesion of dysgerminoma in DSD patients, and no TSPY protein expression in eleven pure dysgerminoma cases.

Next to the presence of TSPY, also presence of OCT3/4 was investigated in this series. OCT3/4 is one of the key regulators of self renewal and pluripotency of embryonic stem cells, and in normal development this protein is only present in primordial germ cells/gonocytes and oogonia [30, 41]. In the testis expression is only seen in GCC (i.e. seminoma and embryonal carcinoma) and its precursor lesion CIS [27, 42]. In DSD patients OCT3/4 expression is present in GB and dysgerminoma [43-44]. OCT3/4 was present in all but one GB analyzed in this study, and also a positive staining was found in all dysgerminomas, in line with previous studies [16, 43]. Case 7 which did not show a positive OCT3/4 staining of the GB, also gave mixed results using other markers (negative TSPY staining amongst others), indicating possible poor quality of the material.

Analyzing the presence of *c-KIT* activating - and *PDGFRA* mutations, either by LightCycler melting curve analysis or conventional sequencing, in the group of sixteen DSD cases showing GB, with or without an invasive tumor, showed that in the majority of cases no mutations could be detected (15 out of 16 cases, 94%). It must be mentioned however, that in a number of cases the percentage of tumor present in the sample was low, possibly leading to false negative results. In three patients a shift in melting curve not corresponding to one of the *c-KIT* c.816 mutations investigated was found, and subsequent sequencing of the LightCycler products revealed a wild type exon 17 sequence in case 1 and 2, and a known synonymous SNP (rs 55789615) in case 4 (I798I), although this latter finding was not confirmed by conventional sequencing of the original DNA sample. Strikingly, these three patients all had bilateral disease. The I798I variant was also detected in a patient with ovarian dysgerminoma (case 30, see above). In one patient (case 16) showing GB and mainly dysgerminoma, missense mutations in *c-KIT* were found in exon 9 and 17, resulting in N505I and D820E respectively, which were not present in

normal adjacent adnexal material. In this case, which was also positive for TSPY, presence of the Y-chromosome was confirmed with fluorescent *in-situ* hybridization on paraffin embedded material of the dysgerminoma lesion using a Y-centromeric probe (data not shown), confirming a 46,XY-DSD diagnosis. A mutation in *c-KIT* codon 816 in a DSD patient presenting with GB and dysgerminoma has also been reported previously [16], indicating that in rare cases these mutations can be found in DSD patients. Interestingly, the phenotypically male patient described here presented with a unilateral cryptorchid testis, which was removed during orchidopexy. He has two sons, who both presented with bilateral cryptorchid testis, which is one of the major risk factors for testicular GCC [45]. If the mutations found are also present in the sons cannot be ascertained as no material is available for analysis. To our knowledge this is the first time a N505I mutation in exon 9 *of c-KIT* has been found. *c-KIT* mutations in exon 9 have been described in GIST [46], and it is thought that these mutations mimic the conformational change that the extracellular c-KIT receptor undergoes when SCF is bound [47]. The activating *c-KIT* D820E mutation has been described together with mutations in exon 9, related to sunitinib resistance in GIST [48]. If the mutations found are located on the same or different alleles cannot be determined, as only paraffin embedded material was available for analysis. Besides the specific *c-KIT* c.816 mutations investigated here, other mutations in exon 17 have been reported in GCC; *c-KIT* gain-of-function D820G and Y823D [2, 10, 20, 37] have been found, next to S821F, C809S, Y823N and D816E together with D820H [12, 20] amongst others, which are not present in the cases analyzed here, and thus do not seem to be involved in ovarian dysgerminomas or DSD. Interestingly, recently genome-wide association studies of have identified SNPs within *KITLG* (SCF) as having the strongest association with an increased risk of developing a testicular GCC, pointing to the importance of the SCF-cKIT pathway in this disease [49-51].

Taken together, *c-KIT* mutations occur in approximately half of pure ovarian dysgerminoma cases, all residing in exon 17, indicating a role in the etiology of the disease. The activated c-KIT, together with prolonged expression of OCT3/4 may allow increased survival and proliferation of undifferentiated gonocytes/oogonia, leading to the development of dysgerminoma. In DSD, presence of Y-chromosomal material leads to the gonadal dysgenesis, in which the germ cells survive because of prolonged expression of both OCT3/4 and TSPY, setting the stage for GB and subsequent dysgerminoma development; although in a minority of cases mutations in *c-KIT* might play a role.

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Chapter 10

General Discussion

Chapter 10

10.1 Introduction

Although the overall incidence of Germ Cell Cancer (GCC) in the general population is low, the type II germ cell tumors/cancer of the testis (TGCC) are the most frequently diagnosed cancer in men aged between 20 and 40 years of age, and the incidence is still rising. These cancers arise from early embryonic germ cells, which by prolonged expression of factors related to pluripotency and proliferation can survive in an unfavorable environment. Disorders of sex development (DSD) are a heterogeneous group of disorders, consisting of three main groups, namely 46,XY-DSD, 46,XX-DSD and sex-chromosome-DSD. DSD patients who bear specific Y-chromosomal material in their karyotype have an increased risk for developing a GCC. The studies described in this thesis illustrate the spectrum of gonadal differentiation present in DSD patients, and deal with specific genetic aberrations involved in the (mal)formation of the gonads, thereby giving a better insight into the micro-environment in which the different GCC precursor lesions develop, possibly leading to an improved identification of patients at risk for developing a GCC. The results will be discussed in light of understanding the pathogenesis of the disease and early identification of patients at risk.

10.2 Gonadal development

The primordial germ cells (PGCs), which are the cells that will later form spermatozoa in males and oocytes in females, arise early in development in the proximal epiblast [1], and migrate through the hindgut to the genital ridges [2]. The stem cell factor (SCF, also known as KITLG)-c-KIT pathway plays an important role in the survival, migration and proliferation of the PGCs during this time [3-5]. Multiple factors are involved in the process of initial formation of the germ cells, amongst others: pluripotency related factors like OCT3/4, NANOG and LIN28 [6-8], bone morphogenetic proteins [9], and others like AP-2gamma [10] and DND1 [11]. The PGCs are characterized by their alkaline phosphatase reactivity (AP) [12]. Depending on the presence of the Y-chromosome and specifically expression of the *SRY* (sex-determining region on the Y-chromosome) gene, the until then indifferent bi-potential gonad will form a testis and the PGCs (then called gonocytes) will associate with (pre-)Sertoli cells, go into mitotic arrest, and differentiate into (pre-)spermatogonia. The expression of embryonic markers like OCT3/4, NANOG, AP and c-KIT is lost during this process and the expression of other factors like TSPY and VASA will be enhanced [13-17]. Full spermatogenesis will be initiated under the influence of testosterone after puberty [18]. In the absence of SRY the bi-potential gonad

will develop into an ovary, and the PGCs will differentiate into oocytes, also losing expression of the aforementioned embryonic markers [15, 19]. In contrast to the male situation, in the female the germ cells will go into meiotic I arrest and meiotic oocytes will be present from post partum onwards.

As mentioned above one of the critical events in the formation of a testis (and ultimately a phenotypical male) depends on the expression of the on the Y-chromosome located *SRY* gene. Expression of *SRY* will induce expression of SOX9, initiating the formation of pre-Sertoli cells, which will further orchestrate the formation of a functional testis [20-23]. It has to be mentioned that the formation of a testis is not depending on the presence of germ cells, this in contrast to the female situation. Anti-Müllerian hormone (AMH) produced by the (pre-)Sertoli, and testosterone and insulin-like factor 3 (INSL3) produced by the Leydig cells, are responsible for further development of the male internal and external genitalia [24-25]. The fate of the bi-potential gonad will be directed towards the ovarian lineage in the absence of a Y-chromosome (functional *SRY*). It has become clear that ovarian development depends on multiple factors working in parallel, including FOXL2, RSPO1 and WNT4. Foxl2 is required for commitment to ovary differentiation, granulosa cell differentiation and ovary maintenance in the mouse (see also below) [26-28], and loss of Wnt4 and Foxl2 leads female to male sex reversal in the mouse [26]. RSPO1 was found to be disrupted in a family showing 46,XX sex reversal [29], and in the mouse loss of Rspo1 causes partial female-to-male sex reversal [30]. Rspo1 and Wnt4 exert their role by regulating β-catenin [30-32], and high levels of β-catenin prevent maintenance of *Sox9* expression [33-35]. The formation of an ovary is, in contrast to testicular development, dependent on the presence of germ cells, absence of which will result in so-called streak gonads (non-functional stroma without germ cells). The absence of both AMH and androgen action in the female will lead to the development of female internal and external genitalia.

Although the formation of a testis or an ovary is determined during early development, the fate of the gonads does not seem to be set in stone after this time. It has been found in mice that FOXL2 is required to prevent transdifferentiation of the adult ovary to a testis. Inducible deletion of *Foxl2* in adult ovarian follicles lead to the up-regulation of testis specific genes including *Sox9*, and the granulosa and theca cell lineages were reprogrammed to Sertoli - and testosterone producing Leydig cell lineages respectively [28]. So the mammalian ovarian phenotype has to be maintained throughout adulthood, mainly by active repression of *Sox9* by FOXL2. Vice versa, in the mouse testis, loss of the DMRT1 transcription factor in Sertoli cells, even in adults, activates

Foxl2 and Sertoli cells are reprogrammed to become granulosa cells. In this environment estrogen producing theca cells are formed and germ cells appear to be feminized [36].

10.3 Disorders of sex development, precursor lesions and germ cell cancer

Patients with DSD have a congenital condition in which the development of chromosomal, gonadal or anatomical sex is atypical (see also Table 1, Chapter 1) [37]. Certain subgroups of these patients have an increased risk of developing a germ cell malignancy, which is directly linked to the presence of a specific part of the Y-chromosome, being the gonadoblastoma on the Y-chromosome (GBY) region [38]. The most likely candidate gene in this region is *TSPY* [14, 39-40].

The type II germ cell tumors/cancers (GCC) of the ovary, testis and dysgenetic gonad originate from an embryonic germ cell, in line with the identified risk factors for this disease as well as the striking overlap between the precursor lesions and PGC characteristics, including morphological characteristics, proteome, epigenome and transcriptome [14, 41-48]. It has been hypothesized that the so-called Testicular Dysgenesis Syndrome (TDS), which links clinical observations like cryptorchidism, infertility and hypospadias with certain environmental factors is the underlying entity in these cancers, although genetic factors are also recognized to play a role [49-53]. The GCC precursor lesions are the co-called carcinoma *in-situ* (CIS) of the testis and gonadoblastoma (GB) in the dysgenetic gonad [54-55]. In **Chapter 3** insight is given into the precursor lesions which can develop in DSD patients. By studying patients with various forms of DSD for which the underlying genetic defect was known a number of conclusions could be drawn: 1) a high risk for GB is found when sex determination is disrupted in an early stage of Sertoli cell differentiation, 2) defects occurring later in gonadal development results in an enhanced risk of CIS as precursor. Both CIS and GB show expression of embryonic germ cell markers like AP, c-KIT, OCT3/4, TSPY and SCF, and it is suggested that they are derived from fetal germ cells which are arrested in early stage of development [48, 56-57]. The transcription factors SOX9 and FOXL2 are required for male and female gonadal development respectively, and in **Chapter 4** the role of these key proteins is investigated in DSD. The results show that next to the expected expression patterns in normal gonads; 1) CIS is always found in gonads with obvious testicular differentiation [58], located at the basal lamina, and associated with Sertoli cells being positive for SOX9. 2) In contrast, when there is no clear testicular differentiation, GB can develop, and

the supportive cells are positive for FOXL2, a granulosa cell marker, although one case has been described with GB associated with supportive cells expressing both FOXL2 and SOX9 (**Chapter 6**). 3) Some DSD patients show expression in the supportive cells of both FOXL2 and SOX9, not associated with precursor lesions, sometimes even within one tubule, pointing to an issue of balance. 4) Both CIS and GB can be found in patients with DSD, in some cases even within one gonad, being the end result of the level of testicular development (see also **Chapters 3** and **5**). These markers nicely show the level of testicularization which can be found in these patients, giving insight into the micro-environment in which the different precursors can develop. Knowledge of this distribution and expression pattern of the markers mentioned above (i.e. OCT3/4, TSPY, FOXL2, and SOX9) is important to identify DSD patients with a high risk to develop GCC. Important in this respect is also the ability to distinguish germ cells delayed in maturation from pre-malignant cells, which can be done by morphological criteria [59-60], but also by presence of SCF (KITLG) [61]. Interestingly, as mentioned above, linkage to this gene has been found related to the development of GCC of the testis [53, 62-63]. The relevance and feasibility of early recognition of TDS and DSD is described in **Chapter 5**.

Taken together, it seems that in DSD and TDS there is a disturbance in the interaction between the germ cells and supporting cells (i.e. the Sertoli and granulosa cells). This initially leads to a delay in maturation, which is possibly followed by malignant transformation of the germ cells. This disturbance seems most prominent in the gonads with the lowest levels of testicularization, possibly linked to the presence of the GBY region and the most likely candidate gene therein, *TSPY*. Presence of TSPY in the germ cells might block them from further maturing in the female direction, even in the presence of supporting granulosa cells, as is the case in GB. In the case of TDS, reduced functionality of the Sertoli cells might have a similar effect on the maturation of the germ cells to pre-spermatogonia, leading to the formation of CIS.

10.4 Gene mutations in disorders of sex development: *SRY and WT1*

Many genetic factors are known to play a role in DSD. Among the genes that have been identified are *SRY* [64] and *RSPO1* [29] in 46,XX DSD, and *SRY* [65], *SOX9* [66], *NR5A1* (*SF1*) [67], *WT1* [68-69], *NR0B1* (*DAX1*) [70], *WNT4* [71], *MAP3K1* [72], and *WWOX* [73] in 46,XY DSD. Aberrations in SRY, and WT1 are the main topic in **Chapters 6, 7,** and **8** and will be further discussed in this paragraph.

10.4.1 *SRY*

As mentioned above the decision if the undifferentiated gonad will form a testis depends on the presence of the Y-chromosome and specifically on the expression of the *SRY* gene, which was first identified by Sinclair *et al.* [64]. The SRY gene encodes a 204 amino acid protein, containing a central DNA binding high-mobility group (HMG) domain, and two nuclear localization signals (NLS) and is involved in the binding and bending of DNA [74-77].

In 10-15% of 46,XY DSD (46,XY male to female sex reversal) patients inactivating mutations in SRY have been identified, with most residing in the HMG domain, and effecting the binding and bending of DNA [78-79]. Next to these, also mutations in the NLS of SRY have been described. It was first found by Li *et al.* [80] that a specific mutation in the HMG domain (R133W) had no effect on DNA binding, but resulted in impaired nuclear localization of SRY. Further mutations in either 1 of the 2 NLSs of SRY were examined in four female 46,XY DSD patients, in which in all cases SRY showed a reduction in nuclear import, linking the C-terminal NLS to importin beta [81]. A mutation in the N-terminal NLS of SRY (W70L) has been described in **Chapter 6**, resulting in a 50% decrease in nuclear accumulation of the mutant SRY. This decrease, which has lead to an impairment of testis differentiation, is reflected by the FOXL2 positive staining of some of the supportive cells, resulting in the micro-environment in which GB could develop [82]. However, several 46,XY DSD cases have been described in which it is not clear how the sequence alterations found contribute to the disorder [83-84], and familial *SRY* mutations have been described in which the mutation present in the patient was also found in family members showing no sign of DSD [78]. Moreover, Domenice *et al.* [85] have described a patient with a *SRY* mutation in the 5' non-HMG region of SRY which was present in normal male relatives. In contrast, Isidor *et al.* [86] describe a family with two sisters with 46,XY DSD, showing GB and dysgerminoma, harboring a SRY frameshift mutation also present in mosaic constitution in their father. He showed signs which are associated with TDS suggesting a possible association between TDS and *SRY* mutations. Shahid *et al.* [87] describe a sex chromosomal DSD patient with GB and a mosaic 45,X/46,XY karyotype with a frameshift mutation in SRY (L94fsX180) also present in mosaic form in the father of the patient, showing mild signs of TDS, again suggesting a link between *SRY* mutations and TDS. It seems that some mutations in *SRY* in a particular genetic background might produce sufficient and timely SRY expression to reach threshold levels required for testis formation [78]. It would be interesting to investigate the presence of variations

in the *SRY* gene in a larger group of patients showing signs of TDS (cryptorchidism, infertility, with or without a testicular GCC) to gain further insight into the possible role SRY plays in this syndrome.

Next to the SRY mutations found in 46,XY DSD, they have also been described in isolated cases of sex chromosomal DSD with a 45,X/46,XY karyotype [87-90] suggesting an additional effect of mutant SRY in the gonadal development of these patients. All studies to date have been performed using conventional sequencing methods; to investigate the presence of possible *SRY* gene mutations in mosaic sex chromosome DSD patients we used a highly sensitive next generation sequencing approach (described in **Chapter 8**). This revealed no variations in the *SRY* gene in a cohort of 14 mosaic sex chromosomal DSD patients, indicating, together with results published by others, the rarity of these mutations (only present in 8% of cases studied).

10.4.2 *WT1*

Wilm's tumor 1 (WT1) plays an important role in early gonadal and kidney development [91], and is expressed earlier in time than *SRY* in the urogenital ridge. WT1 has multiple isoforms, which all contain four C-terminal zinc fingers necessary for DNA/RNA binding. The two major isoforms found differ in an insertion (+KTS) or exclusion (-KTS) of three amino-acids (lysine, threonine and serine) between zinc fingers three and four. It has been shown that the –KTS isoform mainly plays a role in transcription and *AMH* transcriptional activation in Sertoli cells [92]. The +KTS isoform is involved in RNA processing, and plays a role in *Sry* regulation *in vivo* in the mouse [93].

Mutations in *WT1* play a role in 46,XY DSD, i.e. Frasier syndrome (FS), Denys-Drash syndrome (DDS) and WAGR (Wilm's tumor, Aniridia, Genitourinary, mental Retardation) syndrome. Patients with DDS and WAGR syndrome have a high risk to develop Wilms tumor, and all patients with a *WT1* mutation are at risk of developing GB/CIS and GCC. The mutations found in FS mainly consist of *WT1* intron 9 splice site mutations, and these patients have complete 46,XY sex reversal, late onset kidney failure, focal segmental glomerulosclerosis, streak gonads and a high risk for GB [94]. These specific intron 9 splice site mutations lead to a decrease in the +KTS isoform and subsequent reversion in the +/- KTS ratio. This causes defects in the development of glomerular podocytes and male sex-determination, in the end leading to nephritic syndrome and male-to-female sex reversal [69, 94]. **Chapter 7** describes a unique case with bilateral GB and dysgerminoma in an adult woman, who was initially diagnosed with 46,XY DSD, complete gonadal

dysgenesis. Mutation analysis of the SRY gene identified a novel missense mutation (c.383A>G, p.Lys128Arg), which did not have a significant effect on transcriptional activation and nuclear import *in vitro*, although a more dramatic effect *in vivo* cannot be ruled out. As the patient developed progressive renal failure during follow-up, the WT1 gene was analyzed for mutations, showing an exon 9 splice site mutation (IVS9 +5 G>A) resulting in the final diagnosis FS. This combination of a mutation in both *SRY* and *WT1* has not been described before, and sequencing of the *SRY* gene in five additional FS patients did not show any aberrations in the gene. The case presented clearly demonstrates the importance of proper diagnosis of the different variants of DSD.

10.5 *c-KIT*

Recently a set of single nucleotide polymorphisms (SNP) were identified in independent GWAS studies found to be associated with an increased risk of developing a GCC [53, 62-63, 95]. SNPs within KITLG (also known as stem cell factor, SCF), the ligand for c-KIT, were identified as having the strongest association with an increased risk of developing testicular GCC, pointing towards involvement of the c-KIT-SCF pathway in this disease. It is known that activating mutations in c-KIT play a role in the development of testicular GCC (seminomas), and most are missense mutations at codon 816 in exon 17 [96-99]. Activating mutations have, amongst others, also been found in ovarian dysgerminomas, which show the same histology, and a similar pattern of chromosomal aberrations as testicular GCC [100], and gastro-intestinal tumors (GIST). In GIST mutations in *PDGFRA*, a close homologoue of KIT, are also recognized to play a role, and mutations in *c-KIT* and *PDGFRA* are mutually exclusive [101-104]. Moreover, families with both ovarian and testicular GCC have been reported, suggesting a common etiology [105]. The role activating c-KIT and PDGFRA mutations play in the development of (ovarian) dysgerminoma and its precursor lesion in patients with DSD has been studied in **Chapter 9.** The results show that; 1) although a limited series, mutations in *PDGFRA* exon 12, 14 and 18, are not found in ovarian dysgerminomas or DSD. 2) In contrast to its role in ovarian and testicular GCC, activating mutations in c-KIT are not frequently found in DSD. 3) Besides this, next to the c-KIT codon 816 mutations (33% of cases) found, which have also been described in literature [106-107], a significant number (20% of cases) of ovarian dysgerminomas show c-KIT codon 822 mutations. It is conceivable that in ovarian dysgerminomas, the supportive granulosa cells are not able to properly nourish the germ cells and activating mutations in *c-KIT* together with

prolonged expression of OCT3/4 may, in the absence of TSPY, lead to the survival, malignant transformation and ultimately development of a GCC. In DSD, germ cells expressing the Y-chromosome located TSPY, reside in a environment with a low level of testicular differentiation (testicularization), as shown by the FOXL2 or heterogeneous FOXL2 together with SOX9, staining in the supportive cells (see above). The germ cells present in this environment can undergo malignant transformation, depending on the prolonged expression of OCT3/4 and enhanced TSPY expression, ultimately leading to the invasive GCC. In testicular GCC there is a micro-environment in which the SOX9 expressing Sertoli cells are not able to properly support the male germ cells which can undergo malignant transformation, related to the prolonged expression of OCT3/4 and enhanced expression of TSPY, together with, in a number of cases, activating mutations in *c-KIT*. This is in accordance with the earlier finding of Hoei-Hansen *et al.* [107]. The clinical implication is that in absence of DSD characteristics, analysis of activating mutations in *c-KIT* is indicated. The risk for bilateral disease in these specific cases is unresolved so far [108-110].

10.6 Future prospects and challenges

Although a number of genes have been found to play a role in the development of DSD, in a majority of cases it has not been possible to find the causative mutation, hindering genetic counseling and possibly treatment. With the advent of whole-genome copy number (CNV) analysis, whole genome/exome, and next generation deep sequencing has come the possibility to investigate genomic aberrations underlying DSD in an unprecedented manner, which has already resulted in a number of interesting findings.

By analyzing a series of 23 unexplained 46,XY DSD patients by whole-genome CNV analysis, White *et al.* were able to identify three discrete changes in copy number which were likely the cause of the gonadal dysgenesis [111]. They found a large duplication on the X-chromosome, which included *DAX1*, a small deletion immediately downstream of *GATA4* was identified, and a 1.2 Mb deletion was found 300 kb upstream of *SOX9*. This last patient did not show any signs of campomelic dysplasia, a condition associated with mutations in, and deletions and translocations up to 1Mb upstream of the coding sequence of *SOX9*, and 46,XY sex reversal [112-114], suggesting that the deletion only affects *SOX9* expression in the gonads. Further investigation revealed five putative SRY binding sites in this region, indicating that additional sequences next to the known SRY-binding TES-enhancer [21] affect SOX9 expression. Next to these also several previously unreported CNVs were identified affecting the coding

region of genes not known to play a role in gonadal development. It was shown that two of the orthologous mouse genes (*Dnajc15* and *Camkd1*) show a sexually dimorphic expression at the time of sex differentiation. Benko *et al.* [115] using whole-genome CNV analysis identified duplications upstream of the *SOX9* gene in three families with an isolated 46,XX DSD, together with an overlapping deletion in *SOX9* in a family with two probands with 46,XY DSD. A heterozygous multi-exon deletion in *WWOX* removing exons 6-8, was found in a 46,XY DSD patient by whole-genome CNV analysis [116]. However, the deletion was also present in the mother of the patient, not showing any signs of DSD, only irregular menstruation before her first pregnancy. Wwox knock-out mice show gonadal abnormalities, including defects of Leydig cell function [117-118]. The effects of the deletion could be through reduced inhibition of the WNT/β-catenin pathway [119], and/or its possible role in gonadotrophin or sex-steroid biosynthesis. The study by Sutton *et al.* [120] identified *SOX3* as an XX male sex reversal gene in mice and humans, using transgenic mice over-expressing *Sox3* which showed frequent female-to-male sex reversal and combining this with whole-genome CNV analysis of sixteen 46,XX DSD cases, showing rearrangements in the regulatory region of the gene in three patients.

These new tools not only show novel ways to analyze the underlying defects in DSD, but also provide challenges. When aberrations are found resulting in deletion (of part of), or rearrangements in the gene, like in *WWOX*, the effect might easily be explained. But when rearrangements further up- or downstream of a gene are found, identifying regulatory elements residing in that region, and linking them with gene expression will be a daunting task. Whole-genome sequencing can give the same problems as whole-genome CNV analysis, and is (still) costly, and although whole-exome sequencing is a more cost effective method, only aberrations in the coding sequences of genes can be identified. It must be mentioned that advances in this field are developing rapidly, and in the near future whole-genome analysis will be, no doubt, possible in a more cost effective manner. Beneficial in identifying new genes and/or regulatory elements in these patients will be the use of large numbers of well described cases with, whenever possible, DNA obtained from family members. Of importance in this respect are, the diagnosis and treatment of these patients in centers with expertise in DSD, and national and international cooperation of these centers to obtain sufficient numbers of well defined cases, providing a solid basis for doing these kind of studies. This will undoubtedly lead to an improved understanding of the underlying genetic defects in DSD. However, as specific genetic defects can have different effects in individual DSD

patients, no unambiguous answer relating to GCC risk can be given based solely on this knowledge. To asses GCC risk in individual DSD patients additional (interrelated) parameters have to be taken into account. These include, the impact of the genetic defect on gonadal differentiation (i.e. level of testicularization), presence in the gonad of malignant germ cells (positive for OCT3/4, TSPY and SCF), and position of the gonad (abdominal, inguinal, or scrotal). Moreover, in the Caucasian population, genome-wide association studies of testicular GCC and TDS have thus far led to the emergence of a few key pathways involved, being the cKIT-SCF pathway (*KITLG*, *SPRY4* and *BAK1*), telomerase regulation (*TERT* and *ATF7IP*), sex determination (*DMRT1*), and the transforming growth factor ß pathway (*TGFBR3* and *BMP7*) [53, 62-63, 95]. These have to be included as parameters as well. Supporting the fact that DSD is a major risk factor for GCC is the finding that, although this type of cancer is rare in the general Indonesian population, possibly associated with a lower frequency of the high risk alleles, in the DSD patients from this ethnic group, it is as frequently found as in Caucasians (B. Setyawati, unpublished observations). Whatever the underlying defect, important is the long-term surveillance of DSD patients with a high GCC risk, and the need for multi-disciplinary DSD teams in the treatment of these patients, thereby providing the most optimal individualized care possible.

10.7 Concluding remarks

The development of an invasive GCC is the final outcome of a multitude of steps starting with the maldevelopment of the gonad, i.e. leading to a disrupted micro-environment in which the germ cells reside. The risk for malignant transformation depends on a number of factors related to the level of testicular development (testicularization) of the gonads, and the survival and proliferation of the germ cells in this environment. In the case of TDS and DSD environmental and/or genetic/chromosomal abnormalities lead to a lower level of testicularization. The studies performed show that in the case of DSD this can be the result of a reduction in nuclear import of SRY, presence of a *SRY* together with *WT1* mutation, or a mosaic sex-chromosomal constitution in which *SRY* gene mutations do not play a significant role. The role, if any, SRY mutations/variants play in TDS remains to be elucidated. Aberrations in the genes described here and by others, only provide an explanation in a subset of DSD cases, and in many patients the underlying gene defect remains to be identified. Although challenging, next generation -, whole genome -, whole exome sequencing and whole genome CNV analysis might provide ways to

identify these (see above). The germ cells present in this environment are delayed in their maturation and have the ability to undergo malignant transformation, this related to the prolonged expression of OCT3/4, increased TSPY expression and presence of SCF. Double staining for OCT3/4 and TSPY is informative to identify these cells in this microenvironment. This indicates that OCT3/4 is not the driving factor in the development of this type of cancer, just a result of the lack of maturation of the germ cell. Activating mutations in the c-*KIT* gene, which are known to play a role in the development of testicular and ovarian GCC, are not frequently present in the precursor lesions and invasive tumors found in DSD patients. This indicates that the pathways leading to the development of the tumors are differently regulated, and the precursor of dysgerminoma in females without DSD remains to be identified. The malignant germ cells will form the precursor lesions found in TDS and DSD, either GB or CIS, depending on the level of testicularization of the gonad, directly related to the presence of SOX9 and FOXL2 in the supportive cells. In this respect, investigation of possible sub-maturation of the Sertoli cells in the context of TDS remains to be answered. The interplay between genetics and environment, as described above, is referred to by us as genvironment, and needs to be further explored in the context of GCC pathogenesis. A schematic representation of the interrelationship between clinical and pathobiology is shown in Figure 1. Knowledge of these factors allows early identification of and possible intervention in patients at risk for developing GCC.

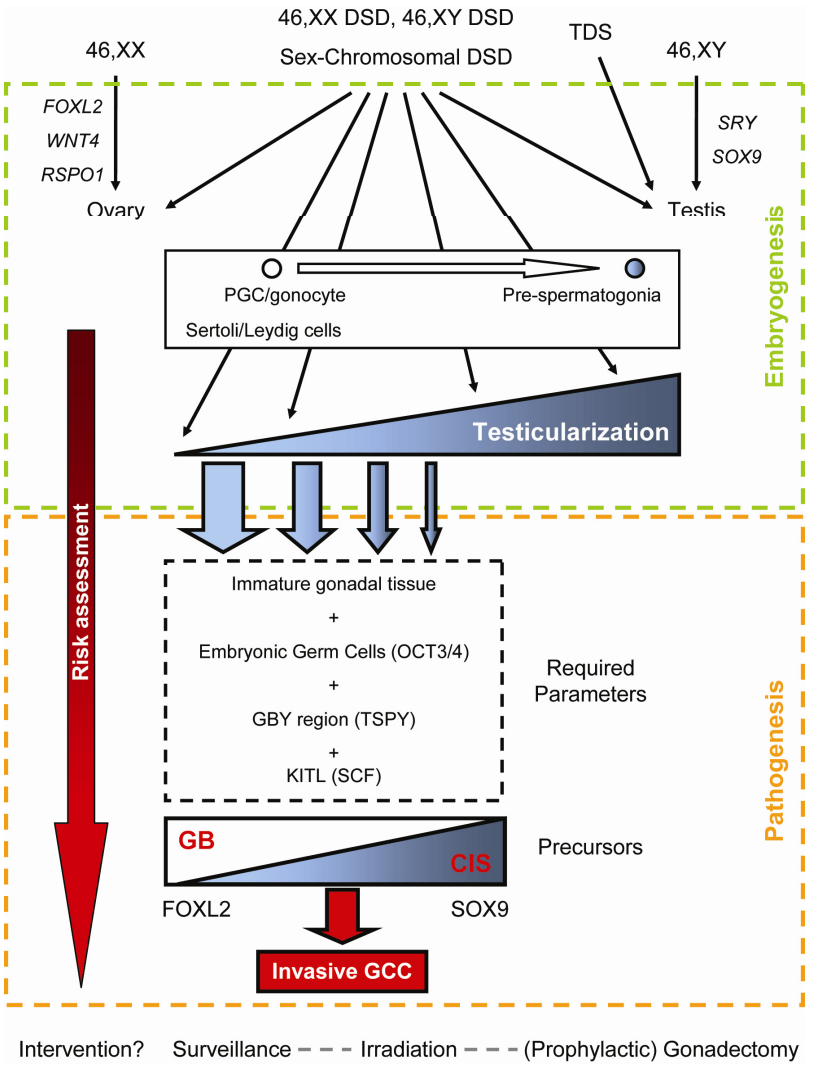

Figure 1. Schematic representation of the interrelationship between clinical and patho-biology. Patients present in the clinic with either a form of disorder of sex development (DSD) or testicular dysgenesis disorder (TDS). The risk of malignant transformation, resulting in an invasive germ cell cancer (GCC), is most likely related to various parameters, including environment, as well as genetic/chromosomal anomalies (genvironment). These lead to a disturbed gonadal development, in which the function of the supportive Sertoli/granulosa cells is impaired. Germ cells residing in this micro-environment might be delayed or blocked in their normal maturation, and prolonged expression of OCT3/4, SCF and increased TSPY expression enhances their chances of survival. A high chance of development of the precursor lesion is present in gonads with a low level of testicularization, which have all the required parameters present. Formation of gonadoblastoma (GB) or carcinoma in situ (CIS) depends directly on the level of testicularization and the expression of FOXL2 or SOX9. Depending on this knowledge, clinical intervention can be performed, ranging from no action at all (absence of hypovirilization or gonadal dysgenesis), surveillance, irradiation (proven presence of CIS), or prophylactic gonadectomy (patient fulfills all criteria to be included in the high risk group).

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Chapter 11

Summary-Samenvatting

Summary

Disorders of sex development (DSD) refer to a congenital condition in which there is an atypical development of chromosomal, gonadal or anatomical sex. DSD consists of three main groups, namely 46,XY-DSD, 46,XX-DSD and sex chromosome-DSD. DSD patients who contain Y-chromosomal material in their karyotype have an increased risk for developing a Type II Germ cell Tumor/Cancer (GCC). The work described in this thesis aims to improve the understanding of the pathobiology of GCC in DSD patients. Giving a better insight in the genes/factors involved in certain forms of DSD, thereby better understanding the micro-environment in which the precursor lesions of the cancer develop, can lead to an improved identification of patients at risk. This in turn will facilitate early detection, diagnosis and treatment, preventing the development of an invasive and possibly metastatic cancer. Such an approach will putatively result in a more localized treatment and thereby excluding long term effects of irradiation and or chemotherapy.

The actual risk for malignant transformation of germ cells in individual DSD patients has been (and still is) hard to predict, partly because of the confusing terminologies which have been used until recently. In **Chapter 3** a large series of DSD patients described in literature and for which the underlying genetic defect was known has been analyzed. It shows that a high risk for gonadoblastoma (GB), the precursor lesion of GCC in the dysgenetic gonad, is present when sex determination is disrupted in an early stage of Sertoli cell differentiation. Important is to remember that GB can only form when the GBY region of the Y-chromosome is present. Defects later in gonadal development results in an enhanced risk of carcinoma in-situ (CIS) as precursor lesion, as can also be found in males without DSD, who will develop a GCC. Both precursor lesions can even be found within a single gonad, and it seems that GB and CIS are a continuum, of which the phenotypic presentation is determined by the micro-environment.

It is known that the SOX9 and FOXL2 transcription factors are necessary for gonadal development in the male and female respectively. In **Chapter 4** the protein expression profiles in gonads of DSD patients was investigated using specific antibodies. In normal gonads, SOX9 was only found in the (pre-)Sertoli cells of the testis, and FOXL2 was only present in the granulosa cells of the ovary. In DSD patients expression patterns were overall in accordance with testicular and ovarian differentiation, as expected based on published data and in line with the normal gonads included in the study. However, some FOXL2 positive cells were detected in semiferous tubule-like

structures in a few DSD cases. Looking at the precursor lesions it was found that testicular CIS was always associated with strong SOX9 staining in the Sertoli cells, and in GB the supportive cells clearly showed FOXL2 positive staining, although it must be mentioned that in the patient described in **Chapter 6** GB with positive staining for both FOXL2 and SOX9 in the supportive cells was seen. It is likely that the level of maturation of the Sertoli cells related to testis formation (i.e., testicularization), due to the SRY-SOX9 pathway, determines whether the malignant counterpart of the primordial germ cells (PGCs) will manifest itself as CIS or GB or even both.

In **Chapter 5** three patients are described, two of which were diagnosed with an invasive GCC, accompanied by presence of both GB and CIS within one gonad. GB is only found in the dysgenetic gonad of DSD patients, retrospectively diagnosing these patients with DSD. This is based on their clinical history showing hypospadias and cryptorchidism. The third patient shows the power of recognizing these parameters, in which the precursor lesion was identified before progression into an invasive GCC. The karyotype was 46,XY in all three patients, and no obvious genetic aberrations were found by whole-genome CNV analysis. These three patients show overlap between the so-called Testicular dysgenesis Syndrome (TDS) and DSD.

A subset of 46,XY DSD cases can be caused by either mutations in the *SRY* gene or mutations in the *WT1* gene. In **Chapter 6** a novel missense mutation is described which was found in a 46,XY female patient showing bilateral GB, which on one side showed staining in the supportive cells for both SOX9 as well as FOXL2. The W70L mutation resides in one of the two nuclear localization signals present within SRY, and resulted in a 50% reduction in nuclear accumulation of the SRY protein, likely explaining the diminished SOX9 expression and improper Sertoli cell differentiation during development. Another novel *SRY* missense – together with a *WT1* mutation, found in a 46,XY female with bilateral GB and invasive GCC, is described in **Chapter 7**. The SRY K128R mutation detected, although present in the central HMG domain, did not lead to a significant reduction in transactivation or a reduction in nuclear import *in vitro*. As the patient had delayed progressive kidney failure the *WT1* gene was analyzed, revealing a pathogenic splice-site mutation in intron 9, know to be the cause of Frasier syndrome (46,XY gonadal dysgenesis). Screening five Frasier syndrome patients with a proven *WT1* mutation did not reveal any sequence variants in *SRY*, indicating that, although a limited series, presence of *SRY* mutations in Frasier syndrome is rare. To our knowledge this is the first patient described with both a *SRY* variant as well as a classical Frasier syndrome

WT1 mutation, demonstrating the importance of proper diagnosis of DSD patients.

Next to the *SRY* mutations found in 46,XY DSD patients, a limited number of mutations in this gene have been described in DSD patients with a mosaic sex-chromosome karyotype, thought to play a role in the etiology of the disease. In **Chapter 8** the presence of *SRY* mutations in a series of fourteen DSD patients with a mosaic sex-chromosome karyotype was, for the first time, analyzed by a highly sensitive next generation sequencing approach, revealing no aberrations. Including this study, a total of 91 patients have been screened for *SRY* mutations, of which only seven showed a variation (8%), indicating that these mutations are rare and only play a role in a minority of cases.

Both in testicular GCC (semimoma) as well as ovarian GCC (dysgerminoma), activating mutations in the *c-KIT* gene are found, although in the latter presence of these mutations has not been extensively explored. In gastro-intestinal stromal tumors next to mutations in *c-KIT* also mutations in *PDGFRA*, a KIT homologue, are found, and these are mutually exclusive. DSD patients with specific Y-chromosomal material in their karyotype are at risk of developing an invasive GCC (dysgerminoma), with GB as precursor lesion. In **Chapter 9**, a series of 16 DSD patients presenting with GB and dysgerminoma and 15 patients presenting with pure ovarian dysgerminomas were analyzed for presence of *c-KIT* and *PDGFRA* mutations. In 53% of ovarian dysgerminomas a mutation in *c-KIT* was found, pointing to a role in the etiology of the disease. No mutations in *PDGFRA* could be detected. No correlation could be found between presence of activating mutations and c-KIT protein expression. In contrast, only one of the DSD patients showed *c-KIT* mutations, and TSPY protein expression was present only in the DSD cases, underlining the importance of presence of (part of) the Y-chromosome in the development of GB. The results point to the fact that in the case of DSD and ovarian dysgerminomas, the pathways leading to development of the tumors are distinct.

In conclusion, the work presented in this thesis gives a better insight into the micro-environment in which the precursor lesions found in DSD (CIS and/or GB) develop, and genetic factors involved in the (mal)development of the gonads, thereby facilitating early diagnosis of these patients.

Samenvatting

Stoornissen in de sex ontwikkeling (Engels: disorders of sex development (DSD) zijn congenitale condities waarin er een atypische ontwikkeling is van de chromosomale, gonadale of anatomische sex. DSD bestaat uit drie hoofdgroepen, namelijk 46,XY-DSD, 46,XX-DSD en sex chromosomale-DSD. DSD patiënten met specifiek Y-chromosomaal materiaal in hun karyotype hebben een verhoogde kans op het ontwikkelen van een type II kiemceltumor/kanker. Het doel van het werk beschreven in dit proefschrift is het beter begrijpen van de pathobiologie van kiemcelkanker in DSD patiënten. Een verbeterd inzicht in de betrokkenheid van genen/factoren die een rol spelen in bepaalde vormen van DSD, daarmee een beter begrip gevend betreffende het micromilieu waarin de voorloper stadia van de kanker zich ontwikkelen, kan tot een verbeterde identificatie van risico patiënten leiden. Dit zal een vroege detectie, diagnose en behandeling vergemakkelijken, en daarmee de ontwikkeling van een invasieve en mogelijk metastatische kanker voorkomen. Deze benadering zal uiteindelijk resulteren in meer gelokaliseerde behandeling, waarmee de lange termijn effecten van bestraling en of chemotherapie voorkomen zouden kunnen worden.

Het werkelijke risico op maligne transformatie van de kiemcellen in individuele DSD patiënten was altijd (en is nog steeds) moeilijk te voorspellen, dit komt mede door de verwarrende terminologie welke tot voor kort werd gebruikt. In **Hoofdstuk 3** is een grote serie DSD patiënten geanalyseerd welke zijn beschreven in de literatuur en voor welke het onderliggende genetische defect bekend is. Het laat zien dat er een hoog risico bestaat voor de ontwikkeling van gonadoblastoom (GB), de voorloper van kiemcelkanker in de dysgenetische gonade, als de sex determinatie verstoord is in een vroeg stadium van Sertoli cel differentiatie. Belangrijk om te onthouden is dat GB alleen kan ontstaan als de GBY regio van het Y-chromosoom aanwezig is. Defecten later in de gonadale ontwikkeling leiden tot een verhoogd risico op het ontstaan van carcinoma in-situ (CIS) als voorloper laesie, deze kunnen ook gevonden worden in mannen zonder DSD welke een kiemcelkanker ontwikkelen. Beide voorloper stadia kunnen zelfs gevonden worden binnen één enkele gonade. Dit geeft aan dat GB en CIS een continuüm zijn waarbij de fenotypische presentatie wordt bepaald door het micromilieu.

Het is bekend dat de transcriptie factoren SOX9 en FOXL2 nodig zijn voor de gonadale ontwikkeling in respectievelijk de man en de vrouw. In **Hoofdstuk 4** zijn in gonaden van DSD patiënten de eiwit expressie patronen onderzocht met behulp van specifieke antilichamen. In normale gonaden werd

SOX9 alleen gevonden in de (pre-)Sertoli cellen van de testis, en FOXL2 was alleen aanwezig in de granulosa cellen van het ovarium. De expressie patronen in patiënten met DSD was, zoals verwacht op basis van gepubliceerde data en in lijn met de normale gonaden geïncludeerd in de studie, in overeenstemming met ovariële en testiculaire differentiatie. Echter, in een beperkt aantal DSD gevallen werd soms FOXL2 positieve cellen gevonden in structuren lijkende op tubuli-seminiferi. In de voorloper stadia werd gevonden dat CIS altijd geassocieerd was met sterke SOX9 aankleuring in de Sertoli cellen, en dat in GB de ondersteunende cellen duidelijk een FOXL2 positieve aankleuring gaven. Hierbij moet wel worden opgemerkt dat in het GB van de patiënt beschreven in **Hoofdstuk 6** de ondersteunende cellen positieve aankleuring lieten zien voor zowel FOXL2 en SOX9. Het is waarschijnlijk dat het niveau van Sertoli cel uitrijping, gerelateerd aan testis formatie (i.e., testicularizatie), als gevolg van de SRY-SOX9 pathway bepaalt of de maligne tegenhanger van de primordiale kiemcel zich manifesteert als CIS of GB of een mix ervan.

Hoofdstuk 5 beschrijft drie patiënten, twee waren gediagnosticeerd met een invasieve kiemceltumor waarbij zowel CIS als GB in één enkele gonade aanwezig waren. GB komt alleen voor in de dysgenetische gonade van DSD patiënten, daardoor werden deze patiënten retrospectief gediagnosticeerd als DSD. Dit is mede gebaseerd op de aanwezigheid van hypospadie en cryptorchisme in hun klinische achtergrond. De derde patiënt, in welke de precursor ontdekt werd voor progressie naar een invasieve kiemceltumor, laat de waarde zien van tijdige herkenning van deze parameters. Alle drie patiënten hadden een 46,XY karyotype, en analyse met “whole-genome CNV analysis” liet geen voor de hand liggende genetische afwijkingen zien. Deze drie patiënten laten overlap zien tussen het zogenaamde testiculaire dysgenese syndroom en DSD.

Een subgroep van 46,XY DSD gevallen kunnen worden veroorzaakt door mutaties in het *SRY* of het *WT1* gen. **Hoofdstuk 6** beschrijft een nieuwe missense mutatie, gevonden in een 46,XY vrouwelijke patiënt met bilateraal GB. Aan één zijde liet het GB in de ondersteunende cellen positieve aankleuring zien voor zowel FOXL2 en SOX9. De gevonden W70L mutatie bevindt zich in een van de twee nucleaire lokalisatie signalen van SRY, en resulteerde in een 50% vermindering in nucleaire accumulatie van het SRY eiwit. Dit verklaart waarschijnlijk de verminderde SOX9 expressie en Sertoli cel differentiatie gedurende de ontwikkeling. Een andere nieuwe *SRY* missense mutatie samen met een *WT1* mutatie, gevonden in een 46,XY vrouwelijke patiënt met bilateraal GB en een invasieve kiemcelkanker, wordt beschreven in **Hoofdstuk 7**. De

gevonden SRY K128R mutatie, hoewel aanwezig in het centrale HMG domein, leidde niet tot een significante vermindering in transactivatie of een reductie in nucleaire accumulatie *in vitro*. Analyse van het *WT1* gen, gedaan vanwege een laat progressieve nier falen in de patiënt, liet een pathogene splice-site mutatie in intron 9, de oorzaak van Frasier syndroom (46,XY gonadale dysgenesie), zien. Analyse van het *SRY* gen in vijf Frasier syndroom patiënten met een bewezen *WT1* mutatie liet geen sequentie varianten zien. Dit is een indicatie dat, hoewel gedaan op maar een kleine serie, de aanwezigheid van *SRY* mutaties in Frasier syndroom zeldzaam is. Zover ons bekend is dit de eerste patiënt beschreven met zowel een *SRY* variant als ook een klassieke Frasier syndroom *WT1* mutatie, dit benadrukt het belang van een juiste diagnose van DSD patiënten.

Naast de *SRY* mutaties gevonden in 46,XY DSD patiënten, worden ook enkele mutaties in dit gen beschreven in DSD patiënten met een mozaïek sex-chromosoom karyotype, welke mogelijk een rol spelen in de etiologie van de ziekte. In **Hoofdstuk 8** wordt voor het eerst, met behulp van een zeer gevoelige "next-generation sequencing" aanpak gekeken naar de aanwezigheid van *SRY* mutaties in een serie van veertien DSD patiënten met een mozaïek sex-chromosoom karyotype. Hierin worden geen afwijkingen gevonden. Samen met deze studie zijn er in totaal 91 patiënten gescreend voor *SRY* mutaties, waarvan er maar 7 (8%) een variatie lieten zien, een indicatie dat deze mutaties zeldzaam zijn en alleen een rol spelen in enkele gevallen.

Zowel in testiculaire kiemceltumoren (seminomen) als in ovariële kiemceltumoren (dysgerminomen) worden mutaties in het *c-KIT* gen gevonden, maar in de tweede groep is de aanwezigheid van deze mutaties niet uitgebreid onderzocht. In gastro-intestinale stromale tumoren worden naast mutaties in *c-KIT* ook mutaties in *PDGFRA*, een homoloog van *KIT*, gevonden en sluiten deze elkaar onderling uit. DSD patiënten met specifiek Y-chromosomaal materiaal in hun karyotype hebben een risico op het ontwikkelen van een invasieve kiemcelkanker (dysgerminoom), met GB als voorloper. **Hoofdstuk 9** beschrijft de analyse van *c-KIT* en *PDGFRA* mutaties in een serie van 16 DSD patiënten met GB en dysgerminoma en 15 patiënten met puur ovariëel dysgerminoma. In 53% van ovariële dysgerminomen werd een mutatie in *c-KIT* gevonden, wijzend op een rol in de etiologie van de ziekte. In *PDGFRA* werden geen mutaties ontdekt. Een correlatie tussen de aanwezigheid van mutaties en eiwit expressie van c-KIT werd niet gevonden. In tegenstelling werd in maar één van de DSD patiënten mutaties in *c-KIT* gevonden, en eiwit expressie van TSPY was alleen aanwezig in de DSD gevallen, dit onderstreept het belang van het aanwezig zijn

van (een deel van) het Y-chromosoom in het ontstaan van GB. De resultaten wijzen op het feit dat in het geval van DSD en ovariële dysgerminomen de routes naar het ontstaan van de tumoren anders zijn.

In conclusie, het werk beschreven in dit proefschrift geeft een beter inzicht in het micromilieu waarin de voorlopers die worden gevonden in DSD (CIS en/of GB) ontstaan, als ook de genetische factoren betrokken bij de (onder)ontwikkeling van de gonaden en vergemakkelijken hierbij een vroege diagnose van deze patiënten.

Supplementary data

Chapter 7

Supplementary Table 1. *SRY* mutations in literature

	Histology description	Phenotype	Mutation	Functionality	Refs
GB	dysgenetic gonads, right microscopic GB	Female (complete GD)	p.E89K	almost completely abolished SRY DNA binding activity	[1]
GB	proliferation atypical germ cells admixed with GB	Female (complete GD)	Q57R	NA	[2]
GB	GB left	Female (complete GD)	C>G pos 184 (arg>gly)	NA	[3]
GB	GB (both patients)	Female (complete GD)	C>T pos 220 (glu>stop)	NA	[3]
GB	GB bilateral	Female (complete GD)	T>C pos 233 (met>thr)	NA	[3]
GB	Li Streak, right GB	Female (complete GD)	A>T pos259, codon 87 Asn>Tyr, (nucl pos 3,258 in HMG box)	NA	[4]
GB	Streaks, right GB	Female but little clitoromegaly	A>G pos 271, codon91 Ser>Gly, (publ: pos 681), S91G	Reduced ability of mutant protein to bind specifically to DNA	[5]
GB	GB	Female (complete GD)	A>T pos 274, codon 92 Lys>Stop	NA	[6]
GB	Streak with bilateral gonadoblastoma in oldest sister (16)	Female (complete GD), 2 sisters (newborn and 16)	C>T pos 289, codon 97 Gln>Stop	Incomplete DNA binding domain	[7]
GB	GB	Female (complete GD)	Y127F, A>T pos 380	Abolished binding capacity	[8]
GB	Bilateral GB	Female (complete GD)	Subst C at +352, A118P	Greatly reduced binding activity	[9]
GB	Bilateral GB	Female (complete GD)	G>T pos 209, W70L	50% reduction nuclear import	[10]
GB	Streaks, right GB	Female (complete GD)	A113T, G>A pos 337, ala>thr, codon113, (publ: pos 747)	NA	[11]
GB	1. bilateral GB +metas 2. encaps GB + streak	Females (2x), (complete GD)	F67V, (pos 199,T>G)	NA	[12]
DG	1. dysgerminoma right. 2.ovarial stroma differentiation of streak gonad, no follicular cells. 3.no histology	Family. Female (all complete GD)	P125L, C>T pos 374, codon 125 Pro>Leu, (publ: pos 681)	Reduced ability of mutant protein to bind specifically to DNA	[5]
GB DG	1. Right GB, Left Dysgerminoma. 2. streak no malignancy	Female (complete GD), 2 sisters	T>A pos 488, codon 163 Leu>stop	NA	[13]
SE GB DG	1. Seminoma. 2. A. left GB, ri DG, B. bilateral GB	Family. 1. father, mosaic state c.72del: hypospadia, cryptorchidism. 2. 2 sisters, Complete GD	c.71delA codon24. stop pos 60	NA	[14]
GB SE	1. bilateral streaks + GB. 2. testicular seminoma	Family. 1. turner, 45x 79%, 46XY 21%. 2. father, mutation in mosaic form	Del C>frame shift L94fsX180	NA	[15]
GB	1. Gonadal dysgenesis. 2. bilateral GB.	Family. 1. and 2. Female (complete GD). Father, mutation in mosaic form	c.347T>C p.Leu>Ser	Strong reduction in DNA affinity.	[54]
YST	Yolk sac tumor (pT4) with chorionic giant cells	Female (complete GD)	G>A pos 284 codon 95 Gly>Glu	NA	[16]
OT	Ovotestis	Female. Ambiguous genitalia. (Ovotesticular DSD)	T>C pos 237 codon 79 Ala>Ala. (silent)	NA	[17]
OT	Ovotestis	Female. Ambiguous genitalia. (Ovotesticular DSD)	T>A pos 302, codon 101, Leu>His	NA	[17]
OT	Ovotestis (unilateral)	Female. (true hermaphroditism)	T>C codon 60 pos 179, Val>Ala	NA	[18]
S	Bilateral streaks	Female. (Turner, 45X) (2x)	S18N, G>A pos 53	NA	[19]
S	Streak gonads	Female (complete GD)	G>A pos 209 codon 70 (Trp>Stop)	NA	[20]
S	Streak	Female (complete GD)	C>A pos 306 (mr in table pos 226), Ser>Arginine pos 76	NA	[21]
S	Streak	Female (complete GD)	C>G pos 270, codon 90 Ile>Met, (publ : nucl pos 680)	N.a. (but in other study by Harley et al 1992 Science, this mutation à reduced DNA binding	[22]
S	Streak	Female (complete GD)	A>T pos 317, codon106 Lys>Ale (publ: nucl pos 727)	NA	[22]
S	Streak	Female (complete GD), 46XY9h+	Deletion A pos 324, codon 108, frameshift. (publ: nucl pos 734)	NA	[22]
S	Streak, no malignancy	Female (complete GD)	G>A pos 320 codon 107 Trp>stop	NA	[23]
S	Streak, no malignancy	Female (complete GD)	AGAG deletion pos 363-366, codon 121-122, frameshift	1/3 protein affected, non functionality is supposed	[24]
S	Gonadal streak removed	Female (complete GD)	Tyr129stop	NA	[25]
S	Ultrasound: streak	Female (complete GD)	A>T +275, K92M	Reduced binding activity	[9]
S	Streak, No GB	Turner, 45x (80%), 46 XY (20%)	Insertion T, frame shift. N82X	NA	[26]
S	Streak, No GB	Turner, 45x (86%), 46 XY (14%)	Insertion A frameshift. L159fsX167 downstream HMG	NA	[26]
S	Streak, No GB	Turner, 45x (89%), 46 XY (11%), also ambiguous genitalia	G>C codon 74, Q74H	NA	[26]
S	Streak, No GB	Female (complete GD)	C>T pos 132, Arg>Gly	NA	[27]
S	Bilateral Streak, no GB	Female (complete GD)	Deletion A in codon 82 at position +244>frame shift	NA	[28]
S	streak	Family, 1. complete GD, 2. healthy father	Pos 8 C>T third codon, S3L	Protein remodelling: could disrupt N terminal a helix	[29]
S DS	Streak (R), dysgenetic testis (L), wollfian and mularian ducts	Male, (ambiguous genitalia, partial GD)	G>A pos 53, S18N	NA	[30]
S T	1 and 2. streak with overialinke stroma and absence GC left, right: prepubertal testis. 3. both gonads ovarian like stroma and absence of GC	Family, Different phenotypes: (compete (1,3) à partial (2) GD)	R30I	affects mainly SRY PKA phosphorylation, therefore reducing DNA-binding activity	[31]
S	Right side consisted entirely of fibroadipose tissue. The left gonad small amount of ovarian sromalike tissue. No follicles were seen; cluster of tubular structures was present. One of the tubules was ciliated, reminiscent of epididymis.	Female, (POF)	Gln2Stop	No protein due to stop codon	[32]
S	Streak gonads with ovarian stroma, no germ cells	Female (complete GD)	G>A pos 192, codon 64 (Met64Leu)	NA	[33]

	Cystic gonad with ovarian stroma and no germ cells	Female (complete GD)	G>C pos 178, codon 60 (Val60Leu)	NA	[33]
ov st	Fragments of mesothelial tissue with a highly fibrotic stroma and cystic epithelial inclusion	Female (complete GD)	GG>AT pos 224-225, codon75 (Arg>Asn)	NA	[34]
ov st	ovarian like stroma, absence germ cells, hilus cell hyperplasia	Female (complete GD)	N65H	No DNA binding activity in vitro	[31]
ov st	Ovarian stroma, no tubules	Female (complete GD)	T>C pos 203 codon 68 (Ile>Thr)	NA	[35]
ov st	Ovarian stroma, no tubules	Female (complete GD)	T>A pos 381 codon 127, (Tyr>stop)	NA	[35]
NA	Germ Cells neg	Female (complete GD)	C>T pos 277, codon 93 (Gln>stop)	NA	[36]
NA	No GB	Female (complete GD)	C>T pos 397 (Arg>Trp)	NA	[3]
NA	No GB	Female (complete GD)	S143C	NA	[2]
NA	NA	Female (complete GD)	I90M	NA	[37]
NA	NA	Female (complete GD)	p.G95R	electrostatic repulsion caused by the proximity of positive charges that could destabilize the tip of helix 2	[1]
NA	NA	Female (complete GD)	T>A pos 12 (Tyr>stop)	NA	[38]
NA	NA	Female, (45x, 47XYY)	T deletion pos 12 (Tyr>stop)	NA	[39]
NA	NA	Female (complete GD)	pos 191 T>G, M64R	NA	[40]
NA	NA	Female (complete GD)	codon 43 pos 127 lys>stop, K43X	NA	[40]
NA	NA	Female (complete GD)	pos 199 T>G, F67V	NA	[40]
NA	NA	Female (complete GD)	G>A pos 209 codon 70 (Trp>stop)	NA	[41][42]
NA	NA	Female (complete GD)	G>C pos 283 codon 95 (Gly>Arg)	NA	[41]
NA	NA	Female (complete GD)	C>T pos 256, codon 86 Arg>Stop	NA	[43]
NA	NA	Female (complete GD)	T>C, pos 326, codon 109 Phe>Ser	No differences in DNA binding compared to WT	[44]
NA	NA	Female (complete GD)	A>G pos 380, codon 127 Tyr>Cys	Abolished SRY protein binding ability (binding specificity in paper..)	[45]
NA	NA	Sex reversal, Partial GD	T>A pos 385, Y129N	Lower degree of cooperativity in binding	[46]
NA	NA	Female (complete GD)	C254T>C, M85T	NA	[47]
NA	NA	Female (complete GD)	c.391C>T, R130X	NA	[47]
NA	NA	Female (complete GD)	A>G codon Glu38Gly	NA	[48]
NA	NA	Female (complete GD)	T>A, nucl pos 387	NA	[48]
NA	NA	Female (complete GD)	T>A codon 129 pos 387, Tyr>stop	NA	[49]
NA	NA	Female (complete GD)	c.294G>A, Trp98stop	NA	[50]
NA	NA	Family. 3 females (complete GD)	c.334G>A, Glu112Leu	NA	[50]
NA	NA	Female (complete GD)	Three base pair deletion promotor SRY in one of the Sp1 binding sites	Abolished Sp1 binding in vitro	[51]
NA	NA	Female (complete GD)	insA 103 stop, F67L	NA	[52]
NA	NA	Female (complete GD)		NA	[53]

NA.: not available, GB: gonadoblastoma, DG: dysgerminoma, SE: seminoma, YST: yolk sac tumor, OT: ovotestis, S: streak, DS: dysgenetic testis, T: testis, ov st: ovarian stroma, GD: gonadal dysgenesis, POF: Premature Ovarian Failure

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Supplementary Table 2. *WT1* mutations in literature

	Histology description	Phenotype	Mutation	Functionality	Refs
GB	Bilateral streak with GB. Right TE (smooth muscle, cartilage, nerve tissue and glial elements)	Female (pure GD)	NA	NA	[1]
GB	Bilateral streak with right side GB	Female (pure GD)	NA	NA	[1]
GB	Streak gonad (Right), GB Left side (removed 19 yrs). Unspecific glomerular changes	Female, normal external genitalia, infantile uterus, normal fallopian tubes and vagina	IVS9+4 C>T	Shift in +/-KTS ratio to 0.39	[2]
GB	Streak gonads GB. FSGS	Female, normal external genitalia	IVS9+4 C>T	Shift in +/- KTS ratio	[3]
GB	Right GB, Left Streak	Female, hypotrophic uterus	IVS9+4 C>T	NA	[4]
GB	Streak gonads, Microscopic GB	Female	IVS9+5 G>A	NA	[5]
GB	GB (bilateral)	Female, normal external genitalia	IVS9+5 G>A	NA	[6]
GB	Streak gonads, Bilateral GB	Female	Mut not given, described as a mutation in intron 9	NA	[7]
GB	Left: GB, Right dysgenetic gonad	Female	IVS9+4 C>T	NA	[8]
GB	GB	Female	Not further specified, intron9 mut	NA	[9]
GB	Streak gonads, bilateral GB. FSGS	Female	IVS9+4 C>T	NA	[10]
GB CIS	Bilateral GB and right side CIS. Testis and sex cords. FSGS	Male (perineal hypospadias, urogenital sinus, bifid scrotum and bilateral cryptorchid testes)	IVS9+4 C>T	Less +KTS (shift in +/-KTS ratio from 2.2 to 0.25)	[11]
GB DG	Complete GD, streak gonads, GB DG FSGS	Female (complete GD), Hypoplastic uterus, normal fallopian tubes	IVS9+4 C>T	NA	[12][13]
GB DG	Left: GB and DG. Right: ovarian stroma (streak).	Female, normal external genitalia (slightly hypertrophied clitoris), normal; vagina, prepubertla uterus	Not assessed	NA	[14]
GB DG	GB and DG	Female	IVS9+5 G>A	NA	[15]
GB DG	Left: GB, Right: DG	Female, hypoplastic uterus	Mut not given, described as a mutation in intron 9	NA	[16]
GB DG	Bilateral GB and left DG(11 yr). Developed Pilocytic Astrocytoma (17yr)	Female, normal external genitalia, normal appearing vagina and uterus	IVS9+5 G>A	NA	[17]
GB	Complete GD, streak gonads, GB and metastatic tumor. FSGS	Female (Complete GD), Vestigial uterus, primitive ovarian tissue (left side), no Wolffian structures	IVS9+5 G>A	Shift in +/-KTS ratio to ~0.5	[12][18]
CIS	Testicular atrophy, CIS, arrest at spermatocyte stage, LC nodules	Male (glandular hypospadias, herniotomy)	IVS9+5 G>A	Less +KTS, less *SRY* and *SOX9* expression	[19]
DG	Right DG (13yr, 20x25cm), Left gonad removed, gonadal dysgenesis Retroperitoneal DG 15yr	Female	IVS9+4 C>T	NA	[20]
d.g.	Left and right: dysgenetic gonad	Female	IVS9+4 C>T	NA	[8]
S	Streak gonads, Focal Glomerular Sclerosis	Female, normal external genitalia	IVS9+2 T>C	Shift in +/- KTS ratio	[3]
S	Complete GD, streak gonads	Female (complete GD)	IVS9+4 C>T	NA	[12]
S	Complete GD, streak gonads	Female (complete GD)	IVS9+4 C>T	NA	[12]
S	Complete GD, streak gonads. FSGS and segmental hyalinosis	Female (Complete GD)	IVS9+4 C>T	NA	[12]
S	Streak gonads (removed 17yrs). Retriperitoneal myofibroblastic tumor (23 yrs). FSGS	Female, normal external genitalia, infantile uterus, normal fallopian tubes and vagina	IVS9+4 C>T	NA	[2]
S	Streak gonads. FSGS	Female, normal external genitalia	IVS9+4 C>T *	Shift in +/- KTS ratio	[3]
S	Streak gonads. FSGS	Female, normal external genitalia	IVS9+4 C>T *	Shift in +/- KTS ratio	[3]
S	Streak gonads (tubules and isolated germ cells? Mentioned in text). Wilms' Tumor 3 yr, no sclerosis of glomeruli	Female, normal external genitalia, hypoplastic uterus	IVS9+4 C>T	NA	[13]
S	Complete GD, streak gonads. FSGS	Female (Complete GD)	IVS9+5 G>A	NA	[12]
S	Complete GD, streak gonads	Female (Complete GD)	IVS9+5 G>A	Shift in +/-KTS ratio to ~0.5	[12][21]
S	Complete GD, streak gonads. FSGS	Female (Complete GD)	IVS9+5 G>A	NA	[12][22]
S	Complete GD, streak gonads. Glomelar Sclerosis	Female (Complete GD), Infantile uterus	IVS9+5 G>A	NA	[12][23]
S	Streak gonads (removed 24 yrs). FSGS	Female, normal external genitalia, infantile uterus, normal fallopian tubes and vagina	IVS9+6 T>A	Shift in +/-KTS ratio to 0.48	[2]
S	Streak gonads	Female, normal external genitalia, vagina, uterus	IVS9+4 C>T	NA	[24]
S	Streak gonads, no malignancy. FSGS	Female	IVS9 +5 G>A	NA	[10]
S	Streak gonads	Female	IVS9+5 G>A	NA	[25]
S	Streak gonads, no signs of malignancy	Female, anfantile uterus	IVS9+4 C>T	NA	[26]
NGT	No gonadal tissue. Bilateral testosterone hilar cell adenoma.	Female, normal external genitalia, small uterus	IVS9+4 C>T	NA	[27]
NA	Focal Glomerular Sclerosis	Female, normal external genitalia	IVS9+5 G>T	Shift in +/- KTS ratio	[3]
NA	Nephrotic syndrome at 9 months. Diffuse mesangial sclerosis	Female, normal external genitalia, Small uterus	IVS9+5 G>A	Shift in +/-KTS ratio to 0.4	[28]
NA	FSGS	Female	IVS9+5 G>A	NA	[29]
NA	Orchidectomy 11 months, testicular tissue?	Female, normal external genitalia, bicornate uterus, normal vagina	IVS9+5 G>A	NA	[30]
NA	NA	4 Female patients	?	?	[31]
NA	NA	Female	Not further specified, intron9 mut	NA	[9]
NA	NA	Female	IVS9+5G>A	NA	[29]
NA	FSGS	Female (Uterus)	IVS9+5G>A	Shift in +/-KTS ratio to 0.67 (1.35 and 1.42 in parents)	[32]

GB: gonadoblastoma, CIS: carcinoma in situ, DG: dysgerminoma, d.g.: dysgenetic gonad, S: streak, NGT: no gonadal tissue, NA.: not available,
GD: gonadal dysgenesis, FSGS: focal segmental glomerulosclerosis, LC: leydig cell

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Chapter 8

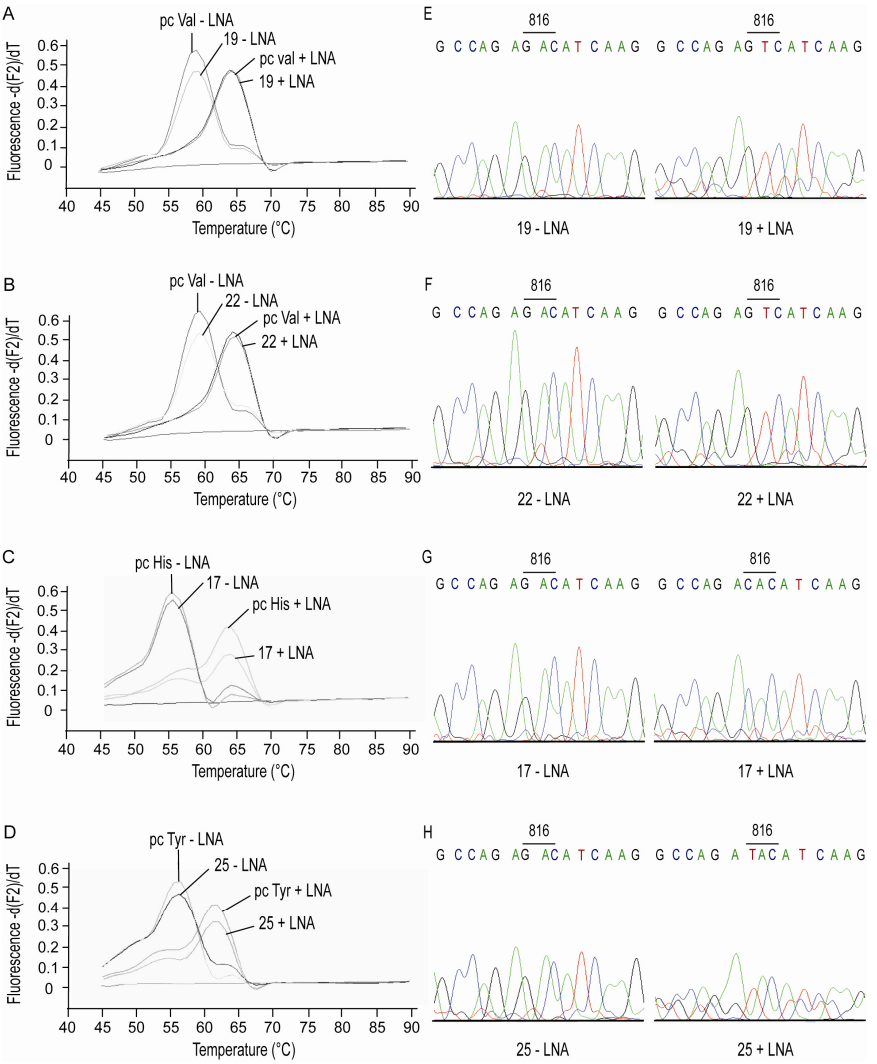
Supplementary Table 1. SRY mosaic amplification primers

Primer Name	Primer sequence
SRY F1_1	CGTATCGCCTCCCTCGCGCCATCAGACGCTCGACATAGCCAATGTTACCCGATTG
SRY F1_2	CGTATCGCCTCCCTCGCGCCATCAGAGACGCACTCTAGCCAATGTTACCCGATTG
SRY F1_3	CGTATCGCCTCCCTCGCGCCATCAGAGCACTGTAGTAGCCAATGTTACCCGATTG
SRY F1_4	CGTATCGCCTCCCTCGCGCCATCAGATCAGACACGTAGCCAATGTTACCCGATTG
SRY F1_5	CGTATCGCCTCCCTCGCGCCATCAGATATCGCGAGTAGCCAATGTTACCCGATTG
SRY F1_6	CGTATCGCCTCCCTCGCGCCATCAGCGTGTCTCTATAGCCAATGTTACCCGATTG
SRY F1_7	CGTATCGCCTCCCTCGCGCCATCAGCTCGCGTGTCTAGCCAATGTTACCCGATTG
SRY F1_8	CGTATCGCCTCCCTCGCGCCATCAGTAGTATCAGCTAGCCAATGTTACCCGATTG
SRY F1_9	CGTATCGCCTCCCTCGCGCCATCAGTCTCTATGCGTAGCCAATGTTACCCGATTG
SRY F1_10	CGTATCGCCTCCCTCGCGCCATCAGTGATACGTCTTAGCCAATGTTACCCGATTG
SRY F1_11	CGTATCGCCTCCCTCGCGCCATCAGTACTGAGCTATAGCCAATGTTACCCGATTG
SRY F1_12	CGTATCGCCTCCCTCGCGCCATCAGCATAGTAGTGTAGCCAATGTTACCCGATTG
SRY F1_13	CGTATCGCCTCCCTCGCGCCATCAGCGAGAGATACTAGCCAATGTTACCCGATTG
SRY F1_14	CGTATCGCCTCCCTCGCGCCATCAGATACGACGTATAGCCAATGTTACCCGATTG
SRY R1_1	CTATGCGCCTTGCCAGCCCGCTCAGACGCTCGACAGCGAAACTCAGAGATCAGCA
SRY R1_2	CTATGCGCCTTGCCAGCCCGCTCAGAGACGCACTCGCGAAACTCAGAGATCAGCA
SRY R1_3	CTATGCGCCTTGCCAGCCCGCTCAGAGCACTGTAGGCGAAACTCAGAGATCAGCA
SRY R1_4	CTATGCGCCTTGCCAGCCCGCTCAGATCAGACACGGCGAAACTCAGAGATCAGCA
SRY R1_5	CTATGCGCCTTGCCAGCCCGCTCAGATATCGCGAGGCGAAACTCAGAGATCAGCA
SRY R1_6	CTATGCGCCTTGCCAGCCCGCTCAGCGTGTCTCTAGCGAAACTCAGAGATCAGCA
SRY R1_7	CTATGCGCCTTGCCAGCCCGCTCAGCTCGCGTGTCGCGAAACTCAGAGATCAGCA
SRY R1_8	CTATGCGCCTTGCCAGCCCGCTCAGTAGTATCAGCGCGAAACTCAGAGATCAGCA
SRY R1_9	CTATGCGCCTTGCCAGCCCGCTCAGTCTCTATGCGGCGAAACTCAGAGATCAGCA
SRY R1_10	CTATGCGCCTTGCCAGCCCGCTCAGTGATACGTCTGCGAAACTCAGAGATCAGCA
SRY R1_11	CTATGCGCCTTGCCAGCCCGCTCAGTACTGAGCTAGCGAAACTCAGAGATCAGCA
SRY R1_12	CTATGCGCCTTGCCAGCCCGCTCAGCATAGTAGTGGCGAAACTCAGAGATCAGCA
SRY R1_13	CTATGCGCCTTGCCAGCCCGCTCAGCGAGAGATACGCGAAACTCAGAGATCAGCA
SRY R1_14	CTATGCGCCTTGCCAGCCCGCTCAGATACGACGTAGCGAAACTCAGAGATCAGCA
SRY F2_1	CGTATCGCCTCCCTCGCGCCATCAGACGCTCGACAGCCATTTTTCGGCTTCAGTA
SRY F2_2	CGTATCGCCTCCCTCGCGCCATCAGAGACGCACTCGCCATTTTTCGGCTTCAGTA
SRY F2_3	CGTATCGCCTCCCTCGCGCCATCAGAGCACTGTAGGCCATTTTTCGGCTTCAGTA
SRY F2_4	CGTATCGCCTCCCTCGCGCCATCAGATCAGACACGGCCATTTTTCGGCTTCAGTA
SRY F2_5	CGTATCGCCTCCCTCGCGCCATCAGATATCGCGAGGCCATTTTTCGGCTTCAGTA
SRY F2_6	CGTATCGCCTCCCTCGCGCCATCAGCGTGTCTCTAGCCATTTTTCGGCTTCAGTA
SRY F2_7	CGTATCGCCTCCCTCGCGCCATCAGCTCGCGTGTCGCCATTTTTCGGCTTCAGTA
SRY F2_8	CGTATCGCCTCCCTCGCGCCATCAGTAGTATCAGCGCCATTTTTCGGCTTCAGTA
SRY F2_9	CGTATCGCCTCCCTCGCGCCATCAGTCTCTATGCGGCCATTTTTCGGCTTCAGTA
SRY F2_10	CGTATCGCCTCCCTCGCGCCATCAGTGATACGTCTGCCATTTTTCGGCTTCAGTA
SRY F2_11	CGTATCGCCTCCCTCGCGCCATCAGTACTGAGCTAGCCATTTTTCGGCTTCAGTA
SRY F2_12	CGTATCGCCTCCCTCGCGCCATCAGCATAGTAGTGGCCATTTTTCGGCTTCAGTA
SRY F2_13	CGTATCGCCTCCCTCGCGCCATCAGCGAGAGATACGCCATTTTTCGGCTTCAGTA
SRY F2_14	CGTATCGCCTCCCTCGCGCCATCAGATACGACGTAGCCATTTTTCGGCTTCAGTA

Supplementary Table 2. SRY reads

sequence	matched reads	variants
1a	2828	
1b	5040	
2a	441	
2b	4600	
3a	1116	
3b	2653	
4a	2872	
4b	2602	
5a	4054	
5b	4210	
6a	4068	
6b	4462	
7a	3348	
7b	2892	
8a	7137	
8b	4000	
9a	4274	
9b	5013	
10a	6830	
10b	4379	
11a	4098	
11b	2850	
12a	3147	
12b	4299	
13a	3195	
13b	3972	
14a	507	
14b	3759	c.49delT 21%
average	3666	
total	102646	

Chapter 9



Supplementary Figure 1. Detection of c-KIT c.816 mutations in patient samples by melting curve analysis. The y-axis represents fluorescence intensity and the x-axis represents temperature. Mutations lead to different melting temperatures of the hybridization probes from the amplification product. (A, B) Melting curves of sample 19 and 22 with and without the addition of LNA are shown together with a positive control harboring the D816V mutation. (C) Melting curves of sample 17 with and without the addition of LNA are shown together with a positive control harboring the D816H mutation. (D) Melting curves of sample 25 with and without the addition of LNA are shown together with a positive control harboring the D816Y mutation. (E, F) Electropherogram showing the A to T mutation in codon 816 in LightCycler products with and without LNA added of sample 19 and 22 respectively. (G) Electropherogram showing the G to C mutation in LightCycler products with and without LNA added of codon 816 in sample 17. (H) Electropherogram showing the G to T mutation in LightCycler products with and without LNA added of codon 816 in sample 25. Note the suppression of wild type c-KIT and the enrichment of the mutant amplification product in the + LNA samples. pc: positive control, Val: valine mutation, His: histidine mutation, Tyr: tyrosine mutation, LNA: locked nucleic acid.

Supplementary Table 1. c-KIT and PDGFRA primer sets

Primers pairs for sequence analysis of *c-kit* in DNA from FFPE tissue

Exon	Primer	Sequence*
8	c-kit 8 M13 for	TGT AAA ACG ACG GCC AGT CAG CAC TCT GAC ATA TGG CC
	c-kit 8 M13 rev	CAG GAA ACA GCT ATG ACC CTC TGC ATT ATA AGC AGT GCC
9A	c-kit 9/1 M13 for	TGT AAA ACG ACG GCC AGT CAG GGC TTT TGT TTT CTT CCC
	c-kit 9/1 M13 rev	CAG GAA ACA GCT ATG ACC GCC ATT GTG CTT GAA TGC AC
9B	c-kit 9/2 M13 for	TGT AAA ACG ACG GCC AGT GCT AGT GGT TCA GAG TTC TA
	c-kit 9/2 M13 rev	CAG GAA ACA GCT ATG ACC CCT AAA CAT CCC CTT AAA TTG G
11	c-kit 11 M13 for	TGT AAA ACG ACG GCC AGT AGG TGA TCT ATT TTT CCC TTT C
	c-kit 11 M13 rev	CAG GAA ACA GCT ATG ACC GTG ACA TGG AAA GCC CCT G
13	c-kit 13 M13 for	TGT AAA ACG ACG GCC AGT GTG CTT TTT GCT AAA ATG CAT
	c-kit 13 M13 rev	CAG GAA ACA GCT ATG ACC GGC AGC TTG GAC ACG GC
17	c-kit 17 M13 for	TGT AAA ACG ACG GCC AGT GTT TTC TTT TCT CCT CCA ACC
	c-kit 17 M13 rev	CAG GAA ACA GCT ATG ACC GAC TGT CAA GCA GAG AAT GG
M13 forward primer		TGT AAA ACG ACG GCC AGT
M13 reverse primer		CAG GAA ACA GCT ATG ACC

* Productsize including primersequence, excluding M13-tail sequence

Primers pairs for sequence analysis of *PDGFRa* in DNA from FFPE tissue

Exon	Primer	Sequence*
12A	PDGFRa 12.1 M13 for	TGT AAA ACG ACG GCC AGT TTC ACC AGT TAC CTG TCC TG
	PDGFRa 12.1 M13 rev	CAG GAA ACA GCT ATG ACC CTT GAG TCA TAA GGC AGC TG
12B	PDGFRa 12.2 M13 for	TGT AAA ACG ACG GCC AGT CCG AGG TAT GAA ATT CGC TG
	PDGFRa 12.2 M13 rev	CAG GAA ACA GCT ATG ACC AGG GAG TCT TGG GAG GTT AC
14	PDGFRa 14.1 M13 for	TGT AAA ACG ACG GCC AGT TGG TAG CTC AGC TGG ACT GAT
	PDGFRa 14.1 M13 rev	CAG GAA ACA GCT ATG ACC CCA GTG AAA ATC CTC ACT CCA
18	PDGFRa 18 M13 for	TGT AAA ACG ACG GCC AGT CTT GAT CCT GAG TCA TTT CTT C
	PDGFRa 18 M13 rev	CAG GAA ACA GCT ATG ACC CTG ACC AGT GAG GGA AGT G
M13 forward primer		TGT AAA ACG ACG GCC AGT
M13 reverse primer		CAG GAA ACA GCT ATG ACC

* Productsize including primersequence, excluding M13-tail sequence

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Curriculum Vitae

Remko Hersmus werd op 8 september 1974 geboren te Hilvarenbeek. In 1992 behaalde hij zijn HAVO diploma aan het St. Odulphus Lyceum te Tilburg. Aansluittend startte hij de Medische Laboratorium opleiding aan de Hogeschool West-Brabant, met als afstudeerrichting biotechnologie. In het kader van deze opleiding werd een stageperiode uitgevoerd op de afdeling celbiologie en genetica. Na het halen van het HLO diploma in 1997 werd hij werkzaam als research analist op de afdeling pathologie voor Prof.dr. J. Trapman, en deed daar onderzoek naar prostaatkanker. Sinds januari 2004 is hij als research analist werkzaam in de werkgroep van Prof.dr L.H.J. Looijenga, afdeling pathologie, in het Josephine Nefkens Instituut, Erasmus MC, Rotterdam.

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Erasmus MC
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PhD Portfolio Summary

Summary of PhD training and teaching activities

Name PhD student: R. Hersmus Erasmus MC Department: Pathology Research School: Molmed	PhD period: 2007 - 2010 Promotor(s): L.H.J. Looijenga, S.L.S. Drop Supervisor: L.H.J. Looijenga

1. PhD training		
	Year	**Workload (Hours/ECTS)**
General academic skills		
- Biomedical English Writing and Communication	2009	2
In-depth courses (e.g. Research school, Medical Training)		
- Molecular Diagnostics III	2008	1
- Molecular Medicine	2008	1.5
Presentations		
- Ghent-Rotterdam DSD workgroup meetings	2007-2010	3
- JNI meetings	2007-2010	3
- LEPO workgroup meetings	2007-2010	3
International conferences		
- 7th Copenhagen Workshop on CIS Testis and Germ Cell Cancer, Copenhagen, Denmark	2010	2
- 3rd Symposium on Disorders of Sex Development, Lübeck, Germany	2011	2
Seminars and workshops		
- Tumor Cell Biology meeting Lunteren	2007-2009	3
- Molmed dagen (posters)	2007-2010	3
2. Teaching activities		
	Year	**Workload (Hours/ECTS)**
Supervising practicals and excursions		
- VO Ontwikkeling, anatomie en pathologie van de placenta	2010 2011	1 1
- VO Microscopische bouw v/d mannelijke genitalien (proces spermatogenese)		
Supervising Master's theses		
- Yvonne van der Zwan		3
Other		
- Supervising students	2007-2011	3