New advances in clinical biomarkers in testis cancer

Vincenzo Favilla, Sebastiano Cimino, Massimo Madonia, Giuseppe Morgia

Department of Urology, University of Messina, Italy

TABLE OF CONTENTS

1. ABSTRACT

Diagnostic work-up when a testicular cancer is suspected includes a clinical examination, determination of risk factors, imaging and serum tumours markers. Tumour markers are useful in the diagnosis and staging of disease, for monitor the therapeutic response and to detect tumour recurrence. The alpha-fetoprotein (AFP) and beta-human chorionic gonadotropin (beta-hCG) are well established as serum markers for GCTs of the testis, lactate dehydrogenase (LDH) and placental-like alkaline phosphatase (PLAP) may be alternative serological markers with less specifity. However, these markers are increased in only about 60% of patients with testicular cancer. Therefore, additional tumour markers would facilitate clinical diagnosis and treatment in these patients. In this review we have evaluated the clinical application of several markers of testis cancers in serum, semen and tissue samples. Immunohistochemistry detection of some of these being solid new markers in addition to the routinely used but none have been shown to be superior to the classical markers in serum and semen samples. Further research is need in this context for the detection of new markers.

2. INTRODUCTION

Cancer of the testis is a relatively rare disease, accounting for about 1% of all cancers in men. Around 24.000 new cases are diagnosed each year in Europe with annual incidence rates (world age-standardised) range between 7.3 (west) and 4.6 (south) per 100,000 (1). In USA, testicular tumours are the most common malignancies among American men between the ages of 20 and 39 years (2, 3). The American Cancer Society has estimated that in the year 2006, 8250 men will develop testicular cancers and 370 men will die of these tumours in the United States (4). Testis tumours are approximately 1% of all cancers in men but only about 0.1% of cancer deaths in males because the majority of these tumours are curable (3-6). More than 90% of testicular tumours originate from germ cells (7). The incidence of testicular germ cell tumours (GCTs) increases shortly after the onset of puberty and peaks in the fourth decade of life with a median age of 34 years at diagnosis (8). The incidence varies among different races and various geographic locations. It is 5 times more frequent in white men compared with African American men (9). Despite several recognized risk factors

are associated in the development of GCTs (e.g., cryptorchidism, prior history of GCT), the pathogenesis of germ cell tumours including the contributing role of environmental factors or genetic susceptibility remains unknown. Testicular GCTs are a heterogeneous group of tumours with diverse histopathology and variable clinical course and prognosis. Currently the most comprehensive and widely accepted system of classification is the one proposed by the World Health Organization (WHO) (10).

The distribution pattern of GCTs in adults and children is different. Testicular tumours in adult patients often consist of seminoma, embryonal carcinoma or mixed testicular GCTs. In the paediatric population, yolk sac tumours and teratoma are the most frequent tumours; on the contrary, seminoma and embryonal carcinoma are rare (11). Non-GCTs, which account for less than 10% of testicular tumours in adults, comprise as high as one third of testis tumours in children. Furthermore, although there is a strong association between post-puberal testicular tumours and intratubular germ cell neoplasia, such association has not been observed in pre-puberal GCTs. Germ cell tumours have been traditionally separated into seminomatous and nonseminomatous tumours. This division, however, is essentially for clinical purposes because of some differences in the management approach and prognosis of these two groups of tumours.

The majority of patients with testicular germ cell tumours (TGCTs) survive with the current treatment regime, but regardless of recent improvements in the outcome of this disease, it is potentially lethal, especially in poor-prognosis patients with disseminated non-seminomas and in patients with relapsed testis cancer, which is often refractory to chemotherapy (12). The subset of patients that require chemotherapy and/o radiotherapy may experience side effects, severe psychological stress and reduction of their reproductive potential (13).

It has been observed that TGCTs of young adults originate from a common precursor, the carcinoma *in situ* (CIS) cell (14), also known as intratubular germ cell neoplasia.

Carcinoma "*in situ*" (CIS o intratubular germ cell neoplasia) gives no symptoms but precedes invasive testicular GCT in all cases of seminoma and non-seminomatous histologies with a median time for progression of CIS to invasive disease of 5 years (16). Because CIS can be cured by low-dose irradiation o unilateral orchidectomy alone, there is a great incentive for non-invasive or minimally invasive methods for detection of CIS. A surgical biopsy is at present the only reliable method for diagnosis of CIS and unilateral or bilateral biopsies are performed in selected patients at risk of CIS, for example those with a history of cryptorchidism and where clinical examination has revealed atrophic testes or ultrasonic microlithiasis with irregular echo pattern. Hence, very few patients are diagnosed at the CIS stage, particularly because most patients with CIS have no symptoms (15). Besides intratubular germ cell neoplasia has been identified in association with GCTs in about 90%

of cases (17, 18). Therefore, strategies for early diagnosis of CIS are essential.

Several serological markers for testicular cancer have been tested and some are available. These markers are useful in the diagnosis and staging of disease, for monitor the therapeutic response and to detect tumour recurrence. Increasing levels of tumour markers during follow-up is an indication that therapy should be initiated, even if no evident disease is found. Besides, the half-life of markers must be taken into consideration when evaluating therapeutic responses. Nevertheless in general the sensivity of tumour markers in GCTs is relatively low. For the pre-invasive CIS stage serological markers are not relevant, as cancer cells are not present in circulating plasma at this stage. Therefore, a less invasive method such as a new tumour marker for early diagnosis of testicular GCTs, preferentially at the non invasive stage or for the detection of GCTs and subtype and for monitoring of these cancers during follow-up is needs.

3. MOLECULAR BIOMARKERS OF TESTICULAR GERM CELL TUMORS IN SERUM AND TISSUE SAMPLES

Serum tumour markers are prognostic factors and contribute to diagnosis and staging (19). Alpha-fetoprotein (AFP) and beta-human chorionic gonadotropin (beta-hCG) are well established as serum markers for GCTs of the testis (12, 20). Overall, there is an increase in these markers in about 51% of cases of testicular cancer (19). **Alpha-fetoprotein (AFP)** is a glycoprotein normally produced by fetal yolk sac, liver and gastroenteric tract. It is increased not only in yolk sac tumours and embryonal carcinoma, but also in hepatocarcinoma, in infective-degenerative liver diseases and in regenerative liver after toxic damage. The level of AFP increases in 50-70% of patients with non-seminomatous germ cell tumour (NSGCT). If an increasing value of AFP is found in pure seminoma, the tumour must be considered and treated as a non seminomatous tumour. **Human chorionic gonadotropin (hCG)** is a glycoprotein produced by syncytiotrophoblastic cells and it consists of two subunits, alfa and beta. The alfa subunit is common to three pituitary trophic hormones: FSH, LH, TSH. The beta subunit makes hCG enzymatically and immunologically distinct. Only the beta subunit (beta-hCG) is measured. It is highly specific for testicular cancer and it is produced specifically by choriocarcinoma cells. A rise in hCG occurs in 40-60% of patients with non-seminomatous germ cell tumours (NSGCTs) but up to 30% of seminomas can present or develop an elevated hCG level during the course of the disease (21).

Measurement of the free beta subunit of human chorionic gonadotropin (beta-hCG) in serum offers additional diagnostic information compared to determination of intact hCG alone in testicular cancer (21). However, these markers (AFP and hCG) are increased in only about 51% of patients with testicular cancer (19). An attempt at increasing sensivity was performed by using nested reverse-transcriptase PCR for the detection of AFP and beta-hCG at the mRNA level but the results did not

Table 1. Comparison of the different biochemical markers

Immunostain Class	PP[1]	OCT4[2]	CD117[3]	AE1/AE3[4]	CAM 5.2[5]	CD30[6]	AFP[7]	hCG[8]	PLAP[9]	HLA-G[10]	Glypican 3[11]
ITGCN[12]/ Classical seminoma	+	+	+	-	-	-	-	+ STC[16]	+	-	-
Spermatocytic seminoma	-	-	-/+	-	-	-	0	-	-	?	-
YST[13]	-	-	-	+	+	-/+	+	-	-/+	-	+
CC[14]	-	-	-	+	+	-	-	+ STC[16]	+	+	+
EC[15]	-	+	-	+	+	+	-/+	-	+	-	-

Abbreviations: Podoplanin[1], POU-family transcription factor[2], cytokeratin[3], cytokeratin[4], cytokeratin[5], cell membrane antigen[6], alfa-fetoprotein[7], human chorionic gonadotropin[8], placental-like alkaline phosphatase[9], human antibody[10], glycoprotein[11], intratubular germ cell neoplasia[12], yolk sac tumor[13], choriocarcinoma[14]; embryonal carcinoma[15], syncytiotrophoblastic cells[16]

correlate with the serum levels of detection at the protein level with conventional measurement in serum, perhaps due to the vulnerability of RNA (22). The mean serum half-life of AFP and hCG is 5-7 days and 2-3 days, respectively. Tumour markers have to be re-evaluated after orchidectomy to determine half-life kinetics. Marker decline in patients with clinical stage I disease should be assessed until normalisation has occurred. Post-orchidectomy markers are important to classify the patient according to the International Germ Cell Cancer Collaborative Group (IGCCCG) risk classification, in fact the persistence of elevated serum tumour markers after orchidectomy might indicate the presence of metastatic disease (macro or microscopically), while the normalisation of marker levels after orchidectomy does not rule out the presence of tumour metastases. Lactate dehydrogenase (LDH) and Placental-like alkaline phosphatase (PLAP) may be considered alternative serological markers. **Lactate dehydrogenase (LDH)** is located in chromosome 12p and serum levels correlate with the total number of copies of the short arm of chromosome 12p (23). The clinical value of LDH is hampered by a relatively low specificity but relevance in the diagnosis of testicular GCTs and a correlation with survival has been found (24). This enzyme is a less specific marker and its concentration reflects the tumour burden, growth rate and cellular proliferation. LDH measurements detect multiple isoenzymes and it is increased in about 80% of advanced seminomas and in 60% of advanced non seminomatous GCTs. The LDH isoenzyme 1 appears to be more specific and sensitive for GCTs than others four isoenzymes (25). Improved detection of tumour cells using Placental-like alkaline phosphatase (PLAP) has been reported (26). **Placental-like alkaline phosphatase (PLAP)** is an enzyme that is normally expressed by placental syncytiotrophoblasts, primordial gonocytes cells (PGCs), foetal oogonia and gonocytes (28, 29). In fact, PLAP is abundantly expressed in classical seminomas/dysgerminomas/germinomas and focally in the majority of embrional carcinoma (EC) and tumours containing yolk-sac tumour (YST) components with a membranous and cytoplasmic pattern, while it is largely negative in GCTs with somatic differentiation such as teratomas and in normal germ cells (27-31). Nevertheless, PLAP is a less specific marker, in fact a recent study applying high performance liquid chromatography (HPLC) based method, showed false-positive results in smokers, it is probably caused by PLAP secretion from lung alveolar tissue (32,33).

According to European Association of Urology guidelines 2009 (EAU 2009) the measurement of serum AFP, hCG and LDH (in advanced tumours) is mandatory, while that of PLAP is optional. However, it is important to understand that although these markers are increased in about 60% of patients with testicular cancer it should be noted that negative marker levels do not exclude the diagnosis of a germ cell tumour (34). Therefore, additional serum markers would facilitate clinical diagnosis and treatment in these patients, especially in the early stage of the disease. In the section below a subset of newly identified markers of serum and sample of tissue will be described.

3.1. C-KIT (KIT)

It is a tyrosine kinase receptor for stem cell factor (SCF) that it is commonly regulated in the gonads, in particular it is present only in immature gonocytes/oogonia (35,36) and is highly expressed in CIS and seminoma/dysgerminoma/germinoma (37,48). KIT is crucial for migration of primordial gonocytes cells (PGCs) (32) and high expression is detectable in GCTs (42, 46, 47). Kit mutations have been found in a varying proportion of testicular seminomas (50-55). One report showed a gain of function mutation in KIT in 1.3% of unilateral testicular GCTs, in contrast to 93% of bilateral testicular GCTs (53), with detection of identical mutations in bilateral cases suggesting that the initiation of the malignant process in PGCs occurs in the early development of the gonads. A different pattern is evident in ovarian GCTs, where similar mutations in KIT were only seen in unilateral dysgerminoma cases (48, 50, 51, 56). This could signify that KIT mutations occur after PGC migration has been completed in dysgerminomas. Besides c-KIT it has also been evaluated in CNS germinomas where mutations in exon 17 were found in 4–6% of germinomas (57, 58), and in primary mediastinal seminomas, where 50% had single or multiple mutations in exon 17 (59).

3.2. OCT3/4 (POU-family transcription factor)

Recently, OCT4 (also known as OCT3 or POU5F1) a transcription factor, has been recognized as fundamental factor of the maintenance of pluripotency in embryonic stem cells and in primordial germ cells (60, 61). It has been proposed as a useful marker for germ cell tumours (GCTs) that can exhibit pluripotentiality, specifically in seminoma/dysgerminoma/germinoma and embryonal carcinoma (EC) (Table 1). The identification of OCT4 protein in formalin-fixed, paraffin-embedded tissues by immunohistochemical methods using commercially

available antibodies has generated considerable interest in characterizing the expression of this transcription factor in GCTs. OCT4 is a POU-domain, octamer-binding transcription factor expressed in human embryonic stem cells (ESCs) and primordial germ cells (PGCs). OCT4 expression is necessary for the maintenance of pluripotentiality in ESC and PGC and it is down regulated in all differentiated somatic cell types *in vitro* as well as *in vivo* (62). Primordial germ cells lacking OCT4 expression have been shown to undergo apoptosis rather than differentiation (63); furthermore, variations in OCT4 levels are responsible for most failures in somatic cell cloning (61). These findings imply a possible different function of OCT4 in ESCs and PGCs, which may have an impact on the understanding of its role in the pathogenesis of GCTs. It has been proposed that the aberrant expression of this transcription factor might contribute to tumour genesis in GCTs (64).

Palumbo *et al* reported the specific mRNA expression of OCT4 in type II GCTs correlated positively with the expression of a 1.5 kb alternative transcript of the platelet-derived growth factor-alpha receptor (65, 66). Subsequently, Looijenga *et al*, in an extensive study, described the expression of OCT4 in GCTs. The testicular tumours analysed in this study included 35 seminomas and 50 non-seminomatous germ cell tumours (NSGCTs), among which 14 had EC components, 21 had teratoma components ,18 had yolk sac components and 5 had elements of choriocarcinoma. Furthermore, 10 spermatocytic seminomas were included in the study. Immunohistochemistry was performed for OCT4 and they have reported a high nuclear expression of OCT4 in neoplastic cells of seminoma and EC (67).

More recently, Jones *et al* applied immunohistochemical analysis for OCT4 in a wide series of primary testicular tumours, among which 64 mixed GCTs and 5 spermatocytic seminomas were reported (68). The results showed that all of the seminoma and EC components were positive in nearly 100% of the cells with strong nuclear staining in all cases except one EC, in which moderate staining was reported. The cells of other GCT components, specifically yolk sac tumours, mature and immature teratomas and choriocarcinomas were consistently immunohistochemical negative. The diagnostic value of OCT4 immunostaining has been further validated by De Jong, who reported an extensive study of OCT4 expression in 223 testicular tumours (209 GCTs and 14 non-GCTs) in consecutive radical orchiectomy specimens GCTs included 3 spermatocytic seminomas and 206 type II GCTs (110 pure seminomas, 50 pure NSGCTs and 46 mixed GCTs containing both seminoma and non-seminoma components (69). About the role of OCT3/4 in the early diagnosis of TGCT, a number of investigators have evaluated the OCT4 immunostaining characteristics of intratubular germ cell neoplasia unclassified (IGCNU) to assess early events in neoplastic transformation and to establish the usefulness of this marker in the diagnosis of this lesion. In the large series of GCTs described by Looijenga, 16 specimens from testicular parenchyma harbouring IGCNU adjacent both to seminomatous and

non-seminomatous components were included in the study. IGCNU tumour cells were all positive regardless of the histological type of adjacent invasive tumour, whereas normal intratubular spermatogenic cells showed no OCT4 immunostaining (67). Eleven additional cases of IGCNU adjacent to invasive tumours were investigated in the study of Rajperts-De Meyts, all cases were reported to be positive with immunostaining of 90–100% in cancer cells, whereas the non-neoplastic germ cell components were negative (70). Immunoreactivity for OCT4 in IGCNU was further defined in a study by Jones, in which 44 IGCNU specimens from patients with GCTs were reported and in all cases a strong nuclear immunostaining for OCT4 in nearly 100% of atypical cells was found (71). Studies involving the immunohistochemical characterization of normal germ cells in human fetal development have shown that OCT4 expression is seen only in the early stages of differentiation, i.e. the gonocyte/PGC stage (70, 72). It has been observed that there is a progressive reduction in OCT4 expression in normal spermatogenic cells in the second and third trimesters of fetal development, with only sporadic immunostaining for OCT4 in the late stages of embryonal development. These normal patterns of OCT4 expression may not apply to germ cells derived from some dysgenetic gonads owing to the post-natal persistence of OCT4-positive gonocytes in these individuals because of a delay in germ cell differentiation (70). In fact, a delay in germ cell maturation has been found in various pathological conditions. In particular, patients with gonadal dysgenesis and hypovirilization demonstrate a delay in germ cell maturation (73). Proper diagnosis of the malignant cells is of great importance in these cases, because these patients are also at risk for development of malignant GCTs. To validate the clinical utility of OCT4 immunohistochemistry in screening for pre-invasive GCT lesions, Jones *et al* have examined 157 testicular tissue samples removed for reasons other than for the management of a suspected malignant tumour, the majority of which were sampled for either infertility or cryptorchidism (74). It must be kept in mind that these conditions are risk factors for the development of GCTs. They found OCT4-positive cells in six patients. Interestingly, the characteristic morphology of IGCNU was apparent by light microscopy in only one of these six cases, while all the others were negative. They concluded that OCT4 immunohistochemistry may be useful in identify early forms of pre-invasive germ cell cancer in patients with risk factors for the development of malignant testicular GCTs (74). Further, another studies have investigated the application of OCT4 detection in advanced disease, in particular, the utility of OCT4 immunohistochemistry in defining a GCT origin for metastatic lesions was documented by Cheng (76). Sixty-two retroperitoneal lymph node dissection specimens containing elements of metastatic GCT from patients who were previously diagnosed with testicular GCTs were studied. In addition, 84 metastatic carcinomas from male patients with known non-germ cell primary tumours were studied for OCT4 immunoreactivity in parallel. All EC components and seminoma components from retroperitoneal lymph node dissection specimens showed strong, intense, diffuse nuclear staining for OCT4. In contrast, yolk sac tumours, choriocarcinomas, mature

teratomas and primitive neuroectodermal tumours were all negative for OCT4. All metastases from non-GCTs showed no OCT4 immunostaining (76). In summary, the immunohistochemical detection of OCT4 may be considered an extremely useful tool in the diagnosis of IGCNU, especially in small biopsy specimens and in screening high risk patients with a history of cryptorchidism, previous controlateral GCT, extragonadal GCTs and in patients with particular types of gonadal dysgenesis (71, 73-75).

3.3. Nanog

The NANOG gene, a member of the homeobox family of DNA binding transcription factors, was recently identified in a screen for pluripotency-promoting genes. The NANOG gene is located on human chromosome 12p13, a region frequently duplicated in human tumours of germ cell origin and in cultured of human embryonic stem cells. In particular, it is a homeobox gene involved in self-renewal and pluripotency of embryonic stem cells (77). It is also expressed in PGCs (78) and is also found in gonocytes, CIS, seminoma and EC (79, 80). A recently study has confirmed with immunohistochemistry and quantitative polymerase chain reaction (PCR) the expression and gene copy number of the human NANOG gene in germ cell tumours. It has been found that NANOG protein was detected in germ line stem cells (gonocytes) within the developing testis. In particular, the authors found that NANOG is highly and specifically expressed in carcinoma *in situ* (CIS), embryonal carcinomas and seminomas but not in teratomas and yolk sac tumours.

The authors concluded that NANOG expression in germ line stem cells (gonocytes), CIS, embryonal carcinoma and seminoma reveals a molecular and developmental link between germ cell tumours and the embryonic cells from which they arise (80). Thus, identification of NANOG as a molecular marker of undifferentiated germ cell tumours provides a novel tool for identification and classification of germ cell tumours.

3.4. Ap-2 gamma

Ap-2 gamma belongs to a family of genes involved in embryonic morphogenesis and regulates the expression of several genes involved in differentiation and cell growth during development (81). This gene it has been identified as a novel markers of CIS and GCTs (83). The presence of Ap-2 gamma in adult testis is restricted to neoplastic lesions with expression in all CIS and seminoma samples in 90-100% of neoplastic cells and in a subset of cells in more differentiated non-seminomatous tumours. It has been observed that Ap-2 gamma is furthermore expressed in the majority of gonocytes until gestational week 22, followed by a rapid down regulation with persistence of a few positive germ cells until 4 months postnatally (83, 84).

A recently study has confirmed that Ap-2 gamma is a very solid marker of CIS because it was expressed in 30/30 analysed tissue samples containing CIS (85). In CNS-located seminomas and in extragonadal metastase from seminomas Ap-2 gamma is expressed with a similar expression pattern to that seen in the testis (46, 47, 84).

3.5. Cell-free circulating DNA

Cell-free DNA represents an interesting universal tumours marker. Increased levels of cell-free DNA were described in 1977 in the plasma of various patients with cancer but the origin of circulating DNA still remains largely unknown (86). Although circulating tumours specific DNA is present in many patients with cancer, it accounts for only less than 5% of total DNA (87, 88). Therefore, it was hypothesized that indirect mechanisms induce apoptotic and/or necrotic death in surrounding and peripheral healthy cells (87–89). The development of PCR based methods has facilitated the quantification of cell-free DNA and today numerous studies have described increased levels in patients with cancer compared to healthy individuals and patients with non-malignant disease (88, 90, 91). DNA fragments, was reported in patients with breast (92), colon (93), ovarian cancer (93), prostate and bladder cancer (88, 89). A recent study has investigated the level of cell-free DNA in samples of the serum of patients with testicular cancer, including seminoma, non-seminoma tumours and in healthy individuals as control. Real-time polymerase chain reaction (PCR) was used to quantify actin-beta DNA fragment and DNA integrity was expressed as the ratio of large to short DNA fragments. It was observed that the actin-beta DNA fragment levels were significantly increased in patients with cancer compared to healthy individuals. DNA integrity, on the contrary, was significantly decreased in patients with cancer. Nevertheless, cell-free DNA fragment levels were not different when comparing patients with nonseminoma and seminoma. PCR analysis demonstrated that cell-free DNA levels distinguished patients with cancer from healthy individuals with 87% sensitivity and 97% specificity. However, in patients in whom the established serum tumours markers alpha-fetoprotein, human chorionic gonadotropin, placental alkaline phosphatase and lactate dehydrogenase were normal, cell-free DNA levels allowed us to distinguish between patients with cancer and healthy individuals with 84% sensitivity and 97% specificity. Furthermore, it was observed that cell-free DNA levels were more frequently increased in patients with clinical stage 3 than in patients with stage 1 or 2 disease (94). It was suggested that an interesting role of circulating DNA may be the identification of stage I cases with occult metastasis and the evaluation of the vitality of residual masses following chemotherapy. Future studies are needed to clarify these important questions. No data exist to date on how long cell-free DNA levels are increased after surgical intervention. It is possible that cell-free DNA levels would reach normal ranges when orchiectomy is performed in patients with localized disease, whereas cell free DNA levels would remain increased in patients with metastatic disease. This hypothesis is supported by the fact that the half-life of cell-free DNA is approximately 16 minutes in plasma (95). Increases in cell-free DNA levels in patients due to trauma and probably also to surgical trauma was reported to attain normal levels within 2 hours after insult (96). Thus, recurrent or persistent increased cell-free DNA levels following orchiectomy may indicate cancer recurrence or the presence of residual metastatic tumour. Even in patients with negative assessment of AFP,

hCG, LDH or PLAP cell-free DNA levels are helpful. In conclusion the analysis of cell-free DNA could facilitate clinical management for testicular cancer but multicenter studies are necessary before cell-free DNA analysis can be implemented in clinical routine investigations.

3.6. Stem cell factor

It has been yet observed that the normal germ cell lineage development depends on the c-KIT–stem cell factor (SCF) pathway (97). Experimental animal study have been demonstrated that c-KIT (the receptor) is present in PGCs, gonocytes, spermatogonia, spermatocytes, round spermatids and Leydig cells (98–102). c-KIT signalling is also important for human spermatogenesis, demonstrated by defects in this pathway. Several studies have demonstrated that c-KIT expression is low and is not detected in adult testis, this makes c-KIT a diagnostic marker for CIS in adults on formalin-fixed, paraffin-embedded material (104, 105). However, false-negative findings have been reported specially in patients with maturation delay or disturbance of spermatogenesis (106, 107). This demonstrates the need for a more specific diagnostic marker to distinguish malignant germ cells from germ cells showing maturation delay. The novel immunohistochemical detection of stem cell factor (SCF), the c-KIT ligand, is informative in this context. In adult testes, the c-KIT ligand, stem cell factor (SCF), is produced by Sertoli cells (103, 108–110), under the control of follicle-stimulating hormone (FSH) (111). Two forms of SCF exist: a membrane bound and a soluble protein (112, 113). Membrane bound SCF is the most efficient in establishing and maintaining germ cells (114–118), while soluble SCF activates c-KIT on Leydig cells to induce testosterone production (119).

A recently study has investigated the presence of SCF in a series of 400 cases of normal (fetal, neonatal, infantile and adult) and pathological gonads. The authors have observed that both the membrane-bound and the soluble form of SCF were absent in normal testis, while all cases of TGCTs, CIS and GB were positive. No SCF was found to be related to germ cells with proven maturational delay, unlike the other known CIS/GB markers. Seminomas showed a heterogeneous staining pattern of positive cells and signal intensity for SCF. Non-seminomas showed different patterns in histological subgroups. All CIS samples, both adjacent to seminoma and adjacent to non-seminoma were positive for SCF. This study demonstrates the additional value of immunohistochemical detection of SCF to identify malignant germ cells, especially in the case of germ cell maturation delay (120).

3.7. Y-encoded TSPY protein

The testis-specific protein Y-encoded (TSPY) gene is the putative gene for the gonadoblastoma locus on the Y chromosome (GBY) that predisposes dysgenetic gonads of intersex patients to gonadoblastoma development. Several studies have documented the expression of TSPY in the more common forms of testicular germ cell tumours (TGCTs) of adult testis, classified as seminomas and nonseminomas (121, 123-126). Various isoforms of TSPY transcripts and proteins have been demonstrated in cancerous samples of TGCTs (124). TSPY is expressed in early gonocytes, in prenatal and postnatal testes (104), in spermatogonia and in spermatids of adult testis (135). It has been postulated to serve a certain role in stem germ cell proliferation and/or male meiosis (122, 123). Currently, the exact mechanisms of TSPY action at the molecular and cellular levels are uncertain; its expression in germ cell tumours suggests that it might exert a proliferative function. Currently, the mechanism by which this premalignant precursor initiates and develops into both seminomas and nonseminomas is unknown. Various genetic studies have demonstrated that CIS/ITGCNU and TGCTs are aneuploid. A gain of complete short arm of chromosome 12 and sometimes amplification of certain portion of it has been the consistent change in the evolving germ cell tumours genome (127-130). Such gain of chromosome 12p genes seems to be associated with advancement of the oncogenic process and increase in pluripotency of the tumours cells (128,129,131,132). Analysis of available microarray data demonstrates a correlation between TSPY expression and up-regulation of certain chromosome 12p13 genes in clinical CIS/ITGCNU and TGCT samples but currently, the exact mechanism by which TSPY alters the expression these chromosome 12p genes is unknown (133, 134). In a recently study it has been valuated in a total of 171 TGCTs consisting of 86 seminomas, 85 non-seminomas and 17 normal testicular tissue the expression pattern of TSPY with reference to those of other germ cell tumours markers, such as OCT4, c-KIT, and placental-like alkaline phosphatase (PLAP), alpha fetal protein (AFP), and human chorionic gonadotropin (hCG). The results have showed that TSPY is predominantly expressed in testicular seminoma and in the precursor carcinoma *in situ* (CIS) or intratubular germ cell neoplasia unclassified (ITGCNU) but not (minimal o negative levels) in various types of nonseminomas, including embryonal carcinoma, teratoma, choriocarcinoma, and yolk sac tumors. Double immunofluorescence analysis on selected testicular seminoma and CIS/ITGCNU specimens for TSPY and other germ cell tumours markers, such as PLAP, OCT4, c-KIT and the proliferative markers Ki-67 have showed that TSPY was co expressed in the same tumours germ cells of both types of TGCTs. TSPY was located in both cytoplasm and nuclei of the tumours germ cells with heterogeneity in staining intensity and sub cellular locations. The results was confirmed by western blotting analyse, TSPY was expressed at high levels in seminoma samples but minimally in mixed TGCT and embryonal carcinomas. Normal testes showed reduced but detectable levels (136). The differential expression pattern of TSPY in seminomatous and nonseminomatous germ cell tumours suggests that it can be used as a diagnostic marker for detection of precursors of germ cell tumours and for subtype of TGCTs. Hence, TSPY in combination with other markers could be an important marker for diagnosis and sub-classification of TGCTs.

3.8. HMGA1/HMGA2

The mammalian high mobility group A (HMGA) family of chromosomal proteins includes HMGA1a and HMGA1b, which are encoded by the same gene, HMGA1,

through alternative splicing (137) and the closely related HMGA2 (138). They are small, non-histone, chromatin-associated proteins that bind DNA in AT-rich regions through three basic domains called 'AT-hooks'. They are proteins that regulate transcription by altering the architecture of chromatin and the assembly of multiprotein complexes of transcriptional factors (139). Both genes are widely expressed during embryogenesis, whereas their expression is low or absent in normal adult tissues (140). It was observed that HMGA2 is a protein that plays a role in embryonic development and adipocytic cell growth (141), on the contrary the HMGA1 protein has a critical role in heart development and growth. Both HMGA1 and HMGA2 proteins are over-expressed in several malignancies (138). Studies have shown that their over-expression has a causal role in malignant cell transformation. In adults testis tissue HMGA1 is present in mitotic cells (spermatogonia and primary spermatocytes), whereas HMGA2 is highly expressed in meiotic and post-meiotic cells (secondary spermatocytes and spermatids) (142, 143); in addition, it has been demonstrated a specific function for HMGA2 in the regulation of spermatogenesis. A recently study has evaluated the expression of HMGA1 and HMGA2 proteins in normal and TGCTs (144). Immunohistochemical assay was first performed on sections of normal human testis, both the spermatogonia and primary spermatocytes showed specific nuclear positivity for HMGA1, whereas only secondary spermatocytes showed specific nuclear positivity for HMGA2. Subsequently, HMGA1 and HMGA2 expression was examined in a series of post-pubertal TGCTs, including 30 seminomas, 15 pure embryonal carcinomas, 10 mixed tumours with relevant yolk sac tumours and 15 mixed tumours with relevant teratoma component. Moreover, HMGA1 and HMGA2 were evaluated in 15 intratubular germ cell tumours (ITGCTs) areas of 30 examined seminomas. The results of this analysis showed an intense HMGA1 immunoreactivity in ITGCTs, seminomas and embryonal carcinomas but neither in epithelial and mesenchymal areas of teratomas or in yolk sac carcinomas, whereas HMGA2 expression was observed in embryonal carcinomas and yolk sac carcinomas but not in ITGCTs, seminomas and teratomas. The expression of HMGA1 and HMGA2 mRNA was confirmed by reverse transcription–PCR that shows an abundant HMGA1 mRNA amplification in seminomas and embryonal carcinoma but not in teratomas and yolk sac tumours. Conversely, HMGA2 mRNA was abundantly amplified from embryonal carcinoma and yolk sac tumours but not in seminomas and teratomas. In conclusion HMGA1 and HMGA2 co expression was observed only in embryonal carcinoma cells. Conversely, no expression of both HMGA1 and HMGA2 proteins was detected in teratomas. In addition, extra-embryonic yolk sac tumours show only expression of HMGA2 protein (144).

This work enhances the current view that HMGA1 and HMGA2, previously believed to play the same role in carcinogenesis, may play different roles in different tissues. The different expression profile of HMGA1 and HMGA2 proteins could be a useful tool for diagnosis of TGCTs. The co expression of HMGA1 and HMGA2 proteins, together with other well-known

molecular markers in cancer cells is suggestive of an embryonal carcinoma diagnosis, while only HMGA1 expression is indicative of seminomas and only HMGA2 expression is specific of yolk sac tumours.

3.9. SOX17/SOX2

The SOX family of transcription factors is involved in development from the early cells in the embryo to differentiated lineages of specialized cells. Currently, 20 SOX proteins have been identified in humans (145). Although different SOX proteins can bind highly similar DNA sequences *in vitro*, they are expressed in a cell-type-specific manner by which they regulate their target genes (146). This regulation is achieved by the collaboration of SOX proteins in association with other proteins (147). Abundantly represented among the protein partners are the POU-proteins (also known as OCT3/4) (148, 149). These transcription factors interact simultaneously with the DNA and stabilizing partner proteins (150,151). SOX and POU protein are in the centre of an intricate network required to maintain pluripotency and to promote differentiation (152, 153). Normally, 1 year after birth, no POU protein are present in the cells of gonads and others tissue. Prolonged expression of POU protein is in fact associated with the development of malignant testicular germ cell tumours of adolescent and young adults (TGCT). In particular, it has been yet shown how POU protein (OCT3/4) appears to be a specific marker in TGCT diagnostics with 100% of CIS, seminoma and EC cells (67,156). The role of SOX2 interaction with POU protein in maintaining pluripotency and preventing differentiation is well established (157); however, how POU protein expression is involved in apoptosis of PGCs is not clear. It has been shown that SOX2 is not present in germ cells of early embryonic gonads to adult testis or in pre-invasive and invasive TGCTs by immunohistochemistry, but it has been confirmed the presence in EC and in a small number of cells of teratoma (36). SOX2 can also be present in Sertoli cells associated or not with CIS; this observation is new because it may result in over diagnosis of intratubular EC as done recently (158). Besides, quantitative RT-PCR has confirmed the elevated expression of SOX2 in sample containing CIS cells, compared with normal testis. This presence of SOX2 in the context of germ cell tumours might be over diagnosed as EC when performed as single staining, because is necessary to perform for a correct diagnosis a double- staining approach for SOX2 and OCT3/4. If SOX2 and OCT3/4 are co-expressed within the same cells it is indicative of EC. A recently study have investigated if another SOX members are expressed in normal and pathological testicular cells. It has been shown that SOX17, instead of SOX2, is present in CIS and seminoma cells and absent in EC cells. These results have been confirmed with quantitative RT-PCR that has showed elevated SOX17 expression in seminoma samples compared with EC samples (163). SOX17 has multiple functions, including a specific role in embryonic develop of haematopoietic stem cells (159-162). Although a differential pattern of expression of SOX17 has been reported (133,128). Immunohistochemistry of SOX17 demonstrated nuclear co-expression with OCT3/4 in fetal gonocytes, CIS and seminoma cells but not in EC and

derived cell lines. The specific nuclear staining for SOX17 is of diagnostic value in discriminating seminoma/dysgerminoma from EC, however SOX17 is not suitable marker for the diagnosis of CIS, because it is found in different maturation stages of spermatogenesis (163). Therefore OCT3/4 is still the best marker for the detection of CIS in the adult testis (69). However, the co-expression of SOX17 and OCT3/4 in CIS and seminoma cells in the absence of SOX2 qualifies SOX17 as potential protein marker in these cells and can be helpful in distinguishing between the invasive components of seminoma and EC.

3.10. Centrosomal kinase Nek2

Regulation of the centrosome cycle is crucial to maintain genome stability and regular cell cycle progression (166-168,177). In many cancers, defects in centrosome regulation have been associated with aneuploidy (167,177-178). In most TGCTs, aberrant centrosome duplication precedes aneuploidy but the mechanisms leading to this defect are still unknown (164). Protein kinases that regulate the centrosome cycle are often aberrantly controlled in cancer cells. Changes in their expression or activity can lead to perturbations in centrosome duplication, potentially leading to chromosome segregation errors and aneuploidy. Most testicular seminomas are polyploid or aneuploid, due to aberrant chromosome segregation in the early stages of the cancer transformation (127,164). Aneuploidy also occurs in non-neoplastic germ cells from infertile males and represents a risk factor for the development of GCTs (127, 164). A study has demonstrated that over duplication of centrosomes is associated with aneuploidy both in type II GCTs and in non-neoplastic germ cells from individuals with aberrant spermatogenesis (164). Nevertheless, the molecular basis of centrosome amplification in testicular seminomas is currently unknown. The centrosome is a cytoplasmatic organelle that serves as a nucleation centre for microtubules throughout the cell cycle. During mitosis, the centrosome organizes the bipolar spindle and is required for equal distribution of replicated chromosomes between daughter cells (165–167). In addition, the centrosome serves as a scaffold to recruit proteins involved in cell cycle-related events, such as protein kinases, phosphatases, and proteins involved in protein degradation by the proteasome (166). Several serine– threonine kinases, such as Cdk1/cyclin B and Nek2 are associated with the centrosome during cell cycle progression and regulate the centrosome cycle and spindle assembly (165-167). Nek2 is a centrosomal kinase highly enriched in male germ cells (169). This centrosomal kinase promotes centrosome separation at the onset of mitosis through phosphorylation and displacement of proteins involved in centrosome cohesion (170-173). Up-regulation of this kinase in human cells causes premature splitting of the centrosome (174) while in contrast, over expression of kinase-dead Nek2 induces centrosome abnormalities that result in monopolar spindles and aneuploidy (175). Hence, regulation of Nek2 abundance and activity is essential to ensure the correct centrosome cycle and aberrant expression of Nek2 has been described in several tumours (176,179-181). A recently study has investigated the expression and regulation of Nek2 in human TGCTs and in normal testis. The authors

have observed that the expression and activity of Nek2 are frequently up-regulated in testicular seminomas; in particular it has been observed that Nek2 was highly expressed in the cancer cells in the entire sample obtained from testicular seminomas compared with normal testis. In addition they found that Nek2 was already expressed at high levels in the early stages of cancer transformation, such as in intratubular germ cell tumours. Interestingly, Nek2 staining was concentrated in the nucleus of cancer cells and up-regulation of Nek2 was a specific feature of testicular seminomas, because other types of TGCTs did not express detectable levels of Nek2. The results were confirmed by western blot analysis (185). Interestingly, other studies have observed that the nuclear localization of Nek2 in testis correlated with the expression of markers for the stemness of germ cells, such as the transcriptional regulator factor OCT4/3 (183, 184). It is possible to speculate that the localisation of Nek2 in the nucleus is an additional marker of the undifferentiated state of seminoma cells. Nevertheless, functional analysis of the role of Nek2 in seminoma cells will be required to determine its role in cancer transformation of germ cells.

3.11. Cytokeratins, glycoproteins and human antibody

Several cytokeratins and glycoproteins have been tested in testicular cancer with different pattern of expression. It was observed that cytokeratins are also helpful in distinguishing seminoma from a solid type of embryonal carcinoma. For example, in a recently study it was observed that seminoma and spermatocytic seminoma were negative when incubated with murine monoclonal antikeratin antibodies **cytokeratin AE1/AE3** (cytoplasmic staining) (186). Several other studies have shown that these antikeratin antibodies had weak expression in seminoma but strongly positive staining in embryonal carcinoma (187,188). The identification of the perinuclear pattern of reactivity with cytokeratin in seminoma may be the first clue in an unusual site (e.g., lymph node of neck) that one is dealing with metastatic seminoma. Studies analyzing the expression of AE1/AE3 in ITGCN are rare. Bruce *et al* have demonstrated a negative staining for this marker in a small series of ITGCN (189) (Table 1). Another low-molecular-weight cytokeratin **CAM 5.2** with cytoplasmic and membrane staining, was analyzed in several immunohistochemical studies and strong positivity was shown in NSGCT. In seminoma and spermatocytic seminoma, the expression was only focal and very weak. The authors concluded that this marker could be very helpful in distinguishing seminoma from NSGCT (187, 190) (Table 1). Nevertheless, there are no data published analyzing the staining of CAM 5.2 in ITGCN. The **CD30** antigen, an antigen located in cell membrane has been used primarily as a diagnostic marker for Hodgkin disease. The solely described non-hematopoietic malignancy with a strong expression for CD30 is embryonal carcinoma. In other types of testicular GCT an equivalent positive staining for CD30 has not been reported (191,192). Hittmair has detected a specific positive immunostaining for CD30 in only few foci of seminoma, in contrast strong reactivity was found in embryonal carcinoma (193). In this study, the staining for CD30 in spermatocytic seminoma was negative. Further studies have confirmed the

expression of CD30 in embryonal carcinoma while in other types of GCT the staining for CD30 was essentially negative (187,188,194, 195). In a small study, Zamecnik and Sultani have demonstrated a negative expression for CD30 in ITGCN (196) (Table 1). Another glycoprotein, **Podoplanin** (clone M2A, located in cell membrane, diagnostically equivalent to D2-40), a small mucin-type transmembrane glycoprotein, has been implicated in tumours progression of a variety of human cancers (197). The expression of podoplanin is up regulated in squamous cell carcinoma of the oral cavity, lung, skin, granulosa cell tumours and mesothelioma (198). Bailey and co-workers have showed an expression of M2A in 100% of the seminoma tumours. In contrast, in non-seminomatous germ cell tumours (NSGCT), the reactivity for M2A was in all cases negative (199). Furthermore, two studies have demonstrated positive expression for podoplanin in ITGCN (47,200) (Table 1). **Glypican 3 (GCP3),** another glycoprotein, has been analysed as a useful marker for subtypes of germ cell tumours (201). The results demonstrated the GCP3 expression in 100% of yolk sac tumours and choriocarcinoma components compared with only 8% of embryonal carcinoma and 38% of teratomas with immature elements. Seminoma and ITGCN were consistently negative. This marker could be promising in identifying non-seminomatous components and distinguishing yolk sac tumours from other germ cell tumours (Table 1). Another useful marker tested is the **HLA-G antibody**, an excellent marker of intermediate trophoblastic cells. This antibody interacts with the non classical major histocompatibility complex class I antigen, located on the cell surface (202). Kurman *et al* have demonstrated in two studies the specificity of this marker for trophoblastic tumours such as choriocarcinomas (203, 204). Therefore, especially in difficult situations such as metastatic high-grade cancer of unknown primary site, this marker could be used for the detection of cells with trophoblastic differentiation (206) (Table 1). Nevertheless, there is not data published about the immunohistochemical staining of HLA-G in spermatocytic seminoma. **TRA-1-60** is an antibody raised against a human EC cell line that recognises a sialylated keratin sulphate proteoglycan (207). TRA-1-60 is expressed by CIS, seminoma and EC (208); this antibody has been evaluated as a serum marker for patients with testicular GCTs (209). TRA-1-60 could be useful in the follow-up of patients with stage 1 non-seminomatous GCTs, as 57.1% of patients with recurrence had elevated serum levels (210). However, a subsequent study have detected elevated levels in most patients with tumours containing EC but elevated levels did not normalised in 15–30% of patients after chemotherapy and the authors concluded that TRA-1–60 is not a useful serum marker of testis cancer (211). Several studies have investigated the expression of **CDH1 (E-cadherin),** a calcium dependent transmembrane adhesion glycoprotein, which is essential for embryonic development and germ cell determination (212). CDH1 is weakly expressed in human foetal testes but not in normal adult testis (213,214). Microarray data have shown a high expression of CDH1 mRNA in CIS as well as in EC but the protein level expression was shown only in a subset of seminomas, in the majority of non-seminomas but not at the CIS stage

(213, 215–218). Discrepancy in expression in CIS between the mRNA and protein levels has suggested that CDH1 expression may be post-transcriptional regulated in testicular GCTs (218). It was observed that in the testis, serum-E-cadherin levels were significantly higher in testicular GCT patients with advanced disease (stage II/III), regardless of tumour histology, when compared to healthy individuals and patients with stage I testicular GCT. An overlap with levels in healthy individuals gave a limited clinical suitability (218). A recent study has analysed the expression of **growth and angiogenesis factors** in serum from 22 testicular GCT patients. The results have showed a marked elevation of pleiotrophin (PTN) and no overlap with control subjects with modest increase for FGF-2, VEGF and EGF in serum of patients with testis cancer (219). Another approach is based on detection of **XIST gene**, in fact the XIST gene is mainly hypomethylated in testicular GCTs irrespective of XIST expression, and a DNA tumour marker has been described based on the unmethylated DNA profile (220). Male somatic cells show complete methylation and a study with 49 patients has showed that identification of an XIST unmethylated fragment in male plasma might be diagnostic for testicular GCTs (221). But at present none of the described markers performed adequately for detection of seminoma either at the initial staging or during follow-up, although combinatory studies of some of the markers have been attempted (222, 223).

4. MOLECULAR BIOMARKERS OF TESTICULAR GERM CELL TUMORS IN SEMEN SAMPLES

Early diagnosis of testicular GCTs, preferentially at the non invasive stage, should be preferable as the disease is potentially lethal, especially in a subset of poor-prognosis patients and in patients that are refractory to chemotherapy. If a patient is diagnosed already at the CIS stage, he can be offered the most gentle and optimal treatment with a high cure rate. Testicular biopsy is still the gold standard in diagnosing CIS, with a false-negative percentage of 0.5% (224). However, at present, there is reluctance by many urologists to perform a surgical biopsy of the controlateral testis at the time of orchiectomy for detection of contralateral CIS. Arguments against a contralateral biopsy are that the procedure is an unnecessary burden in 95% of cases, determines potential complications and the invasive procedure may delay semen production. Therefore a less invasive method is needed. This method could also be applied as screening for CIS performed at andrology or fertility clinics in young men with atrophic testes, history of cryptorchidism or in need for assisted reproduction. Indeed, infertile males have an increased risk of harbouring CIS and could therefore be target for screening. The incidence of CIS in normal healthy males has been estimated as 0.8% - 0.43% (225,226). Many studies have evaluated the possibility for non invasive early diagnosis of CIS in semen using specific immunohistochemical markers. In fact CIS cells are located within the seminiferous tubules and can be exfoliated into semen. The use of semen for detection of neoplastic cells in patients with testicular cancer was already suggested by Czaplicki in 1987 (227) and Giwercman in 1988 (228). It

was observed that patients with testicular GCTs may harbour morphologically abnormal cells in their semen (229) but detection of these may be difficult as the morphology of non-sperm cells are poorly preserved, even with precaution such as adjusting pH and moderate centrifugation (227,230). The abnormal cells in semen from patients with testicular GCTs were shown to have CIS characteristics and they are present in semen due to the fact that seminiferous tubules with CIS are nearly always present close to the tumour (17,231,232). Different methods such as fluorescent *in situ* hybridization (233), immunohistochemistry using Ap-2 gamma and PLAP (234–236) and immunohistochemistry with magnetic beads using the M2A (a transmembrane glycoprotein) antibody (237) proved to be unsuccessful or too laborious. Another study has used flow cytometry, immunochemistry for PLAP and enzymochemistry for alkaline phosphatase for detection of CIS on a surgical biopsy, fine-needle aspirate and semen sample. The results shown that biopsy and fine-needle aspiration cytology were superior to seminal fluid analysis, as malignant cells within semen were undetectable and the seminal plasma placental-like alkaline phosphatase immunoassay failed to discriminate CIS for the high level of background germ cell alkaline phosphatase (238). Another study reported cytological detection of neoplastic cells in the ejaculate or fluid from prostatic massage in three patients with testicular GCTs by cytological microscopy, although no control group was analysed. However, malignant cells were no found in the seminal fluid after orchiectomy (239). Further several studies have investigated the alterations of DNA content in the cells of ejaculate of patients with TGCTs and in healthy controls. A method using the flow cytometry have identified aneuploid cells in 4/8 CIS patients an in 0/26 of controls (240) and by *in situ* hybridisation with a chromosome 1 probe 2/2 CIS patients and 3/6 testicular GCT patients were positive and 0/16 controls (241). Salanova *et al* have quantified the presence of immature hyperdiploid germ cells in the seminal fluid of healthy subjects, oligozoospermic patients with cryptorchidism and of CIS/testis tumour patients. Cell ploidy was evaluated in immature germ cell-enriched seminal cell fractions by *in situ* hybridisation with a chromosome 1 probe, but hyperdiploidy was not a predictive parameter of testis tumour pathologies (242). In a study with fluorescence *in situ* hybridisation for chromosome 12p, nuclei exhibiting P3 chromosome 12 signals were found to be present in a significantly larger number in the patient samples than in control specimens, although a large overlap was seen (233). These studies were hampered by the fact that normal ejaculates contain cells with various numbers of chromosomal copies, such as diploid spermatogonia, spermatocytes, leucocytes and epithelial cells, haploid spermatids, tetraploid spermatogonia and pachytene spermatocytes. Only cells with 3 N could be assigned as possible CIS cells.

Another study has investigated the expression in semen samples of the platelet-derived growth factor alpha-receptor (alpha-PDGF). The authors found that the alpha-PDGF was expressed by CIS and testicular GCTs (243) but analysis of the expression showed that the transcript was also expressed in various other cells and tissue, especially

also in haematopoietic cells (66). After the discovery of a series of novel stem cell-related markers of CIS and TGCTs, several studies have investigated the expression of these nuclear markers in the ejaculate, including AP-2 gamma, NANOG and OCT3/4. In contrast to previous studies which used cytoplasmatic markers of CIS, the nuclear proteins with expression confined to neoplastic cells should be better protected from degradation in semen, since the nucleus is less prone to degradation during passage of the seminal ducts or during processing of the sample of semen. Hoei-Hansen *et al* have demonstrated with non invasive method that the immunostaining of AP-2gamma was able to diagnose CIS like cells in ejaculate from a young infertile patient (234). Subsequent studies have also analysed additional gonocyte markers such as OCT3/4, NANOG and PLAP in semen samples. It has been observed that the immunocytological method is a very promising new approach that it can be applied as a diagnostic analysis (236), as the specifity was 93.6% but the sensitivity only 54.5%, although a negative result did not exclude the presence of germ cell cancer, but on the other hand, despite a low sensitivity, the method was able to detect occasional patients with CIS. Recently Niels J *et al* have performed a study to detect CIS cells in semen of patients with known risk factors for CIS or TGCT using the specific immunohistochemical markers OCT3/4 (244). In this study 41 men at risk for CIS of the testis were found eligible. Indications for inclusions were a suspicious lesion on scrotal ultrasound investigation (n = 14), patients on surveillance after a history of a testicular tumours (n = 14) and 13 patients with bilateral testicular microlithiasis (TM). Three of the 13 men (23%) who underwent testicular biopsies for bilateral TM were histologically diagnosed with CIS (two bilateral), and their semen showed OCT3/4-positive cells in all cases. Twelve of the 14 patients (86%) with a solid mass were diagnosed with a TGCT with adjacent CIS in the parenchyma and in 9 cases (75%) OCT3/4-positive cells were present in the semen. No OCT3/4-positive cells were found in patients with biopsies who did not show any evidence of malignancy. This study demonstrates that OCT3/4-positive cells can be found in semen from the majority of patients with CIS. The most recent report regarding non-invasive detection used an approach of identification of two isoforms of human cytoplasmic isocitrate dehydrogenase in seminal plasma by gel electrophoresis and mass spectrometry. The isoforms were altered in patients with seminoma, but too few samples were analysed to be conclusive on the potential of the biomarker (245).

However, none of the described methods based on examination of semen samples have been shown sufficiently reliable to be used for routine diagnostic purposes, due to too frequent false-negative or false-positive results. CIS and tumour cells are not always detectable in semen, most likely due to a very low number of CIS cells in semen accentuated in testes with an evident tumour, where shedding of CIS cells situated in the vicinity of a tumour may be hindered by compression of tubules by the tumour. Furthermore, it would not be expected that patients where the tumour involves the vas deferens and epididymis have positive semen cytology. There are also

Table 2. Reports on detection of germ cell neoplasia by semen analysis

N CIS/TGCT patients	N control partecipant	Method	Results	Reference
7	50	Cytological examination	Neoplastic cells in 7/7 TGCT[1] patients. No data on wheter controls atypical cells	M. Czaplicki [227]
4	10	M2A[2] immunohistochemistry	M2A[2] positive cells in 4/4 CIS[3]/TGCT[1] patients Non specific staining in 2 controls	A Giwercman [228]
8	26	Flow cytometry	Aneuploid cells in 4/8 CIS[3] patients and 0/26 controls	A Giwercman[240]
18	6	Cytological examination of semen and urine	Suspected or positive in 12/18 TGCT[1] patients and 1/6 control semen, suspected or positive 7/18 TGCT[1] patients and 0/6 controls in urine	J Rojewska[230]
16	9	Flowcytometry, PLAP[4] immunohistochemistry/ enzymochemistry	No positive cells in semen by PLAP[4] staining or aneuploid peaks by flow cytometry, no correlation with CIS[3] by PLAP[4] immunoassay in semen	ET Brackenbury[238]
5	22	M2A[2] immunomagnetic beads and cell sorting	Recovery of a median of 1x10(4) large round cells from CIS[3] patients, from control a median of 2x10(2). No overlap between groups	FJ Meng[237]
10	10	Bicolour fluorescent in situ hybridisation with chromosome 12 probes	> 3 chromosome 12 signals in 13.8% of CIS[3] patients and 0.5% of control patients	FJ Meng[233]
4	41	In situ hybridisation with chromosome 1 probe	Increased germ cell hyperdiploid in semen from CIS[3]/TGCT[1] patients, but overlap with control group	M Salanova[242]
209	294	AP-2gamma [5]and OCT3/4 [6]immunohistochemistry	Diagnosis of CIS[3] in infertile male Sensivity 54.5%, specifity 93.6%	CE Hoei-Hansen[234,236]
41 males at risk for CIS	------	OCT3/4[6] immunohistochemistry	13 had CIS[3] and OCT3/4[6]positive cells in semen. 14 had a TGCT[1], 86% had OCT3/4[6] positive cells in semen. No false positive	J Niels[244]

Abbreviations: testicular germ cell tumours[1], monoclonal antibody[2], carcinoma *in situ*[3], placental-like alkaline phosphatase[4], gene involved in embryonic development[5], POU-family transcription factor[6]

inherent properties of the CIS cells determining if they detach from the basement tubular membrane and can be shed into the semen, or the CIS cells may be partially degraded and injured and lose cell surface antigens in the ejaculatory path. Finally, the rare false positive results seen in the assays could be due to presence of immature germ cells or haematopoietic cells and non-spermatozoal cells are known to be present in seminal fluid in small amounts, including primary spermatocytes, spermatidis, residual bodies, epithelial cells and leucocytes (246). Thus this field still awaits an ultra-sensitive and fail-safe method of diagnosis, which could be applied to routine screening of populations at risk.

5. SUMMARY

Germ cell tumours (GCTs) are a complex entity. Current areas of attention include early detection and avoidance of unnecessary over-treatment. Diagnostic work-up when a testicular neoplasia is suspected includes a clinical examination, determination of risk factors, imaging, serum tumours markers, etc. Recently GCTs have been described as a model of a curable neoplasia with cure rates of about 90%. The main factors contributing to this are: careful staging at the time of diagnosis; adequate early treatment based on chemotherapeutic combinations, with or without radiotherapy and surgery; and very strict follow-up and salvage therapies with cisplatinum based combination chemotherapy that represents, in according to European Association of Urology guidelines 2009 (EAU 2009), the

primary treatment of choice for advanced disease with results in long-term remissions for about 50% of the patients who relapse after first-line chemotherapy. However, the treatment of patients with testicular cancer has a relevant impact on quality of life with a reduction of fertility, risk of cardiovascular disease, reduction in sexual function and a risk of a second malignancy following radio or chemotherapy treatments. Therefore a key role has the early diagnosis of these tumours especially in the CIS stage. Nevertheless the diagnosis of testicular tumours only sporadically occurs at the CIS stage where minimal treatment is necessary, mainly due to the fact that presence of testicular CIS frequently is asymptomatic. However CIS may be suspected in a well-defined group of conditions and recognition of the precursor lesion gives an opportunity for intervention before the invasive tumour has developed. Several serological markers for testicular cancer have been tested and some are available. These markers are useful in the diagnosis and staging of disease, for monitor the therapeutic response and to detect tumour recurrence. The alpha-fetoprotein (AFP) and beta-human chorionic gonadotropin (beta-hCG) are well established as serum markers for GCTs of the testis, lactate dehydrogenase (LDH) and placental-like alkaline phosphatase (PLAP) may be alternative serological markers with less specifity. However, these markers are increased in only about 60% of patients with testicular cancer and it should be noted that negative marker levels do not exclude the diagnosis of a germ cell tumour. Furthermore in the pre-invasive CIS stage serological markers are not relevant, as neoplastic

cells are not present in circulating plasma at this stage and at the present the diagnosis of testicular CIS still is a diagnosis based on histological analysis of a testicular biopsy. Therefore, additional serum markers would facilitate clinical diagnosis and treatment in these patients, especially in the early stage of the disease and may support the diagnosis of the GCT sub-type. In the recent years several new markers have been tested and at the present several cytogenetic and molecular markers are available in specific centres but none have been shown to be superior to the classical markers in serum (TRA 1-60, XIST, CDH1), therefore there is still a need for a seminoma -specific marker. Multiple studies have improved phenotypical characterisation of these tumours and demonstrated that they either de-differentiate or retain several feature of pluripotent embryonic stem cells adding to the hypothesis of a pre-natal origin of these neoplasia and further research is need in this context for the detection of new serum marker. About histological diagnosis of CIS and others TGCTs immunohistochemistry detection of OCT3/4, AP-2 gamma, SOX proteins etc. being solid new markers in addition to the routinely used such as PLAP for CIS. Recently an immunocytological method targeting nuclear stem cell/GCT markers has proven useful for detection of CIS cells in semen in some patients and may become applicable for screening or as an auxiliary test in urology centres that allow a careful follow-up but further researches are needed to optimise semen based method of analysis of several markers. In conclusion progress in knowledge about the aetiology of testicular neoplasm such as involves genetic mutations, polymorphisms and exogenous influences could give the opportunity of the identification of many novel potential markers.

6. ACKNOWLEDGEMENTS

7. REFERENCES

1. DM Parkin, F Bray, J Ferlay, P Pisani: Global cancer statistics, 2002. *CA Cancer J Clin* 55, 74-108 (2005)

2. X Wu, FD Groves, CC McLaughlin, A Jemal, J Martin, VW Chen: Cancer incidence patterns among adolescents and young adults in the United States. *Cancer Causes Control* 16, 309-320 (2005)

3. SS Devesa, WJ Blot, BJ Stone, BA Miller, RE Tarone, JF Jr Fraumeni: Recent cancer trends in the United States. *J Natl Cancer Ins* 87, 175-182 (1995)

4. A Jemal, R Siegel, E Ward, T Murray, J Xu, C Smigal, MJ Thun: Cancer statistics, 2006. *CA Cancer J Clin* 56, 106-130 (2006)

5. LS Krain: Testicular cancer in California from 1942 to 1969: the California Tumor Registry experience. *Oncology* 27, 45-51 (1973)

6. D Raghavan: Testicular cancer: maintaining the high cure rate. *Oncology (Williston Park)* 17, 218-228 (2003)

7. A Talerman. Germ cell tumours. *Ann Pathol* 5, 145-157 (1985)

8. Surveillance, Epidemiology, and End Results (SEER) Program. Available at: www.seer.cancer.gov. SEER*Stat Database: Mortality—All COD, with State, Total U.S. (1969–2002), National Cancer Institute, DCCPS, Surveillance Research Program, Cancer Statistics Branch, released April 2005. Underlying mortality data provided by NCHS (www.cdc.gov/nchs). Accessed April 2005.

9. KA McGlynn, SS Devesa, AJ Sigurdson, LM Brown, L Tsao, RE Tarone: Trends in the incidence of testicular germ cell tumors in the United States. *Cancer* 97, 63-70 (2003)

10. JN Eble, G Sauter, JI Epstein, IA Sesterhenn: Pathology and Genetics of Tumours of the Urinary System and Male Genital Organs. Lyon, France: IARC Press 218-249 (2004). World Health Organization Classification of Tumours.

11. L Weissbach, JE Altwein, R Stiens: Germinal testicular tumors in childhood: report of observations and literature review. *Eur Urol* 10, 73-85 (1984)

12. HJ Schmoll, R Souchon, S Krege, P Albers, J Beyer, C Kollmannsberger, SD Fossa, NE Skakkebaek, R de Wit, K Fizazi, JP Droz, G Pizzocaro, G Daugaard, PHM de Mulder, A Horwich, T Oliver, R Huddart, G Rosti, L Paz Ares, O Pont, JT Hartmann, N Aass, F Algaba, M Bamberg, I Bodrogi, C Bokemeyer, J Classen, S Clemm, S Culine, M de Wit, HG Derigs, KP Dieckmann, M Flasshove, X Garcia del Muro, A Gerl, JR Germa-Lluch, M Hartmann, A Heidenreich, W Hoeltl, J Joffe, W Jones, G Kaiser, O Klepp, S Kliesch, L Kisbenedek, KU Koehrmann, M Kuczyk, MP Laguna, O Leiva, V Loy, MD Mason, GM Mead, RP Mueller, N Nicolai, GON Oosterhof, T Pottek, O Rick, H Schmidberger, F Sedlmayer, W Siegert, U Studer, S Tjulandin, H von der Maase, P Walz, S Weinknecht, L Weissbach, E Winter, C. Wittekind: European consensus on diagnosis and treatment of germ cell cancer: a report of the European Germ Cell Cancer Consensus Group (EGCCCG). *Ann Oncol* 15, 1377-1399 (2004)

13. M Brydoy, SD Fossa, O Klepp, RM Bremnes, EA Wist, T Wentzel-Larsen, O Dahl: Paternity following treatment for testicular cancer. *J Natl Cancer Inst* 97, 1580-1588 (2005)

14. NE Skakkebaek: Possible carcinoma-in-situ of the testis. *Lancet* 2, 516-517 (1972)

15. CE Hoei-Hansen, E Rajpert-De Meyts, G Daugaard, NE Skakkebaek: Carcinoma *in situ* testis, the progenitor of testicular germ cell tumours: a clinical review. *Ann Oncol* 16, 863-868 (2005)

16. NE Skakkebaek, JG Berthelsen, J Muller: Carcinoma-in-situ of the undescended testis. *Urol Clin N Am* 9, 377-385 (1982)

17. GK Jacobsen, OB Henriksen, H von der Maase: Carcinoma *in situ* of testicular tissue adjacent to malignant

germ-cell tumors: a study of 105 cases. *Cancer* 47, 2660-2662 (1981)

18. KP Dieckmann, NE Skakkebaek: Carcinoma *in situ* of the testis: review of biological and clinical features. *Int J Cancer* 83, 815-822 (1999)

19. EA Klein: Tumour markers in testis cancer. *Urol Clin N Am* 20, 67-73 (1993)

20. N Javadpour: The role of biologic tumor markers in testicular cancer. *Cancer* 45, 1755-17561 (1980)

21. A Lempiainen, UH Stenman, C Blomqvist, K Hotakainen: Free beta-subunit of human chorionic gonadotropin in serum is a diagnostically sensitive marker of seminomatous testicular cancer. *Clin Chem* 54, 1840-1843 (2008)

22. AL Hautkappe, M Lu, H Mueller, A Bex, A Harstrick, M Roggendorf, H Ruebben: Detection of germ-cell tumor cells in the peripheral blood by nested reverse transcription-polymerase chain reaction for alpha-fetoprotein- messenger RNA and beta human chorionic gonadotropinmessenger RNA. *Cancer Res* 60, 3170-3174 (2000)

23. FE von Eyben, WE de Graaff, J Marrink, O Blaabjerg, DT Sleijfer, HS Koops, JW Oosterhuis, PH Petersen, J van Echten-Arends, B de Jong: Serum lactate dehydrogenase isoenzyme 1 activity in patients with testicular germ cell tumors correlates with the total number of copies of the short arm of chromosome 12 in the tumor. *Mol Gen Genet* 235, 140-146 (1992)

24. HJ Huijgen, GT Sanders, RW Koster, J Vreeken, PM Bossuyt: The clinical value of lactate dehydrogenase in serum: a quantitative review. *Eur J Clin Chem Clin Biochem* 35, 569-379 (1997)

25. FE von Eyben, G Skude, SD Fossa, O Klepp, O Bormer: Serum lactate dehydrogenase (S-LDH) and S-LDH isoenzymes in patients with testicular germ cell tumors. *Mol Gen Genet* 189, 326-333 (1983)

26. AA Dabare, AM Nouri, JM Reynard, S Killala, RT Oliver: A new approach using tissue alkaline phosphatase activity to identify early testicular cancer. *Br J Urol* 79, 455-460 (1997)

27. GK Jacobsen, B Norgaard-Pedersen: Placental alkaline phosphatase in testicular germ cell tumours and in carcinoma-insitu of the testis. An immunohistochemical study. *APMIS* 92, 323-329 (1984)

28. EJ Nouwen, PG Hendrix, S Dauwe, MW Eerdekens, ME De Broe: Tumor markers in the human ovary and its neoplasms. A comparative immunohistochemical study. *Am J Pathol* 126, 230-242 (1987)

29. J Hustin, J Collette, P Franchimont: Immunohistochemical demonstration of placental alkaline phosphatase in various states of testicular development and in germ cell tumours. *Int J Androl* 10, 29-35 (1987)

30. J Shinoda, Y Miwa, N Sakai, H Yamada, H Shima, K Kato, M Takahashi, K Shimokawa: Immunohistochemical study of placental alkaline phosphatase in primary intracranial germ-cell tumors. *J Neurosurg* 63, 733-739 (1985)

31. B Lifschitz-Mercer, H Walt, I Kushnir, N Jacob, PA Diener, R Moll, B Czernobilsky: Differentiation potential of ovarian dysgerminoma: an immunohistochemical study of 15 cases. *Hum Pathol* 26, 62-66 (1995)

32. N Torabi-Pour, AM Nouri, F Chineguwndo, RT Oliver: Standardization and potential use of HPLC for detection of cellular placental alkaline phosphatase using established tumour cell lines and fresh tumour biopsies. *Urol Int* 63, 234-241 (1999)

33. GH Williams, PJ McLaughlin, PM Johnson: Tissue origin of serum placental-like alkaline phosphatase in cigarette smokers. *Clin Chim Acta* 155, 329-333 (1986)

34. JM Trigo, JM Tabernero, L Paz-Ares, JL Garcia- Llano, J Mora, P Lianes, E Esteban, R Salazar, JJ López-López, H Cortés-Funes: Tumor markers at the time of recurrence in patients with germ cell tumors. Cancer 2000; 88: 162.

35. K Horie, Fujita J, Takakura K, H Kanzaki, H Suginami, M Iwai, H Nakayama, T Mori: The expression of c-kit protein in human adult and fetal tissues. *Hum Reprod* 8, 1955-1962 (1993)

36. LL Robinson, TL Gaskell, PT Saunders, RA Anderson: Germ cell specific expression of c-kit in the human fetal gonad. *Mol Hum Reprod* 7, 845-852 (2001)

37. E Rajpert-De Meyts, NE Skakkebaek: Expression of the c-kit protein product in carcinoma-in-situ and invasive testicular germ cell tumours. *Int J Androl* 17, 85-92 (1994)

38. Y Tsuura, H Hiraki, K Watanabe, S Igarashi, K Shimamura, T Fukuda, T Suzuki, T Seito: Preferential localization of c-kit product in tissue mast cells, basal cells of skin, epithelial cells of breast, small cell lung carcinoma and seminoma/dysgerminoma in human: immunohistochemical study on formalin-fixed, paraffin-embedded tissues. *Virchows Arch* 424, 135-141 (1994)

39. M Inoue, S Kyo, M Fujita, T Enomoto, G Kondoh: Coexpression of the c-kit receptor and the stem cell factor in gynaecological tumors. *Cancer Res* 54, 3049-3053 (1994)

40. N Jorgensen, E Rajpert-De Meyts, N Graem, J Muller, A Giwercman, NE Skakkebaek: Expression of immunohistochemical markers for testicular carcinoma *in situ* by normal human fetal germ cells. *Lab Invest* 72, 223-231 (1995)

41. T Strohmeyer, D Reese, M Press, R Ackermann, M Hartmann, D Slamon: Expression of the c-kit proto-oncogene and its ligand stem cell factor (SCF) in normal and malignant human testicular tissue. *J Urol* 153, 511-515 (1995)

42. H Takeshima, M Kaji, H Uchida, H Hirano, J Kuratsu: Expression and distribution of c-kit receptor and its ligand in human CNS germ cell tumors: a useful histological marker for the diagnosis of germinoma. *Brain Tumor Pathol* 21, 13-16 (2004)

43. PE Hoyer, AG Byskov, K Mollgard: Stem cell factor and c-Kit in human primordial germ cells and fetal ovaries. *Mol Cell Endocrinol* 234, 1–10 (2005)

44. H Nakamura, H Takeshima, K Makino, J Kuratsu. C-kit expression in germinoma: an immunohistochemistry-based study. *J Neurooncol* 75, 163-167 (2005)

45. M Sever, TD Jones, LM Roth, FW Karim, W Zheng, H Michael, EM Hattab, RE Emerson, LA Baldridge, L Cheng: Expression of CD117 (c-kit) receptor in dysgerminoma of the ovary: diagnostic and therapeutic implications. *Mod Pathol* 18, 1411-1416 (2005)

46. C Hoei-Hansen, A Sehested, M Juhler, YF Lau, NE Skakkebaek, H Laursen, E Rajpert-de Meyts: New evidence for the origin of intracranial germ cell tumours from primordial germ cells: expression of pluripotency and cell differentiation markers. *J Pathol* 209, 25-33 (2006)

47. K Biermann, D Klingmuller, A Koch, T Pietsch, H Schorle, R Büttner, H Zhou: Diagnostic value of markers M2A, OCT3/4, AP-2gamma, PLAP and c-KIT in the detection of extragonadal seminomas. *Histopathology* 49, 290-297 (2006)

48. CE Hoei-Hansen, SM Kraggerud, VM Abeler, J Kaern, E Rajpert-De Meyts, RA Lothe: Ovarian dysgerminomas are characterised by frequent KIT mutations and abundant expression of pluripotency markers. *Mol Cancer* 6, 12 (2007)

49. K Molyneaux, C Wylie: Primordial germ cell migration. *Int J Dev Biol* 48, 537-544 (2004)

50. Q Tian, HF Jr Frierson, GW Krystal, CA Moskaluk: Activating ckit gene mutations in human germ cell tumors. *Am J Pathol* 154, 1643-1647 (1999)

51. K Kemmer, CL Corless, JA Fletcher, L McGreevey, A Haley, D Griffith, OW Cummings, C Wait, A Town, MC Heinrich: KIT mutations are common in testicular seminomas. *Am J Pathol* 164, 305-313 (2004)

52. Y Nakai, N Nonomura, D Oka, M Shiba, Y Arai, M Nakayama, H Inoue, K Nishimura, K Aozasa, Y Mizutani, T Miki, A Okuyama: KIT (c-kit oncogene product) pathway is constitutively activated in human testicular germ cell tumors. *Biochem Biophys Res Commun* 337, 289-296 (2005)

53. LH Looijenga, H de Leeuw, M van Oorschot, RJ van Gurp, H Stoop, AJ Gillis, CA de Gouveia Brazao, RF Weber, WJ Kirkels, T van Dijk, M von Lindern, P Valk, G Lajos, E Olah, JM Nesland, SD Fossa, JW Oosterhuis: Stem cell factor receptor (c-KIT) codon 816 mutations predict development of bilateral testicular germ-cell tumors. *Cancer Res* 63, 7674-7678 (2003)

54. EA Rapley, S Hockley, W Warren, L Johnson, R Huddart, G Crockford, D Forman, MG Leahy, DT Oliver, K Tucker, M Friedlander, KA Phillips, D Hogg, MA Jewett, R Lohynska, G Daugaard, S Richard, A Heidenreich, L Geczi, I Bodrogi, E Olah, WJ Ormiston, PA Daly, LH Looijenga, P Guilford, N Aass, SD Fossa, K Heimdal, SA Tjulandin, L Liubchenko, H Stoll, W Weber, L Einhorn, BL Weber, M McMaster, MH Greene, DT Bishop, D Easton, MR Stratton: Somatic mutations of KIT in familial testicular germ cell tumours. *Br J Cancer* 90, 2397-2401 (2004)

55. K Biermann, F Goke, D Nettersheim, D Eckert, H Zhou, P Kahl, I Gashaw, H Schorle, R Büttner: c-KIT is frequently mutated in bilateral germ cell tumours and down-regulated Stem cell markers in detection of germ cell tumour 363 during progression from intratubular germ cell neoplasia to seminoma. *J Pathol* 213, 311-318 (2007)

56. K Pauls, E Wardelmann, S Merkelbach-Bruse, R Buttner, H Zhou: c-KIT codon 816 mutation in a recurrent and metastatic dysgerminoma of a 14-year-old girl: case study. *Virchows Arch* 445, 651-654 (2004)

57. Y Sakuma, S Sakurai, S Oguni, M Satoh, M Hironaka, K Saito: ckit gene mutations in intracranial germinomas. *Cancer Sci* 95, 716-720 (2004)

58. Y Kamakura, M Hasegawa, T Minamoto, J Yamashita, H Fujisawa: C-kit gene mutation: common and widely distributed in intracranial germinomas. *J Neurosurg* 104, 173-180 (2006)

59. RM Przygodzki, AE Hubbs, FQ Zhao, TJ O'Leary: Primary mediastinal seminomas: evidence of single and multiple KIT mutations. *Lab Invest* 82, 1369-1375 (2002)

60. J Nichols, B Zevnik, K Anastassiadis, H Niwa, D Klewe-Nebenius, I Chambers, H Schöler, A Smith: Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* 95, 379-391 (1998)

61. PJ Donovan: High Oct-ane fuel powers the stem cell. *Nat Genet* 29, 246-247 (2001)

62. M Pesce, HR Scholer: Oct-4: gatekeeper in the beginnings of mammalian development. *Stem Cells* 19, 271-278 (2001)

63. J Kehler, E Tolkunova, B Koschorz, M Pesce, L Gentile, M Boiani, H Lomelí, A Nagy, KJ McLaughlin, HR Schöler, A Tomilin: Oct4 is required for primordial germ cell survival. *EMBO Rep* 5, 1078-1083 (2004)

64. S Gidekel, G Pizov, Y Bergman, E Pikarsky: Oct-3/4 is a dosedependent oncogenic fate determinant: *Cancer Cell* 4, 361-370 (2003)

65. Kraft HJ, S Mosselman, HA Smits, P Hohenstein, E Piek, Q Chen, K Artzt, EJ van Zoelen: Oct-4 regulates

alternative platelet-derived growth factor alpha receptor gene promoter in human embryonal carcinoma cells. *J Biol Chem* 271, 12873-12878 (1996)

66. C Palumbo, K van Roozendaal, AJ Gillis, RH van Gurp, H de Munnik, JW Oosterhuis, EJ van Zoelen, LH Looijenga: Expression of the PDGF alpha-receptor 1.5 kb transcript, OCT-4, and c-KIT in humannormal and malignant tissues. Implications for the early diagnosis of testicular germ cell tumours and for our understanding of regulatory mechanisms. *J Pathol* 196, 467-477 (2002)

67. LH Looijenga, H Stoop, HP de Leeuw, CA de Gouveia Brazao, AJ Gillis, KE van Roozendaal, EJ van Zoelen, RF Weber, KP Wolffenbuttel, H van Dekken, F Honecker, C Bokemeyer, EJ Perlman, DT Schneider, J Kononen, G Sauter, JW Oosterhuis: POU5F1 (OCT3/4) identifies cells with pluripotent potential in human germ cell tumors. *Cancer Res* 63, 2244-2250 (2003)

68. TD Jones, TM Ulbright, JN Eble, LA Baldridge, L Cheng: OCT4 staining in testicular tumors: a sensitive and specific marker for seminoma and embryonal carcinoma. Am J Surg *Pathol* 28, 935-940 (2004)

69. J De Jong, H Stoop, GR Dohle, CH Bangma, M Kliffen, JW van Esser, M van den Bent, JM Kros, JW Oosterhuis, LH Looijenga: Diagnostic value of OCT3/4 for preinvasive and invasive testicular germ cell tumours. *J Pathol* 206, 242-249 (2005)

70. E Rajpert-De Meyts, R Hanstein, N Jorgensen, N Graem, PH Vogt, NE Skakkebaek: Developmental expression of POU5F1 (OCT-3/4) in normal and dysgenetic human gonads. *Hum Reprod* 19, 1338-1344 (2004)

71. TD Jones, TM Ulbright, JN Eble, L Cheng: OCT4: a sensitive and specific biomarker for intratubular germ cell neoplasia of the testis. *Clin Cancer Res* 10, 8544-8547 (2004)

72. TL Gaskell, A Esnal, LL Robinson, RA Anderson, PT Saunders: Immunohistochemical profiling of germ cells within the human fetal testis: identification of three subpopulations. *Biol Reprod* 71, 2012-2021 (2004)

73. M Cools, K van Aerde, AM Kersemaekers, M Boter, SL Drop, KP Wolffenbuttel, EW Steyerberg, JW Oosterhuis, LH Looijenga: Morphological and immunohistochemical differences between gonadal maturation delay and early germ cell neoplasia in patients with undervirilisation syndromes. *J Clin Endocrinol Metab* 90, 5295-5303 (2005)

74. TD Jones, GT MacLennan, JM Bonnin, MF Varsegi, JE Blair, L Cheng: Screening for intratubular germ cell neoplasia of the testis using OCT4 immunohistochemistry. *Am J Surg Pathol* 30, 1427-1431 (2006)

75. M Cools, H Stoop, AM Kersemaekers, SL Drop, KP Wolffenbuttel, JP Bourguignon, J Slowikowska-Hilczer, K Kula, SM Faradz, JW Oosterhuis, LH Looijenga: Gonadoblastoma arising in undifferentiated gonadal tissue within dysgenetic gonads. *J Clin Endocrinol Metab* 91, 2404-2413 (2006)

76. L Cheng. Establishing a germ cell origin for metastatic tumors using OCT4 immunohistochemistry. *Cancer* 101, 2006-2010 (2004)

77. YH Loh, Q Wu, JL Chew, VB Vega, W Zhang, X Chen, G Bourque, J George, B Leong, J Liu, KY Wong, KW Sung, CW Lee, XD Zhao, KP Chiu, L Lipovich, VA Kuznetsov, P Robson, LW Stanton, CL Wei, Y Ruan, B Lim, HH Ng: The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat Genet* 38, 431-434 (2006)

78. I Chambers, D Colby, M Robertson, J Nichols, S Lee, S Tweedie, A Smith: Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* 113, 643-645 (2003)

79. CE Hoei-Hansen, K Almstrup, JE Nielsen, S Brask Sonne, N Graem, NE Skakkebaek, H Leffers, E Rajpert-De Meyts: Stem cell pluripotency factor NANOG is expressed in human fetal gonocytes, testicular carcinoma *in situ* and germ cell tumours. *Histopathology* 47, 48-56 (2005)

80. AH Hart, L Hartley, K Parker, M Ibrahim, LH Looijenga, M Pauchnik, CW Chow, L Robb: The pluripotency homeobox gene NANOG is expressed in human germ cell tumors. Cancer 104, 2092-2098 (2005)

81. HJ Auman, T Nottoli, O Lakiza, Q Winger, S Donaldson, T Williams: Transcription factor AP-2gamma is essential in the extra-embryonic lineages for early postimplantation development. Development 129, 2733-2747 (2002)

82. K Almstrup, CE Hoei-Hansen, U Wirkner, J Blake, C Schwager, W Ansorge, JE Nielsen, NE Skakkebaek, E Rajpert-De Meyts, H Leffers: Embryonic stem cell-like features of testicular carcinoma in situ revealed by genome-wide gene expression profiling. Cancer Res 64, 4736-4743 (2004)

83. CE Hoei-Hansen, JE Nielsen, K Almstrup, SB Sonne, N Graem, NE Skakkebaek, H Leffers, E Rajpert-De Meyts: Transcription factor AP-2gamma is a developmentally regulated marker of testicular carcinoma in situ and germ cell tumors. Clin Cancer Res 10, 8521-8530 (2004)

84. K Pauls, R Jager, S Weber, E Wardelmann, A Koch, R Büttner, H Schorle: Transcription factor AP- 2gamma, a novel marker of gonocytes and seminomatous germ cell tumors. Int J Cancer 115, 470-477 (2005)

85. SM Hong, HF Jr Frierson, CA Moskaluk. AP-2gamma protein expression in intratubular germ cell neoplasia of testis. *Am J Clin Pathol* 124, 873-877 (2005)

86. SA Leon, B Shapiro, DM Sklaroff, MJ Yaros: Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res* 37, 646 (1977)

87. S Jahr, H Hentze, S Englisch, D Hardt, FO Fackelmayer, RD Hesch, R Knippers: DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res* 61, 1659 (2001)

88. J Ellinger, PJ Bastian, KI Haan, LC Heukamp, R Buettner, R Fimmers, SC Mueller, A von Ruecker: Noncancerous PTGS2 DNA fragments of apoptotic origin in sera of prostate cancer patients qualify as diagnostic and prognostic indicators. *Int J Cancer* 122, 138 (2008)

89. J Ellinger, PJ Bastian, N Ellinger, P Kahl, FG Perabo, R Büttner, SC Müller, A Ruecker: Apoptotic DNA fragments in serum of patients with muscle invasive bladder cancer: a prognostic entity. *Cancer Lett* 264, 274-280 (2008)

90. HW Chang, SM Lee, SN Goodman, G Singer, SK Cho, LJ Sokoll, FJ Montz, R Roden, Z Zhang, DW Chan, RJ Kurman, IeM Shih: Assessment of plasma DNA levels, allelic imbalance, and CA 125 as diagnostic tests for cancer. *J Natl Cancer Inst* 94, 1697-1703 (2002)

91. PJ Bastian, GS Palapattu, S Yegnasubramanian, X Lin, CG Rogers, LA Mangold, B Trock, M Eisenberger, AW Partin, WG Nelson: Prognostic value of preoperative serum cell-free circulating DNA in men with prostate cancer undergoing radical prostatectomy. *Clin Cancer Res* 13, 5361-5367 (2007)

92. N Umetani, AE Giuliano, SH Hiramatsu, F Amersi, T Nakagawa, S Martino, DS Hoon: Prediction of breast tumor progression by integrity of free circulating DNA in serum. *J Clin Oncol* 24, 4270-4276 (2006)

93. N Umetani, J Kim, S Hiramatsu, HA Reber, OJ Hines, AJ Bilchik, DS Hoon: Increased integrity of free circulating DNA in sera of patients with colorectal or periampullary cancer: direct quantitative PCR for ALU repeats. *Clin Chem* 52, 1062-1069 (2006)

94. J Ellinger, V Wittkamp, P Albers, FG Perabo, SC Mueller, A von Ruecker, PJ Bastian: Cell-Free Circulating DNA: Diagnostic Value in Patients With Testicular Germ Cell Cancer. *J Urol* 181, 363-371 (2009)

95. YM Lo, J Zhang, TN Leung, TK Lau, AM Chang, NM Hjelm: Rapid clearance of fetal DNA from maternal plasma. *Am J Hum Genet* 64, 218 (1999)

96. NY Lam, TH Rainer, LY Chan, GM Joynt, YM Lo: Time course of early and late changes in plasma DNA in trauma patients. *Clin Chem* 49, 1286 (2003)

97. M Ginsburg, MH Snow, A McLaren: Primordial germ cells in the mouse embryo during gastrulation. *Development* 110, 521-528 (1990)

98. K Manova, K Nocka, P Besmer, RF Bachvarova: Gonadal expression of c-kit encoded at the W locus of the mouse. *Development* 110, 1057-1069 (1990)

99. K Manova, RF Bachvarova: Expression of c-kit encoded at the W locus of mice in developing embryonic germ cells and presumptive melanoblasts. *Dev Biol* 146, 312-324 (1991)

100. V Sorrentino, M Giorgi, R Geremia, P Besmer, P Rossi: Expression of the c-kit proto-oncogene in the murine male germ cells. *Oncogene* 6, 149-151 (1991)

101. K Yoshinaga, S Nishikawa, M Ogawa, S Hayashi, T Kunisada, T Fujimoto: Role of c-kit in mouse spermatogenesis: identification of spermatogonia as a specific site of c-kit expression and function. *Development* 113, 689-699 (1991)

102. P Rossi, G Marziali, C Albanesi, A Charlesworth, R Geremia, V Sorrentino: A novel c-kit transcript, potentially encoding a truncated receptor, originates within a kit gene intron in mouse spermatids. *Dev Biol* 152, 203-207 (1992)

103. C Mauduit, S Hamamah, M Benahmed. Stem cell factor/c-kit system in spermatogenesis. *Hum Reprod Update* 5, 535-545 (1999)

104. F Honecker, H Stoop, RR de Krijger, YF Chris Lau, C Bokemeyer, LH Looijenga. Pathobiological implications of the expression of markers of testicular carcinoma *in situ* by fetal germ cells. *J Pathol* 203, 849-857 (2004)

105. E Rajpert-De Meyts, NE Skakkebaek: Expression of the c-kit protein product in carcinoma-in-situ and invasive testicular germ cell tumours. *Int J Androl* 17, 85-92 (1994)

106. E Rajpert-De Meyts, M Kvist, NE Skakkebaek: Heterogeneity of expression of immunohistochemical tumour markers in testicular carcinoma *in situ*: pathogenetic relevance. *Virchows Arch* 428, 133-139 (1996)

107. MA Izquierdo, P Van der Valk, J Van Ark-Otte, G Rubio, JR Germa-Lluch, R Ueda, RJ Scheper, T Takahashi, G Giaccone: Differential expression of the c-kit protooncogene in germ cell tumours. *J Pathol* 177, 253-258 (1995)

108. K Manova, EJ Huang, M Angeles, V De Leon, S Sanchez, SM Pronovost, P Besmer, RF Bachvarova: The expression pattern of the c-kit ligand in gonads of mice supports a role for the c-kit receptor in oocyte growth and in proliferation of spermatogonia. *Dev Biol* 157, 85-99 (1993)

109. H Hakovirta, W Yan, M Kaleva, F Zhang, K Vanttinen, PL Morris, M Söder, M Parvinen, J Toppari: Function of stem cell factor as a survival factor of spermatogonia and localization of messenger ribonucleic acid in the rat seminiferous epithelium. *Endocrinology* 140, 1492-1498 (1999)

110. JI Sandlow, HL Feng, MB Cohen, A Sandra: Expression of c-KIT and its ligand, stem cell factor, in normal and subfertile human testicular tissue. *J Androl* 17, 403-408 (1996)

111. P Rossi, S Dolci, C Albanesi, P Grimaldi, R Ricca, R Geremia: Follicle-stimulating hormone induction of steel factor (SLF) mRNA in mouse Sertoli cells and stimulation of DNA synthesis in spermatogonia by soluble SLF. *Dev Biol* 155, 68-74 (1993)

112. JG Flanagan, DC Chan, P Leder: Transmembrane form of the kit ligand growth factor is determined by alternative splicing and is missing in the Sld mutant. *Cell* 64, 1025-1035 (1991)

113. EJ Huang, KH Nocka, J Buck, P Besmer: Differential expression and processing of two cell associated forms of the kit-ligand: KL-1 and KL-2. *Mol Biol Cell* 3, 349-362 (1992)

114. I Godin, R Deed, J Cooke, K Zsebo, M Dexter, CC Wylie: Effects of the steel gene product on mouse primordial germ cells in culture. *Nature* 352, 807-809 (1991)

115. S Dolci, DE Williams, MK Ernst, JL Resnick, CI Brannan, LF Lock, SD Lyman, HS Boswell, PJ Donovan: Requirement for mast cell growth factor for primordial germ cell survival in culture. *Nature* 352, 809-811 (1991)

116. Y Matsui, D Toksoz, S Nishikawa, D Williams, K Zsebo, BL Hogan: Effect of steel factor and leukaemia inhibitory factor on murine primordial germ cells in culture. *Nature* 353, 750-752 (1991)

117. G Marziali, D Lazzaro, V Sorrentino: Binding of germ cells to mutant Sld Sertoli cells is defective and is rescued by expression of the transmembrane form of the c-kit ligand. *Dev Biol* 157, 182-190 (1993)

118. VC Broudy: Stem cell factor and hematopoiesis. *Blood* 90, 1345-1364 (1997)

119. W Yan, J Kero, I Huhtaniemi, J Toppati: Stem cell factor functions as a survival factor for mature Leydig cells and a growth factor for precursor Leydig cells after ethylene dimethane sulfonate treatment: implication of a role of the stem cell factor/c-Kit system in Leydig cell development. *Dev Biol* 227, 169-182 (2000)

120. H Stoop, F Honecker, GJ van de Geijn, AJ Gillis, MC Cools, M de Boer, C Bokemeyer, KP Wolffenbuttel, SL Drop, RR de Krijger, N Dennis, B Summersgill, A McIntyre, J Shipley, JW Oosterhuis, LH Looijenga: Stem cell factor as a novel diagnostic marker for early malignant germ cells. *J Pathol* 216, 43-54 (2008)

121. F Schnieders, T Dork, J Arnemann, T Vogel, M Werner, J Schmidtke: Testis-specific protein, Y-encoded (TSPY) expression in testicular tissues. *Hum Mol Genet* 5, 1801-1807 (1996)

122. DC Page: Hypothesis: a Y-chromosomal gene causes gonadoblastoma in dysgenetic gonads. *Development* 101, 151-155 (1987)

123. YF Lau: Gonadoblastoma, testicular and prostate cancers, and the TSPY gene. *Am J Hum Genet* 64, 921-927 (1999)

124. YF Lau, HW Lau, LG Komuves: Expression pattern of a gonadoblastoma candidate gene suggests a role of the Y chromosome in prostate cancer. *Cytogenet Genome Res* 101, 250-260 (2003)

125. F Honecker, H Stoop, F Mayer, C Bokemeyer, DH Castrillon, YF Lau, LH Looijenga, JW Oosterhuis: Germ cell lineage differentiation in non-seminomatous germ cell tumours. *J Pathol* 208, 395-400 (2006)

126. M Cools, K van Aerde, AM Kersemaekers, M Boter, SL Drop, KP Wolffenbuttel, EW Steyerberg, JW Oosterhuis, LH Looijenga: Morphological and immunohistochemical differences between gonadal maturation delay and early germ cell neoplasia in patients with undervirilization syndromes. *J Clin Endocrinol Metab* 90, 5295-5303 (2005)

127. JW Oosterhuis, LH Looijenga: Testicular germ-cell tumours in a broader perspective. *Nat Rev Cancer* 5, 210-222 (2005)

128. JE Korkola, J Houldsworth, RS Chadalavada, AB Olshen, D Dobrzynski, VE Reuter, GJ Bosl, RS Chaganti: Down-regulation of stem cell genes, including those in a 200-kb gene cluster at 12p13.31, is associated with *in vivo* differentiation of human male germ cell tumors. *Cancer Res* 66, 820-827 (2006)

129. LH Looijenga, G Zafarana, B Grygalewicz, B Summersgill, M Debiec-Rychter, J Veltman, EF Schoenmakers, S Rodriguez, O Jafer, J Clark, AG van Kessel, J Shipley, RJ van Gurp, AJ Gillis, JW Oosterhuis: Role of gain of 12p in germ cell tumour development. *APMIS* 111, 161-171 (2003)

130. FE von Eyben: Chromosomes, genes, and development of testicular germ cell tumors. *Cancer Genet Cytogenet* 151, 93-138 (2004)

131. G Zafarana, B Grygalewicz, AJ Gillis, LE Vissers, W van de Vliet, RJ van Gurp, H Stoop, M Debiec-Rychter, JW Oosterhuis, AG van Kessel, EF Schoenmakers, LH Looijenga, JA Veltman: 12p-amplicon structure analysis in testicular germ cell tumors of adolescents and adults by array CGH. *Oncogene* 22, 7695-7701 (2003)

132. E Rajpert-De Meyts: Developmental model for the pathogenesis of testicular carcinoma *in situ*: genetic and environmental aspects. *Hum Reprod Update* 12, 303-323 (2006)

133. RI Skotheim, GE Lind, O Monni, JM Nesland, VM Abeler, SD Fossa, N Duale, G Brunborg, O Kallioniemi,

PW Andrews, RA Lothe: Differentiation of human embryonal carcinomas *in vitro* and *in vivo* reveals expression profiles relevant to normal development. *Cancer Res* 65, 5588-5598 (2005)

134. LH Looijenga, R Hersmus, AJ Gillis, R Pfundt, HJ Stoop, RJ van Gurp, J Veltman, HB Beverloo, E van Drunen, AG van Kessel, RR Pera, DT Schneider, B Summersgill, J Shipley, A McIntyre, P van der Spek, E Schoenmakers, JW Oosterhuis: Genomic and expression profiling of human spermatocytic seminomas: primary spermatocyte as tumorigenic precursor and DMRT1 as candidate chromosome 9 gene. *Cancer Res* 66, 290-302 (2006)

135. T Kido, YF Lau: A Cre gene directed by a human TSPY promoter is specific for germ cells and neurons. *Genesis* 42, 263-275 (2005)

136. Y Li, ZL Tabatabai, TL Lee, S Hatakeyama, C Ohyama, WY Chan, LH Looijenga, YF Lau: The Y-encoded TSPY protein: a significant marker potentially plays a role in the pathogenesis of testicular germ cell tumors. *Hum Pathol* 38, 1470-1481 (2007)

137. KR Johnson, DA Lehn, R Reeves: Alternative processing of mRNAs coding for human HMGI and HMGY proteins. *Mol Cell Biol* 9, 2114-2123 (1989)

138. M Fedele, S Battista, G Manfioletti, CM Croce, V Giancotti, A Fusco: Role of the high mobility group A proteins in human lipomas. *Carcinogenesis* 22, 1583-1591 (2001)

139. D Thanos, T Maniatis: The high mobility group protein HMGI(Y) is required for NF-κB-dependent virus induction of human-β gene. *Cell* 71, 777-789 (1992)

140. X Zhou, KF Benson, HR Ashar, KK Chada: Mutation responsible for the mouse pygmy phenotype in developmentally regulated factor HMGI-C. *Nature* 376, 771-774 (1995)

141. S Battista, V Fidanza, M Fedele, AJ Klein-Szanto, E Outwater, H Brunner, M Santoro, CM Croce, A Fusco: The expression of a truncated HMGI-C gene induces gigantism associated with lipomatosis. *Cancer Res* 59, 4793-4797 (1999)

142. P Chieffi, S Battista, M Barchi, S Di Agostino, GM Pierantoni, M Fedele, L Chiariotti, D Tramontano, A Fusco: HMGA1 and HMGA2 protein expression in mouse spermatogenesis. *Oncogene* 21, 3544-3550 (2002)

143. S Di Agostino, M Fedele, P Chieffi, A Fusco, P Rossi, R Geremia, C Sette: Phosphorylation of high-mobility group protein A2 by Nek2 kinase during the first meiotic division in mouse spermatocytes. *Mol Biol Cell* 15, 1224-1232 (2004)

144. R Franco, F Esposito, M Fedele, G Liguori, GM Pierantoni, G Botti, D Tramontano, A Fusco, P Chieffi:

Detection of high-mobility group proteins A1 and A2 represents a valid diagnostic marker in post-pubertal testicular germ cell tumours. *J Pathol* 214, 58-64 (2008)

145. R Grosschedl, K Giese, J Pagel: HMG domain proteins: architectural elements in the assembly of nucleoprotein structures. *Trends Genet* 10, 94-100 (1994)

146. LH Pevny, R Lovell-Badge: Sox genes find their feet. *Curr Opin Genet Dev* 7, 338-344 (1997)

147. M Wegner: From head to toes: the multiple facets of Sox proteins. *Nucleic Acids Res* 27, 1409-1420 (1999)

148. L Dailey, C Basilico: Coevolution of HMG domains and homeodomains and the generation of transcriptional regulation by Sox/POU complexes. *J Cell Physiol* 186, 315-328 (2001)

149. Y Kamachi, M Uchikawa, H Kondoh: Pairing SOX off: with partners in the regulation of embryonic development. *Trends Genet* 16, 182-187 (2000)

150. GJ Veenstra, PC van der Vliet, OH Destree: POU domain transcription factors in embryonic development. *Mol Biol Rep* 24, 139-155 (1997)

151. K Phillips, B Luisi: The virtuoso of versatility: POU proteins that flex to fit. *J Mol Biol* 302, 1023-1039 (2000)

152. H Niwa, J Miyazaki, AG Smith: Quantitative expression of Oct- 3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nature Genet* 24, 372-376 (2000)

153. S Masui, Y Nakatake, Y Toyooka, D Shimosato, R Yagi, K Takahashi, H Okochi, A Okuda, R Matoba, AA Sharov, MS Ko, H Niwa: Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nature Cell Biol* 9, 625-635 (2007)

154. L Cheng, MT Sung, P Cossu-Rocca, T Jones, G Maclennan, J De Jong, A Lopez-Beltran, R Montironi, LH Looijenga: OCT4: biological functions and clinical applications as a marker of germ cell neoplasia. *J Pathol* 211, 1-9 (2006)

155. L Cheng, A Thomas, LM Roth, W Zheng, H Michael, FW Karim: OCT4: a novel biomarker for dysgerminoma of the ovary. *Am J Surg Pathol* 28, 1341-1346 (2004)

156. TD Jones, TM Ulbright, JN Eble, LA Baldridge, L Cheng: OCT4 staining in testicular tumors: a sensitive and specific marker for seminoma and embryonal carcinoma. *Am J Surg Pathol* 28, 935-940 (2004)

157. JL Chew, YH Loh, W Zhang, X Chen, WL Tam, LS Yeap, P Li, YS Ang, B Lim, P Robson, HH Ng: Reciprocal transcriptional regulation of Pou5f1 and

Sox2 via the Oct4/Sox2 complex in embryonic stem cells. *Mol Cell Biol* 2, 6031-6046 (2005)

158. DM Berney, A Lee, J Shamash, RT Oliver: The association between intratubular seminoma and invasive germ cell tumors. *Hum Pathol* 37, 458-461 (2006)

159. J Sohn, J Natale, LJ Chew, S Belachew, Y Cheng, A Aguirre, J Lytle, B Nait-Oumesmar, C Kerninon, M Kanai-Azuma, Y Kanai, V Gallo: Identification of Sox17 as a transcription factor that regulates oligodendrocyte development. *J Neurosci* 26, 9722-9735 (2006)

160. Y Kanai, M Kanai-Azuma, T Noce, TC Saido, T Shiroishi, Y Hayashi, K Yazaki: Identification of two Sox17 messenger RNA isoforms, with and without the high mobility group box region, and their differential expression in mouse spermatogenesis. *J Cell Biol* 133, 667-681 (1996)

161. R Wang, H Cheng, L Xia, Y Guo, X Huang, R Zhou: Molecular cloning and expression of Sox17 in gonads during sex reversal in the rice field eel, a teleost fish with a characteristic of natural sex transformation. *Biochem Biophys Res Commun* 303, 452-457 (2003)

162. I Kim, TL Saunders, SJ Morisson: Sox17 dependence distinguishes the transcriptional regulation of fetal from adult hematopoietic stem cells. *Cell* 130, 470-483 (2007)

163. J de Jong, H Stoop, AJM Gillis: Differential expression of SOX17 and SOX2 in germ cells and stem cells has biological and clinical implications. *J Pathol* 215, 21-30 (2008)

164. F Mayer, H Stoop, S Sen, C Bokemeyer, JW Oosterhuis, LH Looijenga: Aneuploidy of human testicular germ cell tumors is associated with amplification of centrosomes. *Oncogene* 22, 3859-3866 (2003)

165. M Bettencourt-Dias, DM Glover: Centrosome biogenesis and function: centrosomics brings new understanding. *Nature Rev Mol Cell Biol* 8, 451-463 (2007)

166. S Doxsey, D McCollum, W Theurkauf: Centrosomes in cellular regulation. *Annu Rev Cell Dev Biol* 21, 411-434 (2005)

167. W Saunders: Centrosomal amplification and spindle multipolarity in cancer cells. *Sem Cancer Biol* 15, 25-32 (2005)

168. FA Barr, HH Sillje, EA Nigg: Polo-like kinases and the orchestration of cell division. *Nature Rev Mol Cell Biol* 5, 429-440 (2004)

169. K Rhee, DJ Wolgemuth: The NIMA-related kinase 2, Nek2, is expressed in specific stages of the meiotic cell cycle and associates with meiotic chromosomes. *Development* 124, 2167-2177 (1997)

170. AM Fry, T Mayor, P Meraldi, YD Stierhof, K Tanaka, EA Nigg: C-Nap1, a novel centrosomal coiled-coil protein

and candidate substrate of the cell cycle-regulated protein kinase Nek2. *J Cell Biol* 141, 1563-1574 (1998)

171. T Mayor, YD Stierhof, K Tanaka, AM Fry, EA Nigg: The centrosomal protein C-Nap1 is required for cell cycle-regulated centrosome cohesion. *J Cell Biol* 151, 837-846 (2000)

172. S Bahe, YD Stierhof, CJ Wilkinson, F Leiss, EA Nigg: Rootletin forms centriole-associated filaments and functions in centrosome cohesion. *J Cell Biol* 171, 27-33 (2005)

173. S Bahmanyar, DD Kaplan, JG Deluca, TH Jr Giddings, ET O'Toole, M Winey, ED Salmon, PJ Casey, WJ Nelson, AI Barth: Beta-catenin is a Nek2 substrate involved in centrosome separation. *Genes Dev* 22, 91-105 (2008)

174. AM Fry, P Meraldi, EA Nigg: A centrosomal function for the human Nek2 protein kinase, a member of the NIMA family of cell cycle regulators. *EMBO J* 17, 470-481 (1998)

175. AJ Faragher, AM Fry: Nek2A kinase stimulates centrosome disjunction and is required for formation of bipolar mitotic spindles. *Mol Biol Cell* 14, 2876-2889 (2003)

176. DG Hayward, RB Clarke, AJ Faragher, RM Pillai, IM Hagan, AM Fry: The centrosomal kinase Nek2 displays elevated levels of protein expression in human breast cancer. *Cancer Res* 64, 7370-7376 (2004)

177. EA Nigg. Origins and consequences of centrosome aberrations in human cancers. *Int J Cancer* 119, 2717-2723 (2006)

178. GA Pihan, J Wallace, Y Zhou, SJ Doxsey: Centrosome abnormalities and chromosome instability occur together in pre-invasive carcinomas. *Cancer Res* 63, 1398-1404 (2003)

179. T Kokuryo, T Senga, Y Yokoyama, M Nagino, Y Nimura, M Hamaguchi: Nek2 as an effective target for inhibition of tumorigenic growth and peritoneal dissemination of cholangiocarcinoma. *Cancer Res* 67, 9637-9642 (2007)

180. S de Vos, WK Hofmann, TM Grogan, U Krug, M Schrage, TP Miller, JG Braun, W Wachsman, HP Koeffler, JW Said: Gene expression profile of serial samples of transformed B-cell lymphomas. *Lab Invest* 83, 271-285 (2003)

181. DH Wai, KL Schaefer, A Schramm, E Korsching, F Van Valen, T Ozaki, W Boecker, L Schweigerer, B Dockhorn-Dworniczak, C Poremba: Expression analysis of pediatric solid tumor cell lines using oligonucleotide microarrays. *Int J Oncol* 20, 441-451 (2002)

182. W Wu, JE Baxter, SL Wattam, DG Hayward, M Fardilha, A Knebel, EM Ford, EF da Cruz e Silva, AM Fry:

Alternative splicing controls nuclear translocation of the cell cycle-regulated Nek2 kinase. *J Biol Chem* 282, 26431-26440 (2007)

183. RL Brinster. Male germline stem cells: from mice to men: *Science* 316, 404-405 (2007)

184. RA Hess, PS Cooke, MC Hofmann, KM Murphy: Mechanistic insights into the regulation of the spermatogonial stem cell niche. *Cell Cycle* 5, 1164-1170 (2006)

185. F Barbagallo, MP Paronetto, R Franco, P Chieffi, S Dolci, AM Fry, R Geremia, C Sette: Increased expression and nuclear localization of the centrosomal kinase Nek2 in human testicular seminomas. *J Pathol* 217, 431-441 (2009)

186. TM Ulbright, RH Young: Seminoma with tubular, microcystic, and related patterns: a study of 28 cases of unusual morphologic variants that often cause confusion with yolk sac tumor. *Am J Surg Pathol* 29, 500-505 (2005)

187. JC Cheville, S Rao, KA Iczkowski, CM Lohse, VS Pankratz: Cytokeratin expression in seminoma of the human testis. *Am J Clin Pathol* 113, 583-588 (2000)

188. JA Ferreiro: Ber-H2 expression in testicular germ cell tumors. *Hum Pathol* 25, 522-524 (1994)

189. E Bruce, RK Al-Talib, IS Cook, JM Theaker: Vacuolation of seminiferous tubule cells mimicking intratubular germ cell neoplasia (ITGCN). *Histopathology* 49, 194-196 (2006)

190. S Suster, CA Moran, H Dominguez-Malagon, P Quevedo-Blanco: Germ cell tumors of the mediastinum and testis: a comparative immunohistochemical study of 120 cases. *Hum Pathol* 29, 737-742 (1998)

191. H Stein, DY Mason, J Gerdes, N O'Connor, J Wainscoat, G Pallesen, K Gatter, B Falini, G Delsol, H Lemke, R Schwarting, K Lennert: The expression of the Hodgkin's disease associated antigen Ki-1 in reactive and neoplastic lymphoid tissue: evidence that Reed-Sternberg cells and histiocytic malignancies are derived from activated lymphoid cells. *Blood* 66, 848-858 (1985)

192. U Latza, HD Foss, H Durkop, F Eitelbach, KP Dieckmann, V Loy, M Unger, G Pizzolo, Stein H: CD30 antigen in embryonal carcinoma and embryogenesis and release of the soluble molecule. *Am J Pathol* 146, 463-471 (1995)

193. A Hittmair, H Rogatsch, A Hobisch, G Mikuz, H Feichtinger: CD30 expression in seminoma. *Hum Pathol* 27, 1166-1171 (1996)

194. H Durkop, HD Foss, F Eitelbach, I Anagnostopoulos, U Latza, S Pileri, H Stein: Expression of the CD30 antigen in non-lymphoid tissues and cells. *J Pathol* 190, 613-618 (2000)

195. G Pallesen, SJ Hamilton-Dutoit: Ki-1 (CD30) antigen is regularly expressed by tumor cells of embryonal carcinoma. *Am J Pathol* 133, 446-450 (1988)

196. M Zamecnik, K Sultani: Diffuse embryoma with intratubular germ-cell neoplasia. *Histopathology* 45, 427-429 (2004)

197. JT Huse, TL Pasha, PJ Zhang: D2-40 functions as an effective chondroid marker distinguishing true chondroid tumors from chordoma. *Acta Neuropathol* 113, 87-94 (2007)

198. V Schacht, SS Dadras, LA Johnson, DG Jackson, YK Hong, M Detmar: Up-regulation of the lymphatic marker podoplanin, a mucin-type transmembrane glycoprotein, in human squamous cell carcinomas and germ cell tumors. *Am J Pathol* 166, 913-921 (2005)

199. D Bailey, A Marks, M Stratis, R Baumal: Immunohistochemical staining of germ cell tumors and intratubular malignant germ cells of the testis using antibody to placental alkaline phosphatase and a monoclonal anti-seminoma antibody. *Mod Pathol* 4, 167-171 (1991)

200. SB Sonne, AS Herlihy, CE Hoei-Hansen, JE Nielsen, K Almstrup, NE Skakkebaek, A Marks, H Leffers, E Rajpert-De Meyts: Identity of M2A (D2-40) antigen and gp36 (Aggrus, T1A-2, podoplanin) in human developing testis, testicular carcinoma *in situ* and germ-cell tumours. *Virchows Arch* 449, 200-206 (2006)

201. DL Zynger, ND Dimov, C Luan, BT Teh, XJ Yang: Glypican 3: a novel marker in testicular germ cell tumors. *Am J Surg Pathol* 30, 1570-1575 (2006)

202. M McMaster, Y Zhou, S Shorter, K Kapasi, D Geraghty, KH Lim, S Fisher: HLA-G isoforms produced by placental cytotrophoblasts and found in amniotic fluid are due to unusual glycosylation. *J Immunol* 160, 5922-5928 (1998)

203. G Singer, RJ Kurman, MT McMaster, M Shih Ie: HLA-G immunoreactivity is specific for intermediate trophoblast in gestational trophoblastic disease and can serve as a useful marker in differential diagnosis. *Am J Surg Pathol* 26, 914-920 (2002)

204. IM Shih, RJ Kurman: p63 expression is useful in the distinction of epithelioid trophoblastic and placental site trophoblastic tumors by profiling trophoblastic subpopulations. *Am J Surg Pathol* 28, 1177-1183 (2004)

205. F Kommoss, E Oliva, F Bittinger, CJ Kirkpatrick, MB Amin, AK Bhan, RH Young, RE Scully: Inhibin-alpha CD99, HEA125, PLAP, and chromogranin immunoreactivity in testicular neoplasms and the androgen insensitivity syndrome. *Hum Pathol* 31, 1055-1061 (2000)

206. IM Shih, RJ Kurman: Immunohistochemical localization of inhibin-alpha in the placenta and gestational

trophoblastic lesions. *Int J Gynecol Pathol* 18, 144-150 (1999)

207. G Badcock, C Pigott, J Goepel, PW Andrews: The human embryonal carcinoma marker antigen TRA-1-60 is a sialylated keratan sulfate proteoglycan. *Cancer Res* 59, 4715-4719 (1999)

208. A Giwercman, PW Andrews, N Jorgensen, J Muller, N Graem, NE Skakkebaek: Immunohistochemical expression of embryonal marker TRA-1-60 in carcinoma *in situ* and germ cell tumors of the testis. *Cancer* 72, 1308-1314 (1993)

209. J Marrink, PW Andrews, PJ van Brummen, HJ de Jong, DT Sleijfer, H Schraffordt Koops, JW Oosterhuis: TRA-1-60: a new serum marker in patients with germ-cell tumors. *Int J Cancer* 49, 368-372 (1991)

210. ME Gels, J Marrink, P Visser, DT Sleijfer, JH Droste, HJ Hoekstra, PW Andrews, H Schraffordt Koops: Importance of a new tumor marker TRA-1-60 in the follow-up of patients with clinical stage I nonseminomatous testicular germ cell tumors. *Ann Surg Oncol* 4, 321-327 (1997)

211. H Lajer, G Daugaard, AM Andersson, NE Skakkebaek. Clinical use of serum TRA-1-60 as tumor marker in patients with germ cell cancer. *Int J Cancer* 100, 244-246 (2002)

212. D Okamura, T Kimura, T Nakano, Y Matsui: Cadherin-mediated cell interaction regulates germ cell determination in mice. *Development* 130, 6423-6430 (2003)

213. F Honecker, AM Kersemaekers, M Molier, PC Van Weeren, H Stoop, RR De Krijger, KP Wolffenbuttel, W Oosterhuis, C Bokemeyer, LH Looijenga: Involvement of E-cadherin and beta-catenin in germ cell tumours and in normal male fetal germ cell development. *J Pathol* 204, 167-174 (2004)

214. AM Andersson, K Edvardsen, NE Skakkebaek: Expression and localization of N- and E-cadherin in the human testis and epididymis. *Int J Androl* 17, 174-180 (1994)

215. A Heidenreich, IA Sesterhenn, FK Mostofi, JW Moul: Prognostic risk factors that identify patients with clinical stage I nonseminomatous germ cell tumors at low risk and high risk for metastasis. *Cancer* 83, 1002-1011 (1998)

216. T Saito, A Katagiri, R Watanabe, T Tanikawa, T Kawasaki, Y Tomita, K Takahashi: Expression of Ecadherin and catenins on testis tumor. *Urol Int* 65, 140-143 (2000)

217. A Batistatou, CD Scopa, P Ravazoula, Y Nakanishi, D Peschos, NJ Agnantis, S Hirohashi, KA Charalabopoulos: Involvement of dysadherin and E-cadherin in the development of testicular tumours. *Br J Cancer* 93, 1382-1387 (2005)

218. SB Sonne, CE Hoei-Hansen, JE Nielsen, AS Herlihy, AM Andersson, K Almstrup, G Daugaard, NE Skakkebaek, H Leffers, E Rajpert-De Meyts: CDH1 (Ecadherin) in testicular germ cell neoplasia: suppressed translation of mRNA in pre-invasive carcinoma *in situ* but increased protein levels in advanced tumours. *APMIS* 114, 549-548 (2006)

219. A Aigner, P Brachmann, J Beyer, R Jäger, D Raulais, M Vigny, A Neubauer, A Heidenreich, S Weinknecht, F Czubayko, G Zugmaier: Marked increase of the growth factors pleiotrophin and fibroblast growth factor-2 in serum of testicular cancer patients. *Ann Oncol* 14, 1525-1529 (2003).

220. T Kawakami, K Okamoto, H Sugihara, T Hattori, AE Reeve, O Ogawa, Y Okada: The roles of supernumerical X chromosomes and XIST expression in testicular germ cell tumors. *J Urol* 169, 1546-1552 (2003)

221. T Kawakami, K Okamoto, O Ogawa, Y Okada: XIST unmethylated DNA fragments in male-derived plasma as a tumour marker for testicular cancer. *Lancet* 363, 40-42 (2004)

222. AJ Munro, OS Nielsen, W Duncan, J Sturgeon, MK Gospodarowicz, A Malkin, GM Thomas, MA Jewett: An assessment of combined tumour markers in patients with seminoma: placental alkaline phosphatase (PLAP), lactate dehydrogenase (LD) and beta human chorionic gonadotrophin (beta HCG). *Br J Cancer* 64, 537-542 (1991)

223. L Weissbach, R Bussar-Maatz, K Mann: The value of tumor markers in testicular seminomas. Results of a prospective multicenter study. *Eur Urol* 32, 16-22 (1997)

224. KP Dieckmann, M Kulejewski, U Pichlmeier, V Loy: Diagnosis of contralateral testicular intraepithelial neoplasia (TIN) in patients with testicular germ cell cancer: systematic two-site biopsies are more sensitive than a single random biopsy. *Eur Urol* 51, 175-185 (2007)

225. A Giwercman, J Muller, NE Skakkebaek: Prevalence of carcinoma *in situ* and other histopathological abnormalities in testes from 399 men who died suddenly and unexpectedly. *J Urol* 145, 77-80 (1991)

226. J Linke, V Loy, KP Dieckmann: Prevalence of testicular intraepithelial neoplasia in healthy males. *J Urol* 173, 1577-1579 (2005)

227. M Czaplicki, J Rojewska, R Pykalo, K Szymanska: Detection of testicular neoplasms by cytological examination of seminal fluid. *J Urol* 138, 787-788 (1987)

228. A Giwercman, A Marks, NE Skakkebaek: Carcinoma-insitu germ-cells exfoliated from seminiferous epithelium into seminal fluid. *Lancet* 1, 530 (1988)

229. LG Koss: Current status of diagnostic cytology. *Recent Results Cancer Res* 133, 1-16 (1993)

230. J Rojewska, R Pykalo, M Czaplicki: Urine and semen cytomorphology in patients with testicular tumors. *Diagn Cytopathol* 5, 9-13 (1989)

231. NE Skakkebaek: Atypical germ cells in the adjacent ''normal'' tissue of testicular tumours. *APMIS* 83, 127-130 (1975)

232. JW Oosterhuis, AM Kersemaekers, GK Jacobsen, A Timmer, EW Steyerberg, M Molier, PC Van Weeren, H Stoop, LH Looijenga: Morphology of testicular parenchyma adjacent to germ cell tumours. An interim report. *APMIS* 111, 32-40 (2003)

233. FJ Meng, Y Zhou, A Giwercman, NE Skakkebaek, AD Geurts van Kessel, RF Suijkerbuijk: Fluorescence *in situ* hybridization analysis of chromosome 12 anomalies in semen cells from patients with carcinoma *in situ* of the testis. *J Pathol* 186, 235-239 (1998)

234. CE Hoei-Hansen, E Rajpert-De Meyts, E Carlsen, K Almstrup, H Leffers, NE Skakkebaek: A subfertile patient diagnosed with testicular carcinoma *in situ* by immunocytological staining for AP-2gamma in semen samples: case report. *Hum Reprod* 20, 579-582 (2005)

235. ET Brackenbury, KM Grigor, MA McIntyre, GC Howard, TB Hargreave: Negative testicular biopsy and asynchronous bilateral testicular germ cell tumour. *Eur Urol* 25, 79-81 (1994)

236. CE Hoei-Hansen, E Carlsen, N Jorgensen, H Leffers, NE Skakkebaek, E Rajpert-De Meyts: Towards a non-invasive method for early detection of testicular neoplasia in semen samples by identification of fetal germ cell-specific markers. *Hum Reprod* 22, 167-173 (2007)

237. FJ Meng, Y Zhou, NE Skakkebaek, A Marks, A Giwercman: Detection and enrichment of carcinoma-in-situ cells in semen by an immunomagnetic method using monoclonal antibody M2A. *Int J Androl* 19, 365-370 (1996)

238. ET Brackenbury, TB Hargreave, GC Howard, MA McIntyre: Seminal fluid analysis and fine-needle aspiration cytology in the diagnosis of carcinoma *in situ* of the testis. *Eur Urol* 23, 123-128 (1993)

239. DS Yu, J Wang, SY Chang, CP Ma: Differential diagnosis of testicular mass by cytological examination of seminal fluid collected by ejaculation or prostatic massage. *Eur Urol* 18, 193-196 (1990)

240. A Giwercman, OP Clausen, NE Skakkebaek: Carcinoma *in situ* of the testis: aneuploid cells in semen. *Br Med J* 296, 1762-1764 (1988)

241. A Giwercman, AH Hopman, FC Ramaekers, NE Skakkebaek: Carcinoma *in situ* of the testis. Detection of malignant germ cells in seminal fluid by means of *in situ* hybridization. *Am J Pathos* 136, 497-502 (1990)

242. M Salanova, L Gandini, A Lenzi, C Chiarenza, A Filippini, F Dondero, F Di Silverio, M Stefanini: Is hyperdiploidy of immature ejaculated germ cells predictive of testis malignancy? A comparative study in healthy normozoospermic, infertile, and testis tumor suffering subjects. *Lab Invest* 79, 1127-1135 (1999)

243. S Mosselman, LH Looijenga, AJ Gillis, MA van Rooijen, HJ Kraft, EJ van Zoelen, JW Oosterhuis: Aberrant plateletderived growth factor alpha-receptor transcript as a diagnostic marker for early human germ cell tumors of the adult testis. *Proc Natl Acad Sci USA* 93, 2884-2888 (1996)

244. NJ van Casteren, H Stoop, GR Dohle, R de Wit, JW Oosterhuis, LH Looijenga: Noninvasive Detection of Testicular Carcinoma *In situ* in Semen Using OCT3/4. *Eur Urol* 54, 153-158 (2008)

245. M Starita-Geribaldi, M Samson, JM Guigonis, G Pointis, P Fenichel: Modified expression of cytoplasmic isocitrate dehydrogenase electrophoretic isoforms in seminal plasma of men with sertoli-cell-only syndrome and seminoma. *Mol Carcinogen* 47, 410-414 (2008)

246. DC Smith, CL Barratt, MA Williams: The characterisation of nonsperm cells in the ejaculates of fertile men using transmission electron microscopy. *Andrologia* 21, 319-333 (1989)

Abbreviations: TGCT: Testicular Germ Cell Tumour; CIS: Carcinoma *in situ;* AFP: Alpha-Fetoprotein; hCG: Human Chorionic Gonadotropin; LDH: Lactate Dehydrogenase; PLAP: Placental-like Alkaline Phosphatase; EC: Embrional Carcinoma; YST: Yolk Sac Tumour; ESC: Embrional Stem Cell; PGC: Primordial Germ Cell; IGCNU: Intratubular Germ Cell Neoplasia Unclassified; PCR: Polymerase Chain Reaction; SCF: Stem Cell Factor

Key Words: Clinical Markers, Testis Cancer, Neplastic Diseases, Review

Send correspondence to: Giuseppe Morgia, Via Fisichelli 32, 95037 S. G. La Punta (CT), Italy, Tel: 390902213911, Fax: 390957513576, E-mail: gmorgia@unime.it

http://www.bioscience.org/current/vol2E.htm