About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world’s best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org
TESTIS CANCER: GENES, ENVIRONMENT, HORMONES

Testicular cancer (TC) is the most common cancer in males aged 20-40 years, with a worldwide incidence of 7.5 per 100,000, but the rates vary considerably between countries and ethnic groups and there is evidence also for an increasing incidence in last decades. About 95% of all TCs are represented by testicular germ cell tumors (TGCTs), which include seminoma and non-seminoma histological types.

It is generally assumed that the development of TGCT is under endocrine control. In particular, unbalanced androgen/estrogen levels and/or activity are believed to represent the key events for TGCT development and progression. Furthermore, recent evidence has suggested genetic association of TGCT with variations in genes involved in hypothalamic-pituitary-testicular axis and steroidogenic enzymes. This recent evidence expands the current knowledge on the role of genetic contribution in testicular cancer susceptibility, and supports the hypothesis that variations in hormone metabolism genes might change the hormonal environment implicated in testicular carcinogenesis. Therefore, hormonal carcinogenesis is an important and controversial area of current research in TGCT, and further attention is given to genetic factors influencing hormone-related cancer risk.

The genetic component to TGCT is in general strong. In fact, although environmental factors clearly contribute to TGCT development and progression, the proportion of TGCT susceptibility accounted for by the genetic effects is estimated at 25%. TGCT has high familial risks compared with most other cancer types that are generally no more than two-fold: brothers of individuals with TGCT have an 8- to 12-fold increased risk of disease, and sons of affected individuals have a 4- to 6-fold increased risk. Despite this strong familial relative risk, early results from linkage studies identified a limited relationship with genetic factors, suggesting that TGCT is a genetically complex trait.

However, more recently, four genome-wide association studies (GWAS) from the UK and USA have reported association of TGCTs with six new loci (KITLG, SPRY4, BAK1, DMRT1, TERT, and ATF7IP). The strongest association for TGCT susceptibility was found for SNPs in KITLG (ligand for the membrane-bound receptor tyrosine kinase KIT) gene with a greater than 2.5-fold increased risk of disease per major allele, which is the highest reported for any cancer to date. These studies are being now replicated by other researches and attention is given to the relationship between these genetic variations, TGCT risk and frequently associated anomalies of the reproductive tract, such as cryptorchidism and infertility.
Finally, over the past few decades, TCGT research has focused also on external environmental causes acting mainly as endocrine disrupters of androgen and oestrogen pathways, even during the foetal development of the testis. It is well known that the testicular dysgenesis syndrome (TDS) hypothesis, proposed ten years ago, suggests that disturbed testicular development in fetal life may result in one or more of four disorders postnatally, named cryptorchidism, hypospadias, poor semen quality, and TGCT. These four disorders are therefore considered as one clinical entity and are linked together by epidemiological and pathophysiological relations. The relative contribution of genetics and environment in TGCT development, and the interactions between endocrine disruptors and variations in genes involved in hormonal carcinogenesis is therefore another interesting area of research.

<table>
<thead>
<tr>
<th>Page</th>
<th>Title</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>05</td>
<td><strong>Testis cancer: genes, environment, hormones</strong></td>
<td>Alberto Ferlin and Carlo Foresta</td>
</tr>
<tr>
<td>07</td>
<td><strong>Case-control study of anthropometric measures and testicular cancer risk</strong></td>
<td>Fabrizio Giannandrea, Donatella Paoli, Francesco Lombardo, Andrea Lenzi and Loredana Gandini</td>
</tr>
<tr>
<td>12</td>
<td><strong>Epigenetic: a molecular link between testicular cancer and environmental exposures</strong></td>
<td>Aurelie Vega, Marine Baptissart, Françoise Caira, Florence Brugnon, Jean-Marc A. Lobaccaro and David H. Volle</td>
</tr>
<tr>
<td>24</td>
<td><strong>Chemotherapy refractory testicular germ cell tumor is associated with a variant in Armadillo Repeat gene deleted in Velco-Cardio-Facial syndrome (ARVCF)</strong></td>
<td>Chunkit Fung, David J. Vaughn, Nandita Mitra, Stephanie L. Ciosek, Saran Vardhanabhuti, Katherine L. Nathanson and Peter A. Kanetsky</td>
</tr>
<tr>
<td>36</td>
<td><strong>Testicular cancer and HPV semen infection</strong></td>
<td>Andrea Garolla, Damiano Pizzol, Alessandro Bertoldo, Marco Ghezzi, Umberto Carraro, Alberto Ferlin and Carlo Foresta</td>
</tr>
<tr>
<td>41</td>
<td><strong>Germ cell development in the postnatal testis: the key to prevent malignancy in cryptorchidism?</strong></td>
<td>John M. Hutson, Ruili Li, Bridget R. Southwell, Bodil L. Petersen, Jorgen Thorup and Dina Cortes</td>
</tr>
<tr>
<td>52</td>
<td><strong>Genome-wide assessment of the association of rare and common copy number variations to testicular germ cell cancer</strong></td>
<td>Daniel Edsgärd, Marlene D. Dalgaard, Nils Weinhold, Agata Wesolowska-Andersen, Ewa Rajpert-De Meyts, Anne Marie Ottesen, Anders Juul, Niels E. Skakkebaek, Thomas Skot Jensen, Ramneek Gupta, Henrik Leffers and Søren Brunak</td>
</tr>
<tr>
<td>61</td>
<td><strong>Association between polymorphisms in the aryl hydrocarbon receptor repressor gene and disseminated testicular germ cell cancer</strong></td>
<td>Leon J. S. Brokken, Yvonne Lundberg-Giwercman, Ewa Rajpert-De Meyts, Jakob Eberhard, Olof Ståhl, Gabriella Cohn-Cedermark, Gedske Daugaard, Stefan Arver and Aleksander Giwercman</td>
</tr>
<tr>
<td>67</td>
<td><strong>Cryptorchidism and testicular germ cell tumors: comprehensive meta-analysis reveals that association between these conditions diminished over time and is modified by clinical characteristics</strong></td>
<td>Kimberly Banks, Ellenie Tuazon, Kiros Berhane, Chester J. Koh, Roger E. De Filippo, Andy Chang, Steve S. Kim, Siamak Daneshmand, Carol Davis-Dao, Juan P. Lewinger, Leslie Bernstein and Victoria K. Cortessis</td>
</tr>
<tr>
<td>78</td>
<td><strong>Testicular cancer and cryptorchidism</strong></td>
<td>Lydia Ferguson and Alexander I. Agoulnik</td>
</tr>
</tbody>
</table>
Testicular cancer (TC) is the most common cancer in males aged 20–40 years, with a worldwide incidence of 7.5 per 100,000, but the rates vary considerably between countries and ethnic groups and there is evidence also for an increasing incidence in last decades. About 95% of all TCs are represented by testicular germ cell tumors (TGCTs), which include seminoma and non-seminoma histological types. It is generally assumed that the development of TGCT is under endocrine control. In particular, unbalanced androgen/estrogen levels and/or activity are believed to represent the key events for TGCT development and progression.

Furthermore, recent evidence has suggested genetic association of TGCT with variations in genes involved in hypothalamic–pituitary–testicular axis and steroidogenic enzymes (1). This recent evidence expands the current knowledge on the role of genetic contribution in TC susceptibility, and supports the hypothesis that variations in hormone metabolism genes might change the hormonal environment implicated in testicular carcinogenesis. Therefore, hormonal carcinogenesis is an important and controversial area of current research in TGCT, and further attention is given to genetic factors influencing hormone-related cancer risk. The genetic component to TGCT is in general strong. In fact, although environmental factors clearly contribute to TGCT development (and probably to its increasing incidence in some geographical areas), the proportion of TGCT susceptibility accounted for by the genetic effects is estimated at 25%. TGCT has high familial risks compared with most other cancer types that are generally not more than twofold: brothers of individuals with TGCT have an 8- to 12-fold increased risk of disease, and sons of affected individuals have a 4- to 6-fold increased risk (2). Despite this strong familial relative risk, early results from linkage studies identified a limited relationship with genetic factors, suggesting that TGCT is a genetically complex trait. However, more recently, genome-wide association studies (GWAS) have reported association of TGCTs with new loci (such as those in KITLG, SPRY4, BAK1, DMRT1, TERT, and ATF7IP genes). The strongest association for TGCT susceptibility was found for SNPs in KITLG (ligand for the membrane-bound receptor tyrosine kinase KIT) gene with a >2.5-fold increased risk of disease per major allele, which is the highest reported for any cancer to date (3, 4). These studies are being now replicated (5) and attention is given to the relationship between these genetic variations, TGCT risk, and frequently associated anomalies of the reproductive tract, such as cryptorchidism and infertility (6). Finally, over the past few decades, TGCT research has focused also on external environmental causes acting mainly as endocrine disruptors of androgen and estrogen pathways, even during the fetal development of the testis (1). It is well known that the testicular dysgenesis syndrome (TDS) hypothesis, proposed 10 years ago, suggests that disturbed testicular development in fetal life may result in one or more of four disorders postnatally, named cryptorchidism, hypospadias, poor semen quality, and TGCT (7). These four disorders are therefore considered as one clinical entity and are linked together by epidemiological and pathophysiological relations. The relative contribution of genetics and environment in TGCT development, and the interactions between endocrine disruptors and variations in genes involved in hormonal carcinogenesis is therefore another interesting area of research.

The aim of this Research Topic is to give an update on the most controversial issues in research areas of TGCT, as well as original contributions giving novel hypotheses and understandings on the pathogenesis and progression of TGCT. Both clinical and basic researches are reported and many questions are addressed. One of these deals with analysis of anthropometric measures and risk of TGCT (8), an obscure matter that this study clarified, showing that TC is positively associated with height and negatively associated with body mass index, indirectly suggesting that modifications in hormonal factors and food intake during childhood and puberty might influence susceptibility to cancer. The effects of endocrine disruptors during in utero and/or neonatal development have been a matter of discussion and areas of uncertainty exists. One of these is the possible epigenetic modification following exposure to these molecules and Vega and colleagues review this topic showing how chromatin modifications, which can affect testicular physiology might increase susceptibility to TGCT and the potential molecular pathways involved in these alterations in the context of environmental exposures (9).

Not only TGCT development and progression but also treatment outcome and risks of complications from cisplatin chemotherapy seem to be genetically determined. This is well documented by Fung and colleagues, who showed for the first time that a polymorphism in ARVCF gene influences TGCT outcome, carriers of risk allele being exposed to a higher risk of refractory TGCT after initial chemotherapy (10). Furthermore, patients who received chemotherapy had a higher risk of developing semen HPV infection (11), potentially exposing these subjects to HPV-related disorders and worse fertility prognosis.
Physiological and genetic aspects of primordial germ cells, gonocytes, and spermatogenic stem cell transformations are also interesting research areas. One report reviewed the current knowledge on the biology of the postnatal germ cell development (12), another one looked for possible modifications, caused by copy number variations, in genes involved in cell migration of primordial germ cells (13), and Brokken and colleagues showed that polymorphisms in the aryl hydrocarbon receptor repressor gene (that modulates the effects of environmental pollutants) are associated with progression of TGCT to invasive forms (14).

Finally, this Research Topic reports review on the association between cryptorchidism and TGCT (15, 16), clarifying that, regardless of age at orchidopexy, unilateral vs. bilateral forms, or position of undescended testes, patients with history of cryptorchidism are at higher risk of developing TGCT (15), and suggesting that changes in the spermatogonial stem cell self-renewal and differentiation in cryptorchid testes might be an important pathway leading to TGCT (16).

Taken together, the articles presented in this Research Topic report comprehensive review and original articles dealing with most recent research fields in the pathogenesis, progression, and clinical associations of TGCT.

REFERENCES

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 25 August 2014; accepted: 30 September 2014; published online: 21 October 2014.
This article was submitted to Cancer Endocrinology, a section of the journal Frontiers in Endocrinology.
Copyright © 2014 Ferlin and Foresta. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.
Case-control study of anthropometric measures and testicular cancer risk

Fabrizio Giannandrea*, Donatella Paoli, Francesco Lombardo, Andrea Lenzi and Loredana Gandini

Laboratory of Seminology – Semen Bank, Department of Experimental Medicine, University of Rome “La Sapienza,” Rome, Italy

The etiology of testicular germ cell tumors (TGCTs) is poorly understood. Recent epidemiological findings suggest that, TGCT risk is determined very early in life, although the available data are still conflicting. The rapid growth of the testes during puberty may be another period of vulnerability. Body size has received increasing attention as possible risk factor for TC. To clarify the relation of body size and its anthropometric variables to TGCT risk, the authors analyzed data from 272 cases and 382 controls with regard to height (cm), weight (Kg), and body mass index (BMI; kg/m²). Overall, participants in the highest quartile of height were more likely to be diagnosed with TGCTs than participants in the lowest quartile of height, OR 2.22 (95% confidence intervals (CI): 1.25–3.93; adjusted; \( \text{P}_{\text{trend}} = 0.033 \)). Moreover, histological seminoma subgroup was significantly associated with tallness, very tall men (>182 cm) having a seminoma TGCT risk of OR = 2.44 (95% confidence intervals (CI): 1.19–4.97; adjusted; \( \text{P}_{\text{trend}} = 0.011 \)). There was also a significant inverse association of TGCT with increasing BMI ( \( \text{P}_{\text{trend}} = 0.001 \); age-adjusted analysis) and this association was equally present in both histological subgroups. These preliminary results indicate that testicular cancer (TC) is inversely associated with BMI and positively associated with height, in particular with seminoma subtype. Several studies have reported similar findings on body size. As adult height is largely determined by high-calorie intake in childhood and influenced by hormonal factors at puberty, increased attention to postnatal exposures in this interval may help elucidate the etiology of TGCTs.

Keywords: testicular cancer, height, weight, body mass index, body size

INTRODUCTION

Testicular cancer (TC) is the most common type of malignancy in men aged 15–40 years (Dieckmann and Pichlmeier, 2004). The incidence of TC has more than doubled worldwide over the last 40 years in several populations (Purdue et al., 2005; Bray et al., 2006). The reasons of this rise are not entirely clear (McGlynn and Cook, 2009).

Evidence of incidence peak of TC among young adults suggests that causal factors operate very early in life (Bray et al., 2006). One theory attributes it to the increase in endogenous estrogen levels during prenatal life and/or later exposures to various occupational and environmental estrogenic chemicals, termed endocrine-disrupting chemicals (EDCs) (Toppari et al., 1996; Sharpe, 2003; Cook et al., 2011; Giannandrea et al., 2011). According to this hypothesis, these exposures are also risk factors for other male reproductive disorder, such as cryptorchism and impaired sperm quality (Skakkebaek et al., 2001; Storgaard et al., 2006). Recently, it has been suggested that post-natal exposures might increase the risk of developing TC, and that secretion of sex hormones, which are involved in the growth and the development of the testicle, might be instrumental in TC progression (James, 2010; Trabert et al., 2011).

One possible risk factor that has received increasing attention is body size, which is determined by hereditary, nutritional, and hormonal factors (Dieckmann and Pichlmeier, 2002). Height, weight, and body mass index (BMI) have been examined in a number of studies, with mixed results (Lerro et al., 2010). Several studies have reported inverse associations of TC with BMI (Petridou et al., 1997; Bjørge et al., 2006; Lerro et al., 2010). A number of studies have reported that increased adult height may be a risk factor for TC, thereby suggesting that factors related to adult height may also be related to risk of these tumors (Richiardi et al., 2003; Dieckmann et al., 2008; Lerro et al., 2010). One explanation for the height association may be that better childhood nutrition increases both adult height and risk of TC. This hypothesis is supported by epidemiological observations of constantly increasing incidence of TC since the beginning of the twentieth century with the only major interruption in this trend occurring for men born during World War II or immediately thereafter, when food availability had been dramatically reduced (Moller, 1989; Aschim et al., 2005; Giannandrea, 2009).

To clarify the relation of anthropometric variables to TGCT risk and to one another, we analyzed data from 321 cases and 465 controls enrolled at our laboratory with regard to height (cm), weight (Kg) and body mass index (BMI; kg/m²).

MATERIALS AND METHODS

Participants among 321 consecutive patients affected by TC and 465 controls were originally selected at the Laboratory of Seminology, Semen Bank, Department of Experimental
RESULTS
The descriptive tabulation of anthropometric measures distribution of cases and controls is shown in Table 1. The mean ages of the non-seminoma cases was lower than the mean age of the seminoma cases (30.6 years, standard deviation (SD): 6.0 vs. 34.1 years, SD: 5.9; \( p < 0.05 \)). It is well-acknowledged that non-seminoma peak at younger age than seminoma. Therefore, the risk of TC decrease with increasing age at diagnosis, with non-seminomas having a more significant inverse relationship with age (\( p_{\text{trend}} < 0.001 \)) than seminomas (\( p_{\text{trend}} = 0.021 \)) (Table 2).

The relations of anthropometric variables and TC risk are shown in Table 2. In the multivariate analysis, BMI was inversely associated and height was positively associated with risk of TC (Table 2).

Overall, participants in the highest quartile of height were more likely to be diagnosed with testicular germ cell tumors (TGCs) than participants in the lowest quartile of height, OR 2.22 (95% confidence intervals (CI): 1.25–3.93; adjusted; \( p_{\text{trend}} = 0.033 \)). Moreover, histological seminoma subgroup was significantly associated with tallness, very tall men (>182 cm) having a seminoma TGCT risk of OR = 2.44 (95% confidence intervals (CI): 1.19–4.97; adjusted; \( p_{\text{trend}} = 0.011 \)). The multivariate-adjusted odds ratio for the highest quartile group of BMI relative to the lowest was 0.42 (95% CI: 0.24–0.75; \( p_{\text{trend}} = 0.01 \), with no difference by histological subtype (Table 2). There was also a significant inverse association of TGCT with increasing weight (\( p_{\text{trend}} = 0.001; \) age-adjusted analysis) and this association was equally present in both histological subgroups.

DISCUSSION
These preliminary results indicate that TC is positively associated with height, in particular with seminoma subtype, and inversely related with BMI. Therefore, the present study indicated an excess of tall men in the cohort of patients with TC. To our knowledge, this study is the first to explore the relationship between tallness and the risk of TC from a southern European country. Thus far, the majority of studies on tallness in relation to the risk of TC were conducted in US and Nordic countries (Akre et al., 2000; McGlynn et al., 2007; Dieckmann et al., 2008; Cook et al., 2010; Lerro et al., 2010). Measures of adult height, as well as the incidence rate of TC, resulted both elevated among Scandinavian populations (Richiardi et al., 2003; Purdue et al., 2005).
Table 2 | Relation of age, height, weight, and body mass index (BMI) to testicular germ cell tumor risk by histology.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls (n. 382)</th>
<th>All TC (n. 272)</th>
<th>Seminoma (n. 138)</th>
<th>Nonseminoma (n. 134)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. %</td>
<td>No. % OR 95% CI</td>
<td>No. % OR 95% CI</td>
<td>No. % OR 95% CI</td>
</tr>
<tr>
<td>AGE (YEARS)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20–29</td>
<td>56 15.1</td>
<td>82 30.7 1.0</td>
<td>Referent</td>
<td>Referent</td>
</tr>
<tr>
<td>30–34</td>
<td>93 25.0</td>
<td>85 31.8 0.63</td>
<td>0.38, 1.06</td>
<td>0.32, 1.08</td>
</tr>
<tr>
<td>35–39</td>
<td>129 34.7</td>
<td>70 26.2 0.33</td>
<td>0.20, 0.56</td>
<td>0.22, 0.77</td>
</tr>
<tr>
<td>≥40</td>
<td>94 25.3</td>
<td>30 11.2 0.24</td>
<td>0.13, 0.56</td>
<td>0.21, 0.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p trend &lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>HEIGHT (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤173</td>
<td>108 28.3</td>
<td>68 25.0 1.0</td>
<td>Referent</td>
<td>Referent</td>
</tr>
<tr>
<td>174–178</td>
<td>98 25.7</td>
<td>81 29.8 1.62</td>
<td>0.99, 2.66</td>
<td>0.99, 3.54</td>
</tr>
<tr>
<td>179–182</td>
<td>93 24.3</td>
<td>54 19.9 1.37</td>
<td>0.83, 2.25</td>
<td>0.61, 1.98</td>
</tr>
<tr>
<td>&gt;182</td>
<td>83 21.7</td>
<td>69 25.4 2.22</td>
<td>1.25, 3.93</td>
<td>1.19, 4.97</td>
</tr>
<tr>
<td>BMI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤23.15</td>
<td>84 22.0</td>
<td>110 40.4 1.0</td>
<td>Referent</td>
<td>Referent</td>
</tr>
<tr>
<td>23.16–24.97</td>
<td>121 31.7</td>
<td>71 26.1 0.92</td>
<td>0.52, 1.63</td>
<td>0.76, 2.84</td>
</tr>
<tr>
<td>24.98–27.40</td>
<td>128 33.5</td>
<td>65 23.9 0.79</td>
<td>0.44, 1.39</td>
<td>0.69, 2.60</td>
</tr>
<tr>
<td>&gt;27.40</td>
<td>49 12.8</td>
<td>26 9.6 0.42</td>
<td>0.24, 0.75</td>
<td>0.32, 1.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p trend = 0.001</td>
<td></td>
</tr>
<tr>
<td>WEIGHT (Kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤72</td>
<td>94 24.6</td>
<td>102 37.5 1.0</td>
<td>Referent</td>
<td>Referent</td>
</tr>
<tr>
<td>73–80</td>
<td>157 41.0</td>
<td>93 34.1 0.56</td>
<td>0.30, 1.03</td>
<td>0.39, 1.66</td>
</tr>
<tr>
<td>81–87</td>
<td>78 20.4</td>
<td>51 18.7 0.56</td>
<td>0.31, 1.00</td>
<td>0.48, 1.96</td>
</tr>
<tr>
<td>&gt;87</td>
<td>53 13.8</td>
<td>26 9.5 0.27</td>
<td>0.13, 0.52</td>
<td>0.20, 0.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p trend = 0.001</td>
<td></td>
</tr>
</tbody>
</table>

OR adjusted for all continuous variables in the table; significance of p value for trend < 0.05.
Though differences in genetic backgrounds among Scandinavian and southern European populations exist, these preliminary findings imply that possible postnatal exposures may play an important role in determining risk of TC. As adult height is largely determined during the first 2 years of life, it may be postulated that high calorie nutrition after birth could have a role in TC pathogenesis (Frankel et al., 1998; McGlynn et al., 2007; Giannandrea, 2009). In addition, as final adult height is strongly dependent on testis sex-steroids (Veldhuis et al., 2005) a correlation between height and TC risk may suggest that androgen secretion during puberty might be involved in TC progression. One further height-related factor may be insulin-like growth factor (IGF) and the IGF pathway, which is also involved in the control of spermatogenesis (Gunnell et al., 2001; Giovannucci et al., 2004). Increased serum IGF-1 levels were associated with increased height has also been reported in several studies (Chia et al., 2008).

Height has been positively associated with risk of TC in the majority of studies in which it has been examined (Akre et al., 2000; McGlynn et al., 2007; Dieckmann et al., 2008; Cook et al., 2010; Lerro et al., 2010). Akre et al. (2000) found a statistically significant increased risk of TC associated with greater height, and this association was particularly evident in men with seminomas. Dieckmann et al. (2008) found that very tall men (>195 cm) carried a TC risk of OR: 3.35 (95% confidence intervals (CI): 2.88–3.90; adjusted). In the STEED Study, increased height was significantly related to risk of TC (OR: 1.85; CI 95%: 0.71, 1.32) (McGlynn et al., 2007; Lerro et al., 2010).

A number of studies have so far reported an inverse relationship between BMI and TGCTs, although the available results are still conflicting, with some studies reporting null findings (Petridou et al., 1997; Björge et al., 2006; Lerro et al., 2010). A recent meta-analysis conducted by Lerro et al. (2010) has produced a tentative evidence that TC risk was inversely associated with BMI, with a summary OR of 0.92 (95% CI: 0.86–0.98; p = 0.011). The biological reasons of this negative association are likely to be more complex of those contemplated to explain the association of TC with tallness. Some authors have suggested that lower BMI found in previous studies could also be a mathematical artifact as this association may be related to an excess of tall men in TC cohorts rather than to leanness of the patients (Dieckmann and Pichlmeier, 2002). This is due to the fact that height is squared in the denominator of the BMI-formula, lowering the numerical calculation of MBI in very tall men at hight TC risk despite normal weight. However, our study shows additional significant inverse association of TGCT with increasing weight (p_{trend} = 0.001), therefore confuting, at least in part, this suggestion.

Body size is primarily determined by hereditary, nutritional, and hormonal factors (Dieckmann and Pichlmeier, 2002). The hormonal determinants can be divided into growth hormones and sex hormones. Most previous studies on postnatal risk factors for TC have focused on endogenous sex hormones. Obesity is inversely associated with both total testosterone and sex hormone-binding globulin (Akre et al., 2000). Furthermore, obese men have increased levels of both estradiol and estrone coming mostly from extraglandular conversion of androgen precursors (Akre et al., 2000; Dieckmann and Pichlmeier, 2002).

Since testicular germ cell cancer is probably initiated in utero, postnatal hormones are likely to function as promoters. These hormones may continue to exert their effect in advanced stages of tumor development at time of puberty when the testicles grow and develop rapidly.

Adult stature can be considered a proxy of childhood nutrition, although stature is also determined by genetic and hormonal factors. It has been suggested that the trend of increasing adult height and the increasing TC incidence are biologically interconnected with improved nutrition in early life.

In summary, we have found a lower risk of TC among men with high BMI. Further investigation of this inverse relationship may be warranted, for which the present results provide only limited support. Furthermore, tallness was positively associated with risk of TC. The biologic mechanism suggested is the promotion of this cancer by sex hormones and/or growth hormones, such as growth hormone and/or IGF-I.

**FUNDING**

Financial support for this work was provided by a Grant from the Italian Ministry of Education and Research (MIUR-PRIN) and the University of Rome “La Sapienza” Faculty of Medicine.

**REFERENCES**


Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 September 2012; paper pending published: 22 October 2012; accepted: 06 November 2012; published online: 26 November 2012.


This article was submitted to Frontiers in Cancer Endocrinology, a specialty of Frontiers in Endocrinology.

Copyright © 2012 Giannandrea, Paoli, Lombardo, Lenzi and Gandini. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.
In the last decades, studies in rodents have highlighted links between in utero and/or neonatal exposures to molecules that alter endocrine functions and the development of genital tract abnormalities, such as cryptorchidism, hypospadias, and impaired spermatogenesis. Most of these molecules, called endocrine disrupters exert estrogenic and/or antiandrogenic activities. These data led to the hypothesis of the testicular dysgenesis syndrome which postulates that these disorders are one clinical entity and are linked by epidemiological and pathophysiological relations. Furthermore, infertility has been stated as a risk factor for testicular cancer (TC). The incidence of TC has been increasing over the past decade. Most of testicular germ cell cancers develop through a pre-invasive carcinoma in situ from fetal germ cells (primordial germ cell or gonocyte). During their development, fetal germ cells undergo epigenetic modifications. Interestingly, several lines of evidence have shown that gene regulation through epigenetic mechanisms (DNA and histone modifications) plays an important role in normal development as well as in various diseases, including TC. Here we will review chromatin modifications which can affect testicular physiology leading to the development of TC, and highlight potential molecular pathways involved in these alterations in the context of environmental exposures.

Keywords: testicular cancer, physiology, epigenetic, endocrine disrupters

**TESTICULAR CANCERS**

Testicular cancer (TC) is a rare solid tumor which account for 1% of cancers in men. However, it is the most common cancer in men in their 20s and 30s (Ziglioli et al., 2011). It is a real sanitary problem as it affects men during their reproductive years. Interestingly, the development of TC has been associated with urogenital abnormalities (Olesen et al., 2007). TC development has been associated with cryptorchidism, hypospadia, and hypofertility. Indeed, epidemiological studies argue for an increased risk of testicular germ cell tumor in males who suffer for fertility troubles (Burns et al., 2010).

**Epidemiology**

In developed countries, the prognosis for TC is excellent, with a 5-year survival rate >95%. Despite it represents the most frequent solid cancer in young men, it seems a quite rare pathology (Richardson et al., 2012). Thus all these data has led to consider testicular tumors as a curable neoplasm. It has to be noted that there are some geographic and racial variation in the risk of TC development (Richardi et al., 2004). Indeed, in US, Hispanic men had the largest increase in incidence rates for non-germ cellomas (Crawsend et al., 2010). Next to this, the genetic predisposition has been suggested as men with familial history of germ cell TC (TGCC) have a 4- to 10-fold increased risk to develop also such tumor (Greene et al., 2004). The analysis of this genetic risk seems to be associated with autosomal recessive mode of inheritance that must result of the combined effects of several alleles. It was estimated that 25% of TC susceptibility account for by genetic effects (Turnbull and Rahman, 2011). If TCs are quite rare in African men, the morbidity and mortality of TC is quite high in developing countries (Ugwumba and Aghaji, 2010). These data highlight the impact of poverty and paucity of resources to detect and treat cancers. In that line, it has been reported that socioeconomic position has an impact on the risk of TC development as well as the survival rate (Richardson et al., 2012).

The main way to identify TC as soon as possible is auto-palpation by men to look at increased size or the appearance of “hard” tests (Umeh and Chadwick, 2010). After identification of the anomalies, biopsies will help to characterize the type of TC. Then imaging will also play a key role to determine the lymphatic extension and potential metastasis (Brunereau et al., 2012).

There are several kinds of TCs that need to be characterized by the physician using anatopathological approach. There are several kinds depending on cell type at the origin of the development of TC (van de Geijn et al., 2009); thus there are intratubular germ cell neoplasia (IGCN), atypical germ cells with maturation arrest (MA), pseudolymphovascular invasion, real lymphovascular invasion in germ cell tumors, macroscopic Sertoli cell nodules, and Sertoli cell tumors. However, we can thus distinct germ cell tumors versus non-germ cell tumors. The most common TC are germ cell one which represent 95% of these tumors. There are two main types described which are seminoma and non-seminomatous...
germ cell tumors, with a third one which is the spermatoctytic seminoma (Mannuel et al., 2012).

Here we will focus this review on germ cell cancers. Their incidence has been reported to be continuously increasing in the last decades in men of different industrialized countries.

Regarding the two major groups of testicular germ cell cancers which are the seminomas and non-seminomas, each represent approximately 50% of germ cell tumors. It is interesting to note that around 19% of TGCC contain a mix of seminoma and non-seminomatous tumors (Aschim et al., 2006).

Seminomas are managed with orchiectomy and surveillance or radiotherapy in stage I and chemotherapy in more advanced stages (Warde et al., 2011). On the other side, non-seminomas which consist of several histologic types, such as choiosarcoma, teratoma, yolk sac tumor, and embryonal cell carcinoma, each with different tumor marker profile, are less sensitive to radiation and are frequently treated with chemotherapy (Warde et al., 2011).

GENETIC ASSOCIATION

The development of TGCC is associated with many chromosomal abnormalities (Summerill et al., 1998). Indeed, TGCC are aneuploid; with non-seminomatous tumors and seminoma being respectively hypotriploid and hypotriploid. The major association is with the gain of material from the chromosome arm 12p in both seminoma and non-seminomatous tumors (Atkin and Baker, 1982). The analyses of these chromosomal reorganizations lead to the characterization of potential candidates involved in the TGCC pathology. Among them, there is amplification of the KRAS and Cyclin d2 genes (Rodriguez et al., 2003). They are associated with malignant transformation and proliferation. Next to these genes, there are also those implicated in the cell pluripotency such as Stella and Nanog (Clark et al., 2004).

The strongest association for TGCC susceptibility is for single nucleotide polymorphisms (SNPs) at the 12q22 within the androgen receptor (AR) (Kanetsky et al., 2009). It is correlated with a 2.5-fold increased risk of disease. This gene has been involved in several aspect of primordial germ cell (PGC) development. Indeed, it seems to act on PGC migration and survival (Koo et al., 2009). These impacts might rely on the downstream target KRAS which then activate the p110 catalytic subunit of the PI3K pathway which in turn through AKT pathways will act on proliferation, survival, and migration processes.

Next to this, as testicular physiology is under the control of the endocrine functions mainly through the activity of androgen and estrogen receptors, it was deeply studied if polymorphisms of genes involved in hormonal metabolism could be associated with a higher risk of TCs. Even if some reports are contradictory, the studies focused on the androgen receptor (AR), the estrogen receptors and genes involved in either synthesis or degradation of the hormones.

Regarding the estrogen receptors, it was demonstrated that polymorphisms in ERα are associated with azoospermia (Bomanver et al., 2011) and are more likely to be associated with the risk of seminoma and metastasis (Brooklin et al., 2012), whereas polymorphisms in ERβ are more likely to be linked to altered spermatogenesis (Aschim et al., 2005) and with risk of TGCC. New to this, polymorphisms in 17β hydroxysteroid dehydrogenase-4 which convert androgen and estrogen to weaker hormones were associated with TGCC (Chia et al., 2011; Fei et al., 2010). In these metabolic pathways, polymorphisms in cytochrome P450 Cyp-1A1 gene, encoding a hormone-metabolizing protein, were identified and inversely correlated with TC. Their effects were more or less severe regarding the different polymorphisms suggesting that it may contribute to susceptibility to TGCC development (Figueras et al., 2008; Kristiansen et al., 2011).

In this hormonal context, one of the most studied genes in regards to polymorphisms is the AR. The AR gene has two polymorphic regions in exon-1 with CAG codon encoding for glutamine and GCG which encode for glycine. Changes in the length of these polymorphic trinucleotide repeats, (CAG) and/or (GGN), lead to altered transactivation of the AR which has been shown to play a role in several forms of endocrine cancer such as prostate cancer. Regarding TC some studies are a bit contradictory showing either or not link with increased risk of TGCC (Rajpert-De Meyts et al., 2002; Garolla et al., 2009). However, it appears that the increased risk of seminoma was associated with the shorter CAG repeat length. This suggests that an increased AR transactivation may be involved in the development of seminoma and/or progression of carcinoma in situ (CIS) to seminoma (Davis-Dao et al., 2011). It is also demonstrated that the combination of altered number in repeat for both CAG and GCG is important for the correlation with TC. Indeed, Garolla et al. (2009) showed that the combination of CAG (20 repeats) and GCG (17 repeats) was more frequent in patient with TGCC (8 versus 1.7% in control patients).

Like most of the cancer pathologies, TCs seem to be the results of either genetic and environmental factors. It has been stated that TCs derive from a precocious lesion, the CIS of the testis, also known as IGCN or testicular intraepithelial neoplasia (TIN; Sonne et al., 2008). This lesion deserves great attention, because the diagnosis of CIS may lead to a precocious diagnosis of TCs. Usually, the diagnosis of CIS is incidental.

If there is a consensus on the fact that the process of the TC pathology may found its origin during embryonic life of the individual, it can also be suspected that there might also be some other events participating to its appearance. Indeed, it seems quite a long process that TC occurs in the 20s or 30s of life when first event took place in fetal life. Thus it could be hypothesized that there might be a second hit at puberty, like hormone burst that could provoke the definitive occurrence of TC. This clearly highlights the importance of the microenvironment. A study on patients with testicular maldescent shows that there is around a two-fold increased risk to develop TGCC if the orchidopexy is performed after the age of 13, compare to men who had the operation before their 13 (Walsh et al., 2007). Thus placing the tests in the scrotum before puberty decreases the incidence of TGCC, suggesting that intra-abdominal location at puberty promotes testicular tumorogenesis. Gene expression profiling carried out on TGCC samples demonstrate marked differences between the histological subtypes of TGCC. This reflects stages and patterns of differentiation. It also supports a model of differentiation from IGCN to seminomas or embryonal carcinomas. Gene expression patterns and
genomic imprinting analyses in TGCC show similarity to PGCs and gonocytes (Ziglioli et al., 2011).

ENVIRONMENTAL ISSUES

THE TESTICULAR DYSGENESIS SYNDROME HYPOTHESIS

In 1993, Skakkebaek and colleagues proposed that various disorders of male reproductive health, namely cryptorchidism, hypospadias, subfertility, and testicular germ cell tumor, derived from perturbations of embryonal programming and gonadal development during fetal life (Sharpe and Skakkebaek, 1993). Thus they defined it as testicular dysgenesis syndrome (TDS; Skakkebaek et al., 2001). The anomalies may lead to early symptoms, such as hypospadias and undescended testis, as well as late effects such as testis cancer and infertility. The most frequent abnormality due to TDS may be impaired spermatogenesis.

A fetal origin is obvious with regard to two symptoms of TDS: hypospadias and cryptorchidism. Moreover, studies suggested that the precursor cells of testis cancers, CIS testsis, are similar to fetal gonocytes. The accepted etiology for germ cell cancer suggests that developmental arrest of fetal germ cell differentiation is a main event leading to persistence of gonocytes, which in turn develop into CIS. The causes remain unknown, although disturbances in the microenvironment provided by the Sertoli and Leydig cells may play a major role. Moreover, spermatogenesis is strictly controlled and depends on a succession of signals provided by the local environment (Skinner et al., 1991; Verhoeven, 1992; Jégou, 1993).

Among all the studies on testicular functions, there are several windows of time that must be critical for its development. Next to the importance of the fetal development, it appears that puberty must be an important timing. This is when hormone levels will reach optimal concentrations leading to the secondary sexual characters. This will also coincide with the appearance of the TGCC, which is hypothesized to influence TC risk. Thus, alterations in genes involved in the action of sex hormones may contribute to variability of an individual’s susceptibility to TC.

Rodents can be exposed to a growing number of contaminants which can accumulate in their bodies and may have adverse consequences for health. Large range of chemicals (banned or still in use) have been characterized as EDs. Among these EDs, highly produced Bisphenol A (BPA; Rubin, 2011) is present in plastics, including beverage and food storage containers and in the ink used for thermal paper receipts. Some individuals have also been exposed to contaminants with adverse effects originating from medical use (diethylstilbestrol, DES; Bullock et al., 1988).

In mouse ERα have been demonstrated to be involved in the maturation of the spermatocytes (Lubahn et al., 1993). These KO mice present an excess of fluid which increases the pressure within the seminiferous tubules and leads to the germ cell loss (Eddy et al., 1996; Hess et al., 1997). Surprisingly, the ERβ-KO mice show no testicular phenotype (Coule and Korch, 1999).

The deficient male mice for Cyp19 (Cyp19-KO), encoding for the enzyme responsible for the aromatization of testosterone into estrogens, develop normally and their genital tract is anatomically similar to that of the wild-type (Robertson et al., 1999). Males are fertile; however, some of Cyp19-KO males exhibit an altered spermatogenesis by the age of 5 months (Robertson et al., 1999). By the age of 1 year, all males develop abnormal spermatogenesis with a blockage of germ cell maturation at the spermatid stage. This is due to an increase in apoptotic rates when compared to the wild-type animals. The observation of abnormal acrosome development in the Cyp19-KO mouse suggests that acrosome biogenesis could be estrogen-dependent (Robertson et al., 1999). Interestingly, estradiol have been demonstrated to play a role as a survival factor for the human germ cells (Pentikäinen et al., 2008), and also is beneficial for sperm motility (Carreau and Hess, 2010). Moreover, next to these data, deleterious effects of numerous endocrine disruptors on sperm count and male genital tract (cryptorchidism, hypospadias, and infertility) have been documented (Iguchi et al., 2001; Slikk and Wang, 2008) particularly in the context of in utero and/or neonatal exposures.

Imbalanced equilibrium between the estrogen and androgen levels in utero is hypothesized to influence TC risk. Thus, alterations in genes involved in the action of sex hormones may contribute to variability of an individual’s susceptibility to TC. Mutations in testosterone pathway genes may alter the level of testosterone in vivo and hypothetically the risk of developing TC (Kristiansen et al., 2012). In regard with the hypothesis of the TDS and the known impact of steroids on testicular development and functions, it has been hypothesized that endocrine-disrupting chemicals could play a role in these pathologies.

ENDOCRINE DISRUPTERS

The endocrine disrupters (EDs) are compounds which may be of industrial or natural origin and which act to dysregulate steroid function and metabolism. They produce their effects by mimicking, antagonizing, or altering endogenous steroid levels (androgens or estradiol, E2) via changing rates of their synthesis or metabolism and/or expression or action at receptor targets.

The question of environmental endocrine disruption has been a topic of public concern for many years and remains high on the scientific agenda. Indeed the number of chemical used is constantly increasing in developed countries, it is supposed that humans and animals can be exposed to a growing number of contaminants which can accumulate in their bodies and may have adverse consequences for health. Large range of chemicals (banned or still in use) have been characterized as EDs. Among these EDs, highly produced Bisphenol A (BPA; Rubin, 2011) is present in plastics, including beverage and food storage containers and in the ink used for thermal paper receipts. Some individuals have also been exposed to contaminants with adverse effects originating from medical use (diethylstilbestrol, DES; Bullock et al., 1988),
or dietary habits (phytoestrogens; Naiff and Daston, 2004). More surprisingly, in China, human excretions were suspected to be the major contributor of estrogens in municipal wastewater (Zhou et al., 2012).

Moreover, studies have made correlations between elevated levels of phthalates in urines of pregnant women and lower masculinization of their progeny (Suzuki et al., 2012). EDs have also been detected in the maternal milk (Hines et al., 2009).

Regarding the urogenital development and more particularly testis, the impacts of EDs have been quite well described on human and rodent models (Volle et al., 2009; Toppari et al., 2010; Desroses-Lethimonier et al., 2012). This is mainly true for hypospadias, cryptorchidism, and infertility; but the link with TGCC remain to be defined.

It has been demonstrated that EDs with estrogenic activity lead to a decrease of expression of steroidogenic genes which by the end result in lower testosterone concentrations (Joensen et al., 2008). However, the mechanisms seem to differ between species. This highlights the difficulties to find good experimental models. Indeed, one will claim that human cells will not react as murine cells, and either may assume that cell lines or explants tissues might not totally react as in the body. Indeed, endocrine systems are really complex and need to be analyzed in regard of the complexity of integrative physiology.

The effect of such EDs in human pathology is quite difficult to establish. It is really difficult to have standardized cohort between the studies, which is due to the history of patients (environmental and genetic factors). This heterogeneity leads to inconsistent studies which do not allow definitive conclusions on the potential involvement of EDs in TGCC.

It has been demonstrated that there was a six-fold increase in the risk of seminoma among plastic workers exposed to polyvinyl chloride (PVC; Ohlson and Hardell, 2000). However, Hardell et al. (2004a) did not found association between PVC exposure and TC.

Another study from Hardell et al. (2006) show that chlorinated biphenyls (PCBs) could play a role during fetal exposure in the etiology of TC as case mothers were identified to have significantly higher concentrations of these PCBs. However, regarding PCBs, no different risk pattern could be demonstrated for seminoma and non-seminomatous TC (Hardell et al., 2003, 2004b).

However, several studies have demonstrated some effects of these molecules on risk of TGCC (Hardell et al., 2003, 2004b, 2006; McGlynn et al., 2009). In their study McGlynn et al. (2009) associated PCBs with a decrease risk of TGCC development. Different PCBs are either associated with decrease risk of either seminoma or non-seminoma. In the same line of evidence, it was demonstrated that in patients with seminoma there was differences in concentrations of different PCBs (Purdue et al., 2009). Some PCBs (44, 49, and 52) were found to be lower accumulated in patients with seminoma compare to congeners; whereas other PCBs (99, 138, 153, 167, 183, and 195) were significantly higher accumulated in these patients. This suggest that rather than specific concentration of one or another PCB, it may be the relative concentrations of multiple one that might be relevant to established clear correlation with the risk of TC.

Moreover, evidences suggest that exposures to pesticides could be risk factor of TC development. Indeed, a recent study reported a significant association between TC development and use of insecticides such as organochlorine pesticides, namely dichlorodiphenyl dichloroethylene (p,p′-DDE) isomer and hexachlorobenzene (Giannandrea et al., 2011).

Next to this, as TC development seems to be either associated with genetic predisposition and or environmental exposure, it might be of interest to analyze the potential combination of such anomalies. Consistent with this hypothesis, as polymorphisms in AR and some organochlorine pesticides have been associated to risk of TGCC development, and that some of these organochlorine pesticides present anti-androgenic activities, Biggs et al. (2008) have studied the potential interaction of AR polymorphisms and exposure to p,p′-DDE and the association with TC risk. According to their study, they were not able to demonstrate any association between p,p′-DDE exposure and TC risk, either or not in combination with (CAG)n length. However, a point to take in consideration is that p,p′-DDE concentration was measured in adult patients, but as TC is supposed to found its origin in fetal life, this must be the exposition in this period of life that must be significant to define such association. However, it will be difficult for instance to establish such correlation as we do not have access to fetal/neonatal blood of these patients. However, it could be though to perform some clinical trails to collect blood of new borns and make assay for pesticides concentrations. Then it will be necessary to follow these boys and see if they will further develop TC, and perform correlation with fetal/neonatal concentrations of pesticides.

**ED and insulin-like-3**

Next to their steroidogenic function, Leydig cells during development express the insulin-like-3 gene which is responsible for gubernaculum maturation (Ivell and Anand-Ivell, 2011). In human, INSL3 is produced by prenatatal, neonatal, and adult Leydig cells to various extents (Ivell and Anand-Ivell, 2011). INSL3 production seems to be dependent on the state of Leydig cell differentiation, and is stimulated by the long-term trophic effects mediated by luteinizing hormone (LH; Toppari et al., 2007). This finding clearly helps in understanding the complete process of testis descent. The role of the INSL3 on testis descent was highlighted by the fact that mouse KO for the gene encoding INSL3 results in cryptoorchidism (Adham and Agoulnik, 2004). Moreover, animal model of ED exposures demonstrated that INSL3 production is sensitive to estrogenic or anti-androgenic compounds.

This clearly suggests that maternal exposure to EDs during pregnancy can result in cryptoorchidism, a factor that predispose to TC development.

**ED and steriodogenesis**

This link is of particular importance. Indeed, if CIS have been described in boys at birth (Jacobson and Henniges, 1992), TGs appear in young men between 15 and 35 years old, suggesting that puberty and probably the increase of hormone concentrations must be key events.

The impact of EDs on steriodogenesis has been demonstrated for several decades now. EDs have been described to inhibit critical cellular functions involved in steriodogenesis, such as transport of cholesterol into the mitochondria, the expression of sterodogenic genes or the activity of these enzymes (Vanparys...
Epigenetic mechanisms in testicular cancers

Vega et al., 2012). Indeed, it has been demonstrated that exposure of adult rats to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) led to lower production of testosterone in response to human chorionic gonadotropin (hCG), relative to testes from control rats (Kleeman et al., 1990). The addition of pregnenolone is able to restore normal testosterone secretion. This highlights that the impact on steroid synthesis might result from altered cholesterol synthesis or mobilization to the mitochondria (Moore et al., 1991). TCDD was also observed to reduce the number and size of the Leydig cells (Johnson et al., 1994). Moreover, the use of primary culture of Leydig cells also demonstrate that TCDD represses Cyp11a1 expression, through the alteration of the ability of hCG to increase intracellular cAMP levels (Lai et al., 2005).

The impact on the animal phenotypes will depend on the age at exposure. Thus fetal/neonatal exposure will lead to altered internal genital organs. Indeed, experimental models have shown that disruption of the androgen signaling results in feminization of external genitalia (Nagahama et al., 2004). In animal models, exposures to low concentrations of EDs are able to alter testosterone synthesis without any effect of feminization. However, the most interesting thing is that even after neonatal exposure, the adult testosterone concentrations are decreased suggesting long-term impact that is specific to this particular window of exposure (Volle et al., 2009). This has been clearly highlighted as the exposition of adult animal lead to transitory testosterone decrease, associated with temporary germ cell death. In human, majority of the results relies on epidemiological studies and correlative data (Woodruff, 2011). However, several studies using testicular explant models have tried to characterize the impact of EDs on human Leydig cells (Desdoits-Lethimonier et al., 2012). By the end, all these data led to the idea that fetal cells must be more sensitive than adult Leydig cells.

Regarding the signaling pathways involved, and as main regulators of the expression of steroidogenic genes, a reduced activity of the cAMP-mediated PKA pathway would also be expected to reduce the mobilization of cholesterol by cholesterol hydroxylases. However, at the molecular level, it has been demonstrated in mouse that neonatal exposure to EDs with estrogenic activity leads to a decrease of testosterone synthesis through ER receptors and that the orphan nuclear receptor SHP must be a key intermediary for this effect (Volle et al., 2009). Indeed, the SHP KO mice seem to be less sensitive to estrogenic EDs than their wild-type littermates. It was also demonstrated that the impact of SHP on steroidogenesis is due to the repression of either expression and/or activity of the nuclear receptors Sf-1 and Lhr-1 (Volle et al., 2009). It is to note that SHP was not involved in early post-natal decrease of testosterone production induced by estrogenic EDs. This highlights that the involved molecular pathways affected by EDs are multiple and complex.

**ED and germ cell differentiation**

Spermatogenesis, leading to spermatozoa formation, is a complex process with multiple steps involving mitosis, meiosis, and spermiogenesis. It takes place in the seminiferous tubules. There, germ cells are organized from the base of the tubule to the lumen. Germ cells are supported by the nursing Sertoli cells, which extend from the base to the lumen of the seminiferous tubules. Efficient spermatogenesis also relies on the integrity of tight junctions between the Sertoli cells which form the blood–testis barrier (BTB; Cheng and Mruk, 2012). BTB has many functions in the testis such as maintaining a particular immune context and also the control of the flow of nutrients and growth factors that are required for the development of germ cells.

The key role of retinoids in the differentiation process of germ cells was highlighted by two studies demonstrating strab as a key factor (Bowles et al., 2006; Koubova et al., 2006). It also involved the timely regulation of the gene encoding for cyp26b1, an enzyme responsible for retinoid degradation. Indeed, CYP26B1 in Sertoli cells acts as a masculinizing factor to arrest male germ cells in the G0 phase of the cell cycle and prevents them from entering meiosis (Li et al., 2009). This induction of retinoid pathway to induced entry in meiosis seems to be inhibited by the nuclear receptor SHP, a co-repressor of the retinoic acid receptors RAR (Volle et al., 2007). In the opposite manner, the FGF9 signaling pathway acts to determine germ cell fate to enter meiosis (Boisern et al., 2001).

**EPIGENETIC AND TESTICULAR CANCERS**

**TRANSGENERATIONAL EFFECTS: ROLE OF EPIGENETIC MODIFICATIONS**

Epigenetic refers to changes of DNA information without any change of the sequences. It relies on histones post-transductional modification (acetylation, methylation... ) or of DNA methylation levels. Thus, inheritance of these information requires germline transmission of epigenetic patterns between generations. The epigenetic programing of the germine occurs during embryonic development in a sex-specific manner (Western, 2009). These processes are crucial for reproductive functions, as most of mouse models with specific germline invalidation for gene responsible of DNA or histones modifications led to sterile animals (Peters et al., 2001; La Salle et al., 2007). Regarding the male germline, it becomes impaired following sex determination. Thus after puberty when spermatogenesis is fully functional, there are also specific epigenetic modifications along the different steps of germ cell differentiation from spermatogonial precursors up to testicular spermatozoa. Indeed, it has been demonstrated that throughout the different steps of spermatogenesis, germ cells have a dynamic of the epigenetic modifications. This is highlighted by the changes in the expression levels of the enzymes involved in these modifications. If DNA methyltransferases (Dnmts) are mainly expressed in the spermatagonia, histone methyltransferases (HMTs) are mainly expressed at the spermatocyte levels (Godmann et al., 2009). Then other histone modifications such as hyperacetylation of the H4 histone play a key role in the removal of histones and their replacement by protamines during spermatogenesis (Dhar et al., 2012). Such epigenetic modifications have also been demonstrated to be associated with infertility as for example reduced expression of Dnmt3b in patients with spermatogenic arrest (Adiga et al., 2011). If the EDs cannot alter the DNA sequence, there are numerous studies demonstrating that they can impact the epigenome. Indeed, environmental factors can alter the epigenetic programing which will impact the development of the offspring. Interestingly, it has been demonstrated in the last decade that these effects could also potentially impact the subsequent generations.
Huang et al., 2011; Schoevers et al., 2012). Such effect was first demonstrated for the exposure with vinclozolin, a compound with anti-androgenic activities (Anway et al., 2003). Thus exposure during embryonic gonadal sex determination can alter the male germ line epigenetics (e.g., DNA methylation). The epigenetic mechanism involves the alteration of DNA methylation in the germ line that appears to transmit transgenerational adult onset disease, including spermatogenic defects and cancer (Anway et al., 2005). At the molecular level, the authors identify by global transcriptomic approach that the expression of genes involved in these processes such as the Dnmt responsible for histone modifications was affected by vinclozolin treatment (Anway et al., 2005). All these data suggest the main involvement in transgenerational transmission.

Potential link between epigenetic and TGCC?
These modifications in epigenetic programming by EDs are of particular interest as they have been suspected to be responsible of the increased rate of pathologies related to the TDS such as TCs. This potential association between ED and epigenetic in TGCT development is highlighted by the fact that recently, number of studies have demonstrated the modified epigenetic profile of tumor cells compare to “normal” cells (Okamoto and Wakamatsu, 2007; Brait et al., 2012). Indeed, it appears that tumor cells show an epigenetic pattern similar to undifferentiated spermatogonial stem cells (Almstrup et al., 2010). Indeed, CIS cells have a permissive and fetal-like chromatin structure (Almstrup et al., 2010). This is consistent with the fact that CIS, precursor of TGCT have already been identified in tests of embryos.

DNA methylation. DNA methylation is an important event during germ cell development. These enzymatic modifications of DNA rely on Dnmts. Among them, expression profiles of Dnmt3a and Dnmt3l suggest that they might act during prenatal germ cell development for the establishment of de novo methylation. On the other side, Dnmt1 and Dnmt3b rise shortly after birth in the male (Godmann et al., 2009). It was thus hypothesized that these two Dnmts must be involved in the maintenance of methylation patterns in proliferating spermatogonia.

The use of mouse models invalidated for genes encoding these enzymes highlights their crucial involvement during spermatogenesis. Dnmt3a−/−males show a greatly reduced number of spermatocytes (Yaman and Grandjean, 2010; Le Salle et al., 2007). This suggests a major involvement for progression through meiosis. In the same lane, the males invalidated for Dnmt3l participate to the acquisition of DNA methylation at paternally imprinted regions, unique non-pericentric heterochromatic sequences, and interspersed repeats, including transposable elements. Moreover, Dnmt3b−/−mice present alterations of meiotic process leading to spermatogenesis arrest, and spermatocytes apoptosis (Webster et al., 2005). As Dnmt1 expression is restricted to gonocytes, the presence of defects in later stages suggests alteration of processes required for completion of spermatogenesis.

The major role of these epigenetic alterations has been demonstrated in carcinogenesis. Indeed, it has been shown that DNA methylation is associated with repression of tumor suppressor gene expression. This epigenetic process is one of the most studied in the research field, and has been recognized as a major mechanism during TGCC progression (Manton et al., 2003; Ellinger et al., 2009). Moreover, the DNA methylation pattern seems to correlate with histological features of the different types of TGCC. Undifferentiated TGCC (seminomas, IGCC unclassified, and gonadoblastomas) are hypermethylated, whereas more differentiated TGCC (teratomas, yolk sac tumors, and choriocarcinomas) show a higher degree of methylation. Embryonal carcinomas show an intermediate pattern. Thus such parameters could be used to discriminate between seminoma and non-seminoma (Brait et al., 2012).

Such impact involved modifications of genes encoding for enzymes involved in DNA methylation. Consistent with cell type origin of TGCC, the Dnmts are mainly expressed in fetal testis and in the more undifferentiated cell type (spermatogonia) during adult normal spermatogenesis.

Regarding cancers, Dnmt1 was not expressed in seminoma, but upregulated in embryonal carcinoma (Omisano et al., 2007). In contrast, the expression of Dnmt3a was found up-regulated in TGCC compared to non-tumor testicular tissues (Yamada et al., 2003). The expression pattern of Dnmt3b has been deeply studied and show that it could be used as a predictive marker for relapse of stage I seminomas (Arai et al., 2012). Lastly, Dnmt2 was overexpressed in the non-seminoma tumors (Minami et al., 2010).

These changes in Dnmts expression will lead to main alteration of transcriptomic profile between tumors and normal tissues (Alagarsamy et al., 2011). Among them, two targets are of particular interest as they are genes of pluripotency. Indeed, early fetal germ cells and undifferentiated germ cell tumors have in common the expression of pluripotency markers such as the transcription factors Nanog and Oct3/4. Regarding Nanog promoter, it was found hypomethylated in spermatogonia and hypermethylated in sperm (Nettersheim et al., 2011). This selective repression might reflect that the cells need to suppress pluripotency in order to prevent malignant transformation. Finally, methylation of Cpgs in the Nanog promoter in germ cell tumors and derived cell lines correlated to differentiation state. In the same line, the study Oct3/4 showed that seminoma and embryonal carcinoma were hypomethylated (De Jong et al., 2007).

Histone methylation. Many enzymes are involved in histone methylation with specific or redundant level of methylation. These differences rely on the modified histone, targeted amino-acid residues and the number of methyl groups that are added to histone. These modifications are performed by several members of the HMT family. On those involved in spermatogenesis, the Su(var)3 and su(var)2 mediator histone H3 di and/or trimethyla- tion at lysine 9 (Schotta et al., 2003). They are involved in meiosis as double KO mice present defects in male meiosis and highly pronounced apoptosis of stage IV spermatocytes during the transition from mid to late pachytene (O’Carroll et al., 2000; Peters et al., 2001).

G9a, a mammalian HMTag, is a candidate for H3-K9 methyla- tion in non-heterochromatic loci (Tachibana et al., 2007). G9a is essential for early embryonic development and plays a dominant role in H3-K9 methylation of euchromatin. Its role was highlighted as mice lacking G9a are sterile, with germ cells undergoing...
apoptosis during the pachytenic stage (Ishibashi et al., 2007). Interestingly, it was demonstrated that 9α was a target of retinoic signaling pathway, a key regulator of germ cell differentiation, and that it was inhibited in the context of estrogen exposure leading to the increased germ cell apoptosis induced by EDs (Volle et al., 2009).

Another HMT is the enhancer of zeste 2 (EZH2) which trimethylate histone H3 at the lysine 27 (Chang and Hung, 2012). It was demonstrated that during spermatogenesis, EZH2 is restricted to round spermatids in the perinuclear acrosome region (Lambert et al., 2012). This localization is concomitant with the dramatic epigenetic reorganization that occurs during spermiogenesis leading to an extreme compaction of the chromatin.

Histone acetylation. This is another histone modification that must also lead to gene repression. These modifications are implicated in the deregulation of gene expression in cancer. As mentioned above, the C5 cells present epigenetic profile similar to ES cells. Such properties of chromatin have been associated with a high transcriptional and proliferative activity. This is due to lower levels of DNA methylation and of histone methylations (H3K9me2 and H3K27me3). Almstrup et al. (2010). This is consistent with a lower expression of the gene encoding for the EZH2 in the TGCC compared to normal testis tissues (Hinz et al., 2010). This suggests that in TGCC EZH2 does not exert its often assumed oncogenic properties during malignant transformation and progression. High EZH2 levels in normal testicular tissue and the inverse association of its expression levels with the severity of spermatogenic failure point to its potential value as a molecular marker for spermatogenic defects and may indicate an important physiological role of EZH2 during intact spermatogenesis.

It has been suggested that histone H2A and H4 arginine 3 trimelation could be involved in the somatic differentiation observed in non-seminomatous tumors. In healthy testis, the distribution of histone H3 methylation was dependent on the developmental stage of spermatogenic cells and in non-seminoma, histone H3-K4 and K9 methylation was detected in all histological subtypes (Lambert and Kimmins, 2011). This suggested that histone H3-K4 and K9 methylation could be associated with abnormal gene expression in non-seminoma. Histone modifications determine epigenetic patterns of gene expression with methylation of histone H3 at lysine 4 (H3K4), and are often associated with active promoters. LSD1/KDM1 is a histone demethylase that suppresses gene expression by converting dimethylated H3K4 to mono- and unmethylated H3K4 (Wang et al., 2011). Interestingly, LSD1 protein level is highly elevated in pluripotent cancer cells and in human testicular seminoma tissues that express Oct3/4 (Wang et al., 2011).

Histone acetylation. This is another histone modification that must lead to gene repression. These modifications are performed by specific enzymes so-called histone deacetylase (HDCA). Such post-translational modifications are implicated in normal spermatogenesis (Fenici et al., 2008). Indeed, H3K9ac was shown in spermatagonia, spermatocytes, elongating spermatids, and ejaculated spermatozoa of fertile and infertile men (Stellmann et al., 2011). In spermatagonia, the stainings for H3K9ac, H3K18ac, and H3K28ac were strong. Then spermatocytes, the stainings for H3K9ac, H3K18ac, H3K28ac, and H3K4me3 were reduced in the preleptotene to pachytenic stage, but in diplotene stage the stainings for H3K18ac, H3K28ac, and H3K4me3 seemed to become intense in later stages (Song et al., 2011a).

The main involvement of histone acetylation during spermatogenesis is the hyperacetylation of histone H4 during spermiogenesis (Dhar et al., 2012). This signal seems to play crucial role for removal of histones and their replacement by protamines, which is key feature for nucleus condensation, and thus formation of spermatozoa. There are multiple members classified in different subfamilies. Interestingly, choriocarcinomas showed generally high expression for all three class I HDAC isoforms (Gryder et al., 2012). However in contrast with other types of tumors, no diagnostic or prognostic values for HDAC1-3 in TGCC could be inferred (Fritzsche et al., 2011).

Impact of TGCC treatment. Some testis tumors are treated using cisplatin (Koychev et al., 2011). Recent report analyzed the impact of such treatment on the integrity of spermatozoa chromatin in rats (Maselli et al., 2011). As expected, the cisplatin treatment lead to susceptibility of DNA to denaturation and the number of strand breaks were significantly increased in mature sperm. After a recovery period, it was noted that mature sperm did not show significant DNA damages. However, the protamination level of the sperm of these animals was significantly decreased. This was associated with an up-regulation of the histones H1.2, H4, H2A1, and H2B1A. This suggests long-term effect of cisplatin treatment that could have consequences for progenies even after the arrest of cancer cure.

Small non-coding RNA. Next to these well studied epigenetic processes, it also appears that microRNA and small RNA play important roles in both germ cell differentiation and transmission to subsequent generations (Hec et al., 2009; Suh and Biedloch, 2011; Buckley et al., 2012). Indeed, achieving the correct spatial and temporal expression of germ cell-specific genes is fundamental to the production of spermatozoa (Song et al., 2011b). Notably, for the regulation of genes involved in the repression of protein translation is central to many embryonic processes, and is particularly active during spermatogenesis.

The miRNA and siRNA are generated by the nuclear RNase III enzyme Drosha and the cytoplasmic RNase III enzyme Dicer (Papainasnoy and Nef, 2010). The involvement of these particular RNA has been highlighted by the generation of mouse models invalidated for genes encoding Drosha or Dicer (Krothom et al., 2011; Wu et al., 2012). These invalidations led to sterility due to disrupted spermatogenesis characterized by depletions of spermatocytes and spermatids leading to oligoteratozoospermia or azoospermia. miRNAs mostly act by destabilizing target mRNAs or inhibiting their translation. Next to this, the PIWI-interacting RNAs (piRNAs) are predominantly expressed in the germ cell lineage (Khavan et al., 2012). The analyses on this particular class of RNA suggest that they have a potential role in epigenetic
regulation of cell polarization. Moreover piRNA seem to be involved in silencing of transposon expression.

Many miRNA and siRNA have been implicated in the different steps of spermatogenesis. Among them, miR-449 and miR-34 seems to have common targets on the E2F signaling pathway which is mainly involved in the regulation of male germ cell development (Biao et al., 2012). MiR-17-92 (Mirc3) cluster and MiR-106b-25 (Mirc4) cluster miRNAs were suggested to cooperate in regulating spermatogonial development (Tong et al., 2012). The overexpression of miR-184 was demonstrated to promote the proliferation of a germ cell line, GC-1spg (Wu et al., 2011). It was also shown that transient inhibition of miR-21 in SSC-enriched germ cell cultures increased the number of germ cells undergoing apoptosis (Niu et al., 2013).

Interestingly, several miRNAs are unique to testis. Regarding the involvement of miR199a-5p was highlighted by the study showing an inverse relationship between miR199a-5p and embryonal carcinoma antigen podocalyxin-like protein 1 (PODXL) expression (Cheung et al., 2011). This suggests that PODXL must be a downstream effector mediating the action of miR199a-5p. This is of particular interest as PODXL, an anti-adhesive protein, is expressed in aggressive tumors.

Next to this, miR-371-373 and miR-302 clusters are overexpressed in malignant TGCC (Novotny et al., 2012). It downregulates miRNAs involved in biologically significant pathways involved in cellular senescence induced by oncogenic stress.

Among other examples, miR-383 expression is downregulated in the testes of infertile men with MA (Lian et al., 2009). These results suggest that it functions as a negative regulator of proliferation, in part, through inactivation of the pRB pathway. Thus an abnormal expression of miR-383 may potentiate the connections between male infertility and testicular germ cell tumor.

**Therapy issues.** A percentage of tumors are resistant to cisplatin. The downregulation of Oct4 and miR-106b (Koster et al., 2010). Thus, it was hypothesized that modulation of the Oct4/miR-106b/p21 pathway could open new perspectives in the treatment of chemoresis tant TC.

**CONCLUSIONS/PERSPECTIVES** The increasing incidence of reproductive tract diseases and particularly TC, during the last decades, is of concern. Indeed, even if it is a well curable disease with a good 5-year survival rate, it affects men during the time of their reproductive life (between 15 and 40), suggesting that it may affect both fertility and also health of the progeny. It cannot be exclude that germ cells generated at the beginning of carcinogenesis could transmit altered DNA material, due to genetic, epigenetic perturbation. This higher rate of appearance for TC cases is supposed to be associated with exposure to environmental chemicals. This also need to be deeply studied as in our modern society, men are exposed to an increasing amount of chemicals. This suggests that the incidence of TC could be even more important in the future decades. All these potential consequences point out the importance to study the involved mechanisms in appearance, and progression of TGCC. This means that we have to better understand the etiology for such cancers.

In long-term perspective, an increased knowledge of genetic, epigenetic, and gene expression patterns correlated with data of anatomopathology will lead to a better definition and understanding of the pathology. It also suggests that there is a need to analyze patients in a case by case approach in order to identify genetic, epigenetic alterations, and modifications of gene expression patterns. This will help to propose personalized therapy that would probably help in improving survival rate and quality of survivors and avoid relapse.

**ACKNOWLEDGMENTS** Lobaccarò’s lab is supported by Association de Recherche sur les Tumeurs Prostatiques, Ligue contre le Cancer (Comité Allier), Fondation pour la Recherche Médicale (FRM), Fondation BNP-Paribas and Association de Recherche contre le Cancer (ARC), Grant from Ministère de l’Enseignement supérieur et de la Recherche (to Marine Baptissart), Nouveau Chercheur Auvergne (FR12087CC to David H. Voile), ANR Jeune Chercheur (#1103, to David H. Voile).

**REFERENCES**


Vega et al. Epigenetic mechanisms in testicular cancers


Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
Chemotherapy refractory testicular germ cell tumor is associated with a variant in Armadillo Repeat gene deleted in Velco-Cardio-Facial syndrome (ARVCF)

Chunkit Fung1*, David J. Vaughn2, Nandita Mitra3, Stephanie L. Ciosek4, Saran Vardhanabhuti5, Katherine L. Nathanson4 and Peter A. Kanetsky1

1 Department of Hematology and Oncology, Wilmot Cancer Center, University of Rochester Medical Center, Rochester, NY, USA
2 Department of Hematology and Oncology, Abramson Cancer Center, University of Pennsylvania, Philadelphia, PA, USA
3 Department of Biostatistics and Epidemiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA
4 Division of Translational Medicine and Human Genetics, Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA
5 Center for Biostatistics in AIDS research, Harvard School of Public Health, Boston, MA, USA

**Introduction:** There is evidence that inherited genetic variation affects both testicular germ cell tumor (TGCT) treatment outcome and risks of late-complications arising from cisplatin-based chemotherapy. Using a candidate gene approach, we examined associations of three genes involved in the cisplatin metabolism pathway, GSTP1, COMT, and TPMT, with TGCT outcome and cisplatin-induced neurotoxicity. Materials and Methods: Our study population includes a subset of patients (n = 137) from a genome-wide association study at the University of Pennsylvania that evaluates inherited genetic susceptibility to TGCT. All patients in our study had at least one course of cisplatin-based chemotherapy with at least 1 year of follow-up. A total of 90 markers in GSTP1, COMT, and TPMT and their adjacent genomic regions (±20 kb) were analyzed for associations with refractory TGCT after first course of chemotherapy, progression-free survival (PFS), overall survival (OS), peripheral neuropathy, and ototoxicity. Results: After adjustment for multiple comparisons, one Single nucleotide polymorphism (SNP), rs2073743, in the flanking region (±20 kb) of COMT was associated with refractory TGCT after initial chemotherapy. This SNP lies within the intron region of the Armadillo Repeat gene deleted in Velco-Cardio-Facial syndrome (ARVCF). The G allele of rs2073743 predisposed patients to refractory disease with a relative risk of 2.6 (95% CI 1.1, 6.3; P = 0.03). Assuming recessive inheritance, patients with the GG genotype had 22.7 times higher risk (95% CI 3.3, 155.8; P = 0.04) of developing refractory disease when compared to those with the GC or CC genotypes. We found no association of our candidate genes with peripheral neuropathy, ototoxicity, PFS and OS. Discussion: This is the first study to suggest that germline genetic variants of ARVCF may affect TGCT outcome. The result of this study is hypothesis generating and should be validated in future studies.

**Keywords:** GSTP1, COMT, TPMT, ARVCF, testicular germ cell tumor, refractory disease, neuropathy, ototoxicity

INTRODUCTION

Testicular germ cell tumor (TGCT) is the most common solid malignancy that affects men between the ages 15 and 35 (National Cancer Institute, 2011). Cisplatin-based chemotherapy is the standard treatment for patients with metastatic disease and is highly effective with a 5-year survival rate that approaches 95% (Jemal et al., 2008). Since its introduction in the 1970s, there is a large and growing population of long-term survivors with TGCT. However, cisplatin-based chemotherapy causes peripheral neuropathy and ototoxicity in approximately 20–30% of long-term TGCT survivors and risk of these adverse events increases with higher cumulative dose of cisplatin (Fung and Vaughn, 2011).

Germline genetic variation affects both TGCT outcome and late-complications arising from cisplatin-based treatment. Single nucleotide polymorphisms (SNPs) of the bleomycin hydrolase (de Haas et al., 2008) (BLMH) and the high plasminogen-activator inhibitor 1 (de Haas et al., 2010) (PAI-1) genes have been shown to impact survival among TGCT patients (de Haas et al., 2008, 2010). Variants in glutathione S-transferase pi (GSTP1), which encodes an enzyme that detoxifies chemotherapeutic agents by conjugating reactive electrophiles to glutathione (Mannervik et al., 1985), appear to be important in the development of long-term peripheral neuropathy and ototoxicity in adult TGCT patients (Oldenburg et al., 2007a,b). Similarly, SNPs of thiopurine S-methyltransferase (TPMT) and catechol O-methyltransferase (COMT), which encodes enzymes that metabolize thiopurine drugs (Weinshilboum, 2006) and catecholamine containing chemical via methylation (Weinshilboum, 2006), respectively, predispose the pediatric population to increased risk of hearing loss (Ross et al., 2009) after cisplatin therapy.
In an attempt to validate and extend previous findings regarding effects of germline genetic variation on cancer outcome and treatment-related toxicities via their effects on drug metabolism (Coate et al., 2010), we used a candidate gene approach to investigate the associations of GSTP1, COMT, and TPMT, three genes involved in the cisplatin metabolism pathway, with treatment outcome and cisplatin-induced neurotoxicity. We hypothesized that genetic variants in GSTP1, COMT, and TPMT that confer higher intracellular concentration of cisplatin or its metabolites are associated with lower rates of TGCT recurrence and higher incidence of cisplatin-related neurotoxicity. To test this hypothesis, we evaluated a selected panel of SNPs in GSTP1, COMT, and TPMT with cancer outcome, ototoxicity, and peripheral neuropathy in an existing cohort of men with TGCT who have received at least one course of cisplatin-based chemotherapy.

MATERIALS AND METHODS

STUDY POPULATION

Our study cohort is derived from a subset of TGCT case subjects enrolled into an ongoing case-control study designed to evaluate inherited genetic susceptibility to TGCT. Details of the parent case-control study have been described previously (Kanetsky et al., 2009, 2011). In brief, case subjects were recruited from a network of hospitals, including the Hospital of the University of Pennsylvania (HUP), University of Pennsylvania Cancer Network, Fox Chase Cancer Center, and also from the Pennsylvania and New Jersey State Cancer Registries. Only men between ages 18 and 50 years with pathologically confirmed TGCT were included, and those with human immunodeficiency virus, Klinefelter’s or Down syndrome were excluded. All enrolled patients with TGCT who underwent cisplatin chemotherapy at HUP were potentially eligible for the current study. A total of 137 patients had at least one course of cisplatin-based chemotherapy, at least 1 year of follow-up since initiation of cisplatin-based chemotherapy and at least one clinical consultation with an oncologist at HUP and are included in the final analytic cohort.

The study was approved by the institutional review board at the University of Pennsylvania. All patients gave written informed consent.

GENOTYPING FOR SNPs OF GSTP1, COMT, AND TPMT

All study participants had previously been genotyped using the Affymetrix Genome-Wide Human SNP Array 6.0 platform; details of genotyping efforts and quality control measures have previously been reported (Kanetsky et al., 2009, 2011). A total of 90 SNP markers with genotype call rates ≥ 90% that mapped to the genomic regions (± 20 kb) of COMT, TPMT, and GSTP1 (43, 44, and 3 markers, respectively) were used for analysis. A complete listing of annotated SNP markers are available from the authors by request.

TREATMENT RESPONSE ASSESSMENT

A complete review of medical records was performed to abstract information regarding treatment response, patient demographics, tumor characteristics, and treatment information. Monitoring for treatment response included both serum logic (alpha fetal protein (AFP) and beta human chorionic gonadotropin (β-HCG)) and radiographic studies (plain radiography, computed tomography, or magnetic resonance imaging).

We evaluated three treatment efficacy endpoints: (1) refractory disease after first course of chemotherapy, (2) progression-free survival (PFS), and (3) overall survival (OS). Clinical remission was defined as disappearance of all tumors by radiographic methods and normalization of both AFP and β-HCG level for at least 4 weeks after completion of first course of chemotherapy. Cancer recurrence was defined as initial clinical remission with subsequent development of disease evidenced by radiographic imaging or serology. Refractory disease was defined as persistent radiographic or serologic evidence of disease after initial course of chemotherapy.

NEURO- AND OTO-TOXICITY ASSESSMENT

We collected information regarding cisplatin-related peripheral neuropathy and ototoxicity from patients using a self-administered mailed questionnaire instrument. We adapted 12 of the 15 questions from the Michigan Neuropathy Screening Instrument (MNSI) (Moghadder et al., 2006) for assessment of peripheral neuropathy. For ototoxicity, we used 10 questions from an existing screening survey of hearing problem at the National Institute on Deafness and other Communications Disorders (Fung and Vaughn, 2011) with inclusion of one additional question about the presence of tinnitus.

In addition to mailing the questionnaire to all 137 patients in our study cohort, we distributed it on site to patients (n = 31) who had routine clinic visits at the Hospital of the University of Pennsylvania from July 2010 to June 2011. To improve the response rate, we sent reminder letters to non-responders 8 weeks after initial mailing of the questionnaire. At 12 weeks, phone calls were made to all remaining non-responders.

Patients were defined as having peripheral neuropathy if their MNSI score was two or greater. The threshold was based on the results of a MNSI validation study in subjects with diabetic peripheral neuropathy (Feldman et al., 1994). Subjects were defined as having ototoxicity if they answered “yes” to three or more of the 11 questions.

STATISTICAL ANALYSIS

We summarized the baseline characteristics of our population using means and standard deviations for continuous variables (age at diagnosis, age at initiation of chemotherapy, initial cisplatin dose, and cumulative life-time cisplatin dose) and proportions for categorical variables [genetically inferred race from GWAS, histology, International Germ Cell Consensus Classification (IGCCC), primary site of tumor, and type of chemotherapy]. We compared the baseline characteristics between cohorts with and without completed toxicity questionnaires using Fisher’s exact test for categorical variables and t-test for continuous variables. We used logistic regression models to investigate associations between baseline characteristics and peripheral neuropathy, ototoxicity, and refractory disease after first course of chemotherapy. We conducted a time to event analysis using a Cox proportional hazards model to assess the effects of baseline covariates on PFS and OS.
We summarized the major and minor allele frequencies of each SNP marker and determined if their genotypic distributions were in Hardy–Weinberg equilibrium.

We studied associations of SNP markers with endpoints assuming co-dominant, recessive, and dominant genetic models using Pearson's chi-squared tests. We accounted for multiple comparisons by adjusting the p-values of these associations using the method of Benjamini and Hochberg (1995). We used multivariable logistic regression models to assess associations of genetic markers with peripheral neuropathy and ototoxicity, after adjusting for race, IGCCC prognostic group, primary site of disease, type of chemotherapy, and cumulative dose of cisplatin. To assess the association between each SNP and refractory disease after initial chemotherapy, we used multivariable logistic regression models with adjustment for race, IGCCC prognostic group, primary site of disease, type of chemotherapy, and dose of cisplatin during first course of chemotherapy. Since refractory disease accounts for less than 10% of all treatment outcomes, estimates from the logistic regression model are interpreted and reported as relative risks.

To assess the association between each SNP and PFS or OS, we used Cox proportional hazards models. In these time-to-event models, patients contributed to the analyses from the initiation of chemotherapy until date of last follow-up or death in the OS model with inclusion of cancer recurrence as an additional censoring event in the PFS model. In the PFS model, we adjusted for race, IGCCC prognostic group, primary site of disease, type of chemotherapy, and dose of cisplatin during first course of chemotherapy. In the OS model, we adjusted for race, IGCCC prognostic group, primary site of disease, type of chemotherapy, and cumulative dose of cisplatin. We report hazard ratios (HR) and corresponding 95% CI from the Cox models.

All statistical analyses were performed using Stata 12 (StataCorp LP, Texas, 2011).

RESULTS

BASELINE CHARACTERISTICS OF PATIENTS

Baseline characteristics of all 137 patients and the subset of 66 patients (48.2%) who completed the toxicity questionnaire are given in Table 1. As expected, whites account for the majority of patients in both groups (86.8% in the entire cohort and 93.9% in the questionnaire subgroup) and the mean age at TGCT diagnosis is similar (31.0 years and 32.7 years, respectively). Tumor characteristics are similar between the groups: the predominant histology is non-seminoma (81.7% in the entire cohort and 75.8% in the questionnaire subset), the primary site of presentation is testicle (89.0% and 90.9%, respectively), and the majority of tumors are classified as good prognosis at initiation of chemotherapy by the IGCCC criteria (68.6% and 80.3%, respectively). For both groups, bleomycin, etoposide, and cisplatin (BEP) is the most common initial chemotherapy regimen (62.0% of the entire group and 59.1% of the questionnaire subset) and the mean cumulative cisplatin dose (mg/m²) is comparable among them (410.9 and 404.5, respectively).

In general, patients who completed the toxicity questionnaire are similar to the non-responders. There are no differences in terms of histology, primary site of TGCT, types of initial chemotherapy, or cumulative cisplatin dose administered ($P > 0.05$). However, those who completed the questionnaire were older at the time of diagnosis of TGCT ($P = 0.03$) and hence at initiation of chemotherapy ($P = 0.02$). A larger proportion of non-responders were non-whites (19.7% versus 6.1%, $P = 0.02$) and also had poor risk disease (32.4% versus 12.1%, $P = 0.01$) when compared to those who completed the questionnaire.

PERIPHERAL NEUROPATHY AND OTOTOXICITY

Among the 66 patients who completed the toxicity questionnaire, 25 (37.9%) and 30 (45.5%) of them reported peripheral neuropathy and ototoxicity, respectively (Table 2). Patients who were diagnosed with TGCT at an older age were more likely to report developing peripheral neuropathy ($OR = 3.1, 95\% CI 1.5, 6.4$ per 10 year increase in age) as were patients with higher cumulative life-time cisplatin dose ($OR = 1.6, 95\% CI 1.1, 2.2$ per 100mg/m² increased in dose). Those with non-seminoma ($OR = 0.3, 95\% CI 0.1, 0.8$) and BEP ($OR = 0.3, 95\% CI 0.1, 0.95$) as initial treatment had statistically significantly reduced risk of peripheral neuropathy compared to those with seminoma or treatment with etoposide and cisplatin (EP), respectively. Regarding ototoxicity, only higher cumulative life-time cisplatin dose ($OR = 1.5, 95\% CI 1.1, 2.1$ per 100mg/m² increased in dose) and total cisplatin dose ($OR = 2.1, 95\% CI 1.03, 4.3$ per 100mg/m² increased in dose) during initial chemotherapy increased this risk significantly.

TREATMENT OUTCOME

In our cohort of 137 patients, 11 (8.0%) of them had refractory disease after initial course of chemotherapy and 126 (92%) of them achieved clinical remission. Among those with remission, 30 (23.8%) of them experienced disease relapse after a mean follow-up time of 46.8 months, with a total of eight deaths within the entire cohort. Table 3 shows that extra-gonadal primary ($RR = 9.7, 95\% CI 2.5, 37.3$), intermediate/poor risk cancer ($RR = 28.2, 95\% CI 3.5, 228.7$), and initial therapy with etoposide/itisamid/cisplatin (VIP) ($RR = 135.0, 95\% CI 6.7, 2733.6$) were statistically significantly associated with refractory disease. Patients with non-seminoma ($HR = 3.4, 95\% CI 1.03–10.9$), extra-gonadal primary ($HR = 3.5, 95\% CI 1.7, 7.3$), intermediate/poor risk cancer ($HR = 4.1, 95\% CI 2.2, 7.7$), initial VIP ($HR = 27.7, 95\% CI 7.9, 96.7$), and higher total cisplatin dose during initial chemotherapy ($HR = 1.5, 95\% CI 1.01–2.3$ per 100mg/m² increased in dose) all had worse PFS. Patients with extra-gonadal primary ($HR = 5.5, 95\% CI 1.3, 22.9$), intermediate/poor risk cancer ($HR = 17.7, 95\% CI 2.2, 143.5$), VIP as initial chemotherapy ($HR = 253.7, 95\% CI 12.2, 5367.1$), and higher cumulative life-time cisplatin dose ($HR = 1.6, 95\% CI 1.2, 2.1$ per 100mg/m² increased in dose) had worse OS.

ASSOCIATIONS OF VARIANTS IN COMT, TPMT, AND GSTP1 WITH PERIPHERAL NEUROPATHY, OTOTOXICITY, AND TREATMENT OUTCOMES

We found statistically significant associations of four SNP markers (rs4646316, rs4380755, rs5008499, and rs6591256) in or near COMT, TPMT, and GSTP1 with neuropathy and five SNP markers (rs3788306, rs12189790, rs17420046, rs6938294, and rs6912842) with ototoxicity at the nominal p-value of 0.05.
Table 1 | Baseline characteristics of patients.

<table>
<thead>
<tr>
<th>CHARACTERISTICS OF CHEMOTHERAPY</th>
<th>All patients (n = 137)</th>
<th>Patients with completed toxicity questionnaire (n = 66)</th>
<th>Patients without completed toxicity questionnaire (n = 71)</th>
<th>p-valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of initial chemotherapy</td>
<td>EP</td>
<td>46</td>
<td>26</td>
<td>39.4%</td>
</tr>
<tr>
<td></td>
<td>BEP</td>
<td>85</td>
<td>39</td>
<td>59.1%</td>
</tr>
<tr>
<td></td>
<td>TIP</td>
<td>1</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td></td>
<td>VIP</td>
<td>4</td>
<td>1</td>
<td>1.5%</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>1</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td>Mean initial total cisplatin dose ± SD (mg/m²)d</td>
<td>345.3 ± 80.4</td>
<td>337.9 ± 80.0</td>
<td>352.1 ± 82.6</td>
<td>0.30</td>
</tr>
<tr>
<td>Mean cumulative cisplatin dose ± SD (mg/m²)d</td>
<td>410.9 ± 169.2</td>
<td>404.5 ± 166.8</td>
<td>416.9 ± 172.4</td>
<td>0.67</td>
</tr>
<tr>
<td>Mean age at initiation of chemotherapy ± SD (year)</td>
<td>31.5 ± 9.1</td>
<td>33.4 ± 9.3</td>
<td>29.7 ± 8.5</td>
<td>0.02</td>
</tr>
</tbody>
</table>

SD, standard deviation; TGCT, testicular germ cell tumor; EP, etoposide and cisplatin; BEP, bleomycin, etoposide, and cisplatin; TIP, paclitaxel, ifosamide, and cisplatin; VIP, etoposide, ifosamide, and cisplatin.  
*p*-values for comparison of baseline characteristics between cohorts with and without completed toxicity questionnaires. Fisher’s exact test used for categorical variables and t-test used for continuous variables.  
IGCCC prognosis group of TGCT at initiation of first course of chemotherapy.  
Mean total cisplatin dose administered during first course of chemotherapy.  
Mean cumulative life-time cisplatin dose administered by the end of last follow-up visit.  
Italic values indicate *p* ≤ 0.05.

However, after correcting for multiple testing, these associations did not retain statistical significance (Table 4).  
Nine SNPs in or near COMT, TPMT, and GSTP1 had a statistically significant association with refractory disease after initial chemotherapy at the nominal *p*-value of 0.05 (Table 5). After correcting for multiple testing, we found that recessive inheritance of the G allele of rs2073743 confers increased risk (*RR* = 22.7, 95% CI 3.3, 155.8) of refractory disease after first course of chemotherapy. However, this association did not retain statistical significance after adjustment for race, IGCCC, primary site of disease at diagnosis, type of initial chemotherapy, and total dose of cisplatin during first course of chemotherapy.  
The G allele of rs2073743 had a frequency of 22.76% in the cohort and its genotypic distribution was in Hardy–Weinberg proportions (*P* = 0.46). This allele was present in 40.91% of patients with refractory disease compared to 21.14% of those with clinical remission after initial chemotherapy, predisposing patients to refractory disease with a relative risk of 2.6 (95% CI of 1.1, 6.3; *P* = 0.03).  
We found statistically significant association of six SNPs (rs1042781, rs2073743, rs2531706, rs5748505, rs6518598, and rs4380755) in or near COMT, TPMT, and GSTP1 with PFS and six SNPs (rs366148, rs2239395, rs1858770, rs12158214, rs887200, and rs372534) with OS at the nominal *p*-value of 0.05.
ARVCF and refractory TGCT

Table 2 | Risk factors for development of peripheral neuropathy and ototoxicity of 66 patients who completed the toxicity questionnaire.

<table>
<thead>
<tr>
<th></th>
<th>Peripheral neuropathy</th>
<th></th>
<th>Ototoxicity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes N (%)</td>
<td>No N (%)</td>
<td>OR</td>
<td>95% CI</td>
</tr>
<tr>
<td>AGE AT DIAGNOSISa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD (year)</td>
<td>370 ± 8.7</td>
<td>30.0 ± 74</td>
<td>3.1</td>
<td>1.5, 6.4</td>
</tr>
<tr>
<td>RACE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White (n = 62)</td>
<td>23 (92.0)</td>
<td>39 (95.1)</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>Non-white (n = 4)</td>
<td>2 (8.0)</td>
<td>2 (4.9)</td>
<td>0.2</td>
<td>0.12, 9.0</td>
</tr>
<tr>
<td>PRIMARY SITE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testicle (n = 60)</td>
<td>22 (88.0)</td>
<td>38 (92.7)</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>Extra-gonadal (n = 6)</td>
<td>3 (12.0)</td>
<td>3 (73)</td>
<td>0.3</td>
<td>0.1, 9.3</td>
</tr>
<tr>
<td>HISTOLOGY</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seminoma (n = 16)</td>
<td>10 (40.0)</td>
<td>6 (14.6)</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>Non-seminoma (n = 50)</td>
<td>15 (60.0)</td>
<td>35 (85.4)</td>
<td>0.3</td>
<td>0.1, 0.8</td>
</tr>
<tr>
<td>IGCCC PROGNOSIS GROUP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good (n = 53)</td>
<td>20 (80.0)</td>
<td>33 (80.5)</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>Intermediate/poor (n = 13)</td>
<td>5 (20.0)</td>
<td>8 (19.5)</td>
<td>0.3</td>
<td>0.1, 3.6</td>
</tr>
<tr>
<td>TYPES OF INITIAL CHEMOTHERAPY</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EP (n = 26)</td>
<td>14 (56.0)</td>
<td>12 (29.3)</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>BEP (n = 39)</td>
<td>11 (44.0)</td>
<td>28 (68.3)</td>
<td>0.3</td>
<td>0.1, 0.95</td>
</tr>
<tr>
<td>VIP (n = 1)</td>
<td>0 (0)</td>
<td>1 (2.4)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>INITIAL TOTAL CISPLATIN DOSEb</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD (mg/m²)</td>
<td>360.0 ± 81.6</td>
<td>324.4 ± 73.4</td>
<td>2.0</td>
<td>0.9, 4.1</td>
</tr>
<tr>
<td>CUMULATIVE CISPLATIN DOSEc</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD (mg/m²)</td>
<td>476.0 ± 173.9</td>
<td>361.0 ± 148.1</td>
<td>1.6</td>
<td>1.1, 2.2</td>
</tr>
</tbody>
</table>

OR, odds ratio; CI, confidence interval; SD, standard deviation; NA, not applicable; EP, etoposide and cisplatin; BEP, bleomycin, etoposide, and cisplatin; VIP, etoposide, ifosarnide, and cisplation.
aOdds ratio per increase of 10 years of age.
bOdds ratio per increase of 100 mg/m² of total cisplatin dose administered during first course of chemotherapy.
cOdds ratio per increase of 100 mg/m² of cumulative life-time cisplatin dose administered by the end of last follow-up visit.

Bold-italic values indicate p ≤ 0.05.

(Table 6). In particular, patients with the recessive GG genotype of rs2073743 had an inferior PFS with a HR of 3.6 (95% CI 1.1, 11.7) when compared to those with the GC or CC genotypes. However, after correcting the p-values for multiple testing, none of these associations remained statistically significant.

DISCUSSION

This study examined the association between three genes involved in cisplatin metabolism, GSTPI, COMT, and TPMT, with TGCT outcomes and cisplatin-induced ototoxicity and peripheral neuropathy. We found one SNP (rs2073743) in the flanking region (±20 kb) of COMT that was associated with refractory TGCT after initial chemotherapy. This SNP lies within the intron region of the Armadillo Repeat gene deleted in Velo-Cardio-Facial syndrome (ARVCF). This is the first study to show that germline genetic variants of ARVCF may affect TGCT outcome.

Although there is no functional information for SNP rs2073743, we hypothesize that it may affect disease risk by altering the expression of the ARVCF gene. ARVCF gene is a member of the p120 catenin family of proteins (Reintsch et al., 2008) and its over-expression has been shown to cause disruption of cell adhesion (Reintsch et al., 2008), which may facilitate cancer progression. A recent study by Lin et al. (2011) reported that the dominant inheritance of the minor T allele of rs5993891 of ARVCF is associated with a decrease in prostate cancer specific mortality (HR = 0.21, 95% CI 0.07, 0.61). Therefore, it is possible that SNP rs2073743 of ARVCF may predispose patients to higher risk of refractory TGCT by inhibition of cell adhesion, which consequently causes more aggressive tumor biology.

In contrast, SNP rs2073743 may be a marker for genetic variations in COMT that affect the risk of refractory TGCT by altering the level of S-adenosyl-L-methionine (AdoMet) and the concentration of estrogen and its metabolites. COMT encodes a methyltransferase that is critical for the metabolism of endogenous catecholamine containing chemicals and catechol drugs with AdoMet being its intermediary metabolite (Weinshilboum, 2006). There is some evidence to suggest that cisplatin may interact synergistically with AdoMet (Ochoa et al., 2009). Ochoa and colleagues found that administration of both AdoMet and cisplatin causes a 3.0–6.2-fold increase in frequency of renal toxicity in mice while cisplatin alone causes only moderate toxicity and administration AdoMet by itself did not result in any nephrotoxicity (Ochoa et al., 2009). Consequently, it is possible that increased activity of the COMT enzyme causes a decrease in the concentration of AdoMet, which may in turn lead to decreased cytotoxic activity of cisplatin.
Table 3 | Association of baseline characteristics with testicular cancer treatment outcome of 137 patients in the study cohort.

<table>
<thead>
<tr>
<th>Refractory disease</th>
<th>Progression-free survival</th>
<th>Overall survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes N (%)</td>
<td>No N (%)</td>
<td>RR</td>
</tr>
<tr>
<td>AGE AT DIAGNOSIS&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD (year)</td>
<td>270 ± 5.3</td>
<td>314 ± 8.7</td>
</tr>
<tr>
<td>RACE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White (n = 119)</td>
<td>11 (100)</td>
<td>108 (85.7)</td>
</tr>
<tr>
<td>Non-white (n = 18)</td>
<td>0 (0.0)</td>
<td>18 (14.3)</td>
</tr>
<tr>
<td>PRIMARY SITE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testicle (n = 122)</td>
<td>6 (54.5)</td>
<td>116 (92.1)</td>
</tr>
<tr>
<td>Extra-gonadal (n = 15)</td>
<td>5 (45.5)</td>
<td>10 (7.9)</td>
</tr>
<tr>
<td>HISTOLOGY</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seminoma (n = 25)</td>
<td>0 (0)</td>
<td>25 (19.8)</td>
</tr>
<tr>
<td>Non-seminoma (n = 112)</td>
<td>11 (100)</td>
<td>101 (80.2)</td>
</tr>
<tr>
<td>IGCCC PROGNOSIS GROUP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good (n = 94)</td>
<td>1 (9.1)</td>
<td>93 (73.8)</td>
</tr>
<tr>
<td>Intermediate/poor (n = 43)</td>
<td>10 (90.9)</td>
<td>33 (26.2)</td>
</tr>
<tr>
<td>TYPES OF INITIAL CHEMOTHERAPY</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EP (n = 46)</td>
<td>1 (9.1)</td>
<td>45 (35.7)</td>
</tr>
<tr>
<td>BEP (n = 85)</td>
<td>7 (63.6)</td>
<td>78 (61.9)</td>
</tr>
<tr>
<td>TIP (n = 1)</td>
<td>0 (0)</td>
<td>1 (0.8)</td>
</tr>
<tr>
<td>VIP (n = 4)</td>
<td>3 (27.3)</td>
<td>1 (10.8)</td>
</tr>
<tr>
<td>Others (n = 1)</td>
<td>0 (0)</td>
<td>1 (10.8)</td>
</tr>
<tr>
<td>INITIAL TOTAL CISPLATIN DOSE&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD (mg/m²)</td>
<td>345.5 ± 104</td>
<td>345.2 ± 78.6</td>
</tr>
<tr>
<td>CUMULATIVE CISPLATIN DOSE&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD (mg/m²)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RR, relative risk; CI, confidence interval; HR, hazard ratio; NA, not applicable; SD, standard deviation; EP, etoposide and cisplatin; BEP, bleomycin, etoposide, and cisplatin; TIP, paclitaxel, ifosamide, and cisplatin; VIP, etoposide, ifosamide, and cisplatin.

<sup>a</sup>Refractory disease: relative risk per unit increase of 10 years of age; PFS and OS: hazard ratio per unit increase of 10 years of age.

<sup>b</sup>Refractory disease: relative risk per unit increase of 100 mg/m² of total cisplatin dose administered during first course of chemotherapy; PFS and OS: hazard ratio per unit increase of 100 mg/m² of total cisplatin dose administered during first course of chemotherapy.

<sup>c</sup>Refractory disease and PFS: life-time cumulative cisplatin dose is not applicable since status for refractory disease or cancer relapse was ascertained after initial course of chemotherapy only; OS: hazard ratio per unit increase of 100 mg/m² of cumulative life-time cisplatin dose.

Bold-italic values indicate p < 0.05.
Table 4 | Association of genetic variants that reached a nominal p-value of 0.05 with peripheral neuropathy and ototoxicity among 66 patients who completed the toxicity questionnaire.

<table>
<thead>
<tr>
<th>Gene (SNP)</th>
<th>Genotype</th>
<th>Case N (%)</th>
<th>Control N (%)</th>
<th>Univariate model</th>
<th>Multivariate modela</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Odds ratio (95% CI)</td>
<td>p-value</td>
<td>Odds ratio (95% CI)</td>
<td>p-value</td>
</tr>
<tr>
<td>COMT</td>
<td>rs4646316</td>
<td>Co-dominant genetic model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>0 (0)</td>
<td>3 (73)</td>
<td>0.1 (0.02, 0.8)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>T/C</td>
<td>9 (37.5)</td>
<td>23 (56.1)</td>
<td>0.3 (0.1, 0.9)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>15 (62.5)</td>
<td>15 (36.6)</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Dominant genetic model</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T/-</td>
<td>9 (37.5)</td>
<td>26 (63.4)</td>
<td>0.4 (0.1, 0.98)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>15 (62.5)</td>
<td>15 (36.6)</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>TPMT</td>
<td>rs4380755</td>
<td>Co-dominant genetic model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>1 (4.3)</td>
<td>0 (0)</td>
<td>8.7 (1.02, 74.1)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>T/C</td>
<td>9 (39.1)</td>
<td>8 (21.1)</td>
<td>2.9 (1.01, 6.6)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>13 (56.5)</td>
<td>30 (78.9)</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>rs5008499</td>
<td>Co-dominant genetic model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>1 (4.5)</td>
<td>0 (0)</td>
<td>15.0 (1.5, 152.0)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>T/C</td>
<td>8 (36.4)</td>
<td>6 (15.0)</td>
<td>3.9 (1.2, 12.3)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>13 (59.1)</td>
<td>34 (85.0)</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>GSTP1</td>
<td>rs6591256</td>
<td>Dominant genetic model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G/-</td>
<td>12 (52.2)</td>
<td>30 (76.9)</td>
<td>0.3 (0.1, 0.99)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>A/A</td>
<td>11 (47.8)</td>
<td>9 (23.1)</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>COMT</td>
<td>rs3788306</td>
<td>Dominant genetic model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G/-</td>
<td>20 (74.1)</td>
<td>17 (48.6)</td>
<td>3.0 (1.02, 9.0)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>A/A</td>
<td>7 (25.9)</td>
<td>18 (51.4)</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>TPMT</td>
<td>rs12189790</td>
<td>Co-dominant genetic model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>0 (0)</td>
<td>0 (NA)</td>
<td>13.8 (1.1, 90.1)</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>T/C</td>
<td>13 (43.3)</td>
<td>7 (19.4)</td>
<td>3.2 (1.95)</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>17 (56.7)</td>
<td>29 (80.6)</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>rs17420046</td>
<td>Co-dominant genetic model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>1 (3.3)</td>
<td>2 (5.6)</td>
<td>0.1 (0, 0.6)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>T/G</td>
<td>1 (3.3)</td>
<td>12 (33.3)</td>
<td>0.2 (0.1, 0.8)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>G/G</td>
<td>28 (93.3)</td>
<td>22 (61.1)</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>rs6938294</td>
<td>Co-dominant genetic model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G/G</td>
<td>1 (3.3)</td>
<td>2 (5.7)</td>
<td>0.04 (0, 0.6)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>G/A</td>
<td>1 (3.3)</td>
<td>12 (34.3)</td>
<td>0.2 (0.1, 0.8)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>A/A</td>
<td>28 (93.3)</td>
<td>21 (60.0)</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>rs6912842</td>
<td>Co-dominant genetic model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>G/-</td>
<td>2 (6.7)</td>
<td>14 (38.9)</td>
<td>0.1 (0.02, 0.6)</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>A/A</td>
<td>28 (93.3)</td>
<td>22 (61.1)</td>
<td>1.0</td>
<td>–</td>
</tr>
</tbody>
</table>

CI, confidence interval.

*aAdjusted for race, IGCC, primary site of cancer, type of chemotherapy administered, and cumulative life-time cisplatin dose administered.

www.frontiersin.org
Table 5 | Association of genetic variants that reached a nominal $p$-value of 0.05 with refractory disease after initial chemotherapy among 137 patients in the study cohort.

<table>
<thead>
<tr>
<th>Gene (SNP)</th>
<th>Genotype</th>
<th>Yes N (%)</th>
<th>No N (%)</th>
<th>Relative risk (95% CI)</th>
<th>p-value</th>
<th>Relative risk (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>COMT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs2073743</td>
<td>Co-dominant genetic model</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1034564</td>
<td>Co-dominant genetic model</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs366148</td>
<td>Dominant genetic model</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs2531706</td>
<td>Co-dominant genetic model</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs5478505</td>
<td>Dominant genetic model</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1034564</td>
<td>Recessive genetic model</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPMT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs12524744</td>
<td>Co-dominant genetic model</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs9396834</td>
<td>Co-dominant genetic model</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSTP1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs6591256</td>
<td>Co-dominant genetic model</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Relative risk, 95% confidence interval, and $p$-value were calculated.

CI, confidence interval.

*Adjusted for race, IGCCC, primary site of disease at diagnosis, type of chemotherapy administered, and total dose of cisplatin received during initial chemotherapy.

$^b$Corrected p-value for multiple testing: 0.04.

$^c$Corrected p-value for multiple testing: 0.72.
Table 6 | Association of genetic variants that reached a nominal \( p \)-value of 0.05 with progression-free and overall survival among 137 patients in the study cohort.

<table>
<thead>
<tr>
<th>Gene (SNP)</th>
<th>Genotype</th>
<th>Mean PFS/OS (mo)</th>
<th>Univariate model</th>
<th>Multivariate model&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hazard ratio (95% CI)</td>
<td>( p )-value</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COMT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs10427871</td>
<td>Co-dominant genetic model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/G</td>
<td>NA</td>
<td>45.7 (2.5, 845.7)</td>
<td>0.01</td>
<td>9469.3 (62.7, 1429366.1)</td>
</tr>
<tr>
<td>G/A</td>
<td>7.7</td>
<td>6.8 (1.6, 29.1)</td>
<td>0.01</td>
<td>973 (7.9, 1195.6)</td>
</tr>
<tr>
<td>A/A</td>
<td>48.4</td>
<td>1.0</td>
<td>–</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hazard ratio (95% CI)</td>
<td>( p )-value</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs2073743</td>
<td>Recessive genetic model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/G</td>
<td>43.9</td>
<td>3.6 (1.1, 11.7)</td>
<td>0.03</td>
<td>1.2 (0.3, 4.6)</td>
</tr>
<tr>
<td>A/A</td>
<td>48.4</td>
<td>1.0</td>
<td>–</td>
<td>1.0</td>
</tr>
<tr>
<td>rs2531706</td>
<td>Dominant genetic model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/-</td>
<td>7.7</td>
<td>6.8 (1.6, 29.1)</td>
<td>0.01</td>
<td>973 (7.9, 1195.6)</td>
</tr>
<tr>
<td>A/A</td>
<td>48.4</td>
<td>1.0</td>
<td>–</td>
<td>1.0</td>
</tr>
<tr>
<td>rs5748505</td>
<td>Dominant genetic model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T/-</td>
<td>51.1</td>
<td>0.5 (0.3, 0.95)</td>
<td>0.03</td>
<td>0.5 (0.2, 0.9)</td>
</tr>
<tr>
<td>C/C</td>
<td>38.3</td>
<td>1.0</td>
<td>–</td>
<td>1.0</td>
</tr>
<tr>
<td>rs6518598</td>
<td>Dominant genetic model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/-</td>
<td>51.8</td>
<td>0.5 (0.3, 0.9)</td>
<td>0.02</td>
<td>0.4 (0.2, 0.8)</td>
</tr>
<tr>
<td>C/C</td>
<td>38.8</td>
<td>1.0</td>
<td>–</td>
<td>1.0</td>
</tr>
<tr>
<td>TPMT</td>
<td></td>
<td></td>
<td>Hazard ratio (95% CI)</td>
<td>( p )-value</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs4380755</td>
<td>Recessive genetic model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T/T</td>
<td>2.0</td>
<td>61.8 (6.6, 681.9)</td>
<td>&lt; 0.01</td>
<td>273.9 (16.8, 4464.8)</td>
</tr>
<tr>
<td>C/-</td>
<td>49.0</td>
<td>1.0</td>
<td>–</td>
<td>1.0</td>
</tr>
<tr>
<td>COMT</td>
<td></td>
<td></td>
<td>Hazard ratio (95% CI)</td>
<td>( p )-value</td>
</tr>
<tr>
<td>rs366148</td>
<td>Co-dominant genetic model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/A</td>
<td>50.0</td>
<td>7.0 (1.8, 26.8)</td>
<td>&lt; 0.01</td>
<td>4.0 (0.5, 29.7)</td>
</tr>
<tr>
<td>G/G</td>
<td>61.2</td>
<td>1.0</td>
<td>–</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hazard ratio (95% CI)</td>
<td>( p )-value</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs2239395</td>
<td>Dominant genetic model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/-</td>
<td>46.7</td>
<td>8.3 (2.0, 34.8)</td>
<td>&lt; 0.01</td>
<td>4.0 (0.5, 29.7)</td>
</tr>
<tr>
<td>G/G</td>
<td>61.2</td>
<td>1.0</td>
<td>–</td>
<td>1.0</td>
</tr>
<tr>
<td>rs1858770</td>
<td>Co-dominant genetic model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/C</td>
<td>11.2</td>
<td>30.5 (1.5, 608.1)</td>
<td>0.03</td>
<td>26.7 (0.3, 2150.4)</td>
</tr>
<tr>
<td>C/A</td>
<td>53.2</td>
<td>5.5 (1.2, 24.7)</td>
<td>0.03</td>
<td>5.2 (0.6, 46.4)</td>
</tr>
<tr>
<td>A/A</td>
<td>59.8</td>
<td>1.0</td>
<td>–</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hazard ratio (95% CI)</td>
<td>( p )-value</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs12158214</td>
<td>Recessive genetic model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T/T</td>
<td>42.4</td>
<td>5.4 (1.04, 278)</td>
<td>0.05</td>
<td>NA</td>
</tr>
<tr>
<td>C/-</td>
<td>61.4</td>
<td>1.0</td>
<td>–</td>
<td>1.0</td>
</tr>
<tr>
<td>rs887200</td>
<td>Dominant genetic model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/-</td>
<td>46.9</td>
<td>4.1 (1.02, 16.4)</td>
<td>0.05</td>
<td>4.0 (0.8, 18.7)</td>
</tr>
<tr>
<td>A/A</td>
<td>62.5</td>
<td>1.0</td>
<td>–</td>
<td>1.0</td>
</tr>
</tbody>
</table>
TGCTs are thought to develop from totipotent primordial germ cells (Sinisi et al., 2003) and there is evidence to suggest that a combination of high gonadotropin level coupled with unbalanced androgen and estrogen level may be a key event for TGCT development and progression (Garolla et al., 2005). Fotis et al. (1994) reported that 2-methoxyoestriol, which is an endogenous estrogen metabolite, inhibits angiogenesis and suppresses tumor growth in mice. Since COMT degrades catechol metabolites from estradiol and estrone to methylated compounds (Weinshilboum, 2006), genetic variation of COMT may be implicated in the development and progression of TGCT. Recently, Ferlin et al. (2010) studied 17 polymorphic markers in 11 genes involved in hormone metabolism and reported that the minor A allele of rs11205 in the \( \beta \)-hydroxysteroid dehydrogenase type 4 gene (HSD17B4), which is essential for metabolism of estrone to estradiol, is associated with higher risk of TGCT (\( OR = 2.73, 95\% \) CI 1.47, 7.06). In the same study, however, they found no association of rs4680 of COMT with development of TGCT (Ferlin et al., 2010).

Since the association between rs2073743 with refractory disease was no longer statistically significant after adjusting for race, IGCCC, primary site of disease at diagnosis, type of initial chemotherapy, and total dose of cisplatin during first course of chemotherapy, we examined if any of these covariates are associated with rs2073743. Interestingly, we found that patients with the G/G genotype at this marker have higher odds of having both extra-gonadal primary (\( OR = 14.63, 95\% \) CI 2.22, 96.35) and initial chemotherapy with VIP (\( OR = 45.00, 95\% \) CI 2.77, 730.57). However, we found no such association with race, IGCCC, and total dose of cisplatin. Therefore, patients with rs2073742 may have genetic predisposition to more aggressive TGCT at presentation. This may instead explain their increased risk of refractory disease after initial chemotherapy. On the contrary, since VIP is commonly used for treatment of extra-gonadal TGCT, it simply may be a marker for extra-gonadal disease. Finally, our failure to detect any association of rs2073743 with PFS or OS may result from inadequate statistical power due to small sample size (\( n = 137 \)) and the relatively short mean follow-up time of 46.8 months within the context of the excellent 5-year survival rate of TGCT, which approaches 95% (Fung and Vaughn, 2011).

Our study confirms the known established prognostic factors for TGCT, including both IGCCC risk groups and primary site of disease presentation, thus suggesting that our results are internally valid. Based on the original study (\( n = 5862 \)) that validated the IGCCC (Wanderas et al., 2006), the 5-year OS for good, intermediate, and poor risk metastatic GCT are 91%, 79% and 48%, respectively while the 5-year PFS for these respective prognostic groups are 89%, 75%, and 41%. Our study showed slightly better 5-year OS for all risk groups (good risk: 98.7%, intermediate risk: 87.5%, and poor risk: 76.1%) but comparable 5-year PFS except for that of the intermediate risk group (good risk: 80.0%, intermediate risk: 21.8%, and poor risk: 44.1%). These minor differences may be attributed to the smaller sample size of our study cohort. Similarly, our study also confirmed that patients with extra-gonadal primary have worse 5-year OS (testicular: 94.7%, retroperitoneal: 83.3%, and mediastinum 75.0%) and PFS (testicular: 73.0%, retroperitoneal: 22.2%, and mediastinum 33.3%).

Our study confirmed that increased cumulative dose of cisplatin predisposed patients to both hearing loss and peripheral neuropathy. In our study, 82% of patients who received \( >400 \) mg/m\(^2\) of cumulative cisplatin and 38% of those with \( \leq 400 \) mg/m\(^2\) self-reported ototoxicity, which were higher than those previously reported by Bokemeyer et al. (1998). In their study, they showed that approximately 20% of TGCT patients treated with \( \leq 400 \) mg/m\(^2\) of cumulative cisplatin and 50% of those who received \( >400 \) mg/m\(^2\) developed hearing impairment after a median follow-up of 58 months, which were assessed by pure-tone audiometry (Bokemeyer et al., 1998). These differences may be explained by the more objective method of ototoxicity assessment by Bokemeyer and colleagues.

Peripheral neuropathy occurred in 45.5% of our patients, consistent with other studies which showed that 14–43% of TGCT survivors experience persistent symptomatic peripheral neuropathy by cisplatin-based chemotherapy (Hansen et al., 1989;
Aass, N., Kaasa, S., Lund, E., Kaalhus, et al., 1996; Roth et al., 1988; Petersen and Hansen, 1999; von Schlippe et al., 2001). Our study reported that the odds for peripheral neuropathy increased by 1.55 times per 100 mg/m² increase in cumulative cisplatin dose, which is similar to those reported by other studies (Brydoy et al., 2009; Glendenning et al., 2010). For instance, Glendenning et al. (2010) reported that there is a 1.91 times higher odds of developing peripheral neuropathy with each 200 mg/m² increase in cumulative cisplatin dose. Similarly, Brydoy et al. (2009) found that there is a higher prevalence of paresthesias following five or more cycles than following one to four cycles of cisplatin-based chemotherapy in TGCT survivors. Since peripheral neuropathy is expected to be more prevalent in an older population, it is not surprising that age was a significant variable in our regression analysis.

GSTP1, COMT, and TPMT have been implicated in the development of cisplatin-induced hearing impairment (Oldenburg et al., 2007a,b; Ross et al., 2009) and peripheral neuropathy (Oldenburg et al., 2007a,b). Ross et al. (2009) demonstrated that rs12201199 of TPMT (OR = 17.0, 95% CI 2.3, 125.9) and rs9332377 of COMT (OR = 5.5, 95% CI 1.9, 15.9) are associated with cisplatin-induced hearing loss in children. Regarding GSTP1, Oldenburg et al. (2007a,b) found statistically significant associations between rs1695 of this gene with peripheral neuropathy and cisplatin-induced long-term hearing impairment in TGCT survivors. The A > G polymorphism of rs1695 is a missense mutation that leads to amino acid change at codon 105 (Ile > Val) (Oldenburg et al., 2007a,b) and may affect the detoxification capability of this enzyme with chemotherapeutic agents.

In our study, however, we did not find significant associations of ototoxicity and peripheral neuropathy with any of our candidate genes. The following reasons may explain our null results. First, rs12201199 of TPMT and rs1695 of GSTP1 were not included in the Affymetrix Genome-Wide Human SNP Array 6.0 platform and therefore were not examined. Although one SNP of TPMT in our study (rs11964408) was in linkage disequilibrium with rs12201199, its relatively weak linkage with rs12201199, with a correlation coefficient of 0.65, may explain the lack of significant association with peripheral neuropathy. Second, we may have inadequate statistical power due to the small number of patients in our neurotoxicity cohort (n = 66) to detect such associations. For instance, the risk for development of ototoxicity with rs9332377 of COMT in our study was increased with an odds ratio of 1.29 (95% CI 0.54, 3.11) similar to that of Oldenberg and colleague (Ross et al., 2009). However, this association may not have reached statistically significance (P = 0.57) due to the small sample size of our study. Third, the mechanism for development of cisplatin-induced ototoxicity may be different in adults than in children, and therefore genes other than COMT and TPMT may be involved. Finally, unlike the studies by Oldenburg et al. (2007b) and Ross et al. (2009), patients in our study used a questionnaire instead of audiometric testing to screen for hearing impairment. As a result, misclassification of hearing loss may explain our failure to detect any association with ototoxicity.

The association between SNP rs2073743 of ARVCF and refractory TGCT should first be replicated in future studies with a larger number of patients and subsequently validated using a different patient cohort. In particular, studies to elucidate the function of the ARVCF gene in the pathogenesis and progression of TGCT may give insight into development of therapeutic options in TGCT treatment. If indeed presence of the G/G of the ARVCF gene SNP rs2073743 is associated with refractory TGCT, incorporation of this prognostic factor into the IGCCC may be warranted.

In conclusion, our pilot data suggests that patients with the G/G genotype of the ARVCF gene SNP rs2073743 may have increased risk of refractory TGCT after chemotherapy. Although the underlying mechanism for this increased risk of refractory disease is unknown, confirmation of the observed association may have consequences for risk classifications in patients with metastatic disease and may be of use to select patients who will benefit from more aggressive treatment at its onset.

REFERENCES


Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 06 September 2012; paper pending published: 04 October 2012; accepted: 28 November 2012; published online: 13 December 2012.


This article was submitted to Frontiers in Cancer Endocrinology, a specialty of Frontiers in Endocrinology.

Copyright © 2012 Fung, Vaughn, Mitra, Ciosek, Vardhanabhuti, Nathanson and Kanetsky. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.
Testicular cancer and HPV semen infection

Andrea Garolla, Damiano Pizzol, Alessandro Bertoldo, Marco Ghezzi, Umberto Carraro, Alberto Ferlin and Carlo Foresta*

Department of Molecular Medicine, Section of Clinical Pathology, Centre for Human Reproduction Pathology, University of Padova, Padova, Italy

INTRODUCTION

Testicular cancer represents the more frequent solid tumor affecting males aged 15–35 years. In the last decades, its incidence showed a progressive increased probably due to genetic and environmental factors. Despite exposure to some viruses such as HIV, HCV, EBV, and HPV is frequently related to cancer development, there are no studies aimed to evaluate the possible implication of viral infections in the pathogenesis of testicular cancer. In this study, we analyzed sperm parameters and prevalence of HPV on sperm in 155 testicular cancer patients at diagnosis (T−1), after orchiectomy (T0) and after 12 months from surgery or from the end of adjuvant treatments (T12). All patients showed a significantly higher prevalence of semen infection than controls (9.5% and 2.4% respectively,) and altered sperm parameters both at T−1 and T0. Considering sperm parameters, at T−1 we observed a reduction of progressive motility, and after orchiectomy patients showed a reduction of sperm concentration and count and a further worsening of motility. Thereafter, patients were assigned to three groups on the basis of medical option after surgery: S = surveillance, R = radiotherapy, and C = chemotherapy +/- radiotherapy. At T12, untreated patients had an improvement of sperm parameters while R group and even more C group had a strong decrease of sperm number (p < 0.01 both vs. T0 and S group). Moreover, patients who received radio and/or chemotherapy had a very high prevalence of HPV semen infection (S = 7.7%, R = 30.8%, and C = 61.5%). In conclusion, patients with testicular cancer had frequently altered sperm parameters and higher prevalence of HPV semen infection that were worsened after radio and chemotherapy. Because HPV infection is a risk factor for cancer development and it may further reduce fertility, we suggest screening for HPV in testicular cancer patients at diagnosis and particularly after adjuvant treatments.

Keywords: chemotherapy, human papillomavirus, male infertility, radiotherapy, sexual transmitted diseases, sperm infection, sperm parameters, testicular cancer

In particular is well-established the etiologic role of high-risk HPV types in the carcinogenesis of the vulva, vagina, penis, anus, head, neck, and oropharyngeal cavity (Backes et al., 2009; Smith et al., 2009; Guily et al., 2011). Recently, we reported a significant presence of (HPV) DNA in thawed semen samples from patients with testicular cancer who cryopreserved semen (Foresta et al., 2010). This finding was confirmed by other authors showing similar data from patients who banked their sperm because of chemo/radiotherapy (Kaspersen et al., 2011). These data are very important because cryopreserved semen samples, especially when patients undergo therapies that can affect spermatogenesis, are frequently used by assisted reproductive techniques, such as intracytoplasmic sperm injection (ICSI). It has been observed that the bond between virus and sperm is very strong and that conventional washing procedures aimed to select sperm before in vitro fertilization (IVF) have low efficacy in eliminating HPV sperm infection (Foresta et al., 2011a; Garolla et al., 2012). In addition, we demonstrated that artificially infected sperm, both transfected with E6/E7 HPV genes and incubated with viral proteins, are able to enter the oocyte, to deliver HPV genome, and that viral genes are then actively transcribed by the penetrated oocyte (Foresta et al., 2011b). Furthermore, a recent study showed that HPV
infected couples undergoing assisted reproduction techniques (ART) cycles experienced an increased risk of pregnancy loss compared to non-infected ones (Perino et al., 2011). In these couples the more predictive factor of early abortion was represented by HPV sperm infection. An important risk factor for HPV infection is the presence of immunosuppression status. Patients with cancer, particularly after adjuvant therapies, as chemotherapy and radiotherapy can be immunosuppressed and therefore at higher risk for this infection (Rasmussen and Arvin, 1982; Bieber et al., 2006; Fallahian et al., 2010). Despite the large body of literature concerning the role of HPV in many cancer types, very little is known about this viral infection and testicular cancer. In the light of these considerations, we evaluated semen parameters and the prevalence of HPV semen infection in a large group of patients with testicular cancer at diagnosis, after orchiectomy and after further 12 months of surveillance or after the end of radiotherapy and/or chemotherapy. In subjects with HPV semen infection, we evaluated also the percentage of infected sperm.

MATERIALS AND METHODS

PATIENTS

Written informed consent was obtained from all patients, and the study protocol was approved by the local ethics committee. We enrolled 155 consecutive patients affected by testicular cancer candidate to unilateral orchiectomy, who attended the Centre for Human Reproduction Pathology for andrological examination. A medical history including previous circumcision, smoking, and sexual behavior was obtained from each patient. As control subjects, we used a group of 84 healthy proven fertile men. All subjects collected semen for standard semen analysis, detection of HPV semen infection and fluorescence in situ hybridization (FISH) for HPV at diagnosis and 1 month after orchiectomy. Therefore, patients who underwent chemo and/or radiotherapy were reevaluated after 12 months from the end of treatments. Patients who were followed-up just by surveillance repeated the same analysis after 12 months from surgery.

SEMEN PROCESSING

Semen samples were obtained by masturbation after 3 days of sexual abstinence. After liquefaction at room temperature, semen volume, pH, sperm concentration, viability, motility, and normal morphology were determined following World Health Organization guidelines for semen analysis (WHO, 2010). An aliquot was used for further analysis.

HPV DNA DETECTION

The presence of HPV infection was detected by PCR amplification of HPV DNA with SPF10 primers and then genotyped by the INNO-LiPa HPV Genotyping Extra assay (Innogenetics, Gent, Belgium), which can identify the following HPV genotypes: HPV-6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 69/71, 70, 73, 74, and 82 (Eklund et al., 2010).

SPERM FLUORESCENCE IN SITU HYBRIDIZATION (FISH) FOR HPV

Samples containing at least $2 \times 10^6$ ejaculated sperm were fixed in a methanol-acetic acid solution for at least 1 h at $-20^\circ$C. To permeabilize, samples were digested with pepsin diluted 1:25,000 in pre-warmed 0.01 mol/L-1 HCl for 10 min at $37^\circ$C. Permeabilization of the specimens was stopped with 3–5-min washes in PBS 1×; then samples were dehydrated in 70%, 80%, and absolute ethanol for 2 min and finally air-dried. Samples were then overlaid with 20 mL of hybridization solution (Pan Path, Amsterdam), containing biotin-labeled HPV DNA probe (a mix of total genomes containing the conserved HPV region). Each slide was covered with a glass coverslip, and the edges were sealed with nail polish to prevent loss of the mixture during denaturation and hybridization. After a simultaneous denaturation of cellular target DNA and HPV DNA probe on a heating block for 5 min at 95°C, hybridization was performed by incubating the samples at 37°C overnight in a humidified chamber. Thereafter, the coverslips were carefully removed and the slides were washed in PBS 1× for 10 min. After 15 min incubation at 37°C with the differentiation reagent (Pan Path), the slides were washed three times in PBS 1×. The biotin-labeled HPV probe was detected by incubation with 1:200 streptavidin Texas Red® (Vector Laboratories, Burlingame, CA) for 40 min at room temperature. After detection, the slides were washed twice in PBS 1×/0.01% Triton and then twice in PBS 1× and mounted with a solution containing 4’,6-diamidino-2-phenylindole (DAPI) and anti-fade (BioBlue, BioView Ltd., Nes Ziona, Israel). Samples were analyzed using a fluorescence microscope (Nikon ViCo Video Confocal Microscope) equipped with a triple bandpass filter set (FITC, TRITC, DAPI). For each slide, at least 200 spermatozoa were analyzed. Evaluation of nuclear hybridization signals was performed in triplicate by different investigators. In samples that resulted negative during the follow-up, results were confirmed by PCR. When nuclei were completely and homogeneously stained and multiple small spots or single large signals were present, the sperm cells were classified as positive. The method was tested on control slides containing CaSkii cells, a human cervical carcinoma cell line with stably integrated and transcriptionally active HPV genomes, which served as a control for the specific probe. Cells smeared on salinated glass slides were fixed with 4% paraformaldehyde in PBS for 10 min. After fixation, cells were subjected to 3–5-min washes in PBS 1× and then dehydrated with 5-min ethanol washes (30%, 60%, and 95%). Cell smears were then air-dried and stored at 4°C until use.

STATISTICAL ANALYSIS

The values shown are the averages of at least three evaluations performed by different operators. Data regarding sperm parameters were presented as mean ± SD while data on semen infection as percentage. Differences between data were determined by two-tailed Student’s t-test after acceptance of normal distribution with the Kolmogorov-Smirnov test. Comparisons of proportion were performed with a one-sided non-parametric resampling test. P-values (two sided) <0.05 were considered statistically significant.

RESULTS

Mean age of patients with testicular cancer was 31.2 ± 5.4 years, not different from control subjects (30.8 ± 4.7 years). In Table 1 are reported sperm parameters and HPV DNA detection in semen
samples from controls and from patients evaluated before (T−1) and 1 month after orchiectomy (T0). At T−1, patients showed sperm concentration, count, and morphology not different from controls, but a significant reduction of progressive motility (p < 0.05). After orchiectomy, we found a significant reduction of sperm concentration and count (both p < 0.05 vs. T−1) and a further reduction of sperm motility compared to T−1 and control subjects (p < 0.01). Moreover, sperm volume and morphology were not significantly different in patients compared with controls before and after orchiectomy. Interestingly, the percentage of subjects with HPV infection was higher among patients than controls (9.7% vs. 2.4%). This percentage remained unchanged at T0. After orchiectomy, patients were assigned to three groups based on medical option: S = candidate to surveillance, R = candidate to radiotherapy, and C = candidate to chemotherapy with or without radiotherapy. In Table 2, are reported sperm parameters, the percentage of patients with semen infection and percentage of infected sperm observed in the three groups at T0 and after 12 months of surveillance or from the end of radiotherapy or chemotherapy (T12). At T0, considering patients from different groups we found no difference in sperm parameters and prevalence of HPV infection. Six patients were lost during follow-up (4 from group S, 1 from group R, and 1 from group C), therefore data at T12 are referred to the remaining 149 patients. Considering patients as a whole, the mean values of sperm parameters observed at T12 was not different from that at T0. However, patients who underwent surveillance had a significant increase of sperm number and motility compared to T0 (p < 0.05). In contrast, subjects who had radiotherapy showed no increase in sperm number and reduced sperm motility compared to S group (p < 0.05). Moreover, patients who underwent chemotherapy associated or not with radiotherapy, had a reduction of both sperm number and motility vs. T0 vs. patients from groups S (p < 0.05 and p < 0.01 respectively). In the three groups, we found no significant differences in both ejaculate volume and normal morphology comparing T12 with T−1 and T0. Considering all patients, HPV semen infection was significantly higher in patients at T12 respect to T0 (34.9% vs. 9.7%). By the comparison of different groups, the prevalence of infection resulted 7.7%, 30.8% and 61.5% in S, R, and C groups respectively. Finally, patients treated by chemotherapy had also a higher percentage of HPV infected sperm compared to T0 and to S (both p < 0.05). In Figure 1 there is an example of FISH analysis for HPV performed in a semen sample from testicular cancer patients.

### Table 1 | Sperm parameters and HPV DNA detection in semen samples from control subjects and patients at the time of diagnosis (T−1) and 1 month after orchiectomy (T0).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ejaculate volume (mL)</th>
<th>Sperm concentration (million/mL)</th>
<th>Sperm count (million)</th>
<th>Progressive motility (%)</th>
<th>Normal morphology (%)</th>
<th>Positive PCR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n = 84)</td>
<td>3.3 ± 0.8</td>
<td>46.9 ± 11.3</td>
<td>153.0 ± 53.1</td>
<td>53.4 ± 12.1</td>
<td>21.6 ± 6.4</td>
<td>2 (2.4)</td>
</tr>
<tr>
<td>T−1 (n = 155)</td>
<td>3.2 ± 1.3</td>
<td>40.1 ± 45.2</td>
<td>110.1 ± 125.3</td>
<td>34.2 ± 17.7*</td>
<td>18.9 ± 8.3</td>
<td>15 (9.7)*</td>
</tr>
<tr>
<td>T0 (n = 155)</td>
<td>3.1 ± 1.3</td>
<td>19.8 ± 16.6**</td>
<td>61.9 ± 45.5**</td>
<td>31.2 ± 15.1**</td>
<td>18.5 ± 9.3</td>
<td>15 (9.7)*</td>
</tr>
</tbody>
</table>

* p < 0.05 vs. control subjects.
** p < 0.01 vs. control subjects.
### Table 2 | Sperm parameters, HPV DNA detection and FISH for HPV observed in testicular cancer patients 1 month after orchiectomy (T0) and after 12 months (T12) from the end of any treatment.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ejaculate volume (mL)</th>
<th>Sperm concentration (million/mL)</th>
<th>Sperm count (million)</th>
<th>Progressive motility (%)</th>
<th>Normal morphology (%)</th>
<th>Positive PCR (%)</th>
<th>FISH on sperm (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S (n = 46)</td>
<td>2.9 ± 1.3</td>
<td>19.4 ± 14.4</td>
<td>54.6 ± 45.2</td>
<td>30.9 ± 14.1</td>
<td>19.8 ± 9.5</td>
<td>5 (11.1)</td>
<td>22.4 ± 8.3</td>
</tr>
<tr>
<td>R (n = 55)</td>
<td>3.2 ± 1.5</td>
<td>20.7 ± 19.7</td>
<td>67.1 ± 56.7</td>
<td>33.7 ± 17.6</td>
<td>17.9 ± 9.2</td>
<td>4 (7.2)</td>
<td>24.0 ± 3.7</td>
</tr>
<tr>
<td>C (n = 54)</td>
<td>3.1 ± 1.1</td>
<td>19.4 ± 15.2</td>
<td>62.7 ± 60.2</td>
<td>29.1 ± 12.9</td>
<td>17.9 ± 9.3</td>
<td>6 (10.9)</td>
<td>25.3 ± 3.8</td>
</tr>
<tr>
<td>Total (n = 155)</td>
<td>3.1 ± 1.3</td>
<td>19.8 ± 16.6</td>
<td>61.9 ± 45.5</td>
<td>31.2 ± 15.1</td>
<td>18.5 ± 9.3</td>
<td>15 (9.7)</td>
<td>24.0 ± 5.4</td>
</tr>
<tr>
<td>T12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S (n = 42)</td>
<td>3.1 ± 1.5</td>
<td>35.5 ± 25.7*</td>
<td>94.2 ± 93.5*</td>
<td>43.7 ± 18.2*</td>
<td>18.6 ± 10.1</td>
<td>4 (7.7)</td>
<td>24.9 ± 3.7</td>
</tr>
<tr>
<td>R (n = 54)</td>
<td>3.1 ± 1.7</td>
<td>23.8 ± 20.6</td>
<td>69.9 ± 58.7</td>
<td>30.8 ± 15.3#</td>
<td>18.0 ± 13.7</td>
<td>16 (30.8)*#</td>
<td>28.7 ± 6.0</td>
</tr>
<tr>
<td>C (n = 53)</td>
<td>3.3 ± 1.5</td>
<td>11.1 ± 9.9##</td>
<td>24.4 ± 17.4##</td>
<td>20.8 ± 14.2##</td>
<td>17.1 ± 11.2</td>
<td>32 (61.5)*##</td>
<td>39.6 ± 8.2##</td>
</tr>
<tr>
<td>Total (n = 149)</td>
<td>3.2 ± 1.5</td>
<td>22.6 ± 20.6</td>
<td>61.3 ± 50.5</td>
<td>30.7 ± 18.5</td>
<td>17.8 ± 11.5</td>
<td>52 (34.9)*##</td>
<td>29.4 ± 6.9</td>
</tr>
</tbody>
</table>

Data are shown as total and subgrouping patients in: S group = patients who underwent surveillance, R group = patients who underwent radiotherapy, and C group = patients who underwent chemotherapy followed or not by radiotherapy.

* p < 0.05 vs. T0.
** p < 0.01 vs. T0.
# p < 0.05 vs. S.
## p < 0.01 vs. S.
DISCUSSION

It is of concern, especially during last years, the increasing incidence of reproductive tract diseases and particularly testicular cancer (Richardson et al., 2012). The latter disease is particularly found among males aged between 15 and 40 years (Siegel et al., 2012; Viatori, 2012) and, although it is a curable disease with a good prognosis, it is a major risk factor for fertility (Jacobs and Vaughn, 2012). In fact, patients with testicular cancer have frequently a concomitant alteration of spermatogenesis already at diagnosis, that can be further affected by chemo or radiation therapies (Schrader et al., 2001; Bieber et al., 2006). In this paper, we considered sperm parameters and prevalence of HPV semen infection in testicular cancer patients pre- and post-orchiectomy. Moreover, after 12 months we evaluated the effect of different medical options on the same parameters. As previously reported (Selice et al., 2011), in affected patients we observed reduced sperm motility at diagnosis that was still present 1 month after orchiectomy together with a significant reduction of sperm number. Interestingly, we found that patients at diagnosis had a significant increase of HPV semen infection compared to controls. This finding could represent a cause of testicular cancer or an effect of this malignancy. Many hypotheses have been raised regarding the possible cause of testicular cancer development, as in utero and pre-pubertal exposure to endocrine disrupters, genetic predisposition, and environmental conditions. Despite it is well-recognized that some viral infection have a major role in the development of many cancers (EBV, HCV, HPV, HIV etc.), this link has never been proposed for testicular cancer. In particular, HPV infection susceptibility. In this study, we found that after surveillance 7% of patients had HPV semen infection rising to 31% and 61% after radio and chemotherapy respectively. Therefore, drug-mediated immunosuppression seems to be strictly related to HPV seminal infection in testicular cancer patients. Finally considering FISH analysis for HPV, beside a trend to increase in R group, we observed a significantly higher percentage of infected sperm in subjects who received chemotherapy. This is the first study evaluating HPV sperm infection in semen samples from patients with testicular cancer. By our findings we can conclude that HPV infection is more prevalent in affected patients than controls. However, as recently suggested by other authors (Bertazzoni et al., 2013), this infection seems not to be cause of testicular cancer, but rather the effect of a status of higher vulnerability induced by the tumor. More and larger studies of follow-up of HPV infected patients will clarify whether or not there is a cause-effect relationship between HPV and testicular cancer or vice versa. Moreover, our results demonstrated that treatments frequently used in testicular cancer patients and in particular chemotherapy, are able to strongly increase the prevalence of HPV semen infection, probably through their immunosuppressive action. In conclusion, we suggest testing sperm HPV in patients affected by testicular cancer before and particularly after treatments inducing immunosuppression, because this infection can in turn induce cancer in many sites and further reduce male fertility. Further and larger studies are needed to confirm our findings also in patients affected by other tumors.

REFERENCES


Eklund, C., Zhou, T., Dillner, J., and WHO Human Papillomavirus Laboratory Network. (2010). Global proficiency study of

Conflict of Interest Statement: The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 October 2012; accepted: 06 December 2012; published online: 21 December 2012.
This article was submitted to Frontiers in Cancer Endocrinology, a specialty of Frontiers in Endocrinology. Copyright © 2012 Garolla, Pizzol, Bertoldo, Ghezzi, Carraro, Ferlin and Foresta. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.
INTRODUCTION

The right way to treat undescended testes (UDT) remains to be solved. Although much is known about the development of the testes and sperm cells, there is a missing link in our knowledge. It is not known when and how the progenitors of germ cells transform from neonatal gonocytes into spermatogonia or spermatogenic stem cells. Recent research suggests that a critical stage of sperm cell development may occur in the early postnatal period. Germ cell development has been studied extensively in rodents the second phase occurs in the first week after birth (Hutson et al., 1997). The regulation of this two-stage process is not the subject of this review, but interested readers should see some recent reviews (Suell and Hartung, 2005; Adham and Agoulnik, 2004; Amann and Verreamachaneni, 2007; Bay et al., 2011).

In baby boys, the temperature of the intra-scrotal testes drops to 35°C at birth, with all the testicular enzyme systems readjusting presumably within a few weeks to this new environment (Zorziotti, 1991). Between 2 and 4 months of age pituitary gonadotropins stimulate a sudden increase in testosterone production which peaks at about 3–6 months, before it wanes and becomes almost negligible until the onset of puberty. This brief

Germ cell development in the postnatal testis: the key to prevent malignancy in cryptorchidism?

John M. Hutson1*, Ruili Li1, Bridget R. Southwell2, Bodil L. Petersen3, Jorgen Thorup4 and Dina Cortes5

1 Department of Urology, Royal Children’s Hospital, Parkville, VIC, Australia
2 F Douglas Stephens, Surgical Research Group, Murdoch Childrens Research Institute, Melbourne, VIC, Australia
3 Department of Paediatric Surgery and Pathology, Rigshospitalet, Faculty of Health Science, University of Copenhagen, Copenhagen, Denmark
4 Department of Paediatrics, Hobart Hospital, Faculty of Health Science, University of Copenhagen, Copenhagen, Denmark
5 Department of Paediatrics, Hvidovre Hospital, Faculty of Health Science, University of Copenhagen, Copenhagen, Denmark

To permit normal postnatal germ cell development, the mammalian testis undergoes a complex, multi-staged process of descent to the scrotum. Failure of any part of this process leads to congenital cryptorchidism, wherein the malpositioned testis finds itself at the wrong temperature after birth, which leads to secondary germ cell loss and later infertility and risk of cancer. Recent studies suggest that neonatal gonocytes transform into the putative spermatogenic stem cells between 3 and 9 months, and this initial postnatal step is damanged in cryptorchid testes. In addition, it is thought the abnormality high temperature may also impair apoptosis of remaining gonocytes, allowing some to persist and to become the possible source of carcinoma in situ and malignancy after puberty. The biology of postnatal germ cell development is of intense interest, as it is likely to be the key to the optimal timing for orchidopexy.

Keywords: germ cell, gonocyte, spermatogium, testis, cryptorchidism, orchidopexy
FIGURE 1 | Testicular descent. (A) Cranial suspensory ligament (CSL) and gubernaculum (G) hold the testis. Testosterone (T) and MIS/AMH act on Wolffian duct (WD) and Müllerian duct (MD). (B) Insulin 3 (Insl3) causes the gubernacular swelling reaction that holds the testis near the groin during midgestational growth (8–15 weeks). (C) Calcitonin-gene-related peptide (CGRP) released by the genitofemoral nerve, and under the control of testosterone, steers the migrating gubernaculum to the scrotum.

surge of gonadotropins and androgens is known as “mini-puberty” (Job et al., 1988; Hadziselimovic and Zirkovic, 2007). As androgen levels subside there is a surge in production of MIS/AMH by Sertoli cells, which peaks at 6–12 months, but remains high then until puberty when serum levels fall (Baker et al., 1990; Yamanaka et al., 1991; Aks glaede et al., 2010; Figure 2).

ABNORMAL POSTNATAL DEVELOPMENT IN UDT IN HUMANS

Failure of the first or second phase of testicular descent leads to congenital cryptorchidism. The causes of UDT remain largely unknown with some authors suggesting a primary testicular hormonal malfunction (Heyns and Hutson, 1995), while others have suggested a secondary hormonal malfunction, such as hypotalamic-pituitary axis deficiency (Hadziselimovic and Zirkovic, 2007) or a placental failure to produce chorionic gonadotropin (Heyns and Hutson, 1995). Based on twin studies, it has also been suggested that risk factors should be sought in the intrauterine environment and the maternal genes. However, another possibility first suggested in the eighteenth and nineteenth centuries, was that there was an anatomical defect in the mechanism of descent. With the establishment of endocrinology in the early twentieth century, these anatomical concepts were mostly replaced with explanations of hormonal dysfunction, such as described above (Heyns and Hutson, 1995). However, our own research over the last 25 years, along with others, has suggested

FIGURE 2 | “Mini-puberty,” with testosterone (T) and MIS/AMH secreted after birth. Neonatal gonocytes mature into adult dark spermatagonia between 3 and 9 months of age, and primary spermatocytes form about 3–4 years. After a period of quiescence, spermatids form about 10 years of age, with the onset of spermatogenesis. Undescended testis (UDT) interferes with the first step.
that anatomical faults may be a common cause of cryptorchidism (Backhouse, 1964). Cryptorchidism is associated with other congenital abnormalities in less than 20% of the cases, but when present they are often related to abnormalities of the midline and the caudal development field of the body (Cortes, 1998; Jensen et al., 2010; Thorup et al., 2010). Two key observations in favor of this view are the failure to document convincing primary hormonal defects, as well as the realization that the second phase of descent is a very complex anatomical process, that is likely to be prone to subtle anomalies (Amann and Verramacheni, 2007; Thorup et al., 2012). This is further supported by the fact that the anomaly most often occurs unilaterally, and in cases where associated Wolfian duct and/or ureteric bud malformations are present, these are predominantly found ipsilaterally (Cortes et al., 1998a).

If something goes wrong with migration of the gubernaculum from the external inguinal ring in the groin across the pubis and into the scrotum, the initially normal postnatal testis is retained deep to the inguinal fat pad, which is an effective insulator, keeping the UDT at 34–37°C (Mieusset et al., 1993), rather than the normal scrotal temperature of 33°C, and this is believed to trigger the progressive postnatal testicular dysfunction, as the postnatal testis function is only optimal at 33°C (Zorgniotti, 1991). The exquisite temperature sensitivity of the testis has been well documented for a long time, but is currently not a fashionable area of research in this era of molecular biology. However, the phenomenon has been solidly proven despite the genetic regulation of it not being fully described. This abnormal postnatal development in the testis also leads to inhibited postnatal androgen production (Raivo et al., 2003) and MIS/AMH production at 6–12 months of age (Yamanaka et al., 1991).

The net effect of cryptorchidism is germ cell loss, leading to infertility (Mengel et al., 1974; Hadziselimovic, 1983). This has been well described in many long-term outcome studies in men with a previous history of cryptorchidism in childhood (Cortes, 1998; Gracia et al., 2000; Cortes et al., 2001; Vincardi et al., 2001). However, the cause for this germ cell loss and its timing have remained elusive until relatively recently. In the 1950s, orchidopexy was recommended in boys aged 10–15 years if the cryptorchid testes failed to descend spontaneously into the scrotum at the onset of puberty (Gross and Jewett, 1956; Vincardi et al., 2001). This recommendation was based on the clear observation that a significant number of cryptorchid testes descended spontaneously at puberty. In addition, it was assumed that germ cell development was totally quiescent in childhood. Indeed, it was thought that the testis was effectively in “hibernation” until the onset of spermatogenesis in early childhood.

Current long-term follow-up studies of men with a previous history of cryptorchidism show a 5- to 10-fold increase in malignancy risk (Whitaker, 1988; Wood and Eldridge, 2009). However, it is easy to forget that because of the very long lag-time (i.e., 30–40 years) between intervention and measurement of the outcome, in this case development of testicular malignancy, that the age of orchidopexy in most of these studies in the current literature is about 5–15 years of age. This has led to the widespread view in adult endocrinology and oncology that orchidopexy is unable to prevent development of cancer in men (Gracia et al., 2000). However, the age for orchidopexy is now much younger, and it remains to be seen whether this will alter outcomes in adulthood. Recent studies actually indicate that operation for cryptorchidism at a young age lowers the risk of testicular cancer in adulthood (Petersson et al., 2007).

Meanwhile, in pediatrics, it first became apparent in the 1970s that degeneration of the UDT was occurring in early childhood (Mengel et al., 1974). Initially it was noted that there was macroscopic atrophy of the testis in early primary school years leading to the view that maybe orchidopexy should be done in 5- to 6-year-old boys rather than at 10–15 years, based on the assumption that this might prevent the obviously visible development of atrophy. During the 1970s and early 1980s there was histological evidence accumulating of degeneration visible in boys of 2 years of age with UDT, leading to the recommendation in pediatric surgery that orchidopexy might be optimally done in 2-year-old boys (Hadziselimovic et al., 1975). It was then appreciated that there were signs of early degeneration in the testis on electron microscopy at about 12 months of age (Hadziselimovic, 1983, Figure 3).

Furthermore, it was reported that all UDT harbored germ cells at the time of birth, but the number of germ cells was decreased in about one-fourth of cryptorchid newborns (Cortes, 1998). Lack of germ cells has been reported from 12, and especially from 18 months of age, and therefore surgery has been recommended before 12 or 18 months of age (Rütten et al., 2007).

Recent studies have implicated the very first phases (i.e., in the first year) of postnatal germ cell development in the etiology of the subsequent infertility and risk of malignancy. However, we first need to describe normal germ cell development so that the effects of cryptorchidism can be understood in contrast.

NORMAL GERM CELL DEVELOPMENT

Around 4–6 weeks of gestation within the embryonic urogenital ridge the primitive gonad forms on the anteromedial surface of the middle kidney or mesonephros. Primordial germ cells form from endodermal cells in the caudal edge of the yolk sac stalk, and then migrate into the embryonic celom around the wall of the midgut and into the urogenital ridge, eventually colonizing the ambisexual gonad and differentiating into gonocytes. By 22 weeks of gestation, these primitive germ cells mature into fetal spermatogonia (Hadziselimovic, 1983). Mesenchymal cells in the 7- to 8-week ambisexual gonad surrounding the arriving germ cells form the Sertoli cells with the onset of sexual differentiation to create the testicular cords, inside a basement membrane. Outside the cords some mesenchymal cells form Leydig cells and begin hormone synthesis to produce androgen and insulin-like hormone 3 (INSL3), while others form into myoepithelial cells around the cords.

At birth, the fetal spermatogonia or neonatal gonocytes are located in the center of the spermatic cords. Throughout childhood it appears that the germ cells remained in the center of the cords, and seemed to be in “hibernation” until the onset of spermatogenesis at puberty, as there was little histological change in their appearance or location. However, in recent years, it became apparent that by 3–4 years of age the cell in the center of the
have been classified as dark type-A (Ad), pale type-A (Ap), and type-B. Ad-spermatogonia have nuclei showing a homogenous, dark stainable chromatin and one or more clear cavities of vacuole like appearance (Clermont, 1963). The type-A spermatogonium has become the likely stem cell of spermatogenesis (de Rooij and Russell, 2000; Drummond et al., 2001; Biers and Malone, 2010). Interestingly not all neonatal gonocytes transform into type-A spermatogonia, so that by 1–2 years of age the total germ cell number is less than half that at birth (Hadziselimovic, 1983). The remaining gonocytes are thought to undergo apoptosis, or programmed cell death, so that by 2 years of age there are none left in the testis (Huff et al., 2001b). This process can be seen readily in the postnatal rat testis, as signs of apoptosis are rare until day 3, but by day 4 analysis shows that one-third of them are lost (Roosen-Ruige and Leik, 2000).

**THE SPERMATOCYTOGENIC STEM CELL**

As neonatal gonocytes seem to differentiate into unipotent stem cells for subsequent spermatogenesis, their change in appearance, location within the cord, and function suggest dramatic changes in germ cellular physiology. These changes are regulated by a precisely coordinated expression of key proteins, some of which are known. In early embryos, germ cells and pluripotent embryonic stem cells can form any type of cell. However, as development proceeds, the ability of germ cells to form different cells becomes restricted. In tests a subpopulation of germ cells retain the ability to differentiate, but only as unipotent spermatogenic stem cell (SSCs; Hofmann et al., 2005).

Huckins and Oakberg have proposed a widely accepted model for spermatogenic development which is useful to describe here (Huckins, 1971; Oakberg, 1971; de Rooij and Russell, 2000). In this model, single type-A spermatogonia are the putative SSCs, which can self-renew, while paired type-A spermatogonia are differentiating paired daughter cells connected by an intercellular bridge. Paired type-A spermatogonia divide into chains of aligned cells, which then become type A1, A2, A3, and then A4 spermatogonia. The latter cells (A4) divide to form intermediate spermatogonia and the type-B spermatogonia. Type-B cells then divide to form primary spermatocytes that enter meiosis (Figure 4). All these steps are regulated by growth factors from Sertoli and possibly the peritubular myoid cells (Skinner, 1991; Jegou, 1993). A recent morphological study (Drummond et al., 2001) showed that postnatal development of type-A spermatogonia may occur more rapidly than in mature spermatogenesis.

The type-A spermatogonia and SSCs have been shown recently to express a range of different markers (Figure 5). Undifferentiated SSCs express the integrins β-1 and α-6 (Shinohara et al., 1999), and the receptors for glial-derived neurotrophic factor (GDNF), such as GDNF family receptor alpha-1 (GFRα-1) and receptor tyrosine kinase (RET) (de Rooij and Russell, 2000). In addition undifferentiated SSCs express Zfhb-16 (previously known as Pfl; Buas et al., 2004; Costoya et al., 2004). Once SSCs start to differentiate, they begin expressing a range of different markers, including c-Kit, the receptor for stem cell factor (Besmer et al., 1993; Yoshida et al., 2006). They also express Sohlh1 and Sohlh2, as well as neurogenin 3 (Ballow et al., 2006; Filippini et al., 2007; Hase et al., 2008).

As can be seen from this brief overview, most of what we know about SSCs...
FIGURE 4 | Postnatal germ cell development in humans. (A) Gonocytes migrate from the center of the cords to the basement membrane around 6 months, and become type-A spermatogonia. By 3–4 years of age the center of the cords becomes recolonized with primary spermatocytes. (B) Numbers of germ cells/tubule in a normal testis and undescended testis (UDT) relative to age in years. Note the normal fall in total numbers between birth and 2 years, but failure of this to recover in UDT. The shaded area shows the normal range and the dotted lines show the average numbers.

FIGURE 5 | Germ cell development in the first week postnatally in the rat, showing the germ cells labeled with MVH (mouse homolog of Drosophila Vasa), and the Sertoli cells labeled with MIS/AMH (bar = 10 μm). (A) Day 0, (B) Day 4, (C) Day 6, (D) Day 10.
comes from studies of adult spermatogenesis or stem cell research, rather than a direct study of the type-A spermatogonium in the postnatal testes.

**ABNORMAL GERM CELL DEVELOPMENT**

All cryptorchid testes of newborns contain germ cells, although in some testes the number is impaired compared to normal. So significant deranged germ cell development in cryptorchid testes probably begins postnatally, except in the dysembryonic testis in disorders of sex development (DSD). The first step to fail is transformation of gonocytes into type-A spermatogonia, which is delayed or interrupted. Evidence for this disruption is found in the persistence of large numbers of gonocytes in the center of the testicular cords well beyond 6 months of age (Figure 6), and a decreased number of type-A spermatogonia (Hadziselimovic et al., 1986; Huff et al., 1989, 1991). After the first year or so the number of spermatogonia decrease, while those that remain are gonocytes with bizarre nuclei (Hadziselimovic, 1983). By 3–4 years of age the abnormality has become even more obvious, with failure of primary spermatocytes to appear (Huff et al., 1989).

The abnormally high temperature of the cryptorchid human testis is considered by most authors to be the cause of this germ cell maldevelopment. Considerable studies have been performed in various animal models showing that heat stress leads to a combination of both indirect and direct effects on the germ cells, causing impaired transformation and maturation as well as inhibited apoptosis. This thermal injury is mediated by reactive oxygen species and certain heat-shock proteins, which damage the germ cells as well as Sertoli cells (Setchell, 1998; Zini et al., 1999; Bell and Hartung, 2003).

Defective transformation of gonocytes into type-A dark spermatogonia between 3 and 9 months of age correlates with abnormal sperm counts after puberty (Hadziselimovic and Herzog, 2000a; Huff et al., 2001a). In cases of no adult dark spermatogonia, the age of orchidopexy has not affected a poor result for spermatogenesis in adulthood, at least so far (Hadziselimovic and Herzog, 2001b). The degree of risk of subsequent infertility can be quantitated, as it is almost inevitable if there are fewer than 1% of the number of spermatogonia per tubular cross-section, seen in biopsies from age-matched controls (Cortes et al., 1996). Under normal physiological circumstances, the surge of gonadotropins and testosterone, and possibly other as yet unknown hormones, occurs at 2–3 months of postnatal age. This hormonal surge stimulates the gonocytes in the testis to develop into adult dark spermatogonia. In cryptorchidism there has been reported evidence for a beneficial role of adjuvant gonadotropin-releasing hormone to improve fertility in UDT (Biers and Malone, 2010). It has been questioned as a general recommendation (Hutson, 2010). Recently there have been described different groups of cryptorchid boys, and if the patients at the time of surgery have a decreased number of germ cells and/or inhibit B antibodies may be involved in the germ cell loss in cryptorchidism, but there is no evidence for postpubertal antibodies after previous surgery in childhood for cryptorchidism (Miralas et al., 2003). All these studies suggest that the postpubertal poor fertility is likely to be caused by an abnormally small pool of SSCs, the type-A spermatogonia.

The risk of cancer after cryptorchidism in infancy is less easily correlated with abnormal germ cell development after birth. It has been described that the risk of developing a testicular germ cell cancer is about 5– to 10-fold higher in men with a previous history of cryptorchidism compared with those with descended testis in childhood (Chillers et al., 1986; Gwentman et al., 1987; Moller et al., 1998; Wood and Elder, 2009). Testicular germ cell tumors are extremely common, affecting 1% of young men (Borth et al., 2000).

The common precursor of those tumors is the premalignant CIS cell, which is proposed to be a fetal germ cell that failed to differentiate, and remains, dormant in the testis until after puberty (Skakkebaek et al., 1982). The evidence supporting this view comes from the many similarities between CIS cells and fetal gonocytes, which share many markers and morphological characteristics (Holstein and Korner, 1974; Skakkebaek et al., 1982; Borth et al., 2000; Rappert De Meyts et al., 2003). CIS cells may well originate during fetal development in DSD, where the germ cell physiology is deranged by the underlying genetic anomaly. By contrast, this seems less plausible in cryptorchidism if the maldescent is caused by extraneous mechanical defects.

In cryptorchidism, therefore, the CIS cell may arise from the neonatal gonocytes, which are abnormally abundant. The gonocytes have pluripotent stem cell properties, which if they persist until after puberty, with or without mutation, may develop into cancer.

Carcinoma in situ cells are more likely to be present where there are specific genetic defects, and there may be developmental arrest (Skakkebaek et al., 1987, 1998; Honcser et al., 2004). Expression profiles of CIS cells and embryonic stem cells are very similar, which supports the view of CIS being a multipotent stem cell (Almstrup et al., 2004). A unifying hypothesis has been proposed to link testicular maldevelopment, testicular cancer, low sperm...
Hutson et al. Postnatal germ cell development

counts, and cryptorchidism, known as the testicular dysgenesis syndrome (TDS, Asklund et al., 2004).

Carcinoma in situ is more common in men with previous cryptorchidism, and is documented in 2–3% of adult patients (Givarcman et al., 1987, 1989). However, the prevalence of CIS is higher in those men with macroscopic testicular atrophy (Harland et al., 1998) or those with bilateral cryptorchidism (Swezlow et al., 1997). Where cryptorchidism is unilateral, the contralateral descended testis has a slightly increased risk of cancer (Forman et al., 1994). Not surprisingly, cancer risk is also increased where testes were intra-abdominal, the genitalia were abnormal or there was a known abnormal karyotype (Cortes et al., 1999).

In a pediatric collective series of six studies, the risk of CIS in 2800 boys with cryptorchidism was 0.36% only (Cortes, 1998). When compared to higher incidence data from adults these pediatrnic figures support the hypothesis that the characteristics of CIS cells and later testicular cancer develops over time, and are not present from fetal life. Generally CIS in pediatric series is described in later childhood (Cortes et al., 1999). However, in this paper we present a 12-month-old boy with suspected CIS in bilateral cryptorchid tests (Figure 7). It has to be emphasized that positive immunohistochemical markers indicating CIS in the adult testis do not have such predictive value when applied to the childhood cryptorchid tests. Positive staining by such markers of persistent fetal germ cells is seen in almost half of the cryptorchid testes of boys younger than 5 years (Thorup et al., 2012). Presence of placental alkaline phosphatase (PLAP) positive immunohistochemical stained gonocytes in bilateral cryptorchid tests of infant boys indicates the ability of delayed germ cell transformation and a preserved good fertility potential (Cortes et al., 2012). Further research in this field is therefore needed.

It has been suggested that testicular biopsy itself at the time of orchidopexy is a risk factor for postpubertal cancer (Swezlow et al., 1997). In the review by Swezlow et al. (1997) only 9% of the operated tests were subjected to biopsy, consistent with a possible selection bias by the surgeon, who may have only picked the more atrophic tests for analysis. This view is supported by a large study of 838 cryptorchid patients having routine biopsy, where there was no correlation between the biopsy itself and subsequent malignancy (Moller et al., 1998).

Some authors have suggested that adolescents with a previous history of cryptorchidism and orchidopexy should be offered a biopsy to identify if CIS is present, before invasion has occurred (Givarcman et al., 1989). Alternative screening methods such as reliable blood or semen analysis are not yet available, although testicular ultrasonography may have a place (Holm et al., 2001). Ultrasonography shows a relationship between microscleroses and CIS, but the significance of this is uncertain (Lentz et al., 1996), particularly as more recent studies of testicular microsclerosis show that it is quite common in normal adolescents (Goede et al., 2009). CIS is also associated in some patients with cryptorchidism with multinucleated germ cells, consistent with aberrant germ cell development (Cortes et al., 2003).

There is inconclusive evidence that an early age of surgery leads to a reduction in risk of cancer (Pike et al., 1986, Swezlow et al., 1997; Wood and Elder, 2009). However, because of the long lag-time between treatment and outcome, the age at surgery in these follow-up studies averaged more than 2 years old. In the context of the proposal, that early germ cell development is the key, these long-term results are consistent with either no effect of age at all, or with the suggestion that lack of neonatal gonocyte transformation and impaired apoptosis predisposes to malignancy.

FIGURE 7 | Testicular tissue from a 12-month-old boy with bilateral cryptorchidism. Carcinoma in situ (CIS) is suspected. (A) HE staining: Sertoli cells are normal and the germ cell number is at the upper range for age. Some germ cells have two nuclei, and some nuclei have irregular morphology and some nucleolus. A microlith is clearly seen within a tubule. (B) Strong PLAP expression seen in all germ cells. (C) OCT4 expression in germ cells also in the periphery of tubules, which is not often seen so impressively in most cryptorchid testes at a similar age.
One of the problems that has bedeviled the relationship between cryptorchidism and malignancy is the lack of an animal model, as rodents with UDT do not develop testicular tumors. Another problem is that evidence is accumulating that there are some testes that become undescended after birth, and that this group, now known as acquired cryptorchidism, have a different risk of malignancy compared with congenital UDT.

**ACQUIRED CRYPTORCHIDISM**

After birth, the distance from the inguinal canal to the scrotum increases from 4–5 to 8–10 cm in early adolescence (Smith et al., 1989). This means that the spermatic cord must double in length between birth and puberty (Hutson et al., 2010). We have previously proposed that failure of this elongation is the cause of ascending testes or acquired UDT (Hutson and Hasthorpe, 2005), and this is the likely explanation for many children still having orchidopexy at 5–10 years, despite recommendations for surgery in infancy (Donaldson et al., 1996).

Realization that there are two types of UDT has triggered a re-evaluation of the cause and effect of UDT. First, it is becoming apparent that men who had untreated, acquired UDT have no increased cancer risk, but still have impaired fertility (Cortes et al., 1998b). Secondly, it means that current long-term follow-up studies in adults are likely to contain a mixture of patients with both congenital and acquired UDT (Bone et al., 2008), making their interpretation difficult.

Investigators have puzzled over why UDT in rodents caused infertility but not cancer, as in humans. We propose that this is the result of differences in timing in when the testis reaches the low-temperature scrotal environment. In humans, the testis is at 33°C from birth, so that congenital UDT has the potential to damage neonatal gonocytes by high temperature. By contrast, rodent testes only reach the scrotum after 12 days of age (Bergh, 1991), which is well after normal gonocyte transformation and/or apoptosis is complete (Figure 8).

Undescended testis in rodents, therefore, is actually a model for acquired UDT in humans, with high temperature damaging the SSCs, but with no cancer risk as the gonocytes will have developed or disappeared normally. Once this key difference in timing of testicular descent is understood, extrapolation between rodent models and the human situation is straightforward, as other key processes are the same. Both species groups have a postnatal “mini-puberty” at 3–6 months in humans and 2–4 days in mice, when there is burst of testicular hormones and cytokines which are likely to be involved in gonocyte development. In addition, gonocyte migration from the center of the cords to the basement membrane to form SSCs is similar in both timing and general morphology.

A temperature difference between undescended and normally descended testes in rats is not present until after 12 days of age (Zorgniotti, 1991), which is well after the gonocytes should have transformed normally into SSCs or have undergone apoptosis. If excess persisting gonocytes are the cause of CIS and cancer in cryptorchidism, then none will be present in the tests when the temperature becomes abnormal in rodent UDT, consistent with the lack of cancer in these models. However, the high temperature of the UDT will still damage SSCs leading to subsequent infertility (Figure 8).

**Figure 8** | Testicular temperature in humans and rodents.

(A) Human gonocytes mature in the first year when the testicular temperature is 33°C. Congenital UDT have abnormal temperature early, which interferes with gonocyte development. By contrast, acquired UDT only interferes with subsequent survival of stem cells. (B) In rodents, gonocytes mature before a change in temperature occurs, so that gonocyte development is unaffected, similar to acquired UDT in humans.
CONCLUDING REMARKS

All the evidence available points to early germ cell maturation being the “missing link” in the disconnection between the timing of orchidopexy and the subsequent risks of both infertility and malignancy. This suggests that infants getting real early operation may have significantly improved outcome compared to surgical delay. Furthermore, in some cases supplemental hormonal treatment may be needed to achieve the normal transformation to adult spermatogenesis.

The key role of heat in interfering with the signaling that controls both gonocyte transformation and/or apoptosis needs to be reinvestigated fully, as it is likely to lead to changes in clinical management of congenital cryptorchidism.

REFERENCES


macological, serum and hormonal data in 251 adults operated on for cryp-
Hadziselimovic, F., and Herzog, B. (1983). Cryp-
Hadziselimovic, F., and Herzog, B. (2001a). Importance of early postna-
Hadziselimovic, F., Herzog, B., and Seguda, H. (1975). Surgical cor-
nexion of cryptorchid testis at 2 years: electron microscopic and histo-
Hadziselimovic, F., Thommen, L., Gurtner, G. C., and Herzog, B. (2000). The significance of postnat-
al gonadotropin surplus for testicu-
Hadziselimovic, F., and Zevkovich, D. (2007). Is the prohibition of hor-
Hao, E., Jonsson, M., Richardson, T. E., Chapman, K. M., Daneels, R. S., Hammer, R. E., et al. (2006). Sertoli cell-derived mIAP is male-
sterile because of degeneration of differentiating-type A spermatogonia. Stem Cells 26, 1547–1557.
cept of testicular tumor in testicular cancer: defin-
ing a high risk group. J. Urol. 159, 1533–1537.
germ line stem cells. Stem Cells 23, 208–213.
Iones, M. S., Toh, G., Thinhung, A. M., Hansen, T. H., Olsen, I., Christensen, K., et al. (2010). Crypt-
torchidism concordance in monozy-
gotic and dizygotic twin brothers, full-
brothers, and half-brothers. J. Urol. 185, 154–158.
Lenz, S., Skakkebaek, N. E., and Her-
tel, N. T. (1996). Abnormal ultra-
Molander, J. (1987). Sertoli cell-derived mIAP is male-
sterile because of degeneration of differentiating-type A spermatogonia. Stem Cells 26, 1547–1557.
Molander, J., Van, M., Richardson, T. E., Chapman, K. M., Daneels, R. S., Hammer, R. E., et al. (2006). Sertoli cell-derived mIAP is male-
sterile because of degeneration of differentiating-type A spermatogonia. Stem Cells 26, 1587–1589.
Molander, J., Van, M., Richardson, T. E., Chapman, K. M., Daneels, R. S., Hammer, R. E., et al. (2006). Sertoli cell-derived mIAP is male-
sterile because of degeneration of differentiating-type A spermatogonia. Stem Cells 26, 1587–1589.
Molander, J., Van, M., Richardson, T. E., Chapman, K. M., Daneels, R. S., Hammer, R. E., et al. (2006). Sertoli cell-derived mIAP is male-
sterile because of degeneration of differentiating-type A spermatogonia. Stem Cells 26, 1587–1589.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 16 October 2012; accepted: 11 December 2012; published online: 03 January 2013.


This article was submitted to Frontiers in Cancer Endocrinology, a specialty of Frontiers in Endocrinology.

Copyright © 2013 Hutson, Li, Southwell, Petersen, Thorup and Cortes. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

“fendo-03-00176” — 2012/12/31 — 11:26 — page 11 — #11
Genome-wide assessment of the association of rare and common copy number variations to testicular germ cell cancer

Daniel Edsgård¹, Marlene D. Dalgaard², Nils Weinhold³, Agata Wesolowska-Andersen¹, Ewa Rajpert-De Meyts¹, Anne Marie Ottesen², Anders Juul², Niels E. Skakkebæk², Thomas Skot Jensen¹, Ramneek Gupta¹, Henrik Leffers² and Søren Brunak¹*¹

1 Department of Systems Biology, Technical University of Denmark, Lyngby, Denmark
2 Department of Growth and Reproduction, Rigshospitalet, Copenhagen, Denmark

INTRODUCTION

Testicular germ cell cancer (TGCC) is one of the most heritable forms of cancer. Previous genome-wide association studies have focused on single nucleotide polymorphisms, largely ignoring the influence of copy number variants (CNVs). Here we present a genome-wide study of CNV on a cohort of 212 cases and 437 controls from Denmark, which was genotyped at ~1.8 million markers, half of which were non-polymorphic copy number markers. No association of common variants were found, whereas analysis of rare variants (present in less than 1% of the samples) indicated a single gene with significantly higher accumulation of rare CNVs in cases as compared to controls, at the gene PTPN1 (P = 3.8 x 10^-2, 0.9% of cases and 0% of controls). However, the CNV could not be verified by qPCR in the affected samples. Further, the CNV calling of the array-data was validated by sequencing of the GSTM1 gene, which showed that the CNV frequency was in complete agreement between the two platforms. This study therefore disconfirms the hypothesis that there exists a single CNV locus with a major effect size that predisposes to TGCC. Genome-wide pathway association analysis indicated a weak association of rare CNVs related to cell migration (false-discovery rate = 0.021, 1.8% of cases and 1.1% of controls). Dysregulation during migration of primordial germ cells has previously been suspected to be a part of TGCC development and this set of multiple rare variants may thereby have a minor contribution to an increased susceptibility of TGCCs.

Keywords: copy number variation, rare variants, testicular germ cell cancers

Testicular germ cell cancer (TGCC) is the most common malignancy in young men aged 15–45 years. The incidence has increased over the last decades and is highest in the Nordic countries with 8–9 cases per 100,000, whereas the incidence in men of African and Asian ancestry is five-fold lower (Chia et al., 2010). Environmental exposure partly explains the increasing incidence and ancestral disparity (Skakkebæk et al., 2001), but there is also evidence of a substantial genetic contribution to TGCC susceptibility. The familial aggregation of TGCC is one of the highest of all cancers. Brothers and sons of TGCC patients have an 8–9 times and 4–6 times higher risk to develop the disease, while the risk increases 75- and 35-fold for monozygotic and dizygotic twins, respectively (Swerdlow et al., 1997; Hemminki and Li, 2004). Despite the relatively high degree of heritability, genome-wide familial linkage analyses have not identified any loci predisposing for TGCC, and candidate studies have only found one rare deletion (2–3%) at the Y chromosome that confers a modest 2–3 fold increased risk (Nathanson et al., 2005). Recently, genome-wide association studies that search for common single nucleotide variants associated to TGCC have identified susceptibility loci at the genes KITLG, SPRY4, BAK1, DMRT1, TERT, and ATF7IP (Kanetsky et al., 2009; Rapley et al., 2009; Turnbull et al., 2010; Dalgaard et al., 2012). The strongest association was found at KITLG with a greater than 2.5-fold increased risk of disease. Consistent with the relatively high familial relative risk, this is the largest effect size found for any single loci among cancers. However, a considerable portion of the heritability remains to be explained.

Here we investigate constitutional DNA copy number variations (CNVs) as another source of genetic variability that may contribute to the development of TGCC. Recent studies have described associations of common CNVs with neuroblastomas (Diskin et al., 2009), systemic autoimmunity (Fanciulli et al., 2007), psoriasis (Hollox et al., 2007), and osteoporosis (Yang et al., 2008). Rare variants, typically originating from recent and de novo events, constitute a significant portion of genomic variation. The thousand genomes project indicates that there are about 20,000 CNVs with allele frequencies down to 1% (1000 Genomes Project Consortium, 2010; Mills et al., 2011). The contribution of such rare, or even rarer, variants, to complex disease susceptibility is to a large extent unknown, but they seem to play an important role in psychiatric disorders (International Schizophrenia Consortium, 2008; Pinto et al., 2010) and they have been indicated to influence childhood obesity (Glessner et al., 2010). Further, identification of
de novo mutations is possible in studies of family-trios and recently three de novo CNVs were found in 3 out of 43 TGCC trios, a frequency higher than found in two other cancer types (Stadler et al., 2012), highlighting the paradigm of rare genetic events influencing susceptibility to TGCC. To date, case-control association studies of individual rare CNVs have insufficient power to identify disease-causing variants. To evaluate the impact of rare CNVs with respect to risk for TGCC, we therefore compared the genome-wide burden of rare CNVs and investigated whether any genes or pathways were targeted by multiple rare CNVs such that their aggregated frequency was higher in cases than in controls.

In summary, to assess the effect of CNVs on TGCC we genotyped a Danish case-control cohort (Dalgaard et al., 2012) and analyzed the resulting data with respect to the association of both common and rare germline CNVs to TGCC.

**RESULTS**

To identify CNVs that confer a risk to TGCC, we analyzed common and rare variants in a genome-wide dataset of approximately 1.8 million markers in a Danish cohort constituting 212 TGCC cases and 437 controls. Application of stringent quality control criteria for reliable CNV identification (Figure 1) resulted in a final discovery set of 189 cases and 380 controls. Common variants were defined as CNVs present in more than 1% of the study population, and rare variants as CNVs present in no more than 1% of the studied subjects. Common variants were analyzed with respect to individual locus association, and rare variants with respect to overall genetic burden, gene association, and pathway association.

**LOCUS ASSOCIATION ANALYSIS**

In order to identify common CNVs associated with TGCC, binary copy number state frequencies of the case and control cohorts were compared at all loci with CNV frequencies above 1%. We observed one genome-wide significant deletion at 1p13.3 covering the gene GSTM1 (P = 0.02, 37.2% cases, 19.5% controls), but downstream quality control by manual inspection of the copy number intensity histogram at this locus, and application of histogram-based association analysis (Barnes et al., 2008) suggested a false positive

![Figure 1: CNV quality control and analysis.](image)
finding (nominal $P = 0.26$, 51.3% cases, 58.2% controls). A histogram of the copy number signal showed three distinct clusters with 51, 40, and 8% of the cases, respectively, and 58, 37, and 5% of the controls. The three clusters were assumed to correspond to copy numbers of 1, 2, and 3. Further, the deletion allele frequency at this locus has been estimated to be $\sim 40\%$ in the International HapMap Phase 3 population study (Altshuler et al., 2010).

Given the varying deletion frequencies reported, and to assess the quality of the array-data CNV calling, we performed targeted sequencing of the GSTM1 (chr1p13) gene in 62 patients. The sequencing data showed three clusters with 52, 40, and 8% of the samples, and 55 out of the 62 patients were present in the same clusters as in the array-data. The CNV frequencies of the three clusters were in perfect agreement with those from the histogram analysis of the array-data, thereby corroborating that deletion of GSTM1 had no association to TGCC. The sequencing also revealed that the actual copy numbers were 0, 1, and 2 rather than 1, 2, and 3 since the majority of samples had no DNA present (0 sequence reads). The reason for that mis-assignment is that a copy number state, having the major allele frequency, whereas in this case only 5–10% of the population carry two copies of the gene.

**CNV BURDEN ASSOCIATION ANALYSIS**

Testing whether individuals with TGCC had a greater genomic burden of rare CNVs than controls, we observed a weak indication of increased burden with respect to the number of CNVs per sample, the number of affected genes per sample, and the average length of CNVs per sample (case/control ratio: 1.08, 1.10, and 1.11 respectively), and a significant difference with respect to the total length of all CNVs per sample (case/control ratio: 1.19, $P = 0.03$; Table 1).

**GENE ASSOCIATION ANALYSIS**

Next, we explored if there were any specific genes where rare CNVs were more common in cases than controls. This analysis did not require that CNVs found in different samples overlapped each other, rather, it was sufficient that they were located within the same genic region. Two genes were found to have genome-wide significance, PTPN1 ($P_{emp.} = 0.038$) and KCNB2 ($P_{emp.} = 0.022$), affecting 0.9 and 1.2% of cases, respectively, whereas no occurrence in controls was observed at these loci (Table 2). The CNV at PTPN1 involved five cases, all found to have a heterozygous deletion at the same intronic region (Figure 2). CNVs at KCNB2 were found at three different loci: four and one deletions at two different introns and one deletion and one duplication at the promoter (Figure 2). Several CNVs have previously been reported in healthy individuals at KCNB2, but not at PTPN1 (Database of Genomic Variants, v. 10), corroborating a true TGCC association at PTPN1, but weakening the possibility of an actual association to KCNB2.

We attempted to verify the CNV at PTPN1 by performing qPCR on the affected samples, but all five samples with an indication of a heterozygous deletion in the array-data were observed to have two copies in the qPCR. Thus, the CNV calls from the array-data of PTPN1 are likely to be false positives and no association between PTPN1 and TGCC can be inferred at this stage.

**PATHWAY ASSOCIATION ANALYSIS**

Proteins tend to act in concert and perturbations of different components in a set of proteins that are interacting in a network may result in a dysregulation with similar outcome (Lage et al., 2007; Navlakha and Kingsford, 2010; Pers et al., 2011). We therefore conducted association analysis on the level of pathways and protein–protein interaction networks. Association was assessed in the same fashion as for genes and loci described above, that is, by comparing case and control cohorts with respect to the total frequency of rare CNV events targeting a pathway. We compiled comprehensive collections of gene sets, where a set of genes either share function or operate in the same pathway, and performed one thousand random permutations (shuffling case and control status) to estimate a local false-discovery rate (FDR) of each gene set (Edsgärd et al., CNV associations to testicular cancer).

---

**Table 1 | Global burden of rare CNVs in cases versus controls.**

<table>
<thead>
<tr>
<th>Type</th>
<th>Burden</th>
<th>$P$</th>
<th>Case:control ratio</th>
<th>Baseline (control)</th>
<th>Baseline (case)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>Rate</td>
<td>0.09</td>
<td>1.08</td>
<td>4.8</td>
<td>5.17</td>
</tr>
<tr>
<td></td>
<td>Gene rate</td>
<td>0.19</td>
<td>1.10</td>
<td>3.3</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>Mean length (kb)</td>
<td>0.14</td>
<td>1.11</td>
<td>76.9</td>
<td>85.1</td>
</tr>
<tr>
<td></td>
<td>Total length (kb)*</td>
<td>0.03</td>
<td>1.19</td>
<td>372.5</td>
<td>444.3</td>
</tr>
<tr>
<td>Duplications only</td>
<td>Rate</td>
<td>0.36</td>
<td>1.03</td>
<td>1.9</td>
<td>1.93</td>
</tr>
<tr>
<td></td>
<td>Gene rate</td>
<td>0.29</td>
<td>1.09</td>
<td>1.9</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>Mean length (kb)*</td>
<td>0.01</td>
<td>1.33</td>
<td>112.3</td>
<td>149.0</td>
</tr>
<tr>
<td></td>
<td>Total length (kb)</td>
<td>0.07</td>
<td>1.22</td>
<td>279.4</td>
<td>340.8</td>
</tr>
<tr>
<td>Deletions only</td>
<td>Rate</td>
<td>0.09</td>
<td>1.12</td>
<td>2.9</td>
<td>3.24</td>
</tr>
<tr>
<td></td>
<td>Gene rate</td>
<td>0.23</td>
<td>1.11</td>
<td>1.4</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>Mean length (kb)</td>
<td>0.49</td>
<td>1.00</td>
<td>49.9</td>
<td>49.9</td>
</tr>
<tr>
<td></td>
<td>Total length (kb)</td>
<td>0.21</td>
<td>1.09</td>
<td>151.6</td>
<td>165.0</td>
</tr>
</tbody>
</table>

*Significant difference ($P < 0.05$). Genome-wide $P$-values were estimated by 10,000 permutations of case-control status.
The three remaining gene sets, which did not include PTPN1, were: "regulation of cell migration" [go:0030334, 1.8% versus 1.1%, FDR = 0.021, OR = 3.47 (1.12−11.82)], "positive regulation of catalytic activity" [go:0043085, 1.4% versus 0.5%, FDR = 0.04, OR = 5.54 (1.31−32.78)], and "macromolecular complex disassembly" [go:0032984, 2.3% versus 1.9%, FDR = 0.047, OR = 2.47 (1.00−6.23)]. The most significant gene set among all sets tested was "regulation of cell migration." A total of 16 individuals harbored CNVs that altogether overlapped 14 genes in this pathway, at 13 unique CNV loci (Table 4). Genes in this gene set that were affected in cases but not controls included: BCL2, CDH13, CORO1A, KDR, MUC2, MUC5AC, ONECUT2, and PTPRK.

**DISCUSSION**

We assessed the effect of common and rare CNVs in a TGCC case-control cohort. No single locus was found to be associated to TGCC, but one potential gene network of interest with a weak significant association was identified, having an elevated frequency of rare CNVs among cases. The absence of any single locus CNV associated to TGCC is in line with the relatively few findings for other diseases, including a screening of ~3,400 common CNVs in eight common diseases (Craddock et al., 2010). Furthermore, common CNVs are typically ancient variations, which are tightly correlated with single nucleotide polymorphisms (SNPs), and can therefore be detected by genome-wide association studies of common SNPs (Lander, 2011). However, one should not neglect the importance of common CNVs in gene-phenotype association studies, since there exists evidence that disease associated SNPs have a tendency to tag CNVs more often than random, and that such CNV-tagging SNPs are enriched for expression quantitative trait loci (eQTL; Gamazon et al., 2011). Further, rare CNVs are
not tagged by common SNPs implicated by GWASs and they have been found to play a major role in neurodevelopmental disorders (Merikangas et al., 2009) and rare de novo CNVs were recently found in 3 out of 43 TGCC family-trios (Stadler et al., 2012). We have previously searched for CNVs associated to familial TGCTs, where the inheritance component is much higher than among sporadic cases, but failed to find a CNV that was significant across the small set of studied families. We only identified a handful of CNVs that segregated with TGCT, but these were either family-specific or relatively common variants, such as RLN1 (Edsgård et al., 2011).

The rare association analysis presented in this study initially indicated two genes to be associated with TGCC, PTPN1, and KCNB2. KCNB2 was considered a false positive due to the amount of previously reported CNVs in independent control cohorts at this locus, being on par with that of the frequency in the case group of this study. PTPN1 appeared as a good candidate since it has previously been implicated with the progression of prostate cancer along with evidence that the androgen receptor is a transcriptional regulator of PTPN1 (Lessard et al., 2010, 2012). A loss of PTPN1 may thereby be associated with impaired responsiveness to androgens, which would be consistent with the fact that low androgen status during development is a risk factor for TGCT (Rajpert-De Meyts and Skakkebaek, 1993; Sonne et al., 2008). Further, CNVs at the 20q13 chromosomal region have been observed in several other cancers (Nishizaki et al., 1998; Schaid, 2004; Furukawa et al., 2006). Furthermore, all affected probands in this study presented a deletion, consistent with a tumor suppressing function of oncogenic kinases, and PTPN1 has been shown to be able to play both a pro- and anti-oncogenic role (Stuible et al., 2008). A considerable effort was made to ensure high quality of the CNV calls: by using two complementary CNV calling methods, by applying several strict QC criteria, and by manual inspection of the raw probe signals. However, despite the stringent QC the qPCR did not verify the heterozygous deletion of PTPN1 in any of the samples. This highlights the difficulty of assessing the reliability of CNV calls from genome-wide short-nucleotide microarrays.

Our pathway analysis identified the gene set “regulation of cell migration” as having the highest difference in proportion of rare CNVs in cases compared to controls. There is a growing body of information that strongly suggests a crucial role of primordial...
germ cell (PGC) biology in TGCC oncogenesis. PGCs are embryonic cells which during mid-gestation migrate from the base of the yolk sac, along the hind-gut, to the genital ridge, one of the longest migrations of all mammalian cells. Four recent SNP GWAS TGCC studies associated KITLG and a number of other genes related to the KIT-KITLG pathway (Kanetsky et al., 2009; Rapley et al., 2009; Turnbull et al., 2010; Dalgaard et al., 2012), a regulatory network which is believed to be of crucial importance in the determination of the fate of PGCs (Rapley and Nathanson, 2010). For instance, mutations of the KIT receptor, or the KIT ligand, in the mouse, blocks PGC migration, resulting in infertility (Matzuk and Lamb, 2002). In addition, a disturbance of the migration of PGCs during early fetal development may cause extragonadal germ cell cancers along the midline of the body (Oosterhuis and Looijenga, 2005).

One of the afflicted genes in the “regulation of cell migration” gene set was PTPRK, at which two samples had a deletion. Like PTPNI, PTPRK belongs to the family of protein tyrosine phosphatases, but it is a membrane-bound receptor. PTPRK is also implicated in TGFβ-signaling (Xu et al., 2010), and a recent GWAS study indicated the involvement of TGFβ superfamily signaling in testicular dysgenesis (Dalgaard et al., 2012). In mouse, PGCs divide rapidly under the influence of TGFβ signaling factors, and defects in PGC development is observed in knockout models of bone morphogenetic proteins (BMPs; Lawson et al., 1999; Ying et al., 2000). In total there were 14 genes which were part of the “regulation of cell migration” pathway and that harbored CNVs. It would be of high interest to further elucidate the role of these genes by studying how the CNVs affect the expression levels of the corresponding genes. What may complicate matters is if the critical period of time is the development of the fetus, which may force one to revert to the study of animal models.

In the analysis of common CNVs we made a further assessment of the GSTM1 CNV frequency by targeted sequencing. The main reason for this was to assess the quality of and verify the array-data CNV calls of a seemingly problematic locus. However, GSTM1 was also partly chosen due to that the risk of developing TGCC has been shown to increase by the exposure to certain environmental factors (Skakkebaek et al., 2001) and GSTM1 is known to be involved in the detoxification of xenobiotic compounds (Hengstler et al., 1998). Hypothetically, it is not unlikely that there exists a genetic variation that may affect the ability to metabolize environmental chemicals, which in turn affects the risk of TGCC. Apart from such gene-environment interactions it is probable that part of the unexplained heritability can be explained by gene–gene interactions, where a combination of several genetic variations cause a greater effect on the phenotype than the sum of their individual effects (epistasis). Further research is required to assess the effect of rare variants in varying genetic backgrounds.

In conclusion, this study corroborates the rejection of the hypothesis that a single CNV locus mediates a major risk for TGCC. It suggests that several rare CNVs may contribute to the oncogenesis of a subset of TGCC subjects, but the frequency after aggregation of CNVs on the implicated gene and pathways is still low, and these CNVs therefore only provide a minor contribution to the overall heritability. Larger cohorts are needed to further explore the impact of rare variants in TGCC.

MATERIALS AND METHODS

SAMPLE COLLECTION

Two hundred and twelve men with TGCCs and 439 healthy young men with semen concentrations above 60 million sperm/ml were collected at the Department of Growth and Reproduction, Rigshospitalet, Denmark. All DNA samples were obtained from men of Danish ancestry, who provided informed consent for genetic analysis. The samples were coded during the entire analysis. The project has been approved by the Regional Medical Ethics Committee (Nr H-KF-265848) and the Danish Personal Data Protection Agency (Nr 2008-41-2158). This cohort has previously been analyzed with respect to single nucleotide variation (Dalgaard et al., 2012). Sixty-two of the TGCC patients were selected for sequencing of the GSTM1 gene.

GENOTYPING AND SNP-BASED SAMPLE QUALITY CONTROL

Germline DNA was extracted from peripheral blood using QuickGene DNA whole blood kits from FujiFilm Life Science according to the manufacturer’s manual. Samples were genotyped using the Affymetrix Genome-wide Human SNP Array 6.0. Here we present the analysis of CNVs, SNP association analysis and details of the cohort are described elsewhere (Dalgaard et al., 2012), but an initial sample quality control was performed using SNP genotypes called by the Birdseed algorithm [Affymetrix Power Tools (APT) v. 1.10.2]. We excluded samples based on a genotyping call rate below 96%; QC contrast below 0.4 as according to the Affymetrix GCSO software; non-European ancestry by inspection of a plot of the first two principal components of the cohort and the HapMap phase III samples (Alshuler et al., 2010); high degree of relatedness based on identity-by-descent where one individual was kept among related samples; and samples with an outlying inbreeding coefficient.

CVN DETECTION AND QUALITY CONTROL

For samples that passed the SNP quality control described above we ran two CNV calling algorithms, BirdSuite (v. 1.5.5) and PennCNV (v. 2010May01). PennCNV requires a signal intensity file and a SNP genotyping file and these were generated by quantile normalization of PM-only probes with median polish probe summarization (APT v. 1.12.0) and Birdseed (v. 2 in APT 1.12.0), respectively. We excluded CNVs which failed any of the four following criteria: (1) A CNV was not called by both algorithms. A histogram of the percentage of overlap indicates that the vast majority has >90% overlap, but we set the threshold to at least 10%. (2) A CNV log odds confidence score larger than two, as recommended by BirdSuite (Korn et al., 2008). (3) CNV size was less than five markers or four kilobases, in effect excluding the 25% short-length quantile of CNVs. (4) A CNV was longer than one megabase (three outliers based on histogram). Further, samples were excluded with respect to the three following criteria: (1) extreme sample burden in terms of more than 110 CNVs (four outliers); or (2) a total length of CNVs larger than 7.5 megabases (two outliers); (3) bad sample quality in terms of high variance of copy number signal (median copy number variance larger than two, as recommended by BirdSuite, or a Log R Ratio standard deviation (LRR SD) obtained from PennCNV larger than 0.4). LRR SD was set according to PennCNV guidelines when CNV calling
are performed on Affymetrix samples. Finally, rare CNVs that had more than 50% overlap with segmental duplication regions (retrieved from UCSC hg18) were removed, since such regions have been shown to generate more false CNV calls (Pinto et al., 2010). A total of 189 TGCC cases and 380 controls remained after the completion of all quality control steps, harboring a total of 1088 and 1872 rare CNVs, respectively.

**CNV ASSOCIATION ANALYSIS**

Copy number variant association analysis was performed using plink (v. 1.07) and custom R (v. 2.12) scripts. Common and rare CNVs were defined as those with an allele frequency above or below 1%, respectively. The allele frequency of a CNV was determined using the locus within a CNV region with the maximum number of overlapping individual CNVs.

**LOCUS ASSOCIATION**

Common CNVs were evaluated by searching the whole genome for loci with a significantly higher degree of affected cases as compared to controls. Binary state frequencies were used, and a Fisher test was performed for deletion, amplification, and any type of aberration, respectively. Genome-wide significance was estimated by generating a null distribution based on one thousand case-control status permutations. For each permutation the minimal P-value was selected, thereby providing control of the family-wise error rate (FWER). Loci with significant associations were further verified by CNVtools (Barnes et al., 2008) which uses a complementary CNV calling strategy, since it employs a statistical model based on density-based clustering rather than a hidden Markov model. Furthermore, loci residing at the edges of a common CNV and associations from variation of boundary truncation were excluded.

**GLOBAL BURDEN ANALYSIS**

The impact of rare CNVs was assessed by three approaches: genome-burden analysis, gene association, and pathway association analysis.

The global burden of rare CNVs in cases compared to controls were assessed with respect to (i) the number of CNVs per sample, (ii) the number of affected genes per sample, (iii) the average length of CNVs per sample, and (iv) the total length of CNVs per sample.

**GENE ASSOCIATION**

Gene association analysis was performed using the number of case and control samples harboring a rare CNV that overlapped the gene of interest. Genes were retrieved from UCSC (hg18). CNV frequencies were compared with Fisher’s test and multiple testing corrected P-values were obtained based on case-control permutation as described above for the locus association of common CNVs. Significant CNVs which were found to have a lower allele frequency in our case cohort than in the Database of Genomic Variants (DGV, v. 10), were considered false positives.

**PATHWAY ASSOCIATION**

Pathway association analysis was performed based on the number of case and control samples that had a rare CNV in any of the genes of a pathway. The R package “siggenes” was used to obtain P-values corrected for multiple testing across all tested gene sets. The package provides a FDR, based on randomized case-control sampling, as well as an adjustment of the variance of an individual pathway using information from the observed variances of all pathways (Efron and Tibshirani, 2002). Gene sets were retrieved from KEGG (Kyoto Encyclopedia of Genes and Genomes; Ogata et al., 1999), Reactome (D’Eustachio, 2011), BioCarta¹, NCI-Nature curated pathways (Pathway Interaction Database; Schaefer et al., 2009), GO (Gene Ontology; Ashburner et al., 2000), COSMIC (Catalog of Somatic Mutations In Cancer; Forbes et al., 2011), Cyclebase (Gauthier et al., 2010), protein–protein interaction complexes (Lage et al., 2007), OMIM (Online Mendelian Inheritance in Man²), MGI (Mouse Genome Informatics³) and a set of candidate infertility genes from a recent review (Matzuk and Lamb, 2008). Terms annotating more than 700 or less than 5 genes were discarded, since they do not produce meaningful statistical results.

**VERIFICATION OF PTPN1 COPY NUMBER**

Copy number verification of PTPN1 was done using Quantitative PCR on the Mx3000P platform from Stratagene (Agilent Technologies, Santa Clara CA, USA). The protocol has been described previously (Ottesen et al., 2007). Primers for the CNV at PTPN1 were designed using Primer3 (Rozen and Skaletsky, 2000). Primer sequences for PTPN1 were: forward 5′-TTC AAC CCT AAC TAG GTA TGC A-3′ and reverse 5′-CTA AAA TGC TGG AGA CTT AGG T-3′ and primers for GAPDH: forward 5′-CTC CCC ACA CAC ATG CAC TTA-3′ and reverse 5′-TGG CCA AGT TGC CTT TCC TT-3′ (DNA Technology A/S, Aarhus, Denmark). GAPDH was used as endogenous control gene. Mixtures of forward and reverse primers were denatured for 3 min at 95°C and incubated on ice until use. DNA concentrations were 8–17 ng, 15 µL. Brilliant SYBR Green QPCR Master Mix (Stratagene), 7.0 µL primer mixture of PTPN1 (final conc.: Fw and Rev 100 nm) or GAPDH (final conc.: Fw and Rev 100 nm), and a total volume of 30 µL. Conditions for amplification were as follows: one cycle at 95°C for 10 min and 40 cycles at 95°C for 1 min, 62°C for 1 min and 72°C for 1 min. The PTPN1:GAPDH ratio was calibrated to a normal male reference control of DNA, as previously described for other genes (Ottesen et al., 2007; Mau Kai et al., 2008). All specimens were analyzed in triplicate and the mean-ratio was used to infer integer copy number.

**TARGETED SEQUENCING OF GSTM1**

**Design of targeted capture baits**

Target capture was designed on the Agilent SureSelect capture system (Santa Clara CA, USA). Baits for capture were designed by tiling the genomic region harboring GSTM1 with an approximately 50% overlap between consecutive baits. The bait sequences were optimized to avoid regions with extreme GC content or highly variable regions, and to avoid possible cross-hybridization or self-folding of the baits, which could decrease the hybridization efficiency.

¹http://www.biocarta.com
³http://www.informatics.jax.org
specificity. The principle of this method has been described previously (Wesolowska et al., 2011).

**Library preparation, pooling, target enrichment, and sequencing**

DNA shearing, library preparation, and pooling were performed using a modified protocol of the SureSelect Target Enrichment System (Agilent Technologies) to allow sample multiplexing as described elsewhere (Wesolowska et al., 2011). Three micrograms of genomic DNA was sheared by ultrasound (Covaris; Woburn, MA, USA) to yield fragments of an average size of ∼200 bp. The sheared fragments were ligated with custom made adaptors containing a unique four-base barcode, and subsequently purified and amplified by ligation-mediated PCR (LM-PCR). Amplified DNA was pooled in groups of 10 with equal amount of each, after which enrichment was performed by hybridization to custom SureSelect target capture baits (Agilent Technologies). The captured libraries were processed with Illumina Cluster Generation Station (Illumina, San Diego CA, USA) following the manufactures recommendations. We performed 100 nt single-end read sequencing on the Illumina HiSeq 2000 sequencing platform.

**Data analysis**

Sequence reads were trimmed and high quality reads were mapped to the human reference genome build 37 (GRCh37) using Burrow-Wheeler Alignment algorithm (Li and Durbin, 2010). Alignments with mapping score below 30 were discarded and PCR duplicates were removed with Picard MarkDuplicates.

**ACKNOWLEDMENTS**

We would like to thank Betina F. Nielsen for skillful microarray work and clinicians for handling of patients and controls (especially Dr Niels Jørgensen and his team). We would like to thank the DTU Multi-Assay Core facility for automated library preparation infrastructure. The work was supported by the Villum Kann Rasmussen Foundation, the Danish childhood cancer research foundation, and the Danish Cancer Research Foundation.

**REFERENCES**


This article was submitted to Frontiers in Cancer Endocrinology, a specialty of Frontiers in Endocrinology. Copyright © 2013 Edsgärd, Dalgaard, Weinhold, Wesolowska-Anderwens, Raijter-De Meys, Ottesen, Andal, Skakkebaek, Skakkebaek, Skakkebaek, Skakkebaek, and Brunak. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are cred- ited and subject to any copyright notices concerning any third-party graphics etc.
Association between polymorphisms in the aryl hydrocarbon receptor repressor gene and disseminated testicular germ cell cancer

Leon J. S. Brokken1*, Yvonne Lundberg-Giwercman1, Ewa Rajpert-De Meyts2, Jakob Eberhard3, Olof Ståhl3, Gabriella Cohn-Cedermark4, Gudske Daugaard5, Stefan Arver6 and Aleksander Giwercman1,7

1 Department of Molecular Reproductive Medicine, Lund University, Malmö, Sweden
2 Department of Growth and Reproduction, Rigshospitalet, Copenhagen, Denmark
3 Department of Oncology-Pathology, Radiumhemmet, Karolinska Institute and University Hospital, Stockholm, Sweden
4 Department of Oncology–Pathology, Radiumhemmet, Karolinska Institute and University Hospital, Stockholm, Sweden
5 Department of Oncology, Rigshospitalet, Copenhagen, Denmark
6 Centre for Andrology and Sexual Medicine, Karolinska University Hospital Huddinge, Department of Medicine, Stockholm, Sweden
7 Reproductive Medicine Centre, Skåne University Hospital, Malmö, Sweden

*Correspondence: Leon J. S. Brokken, Molecular Reproductive Medicine, Clinical Research Centre, Lund University, House 91, Floor 10, Jan Walderströms gata 35, 20502 Malmö, Sweden. e-mail: leon.brokken@med.lu.se

In the Western world, testicular germ cell cancer (TGCC) is the most common malignancy of young men. The malignant transformation of germ cells is thought to be caused by developmental and hormonal disturbances, probably related to environmental and lifestyle factors because of rapidly increasing incidence of TGCC in some countries. Additionally, there is a strong genetic component that affects susceptibility. However, genetic polymorphisms that have been identified so far only partially explain the risk of TGCC. Many of the persistent environmental pollutants act through the aryl hydrocarbon receptor (AhR). AhR signaling pathway is known to interfere with reproductive hormone signaling, which is supposed to play a role in the pathogenesis and invasive progression of TGCC. The aim of the present study was to identify whether AhR-related polymorphisms were associated with risk as well as histological and clinical features of TGCC in 387 patients and 537 controls. Haplotype-tagging single-nucleotide polymorphisms (SNPs) were genotyped in genes encoding AhR and AhR repressor (AHRR). Binary logistic regression was used to calculate the risk of TGCC, non-seminoma versus seminoma, and metastasis versus localized disease. Four SNPs in AHRR demonstrated a significant allele association with risk to develop metastases (rs2466287: OR = 0.43, 95% CI: 0.21–0.90; rs2672725: OR = 0.49, 95% CI: 0.25–0.94; rs6879758: OR = 0.43, 95% CI: 0.08–0.92; rs6896163: OR = 0.34, 95% CI: 0.12–0.98). This finding supports the hypothesis that compounds acting through AhR may play a role in the invasive progression of TGCC, either directly or through modification of reproductive hormone action.

Keywords: aryl hydrocarbon receptor, aryl hydrocarbon receptor repressor, testicular germ cell cancers, genetic polymorphisms, genetic variation, association studies, metastasis, histology

INTRODUCTION

Testicular germ cell cancer (TGCC) represents the most frequently diagnosed cancer in young men between 20 and 40 years of age. The rates vary considerably according to region and ethnicity, which suggests that both environmental and genetic factors contribute to TGCC. In Europe, a three- to fourfold increased incidence has been noted during the past 30–40 years, which suggests that an increased exposure to persistent environmental pollutants may play an important role.

Common persistent environmental pollutants include dioxins and polycyclic aromatic hydrocarbons, which exert their toxic and carcinogenic effects through the ligand-activated transcription factor aryl hydrocarbon receptor (AhR). In complex with its binding partner AhR nuclear translocator (ARNT), it mediates cellular responses to xenobiotic compounds. The AhR/ARNT heterodimer stimulates the expression of AhR repressor (AhRR), which provides a negative feedback regulation by competing with ARNT for binding to AhR (Mimura et al., 1999).

Testicular germ cell cancer derives from carcinoma in situ (CIS) cells, also known as intratubular germ-cell neoplasia unclassified (Skakkebaek et al., 1982; Oosterhuis and Looijenga, 2005), that are believed to arise from primordial germ cells that are blocked in differentiation. The initial malignant transformation of germ cells is thought to be caused by hormonal disturbances in the microenvironment of differentiating germ cells (Skakkebaek et al., 1987; Sharpe and Skakkebaek, 1993; Rajpert-De Meyts et al., 1998). An association of TGCC with maternal estrogen and androgen levels in early pregnancy has recently been reported, supporting this hypothesis (Holl et al., 2009). In addition to altering transcription of steroidogenic genes, such as cytochrome P450 enzymes, the
AHR signaling pathway has been shown to interface with estrogen and androgen receptor signaling (Jana et al., 1999; Kiru et al., 2003; Safo and Wornike, 2003; Rew et al., 2006; Reischlag et al., 2006; Kollara and Brown, 2010). Additionally, AHR plays a significant role in tumor promotion and progression by deregulation of cell–cell contact and inhibition of apoptosis (Mulero-Navarro et al., 2005; Dry et al., 2006; Carvajal-Gonzalez et al., 2009; Chopra et al., 2009; Dietrich and Kaina, 2010).

The risk of environment-related carcinogenesis depends not only on the magnitude of the exposure, but also on individual susceptibility to the pollutant. Therefore, we aimed to analyze the association between polymorphic variants of genes encoding AHR and AHRR and the risk as well as histological and clinical features of TGCC.

MATERIALS AND METHODS
The cohort consisted of TGCC patients from Sweden and Denmark, two neighboring Scandinavian countries, with populations sharing largely the same genetic background as both descended from a common ancestral population.

SWEDISH TGCC PATIENTS
All TGCC patients that were referred to the Department of Oncology, Skåne University Hospital, Lund, the Department of Oncology, Radiumhemmet or Södersjukhuset, Karolinska University Hospital, Stockholm since March 1996 and November 1998 until October 2006, respectively, were asked to participate in a study focusing on reproductive function. In total 460 patients were eligible for the study, of which 75 declined to participate and 45 were excluded due to linguistic problems, bilateral testicular cancer, or moving to another region. Seven patients were excluded due to contra-lateral testicular cancer diagnosed after inclusion and 3 died of progressive disease before blood sampling. In total, 278 patients were random selected for this study; the criterion for selection being sufficient DNA amount. In total, 11 patients were excluded because of mistaken diagnosis (n = 6), purely extra-gonadal germ cell cancer, physical handicap, or moving to another region. Seven patients were excluded due to compromising mental conditions, 18 were excluded due to contra-lateral testicular cancer diagnosed after inclusion and 3 died of progressive disease before blood samples were obtained. Among the 320 participants in the study on reproductive function, no DNA was available for 39. Additionally, 3 patients were excluded due to extra-gonadal germ cell cancer, leaving the total at 278. All patients gave their informed written consent and the study was approved by the Ethical Boards of Lund University and Karolinska Institute.

DANISH TGCC PATIENTS
In the period between 1999 and 2008, DNA was collected from approximately 300 TGCC patients on the occasion of semen banking prior to surgery or fertility assessment after treatment. One hundred samples were random selected for this study; the criterion for selection being sufficient DNA amount. In total, 11 patients were excluded because of mistaken diagnosis (n = 6), purely extra-gonadal tumor (n = 4), and in one case because the genetic SNP analysis failed. Among the remaining 89 patients, 5 presented with CIS only and were therefore not included in the analysis of distribution of seminomas (SE) versus non-seminomas (NSE). For six patients no information on stage was available.

CONTROL SUBJECTS
Control subjects were recruited in a study of reproductive function among Swedish military conscripts aged 18–20 years in the period 2000–2001 (Richhoff et al., 2002). As part of the investigation, scrotal palpation and ultrasound was performed in order to exclude testicular tumors or microcalcifications, which are indicative of an increased risk of CIS. Furthermore, they delivered one ejaculate for semen analysis as well as a blood sample for assessment of hormone levels. Among the 305 men that were eligible, 214 men with a Swedish mother were selected as control subjects (Table 1).

GENOTYPING
Genomic DNA was prepared from peripheral leukocytes using QIAamp DNA Maxi Kit (Qiagen, Germany). All samples were normalized to the same DNA concentration and the genotypes were determined using the Sequenom MassArray MALDI-TOF mass spectrometry. Eleven SNPs in the gene encoding aryl hydrocarbon receptor (AHR) and 18 SNPs in the gene encoding aryl hydrocarbon receptor repressor (AHRR) with a minor allele frequency >0.05 that were identified as haplotype-tagging SNPs were selected using dbSNP (available at: http://www.ncbi.nlm.nih.gov/SNP) and SNP assays were designed using MassArray Assay Design ver. 2 software (Sequenom Inc., USA). Primers were obtained from Metabon GmbH (Germany) and all reactions were run under the same conditions, except for the primer annealing temperature of the primary PCR. PCR reactions were performed in a total volume of 6 μL containing 2.5 ng template DNA, 1.25× Taq PCR buffer (Hotstart, Qiagen), 0.15 U Taq polymerase (Hotstart, Qiagen), 3.5 mM MgCl₂, 0.5 mM dNTP, and 100 nM of each primer. Amplifications were performed using Taq polymerase.

Table 1 | Characteristics of controls and case patients.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>TGCC-SE</th>
<th>TGCC-DK</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (%)</td>
<td>214</td>
<td>278</td>
<td>89</td>
</tr>
<tr>
<td>Age (year)</td>
<td>18 ± 0</td>
<td>31 ± 7</td>
<td>31 ± 8</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSE ( %)</td>
<td>153 (65.0)</td>
<td>34 (38.2)</td>
<td></td>
</tr>
<tr>
<td>SE ( %)</td>
<td>125 (45.0)</td>
<td>50 (56.2)</td>
<td></td>
</tr>
<tr>
<td>CIS only ( %)</td>
<td>5 (5.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Localized</td>
<td>200 (71.9)</td>
<td>65 (73.0)</td>
<td></td>
</tr>
<tr>
<td>Metastasis</td>
<td>78 (28.1)</td>
<td>23 (26.2)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>19 (6.5)</td>
<td>5 (5.2)</td>
<td></td>
</tr>
<tr>
<td>Family history of TGCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes ( %)</td>
<td>91 (3.2)</td>
<td>7 (2.3)</td>
<td></td>
</tr>
<tr>
<td>No ( %)</td>
<td>214 (100)</td>
<td>250 (90.3)</td>
<td>77 (88.5)</td>
</tr>
<tr>
<td>Unkown ( %)</td>
<td>19 (6.5)</td>
<td>5 (5.2)</td>
<td></td>
</tr>
<tr>
<td>History of cryptorchidism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes ( %)</td>
<td>7 (3.3)</td>
<td>23 (8.3)</td>
<td>13 (14.4)</td>
</tr>
<tr>
<td>No ( %)</td>
<td>207 (96.7)</td>
<td>247 (88.9)</td>
<td>63 (70.6)</td>
</tr>
<tr>
<td>Unknown ( %)</td>
<td>8 (3.3)</td>
<td>13 (14.4)</td>
<td></td>
</tr>
</tbody>
</table>
GeneAmp 9700 machines with dual-384 heads as follows: 95°C for 15 min, 45 cycles at 95°C for 20 s, 56°C, 60°C, or 64°C for 30 s, 72°C for 60 s, and finally 72°C for 3 min. Dephosphorylation of unincorporated dNTP was achieved using shrimp alkaline phosphatase. Concentrations of individual homogenous MassEXTEND (hmE) primers were adjusted to even out peak heights in the mass spectrum. The extension reactions were carried out by mixing the adjusted primer mix (containing approximately 1 μM of each primer) with hmE mix containing buffer and 50 μL of each di/dNTP mix and 1:25 U of Thermo Sequenase (Amersham Biosciences, Uppsala, Sweden). PCR amplification of hmE reactions was performed as follows: 94°C for 2 min and 99 cycles at 94°C for 3 s, 52°C for 3 s, and 72°C for 3 s. The samples were then manually desalted by using 6 mg of Clean Resin and a dimple plate and subsequently transferred to a 384-well SpectroCHIP using a nanodispenser. Randomly selected samples of each genotype were directly sequenced in order to validate the SNP assays.

**STATISTICAL ANALYSIS**

Single-nucleotide polymorphisms data was processed and analyzed using the web-based SNPator data analysis suite (Morcillo-Suarez et al., 2008). Agreement with Hardy–Weinberg equilibrium was tested using the web-based SNPator data analysis suite (Morcillo-Suarez et al., 2008). Agreement with Hardy–Weinberg equilibrium was used to calculate the odds ratio (OR) and 95% confidence interval for developing TGCC, for developing either SE or NSE, and for developing disseminated disease [defined by stages II, III, and IV according to the Royal Marsden Hospital staging classification (Husband and Koh, 2004), with stage I disease defined as tumor confined to the testis, with no evidence of metastases]. In the latter analysis histological subtype was included as a covariant in the logistic regression. Associations were considered statistically significant at \( p < 0.05 \). Assuming a co-dominant additive model, we tested a linear trend of increasing effect in the different genotypes using linear-by-linear association \( \chi^2 \) statistics (SPSS ver. 21). Linkage disequilibrium (LD; i.e., non-random association of alleles) was assessed for each gene. The LD between identified SNPs was determined by pairwise comparisons of correlation coefficient between SNPs \( r^{2} \). The likelihood that recombination has occurred between SNPs \( (L') \). Since we expected that the selected SNPs have a small contribution to TGCC and we regard our study to be exploratory rather than confirmatory, we have chosen to avoid correction for multiple testing. This may increase the risk of type 1 errors, but it prevents type 2 errors.

**RESULTS**

No significant deviations from Hardy–Weinberg equilibrium were detected for the SNPs included in the analysis. We did not find a statistically significant association between the studied polymorphisms and the risk of TGCC.

For four SNPs in **AHRR** a significant allele association with the occurrence of disseminated disease was observed (Table 2).

<table>
<thead>
<tr>
<th>SNP</th>
<th>Risk Ratio (OR)</th>
<th>95% CI</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2672725</td>
<td>1.35</td>
<td>1.02 to 1.79</td>
<td>0.042</td>
</tr>
<tr>
<td>rs6879758</td>
<td>1.39</td>
<td>1.07 to 1.82</td>
<td>0.014</td>
</tr>
<tr>
<td>rs6896163</td>
<td>1.42</td>
<td>1.10 to 1.85</td>
<td>0.007</td>
</tr>
<tr>
<td>rs698746</td>
<td>1.50</td>
<td>1.13 to 2.01</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Patients with metastatic disease had significantly lower frequencies of the minor rs2466287 G, rs2672725 G, rs6879758 C, and rs6896163 G alleles compared to patients with localized disease.

**DISCUSSION**

In this study we have analyzed associations between SNPs in genes encoding AHR and AHRR and the risk of developing TGCC, histological subtype, and the occurrence of metastasis. Whereas no associations were found with SNPs in AHR, four variants in **AHRR** associated significantly with the occurrence of metastatic disease. Both AHR and its negative regulator **AHRR** are ligand-activated transcription factors that belong to the family of basic-helix-loop-helix Per-Arnt-Sim (bHLH-PAS) proteins. They are ubiquitously expressed in almost all human tissues, with levels particularly high in the testis (Burbach et al., 1992; Tsuchiya et al., 2003).

Several epidemiological studies have shown that TCDD promotes cancer, and experiments in AHR-deficient mice have shown that AHR is essential for the tumor-promoting effects of TCDD (Andersson et al., 2002; McMillan and Bradfield, 2007). Conversely, transgenic mice with a constitutively active AHR spontaneously develop tumors, their weights of testis and ventral prostate are decreased and the epididymal sperm reserve is reduced (Brunberg et al., 2011). Furthermore, AHRR, the negative regulator of AHR signaling, functions as a tumor suppressor in multiple human tumors (Zudaire et al., 2008; Li et al., 2012).

While SNPs in either AHR or AHRR did not associate with the risk of TGCC, it is interesting that an association was observed between AHRR and dissemination of TGCC, since AHR signaling...
<table>
<thead>
<tr>
<th>SNP</th>
<th>Allele and genotype</th>
<th>Combined</th>
<th>Sweden</th>
<th>Denmark</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Localized</td>
<td>Metastasis</td>
<td>P-value</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>rs2466287</td>
<td>C 472 (90) 181 (95) Referent</td>
<td>356 (90) 147 (95) Referent</td>
<td>116 (8) 3.4 (94) Referent</td>
<td></td>
</tr>
<tr>
<td>(AHRR)</td>
<td>G 54 (10) 9 (5)</td>
<td>40 (10) 7 (5)</td>
<td>0.036 0.42 (0.19–0.97)</td>
<td>14 (11) 2 (6)</td>
</tr>
<tr>
<td></td>
<td>CG 46 (17) 9 (5)</td>
<td>34 (17) 7 (5)</td>
<td>0.089 0.47 (0.20–1.12)</td>
<td>12 (18) 2 (11)</td>
</tr>
<tr>
<td></td>
<td>GG 4 (2) 0 (0)</td>
<td>3 (2) 0 (0)</td>
<td>–</td>
<td>1 (2) 0 (0)</td>
</tr>
<tr>
<td>Trend</td>
<td>0.024 0.041</td>
<td>0.248 0.359</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs2672725</td>
<td>C 467 (89) 179 (64) Referent</td>
<td>351 (89) 146 (95) Referent</td>
<td>116 (8) 3.3 (92) Referent</td>
<td></td>
</tr>
<tr>
<td>(AHRR)</td>
<td>G 59 (11) 11 (6)</td>
<td>45 (11) 8 (5)</td>
<td>0.028 0.43 (0.20–0.93)</td>
<td>14 (11) 3 (6)</td>
</tr>
<tr>
<td></td>
<td>CC 230 (28) 84 (68) Referent</td>
<td>157 (79) 69 (80) Referent</td>
<td>52 (80) 1.5 (69) Referent</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CG 49 (18) 11 (2)</td>
<td>37 (19) 8 (10)</td>
<td>0.088 0.49 (0.22–1.11)</td>
<td>12 (18) 3 (7)</td>
</tr>
<tr>
<td></td>
<td>GG 5 (2) 0 (0)</td>
<td>4 (2) 0 (0)</td>
<td>–</td>
<td>1 (2) 0 (0)</td>
</tr>
<tr>
<td>Trend</td>
<td>0.035 0.041</td>
<td>0.248 0.359</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs6879758</td>
<td>G 499 (95) 187 (98) Referent</td>
<td>375 (94) 152 (99) Referent</td>
<td>124 (8) 3.5 (97) Referent</td>
<td></td>
</tr>
<tr>
<td>(AHRR)</td>
<td>C 29 (5) 3 (2)</td>
<td>23 (6) 2 (1)</td>
<td>0.023 0.21 (0.05–0.92)</td>
<td>6 (5) 1 (0)</td>
</tr>
<tr>
<td></td>
<td>GG 235 (88) 92 (87) Referent</td>
<td>174 (88) 75 (97) Referent</td>
<td>52 (80) 1.5 (69) Referent</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GC 29 (10) 3 (3)</td>
<td>23 (12) 2 (1)</td>
<td>0.034 0.20 (0.047–0.99)</td>
<td>6 (9) 1 (6)</td>
</tr>
<tr>
<td></td>
<td>CC 0 (0) 0 (0)</td>
<td>0 (0) 0 (0)</td>
<td>–</td>
<td>0 (0) 0 (0)</td>
</tr>
<tr>
<td>Trend</td>
<td>0.022 0.022</td>
<td>0.022 0.022</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs6891613</td>
<td>A 497 (94) 188 (98) Referent</td>
<td>373 (94) 153 (98) Referent</td>
<td>124 (8) 3.5 (97) Referent</td>
<td></td>
</tr>
<tr>
<td>(AHRR)</td>
<td>G 31 (6) 4 (2)</td>
<td>25 (6) 3 (2)</td>
<td>0.035 0.29 (0.087–0.98)</td>
<td>6 (5) 1 (0)</td>
</tr>
<tr>
<td></td>
<td>AA 233 (88) 92 (87) Referent</td>
<td>174 (88) 75 (97) Referent</td>
<td>52 (80) 1.5 (69) Referent</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AG 31 (12) 4 (4)</td>
<td>25 (13) 3 (4)</td>
<td>0.041 0.28 (0.082–0.95)</td>
<td>6 (6) 1 (6)</td>
</tr>
<tr>
<td></td>
<td>GG 0 (0) 0 (0)</td>
<td>0 (0) 0 (0)</td>
<td>–</td>
<td>0 (0) 0 (0)</td>
</tr>
<tr>
<td>Trend</td>
<td>0.032 0.031</td>
<td>0.022 0.022</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In this group, the same trends with similar relative risks were separately, most probably due to the limited number of cases the association between AHRR polymorphisms and metastatic hormones could therefore be an alternative mechanism underlying these genes encode. Yet, polymorphisms in non-coding regions can still play an important role in the regulation of gene expression, e.g., by affecting mRNA stability, regulation by intronic micro RNAs, or potential splice sites. The identified SNPs could also be in LD with other genetic polymorphisms that are causally related to TGCC.

In summary, we have identified polymorphic variants in the gene encoding AHRR that associated with risk of disseminated TGCC. This supports our hypothesis that the AHRR signaling pathway may affect the progression of TGCC, possibly by interfering with reproductive hormone actions.

ACKNOWLEDGMENTS

The authors would like to thank Marlene Dalggaard for help with collection of the patient data. This study was supported by grants from the Swedish Research Council (grants K2009-54X-20095-01-3 and K2009-54X-20095-04-3), the Swedish Cancer Society (CAN 2008:520 and 5148-B10-04PDS), the Research Fund and Cancer Research Fund of Malmö University Hospital, the Gunnar Nilsson Cancer Foundation, and King Gustaf V's Jubilee Fund for Cancer Research, Stockholm (grants 44052 and 74061) and the Danish Cancer Society (460-A2127-11-S2).

REFERENCES


Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 November 2012; accepted: 07 January 2013; published online: 24 February 2013.


This article was submitted to Frontiers in Cancer Endocrinology, a specialty of Frontiers in Endocrinology.

Copyright © 2013 Brokken, Lundberg-Giwercman, Rajpert-De Meyts, Eberhard, Ståhl, Cohn-Cedermark, Daugaard, Arver and Giwercman. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.
Cryptorchidism and testicular germ cell tumors: comprehensive meta-analysis reveals that association between these conditions diminished over time and is modified by clinical characteristics

Kimberly Banks1,2,3, Ellenie Tuazon1, Kiros Berhane1, Chester J. Koh1,4, Roger E. De Filippo1,4, Andy Chang1,4, Steve S. Kim1,4, Siamak Daneshmand1, Carol Davis-Dao1, Juan P. Lewinger1, Leslie Bernstein1,2 and Victoria K. Cortessis1*

1 Keck School of Medicine, University of Southern California, Los Angeles, CA, USA
2 City of Hope National Medical Center, Duarte, CA, USA
3 St. Joseph Hospital, Orange, CA, USA
4 Children’s Hospital Los Angeles, Los Angeles, CA, USA

Edited by:
Carlo Foresta, University of Padova, Italy
Reviewed by:
Marialuisa Appetecchia, Regina Elena National Cancer Institute IRCCS, Italy
Francesco Lombardo, University of Rome “La Sapienza,” Italy

*Correspondence:
Victoria K. Cortessis, Department of Preventive Medicine, University of Southern California, 1441 Eastlake Avenue, MC-9175, Los Angeles, CA 90089-9175, USA.
e-mail: cortessi@usc.edu

Introduction: Risk of testicular germ cell tumors (TGCT) is consistently associated with a history of cryptorchidism (CO) in epidemiologic studies. Factors modifying the association may provide insights regarding etiology of TGCT and suggest a basis for individualized care of CO. To identify modifiers of the CO-TGCT association, we conducted a comprehensive, quantitative evaluation of epidemiologic data.

Materials and Methods: Human studies cited in PubMed or ISI Web of Science indices through December 2011 and selected unpublished epidemiologic data were reviewed to identify 35 articles and one unpublished dataset with high-quality data on the CO-TGCT association. Association data were extracted as point and 95% confidence interval estimates of odds ratio (OR) or standardized incidence ratio (SIR), or as tabulated data. Values were recorded for each study population, and for subgroups defined by features of study design, CO and TGCT. Extracted data were used to estimate summary risk ratios (sRR) and evaluate heterogeneity of the CO-TGCT association between subgroups.

Results: The overall meta-analysis showed that history of CO is associated with four-fold increased TGCT risk [RR = 4.1(95% CI = 3.6–4.7)]. Subgroup analyses identified five determinants of stronger association: bilateral CO, unilateral CO ipsilateral to TGCT, delayed CO treatment, TGCT diagnosed before 1970, and seminoma histology.

Conclusions: Modifying factors may provide insight into TGCT etiology and suggest improved approaches to managing CO. Based on available data, CO patients and their parents or caregivers should be made aware of elevated TGCT risk following orchidopexy, regardless of age at repair, unilateral vs. bilateral non-descent, or position of undescended testes.

Keywords: testicular neoplasms, cryptorchidism, seminoma, non-seminoma, meta-analysis

INTRODUCTION
Cryptorchidism (CO), or undescended testis, affects approximately 3% of all male live births, making it one of the most common congenital disorders. Despite a high rate of spontaneous resolution during the first year of life, CO is firmly established as the primary risk factor for subsequent development of testicular germ cell tumors (TCGT) (John Radcliffe Hospital Study Group, 1992; Berkowitz et al., 1993; Thong et al., 1998; Paulozzi, 1999). TGCT are the most common form of malignancy among young men in the United States. Advances in systemic therapy have improved overall TGCT survival from 83% in 1975–1979 to 96% in 1999–2005 (Jemal et al., 2010). However, incidence of TGCT has nearly doubled during the same time period (Fast Stats), and it is now evident that significant sequelae include subfertility, (Walsh et al., 2009) sexual dysfunction, (Magelssen et al., 2006) and elevated risk of second malignancy (Moller et al., 1993; Fossa et al., 2005; Travis et al., 2005; Van den Belt-Dusebout et al., 2007). Therefore, a clear understanding of etiologic risk factors and more comprehensive risk stratification is a priority of TGCT research.

Risk of CO and TGCT are associated with additional disorders of the male reproductive system, hypospadias, and impaired spermatogenesis. This pattern is postulated to reflect origins of all of these conditions in errors of development of the fetal testis according to the testicular dysgenesis syndrome hypothesis, which elegantly accounts for experimental research identifying genetic and early environmental factors predisposing to these phenotypes in animal models (Skakkebæk et al., 2001). Little is currently
known regarding the specific insults that may lead to elevated risk of individual and joint phenotypes in humans, or the stages of testicular development when such factors may act. In the present report we comprehensively reviewed the rich set of published observational data on co-occurrence of CO and TGCT as a first step in disentangling the complex associations among these related conditions.

Currently, little is known about the overall characterization of tumor risk in patients with a prior history of CO. Individual estimates of relative risk from the literature range from 1.35 (95% CI = 0.73–2.48) to 18 (95% CI = 12–26), (Miller and Seljelik, 1971; Mostofi, 1973; Morrison, 1976; Henderson et al., 1979; Loughlin et al., 1980; Schottenfeld et al., 1980; Wobbes et al., 1980; Forger et al., 1981; Coldman et al., 1982; Depue et al., 1983; Mills et al., 1984; Pottern et al., 1985; Moss et al., 1986; Giwercman et al., 1987; Swerdlow et al., 1987, 1997; Gershman and Stolley, 1988; Strader et al., 1988; Thornhill et al., 1988; Haughey et al., 1989; Benson et al., 1991; Stone et al., 1991; United Kingdom Testicular Tumor Study Group (UK), 1994; Gallagher et al., 1995; Davies et al., 1996; Moller et al., 1996; Prener et al., 1996; Petridou et al., 1997; Sabroe and Olsen, 1998; Sigurdson et al., 1999; Weir et al., 2000; Stang et al., 2001; Bonner et al., 2002; Herrinton et al., 2003; Dieckmann and Pichlmeier, 2004; Kanto et al., 2004; Hardell et al., 2007; McGlynn et al., 2007; Myrup et al., 2007; Pettersson et al., 2007; Walschaerts et al., 2007; Dusek et al., 2008) suggesting significant differences in study design and/or heterogeneity of the effects of clinical characteristics which may impact risk of TGCT amongst males with CO. Potential modifying factors include anatomic location (abdominal vs. inguinal vs. ectopic) and laterality (unilateral vs. bilateral) of undescended testes, age at treatment, mode of treatment (spontaneous descent vs. hormones or orchiopexy), as well as temporal trends in TGCT risk, and tumor histology. Previous meta-analyses evaluating the CO-TGCT association have been limited to specific subsets of these factors (Castejon Casado et al., 2000; Walsh et al., 2007; Tuazon et al., 2008; Akre et al., 2009).

We report a broader systematic review and meta-analysis of the overall association between CO-TGCT, and explore the possible impact of study design, temporal trends, and clinical features on this association.

MATERIALS AND METHODS

The analysis followed specifications for meta-analysis of observational studies in epidemiology (Stroup et al., 2000) and adhere to PRISMA guidelines (Moher et al., 2009). The outcome was TGCT. The exposure was CO, defined as a testicle undescended at birth that subsequently descended spontaneously, was repositioned into the scrotum by orchiopexy or hormone therapy, or remained undescended. Subgroups were defined by features of study design, CO, and TGCT.

STUDY SELECTION

We searched the MEDLINE (National Library of Medicine, Bethesda, MD, USA) Pubmed interface without language restrictions for human studies published through December 2011, using key words “CO,” “undescended testicle,” “undescended testis,” “undescended testes,” “case-control study,” “cohort study” in combination with “testicular cancer,” “testicular carcinoma,” “testicular neoplasia,” “testis cancer,” “testis carcinoma,” “testis neoplasia,” reviewing also reports cited in retrieved articles and review articles, and by citation indices (ISI Web of Science) for these reports. We also sought high-quality unpublished data. The Review Protocol has not been registered.

DATA EXTRACTION AND CODING

A genetic counselor and a statistician reviewed retrieved articles to determine eligibility for the meta-analysis, resolving conflicts by consensus among themselves and an epidemiologist. Data on study design, overall CO-TGCT associations, and subgroup-specific CO-TGCT associations were extracted systematically by a single reviewer and confirmed by two others. We eliminated redundant data arising from repeated publication, consulting original authors whenever possible.

We extracted published information on RRs relating CO to TGCT as follows: when provided, we recorded point estimates of the odds ratio (OR) for case-control studies and standardized incidence ratio (SIR) for cohort studies, with corresponding standard error or information from which it could be calculated (variance, CI, p-value). When only a point estimate was reported, we requested corresponding variance term from original authors. If the OR estimate was not provided, we calculated it from published tabular data.

Features of study design

We noted the following features of each study: data structure (case-control study of TGCT, cohort study of males born with CO, TGCT cases for whom frequency of CO was compared to external population), country where study was conducted, race/ethnicity of participants, and source of CO data (birth record, medical record before TGCT diagnosis, medical record at/following TGCT diagnosis, reported by participant and/or his mother). For case-control and cohort studies we noted source of reference group (population or population-based registry, hospital or neighborhood, friend). We distinguished published RR estimates extracted from those we calculated from published data; for published estimates, we tabulated covariates and matching variables in original analyses.

Features of cryptorchidism

When possible we extracted or calculated RR estimates of CO-TGCT association for subgroups defined by each of several features of CO: laterality of undescended testicle relative to TGCT (unilateral CO contralateral to tumor, unilateral CO with relation to tumor unspecified, unilateral CO ipsilateral to tumor, bilateral CO), level of maldescent (ectopic, inguinal, abdominal), whether definition of CO included spontaneous descent, means of resolving CO (spontaneous, orchiopexy or hormone therapy, remained undescended), and age at resolution. For this variable we used frequently published categories (0–9 years of age, 10–14 years, 10 years or older, 15 years or older) (United Kingdom Testicular Cancer Study Group (UK), 1994; Moller et al., 1996; Swerdlow et al., 1997) combining data from smaller intervals if provided (Swerdlow et al., 1997). Among studies with alternate cut points, one provided raw data from which
we recalculated estimates within above strata (Herrinton et al., 2003); for four others (Pottern et al., 1985; Strader et al., 1988; Myrup et al., 2007; Pettersson et al., 2007) we assigned data to closest category corresponding to above strata (Table S3, footnotes b–d).

Features of testicular germ cell tumors
We extracted or calculated RR estimates for subgroups defined by histology (non-seminoma, non-seminoma/mixed germ cell tumor (GCT), mixed GCT, seminoma). A separate code for mixed GCT was introduced in 1990 (ICD-0-2), and tumors of mixed histology were previously coded as non-seminoma. Therefore, for studies including diagnoses before 1990, if authors did not specify that non-seminoma excluded mixed histology, we coded reported non-seminoma as "non-seminoma/mixed GCT." As a measure of year of TGCT diagnosis, we determined midpoint of range of years of diagnosis among cases participating in each study.

Unpublished data
Original population-based case-control data (Lacson et al., 2012) were provided before publication. Briefly, 163 TGCT cases identified by the Los Angeles County Cancer Surveillance Program (CSP) and 284 age-matched neighborhood controls were enrolled. TGCT data (histologic type, laterality, age at diagnosis) were provided by the CSP, and CO data (laterality, age at resolution, mean of resolution) were provided by participants and their mothers during in-person interviews.

STATISTICAL ANALYSES
Estimating overall and stratum-specific summary RR of CO-TGCT association
We conducted all meta-analyses using a random effects model, which accounts for between- and within-study variance thereby incorporating the conservative assumption that individual studies estimate different effect sizes (Sutton et al., 2000). We used STATA 8.0 (Stata Corp, College Station, TX) to weight natural log of each contributing OR or SIR estimate by the reciprocal of the corresponding variance. We used this technique to estimate overall and stratum-specific summary relative risk (sRR) estimates. Forest plots were graphed displaying each study’s contribution to sRRs.

Meta-analyses were performed separately for case-control (Tables S2–S4, columns A) and cohort (Tables S2–S4, columns B) studies, with summary measures subsequently pooled. Resulting sRR estimates [Figures 1A, 2A–C and 3A–C; Tables S2–S4, columns C (boldface)] summarize available data on CO-TGCT associations from studies with reference groups judged comparable to cases. Other articles compared CO prevalence of TGCT cases with CO prevalence from external

---

**TABLE S3**

<table>
<thead>
<tr>
<th>Feature</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology</td>
<td>Non-seminoma</td>
</tr>
<tr>
<td>Laterality</td>
<td>Right</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>25 years</td>
</tr>
</tbody>
</table>

**FIGURE 1** Forest and funnel plots for overall association of testicular germ cell tumor (TGCT) risk with history of cryptorchidism (A) Forest plot for combined cohort and case-control data; (B) Funnel plot for combined cohort and case-control data; (C) Forest plot for TGCT case series data; (D) Funnel plot for TGCT case series data.
populations, rather than source population of the cases; limited comparability in such studies is now recognized as a potential source of severe bias (Rothman et al., 2008). We therefore summarized these data separately (Figure 1C; Tables S2–S4, column D).

We implemented Egger’s test of publication bias, assessed heterogeneity by appropriate p-value and I² (Berkowitz et al., 1993) estimates, and assessed trends as described in Appendix Methods, along with procedures for imputation and sensitivity analysis.

ROLE OF THE FUNDING SOURCE
Sponsors had no role in the design, implementation, or reporting of the research. Drs. Victoria K. Cortessis and Leslie Bernstein had full access to all of the original data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

RESULTS
We identified 147 published articles reporting on both CO and TGCT in humans, eliminating those that did not provide data relating CO to TGCT risk (N = 95, not cited), or were duplicate reports of data included in the analysis (10 reports, Table S1). Data from 35 of the remaining 42 reports and raw data from one unpublished case-control study contributed to meta-analyses on which we base our inferences (Table 1A; Figure S1). We requested from authors of three of these reports (Moss et al., 1986; Strader et al., 1988; Swerdlow et al., 1987) standard error data corresponding to published histology stratum-specific point estimates, and received these for one (Strader et al., 1988). In all, data on 9542 TGCT cases contributed to the analyses. For historical interest, we separately summarized estimates from the remaining seven reports, whose authors compared CO among TGCT case-series with...
frequencies of CO measured outside source populations of the cases (Table 1B).

Sensitivity analyses revealed that no single case-control or cohort study influenced either overall or stratum-specific estimates of RR sufficiently to alter interpretation (results not shown). Egger’s tests revealed no evidence of publication bias among cohort data (p = 0.68), case-control data (p = 0.34), or these data types combined (p = 0.40); however, addition of data from the TGCT case series introduced an impression of substantial publication bias (p = 0.01). Visual inspection of Begg’s funnel plots showed that while magnitude and standard error of RR estimates from most cohort and case-control studies (34 of 36) are within the 95% confidence limits (Figure 1B), this is true of a far smaller proportion of TGCT case-series (4 of 7, Figure 1D).

OVERALL CO-TGCT ASSOCIATION AND EFFECTS OF STUDY DESIGN
We estimated sRR of developing TGCT following a history of CO to be 4.0 (95% CI = 3.4–4.6) in case-control studies (Table S2A1) and 4.8 (95% CI = 3.2–7.2) in cohort studies (Table S2B1). The sRR estimated by pooling these results was 4.1 (95% CI = 3.6–4.7), with 14% of the variance ($I^2$) attributed to between-study heterogeneity (Figures 1A,B; Table S2C1). By contrast, sRR estimated from studies that compared CO frequencies between TGCT case series and external populations was...
Table 1 | Epidemiologic studies of cryptorchidism and testicular germ cell tumors (TGCT) included in the meta-analysis.

<table>
<thead>
<tr>
<th>First author</th>
<th>Year published</th>
<th>Country</th>
<th>Ethnicity</th>
<th>Number of TGCT cases</th>
<th>Number of controls</th>
<th>Source of cases</th>
<th>Source of controls</th>
<th>Spontaneous descent included</th>
<th>Number of variables matched and/or adjusted for</th>
<th>Source of cryptorchidism data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morrison (1976)</td>
<td>1976</td>
<td>USA</td>
<td>NS</td>
<td>596</td>
<td>602</td>
<td>Reg</td>
<td>Pop</td>
<td>No</td>
<td>0</td>
<td>TCMR</td>
</tr>
<tr>
<td>Henderson (1979)</td>
<td>1979</td>
<td>USA</td>
<td>WP</td>
<td>79</td>
<td>79</td>
<td>Reg</td>
<td>Negh</td>
<td>NS</td>
<td>2</td>
<td>M</td>
</tr>
<tr>
<td>Pottern (1985)</td>
<td>1985</td>
<td>USA</td>
<td>WP</td>
<td>271</td>
<td>259</td>
<td>Hosp</td>
<td>Hosp</td>
<td>Yes</td>
<td>2</td>
<td>C + M</td>
</tr>
<tr>
<td>Moss (1986)</td>
<td>1986</td>
<td>USA</td>
<td>WP</td>
<td>246</td>
<td>252</td>
<td>Reg + Hosp</td>
<td>Friends</td>
<td>NS</td>
<td>3</td>
<td>C, M</td>
</tr>
<tr>
<td>Gershman (1988)</td>
<td>1988</td>
<td>USA</td>
<td>NS</td>
<td>79</td>
<td>79</td>
<td>Reg</td>
<td>Pop</td>
<td>NS</td>
<td>2</td>
<td>C</td>
</tr>
<tr>
<td>Gallagher (1995)</td>
<td>1995</td>
<td>Canada</td>
<td>WP</td>
<td>506</td>
<td>994</td>
<td>Reg</td>
<td>Pop</td>
<td>NS</td>
<td>1</td>
<td>C</td>
</tr>
<tr>
<td>Prener (1996)</td>
<td>1996</td>
<td>Denmark</td>
<td>NS</td>
<td>171</td>
<td>366</td>
<td>Reg</td>
<td>Pop</td>
<td>Yes</td>
<td>4</td>
<td>PTCMR</td>
</tr>
<tr>
<td>Moller (Moller et al., 1996)</td>
<td>1996</td>
<td>Denmark</td>
<td>NS</td>
<td>514</td>
<td>720</td>
<td>Reg</td>
<td>Pop</td>
<td>Yes</td>
<td>1</td>
<td>C</td>
</tr>
<tr>
<td>Petridou (1997)</td>
<td>1997</td>
<td>Greece</td>
<td>NS</td>
<td>97</td>
<td>198</td>
<td>Hosp</td>
<td>Pop</td>
<td>NS</td>
<td>2</td>
<td>C + M</td>
</tr>
<tr>
<td>Sabroe (1998)</td>
<td>1998</td>
<td>Denmark</td>
<td>NS</td>
<td>357</td>
<td>704</td>
<td>Reg</td>
<td>Pop</td>
<td>Yes</td>
<td>2</td>
<td>BR</td>
</tr>
<tr>
<td>Sigurdson (1999)</td>
<td>1999</td>
<td>USA</td>
<td>WP</td>
<td>160</td>
<td>136</td>
<td>Hosp</td>
<td>Friends</td>
<td>NS</td>
<td>4</td>
<td>C</td>
</tr>
<tr>
<td>Weir (2000)</td>
<td>2000</td>
<td>Canada</td>
<td>NS</td>
<td>325</td>
<td>490</td>
<td>Reg</td>
<td>Pop</td>
<td>No</td>
<td>5</td>
<td>C</td>
</tr>
</tbody>
</table>

(Continued)
### Table 1 | Continued

| First author | Year published | Country         | Ethnicity | Number of TGCT cases | Number of controls | Source of cases | Source of controls | Spontaneous descent included | Number of variables matched and/or adjusted for | Source of cryptorchidism data
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Stang (Stang et al., 2001)</td>
<td>2001</td>
<td>Germany</td>
<td>NS</td>
<td>262</td>
<td>797</td>
<td>Reg</td>
<td>Pop</td>
<td>Yes</td>
<td>2</td>
<td>C, M</td>
</tr>
<tr>
<td>Bonner (Bonner et al., 2002)</td>
<td>2002</td>
<td>USA</td>
<td>WO</td>
<td>116</td>
<td>328</td>
<td>Hosp</td>
<td>Hosp</td>
<td>NS</td>
<td>1</td>
<td>C</td>
</tr>
<tr>
<td>Herrinton (Herrinton et al., 2003)</td>
<td>2003</td>
<td>USA</td>
<td>WP</td>
<td>183</td>
<td>551</td>
<td>Hosp</td>
<td>Hosp</td>
<td>Yes</td>
<td>2</td>
<td>PTCMR</td>
</tr>
<tr>
<td>Dieckmann (Dieckmann and Pichlmeier, 2004)</td>
<td>2004</td>
<td>Unknown</td>
<td>Unknown</td>
<td>538</td>
<td>551</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>Hardell (Hardell et al., 2007)</td>
<td>2007</td>
<td>Sweden</td>
<td>NS</td>
<td>888</td>
<td>870</td>
<td>Reg</td>
<td>Pop</td>
<td>NS</td>
<td>1</td>
<td>C</td>
</tr>
<tr>
<td>McGlynn (McGlynn et al., 2007)</td>
<td>2007</td>
<td>USA</td>
<td>WP</td>
<td>767</td>
<td>928</td>
<td>Reg</td>
<td>Pop</td>
<td>NS</td>
<td>3</td>
<td>C</td>
</tr>
<tr>
<td>Walschaerts (Walschaerts et al., 2007)</td>
<td>2007</td>
<td>France</td>
<td>NS</td>
<td>200</td>
<td>585</td>
<td>Hosp</td>
<td>Hosp</td>
<td>NS</td>
<td>1</td>
<td>C</td>
</tr>
<tr>
<td>Dusek (Dusek et al., 2008)</td>
<td>2008</td>
<td>Czech Republic</td>
<td>WO</td>
<td>356</td>
<td>317</td>
<td>Hosp</td>
<td>Hosp + Neigh</td>
<td>NS</td>
<td>1</td>
<td>C</td>
</tr>
<tr>
<td>Lacson (Lacson et al., 2012)</td>
<td>2012</td>
<td>USA</td>
<td>WP</td>
<td>163</td>
<td>284</td>
<td>Reg</td>
<td>Neigh</td>
<td>Yes</td>
<td>3</td>
<td>C</td>
</tr>
</tbody>
</table>

**ii. Cohort studies of boys with Cryptorchidism**

| Giwercman (Giwercman et al., 1987) | 1987 | Denmark | NS | 6 | – | Hosp | – | No | 2 | PTCMR |
| Benson (Benson et al., 1991) | 1991 | USA | NS | 2 | – | Hosp | – | Yes | 1 | PTCMR |
| Sverdlov (Sverdlov et al., 1997) | 1997 | England | NS | 11 | – | Hosp | – | No | 2 | PTCMR |
| Pettersson (Pettersson et al., 2007) | 2007 | Sweden | NS | 56 | – | Reg | – | No | 2 | PTCMR |
| Myrup (Myrup et al., 2007) | 2007 | Denmark | NS | 110 | – | Reg | – | No | 2 | PTCMR |

**B. STUDIES THAT PROVIDED DATA ON TGCT CASE SERIES, ESTIMATING RISK RATIOS USING CRYPTORCHIDISM FREQUENCY FROM EXTERNAL POPULATION**

| Fonger (Fonger et al., 1981) | 1981 | Canada | NS | 646 | – | Hosp | – | Yes | – | TCMR |
| Kanto (Kanto et al., 2004) | 2004 | Japan | J | 240 | – | Hosp | – | Yes | – | TCMR |

*NS, not specified; WO, white only; WP, white plus other ethnicities; J, Japanese.
*Reg, cancer registry; Hosp, hospital; Neigh, neighborhood; Pop, population.
*NS, not specified.
*dC, case/control self report; M, case/control mother’s report; C + M, case/control self report supplemented by mother’s report; BR, birth record; PTCMR, pre-testicular cancer medical record, TCMR: testicular cancer medical record.
14.8 (95% CI = 10.7–20.4), with 86% of variance attributed to between-study heterogeneity (Figures 1C,D; Table S2Dii). Two additional features of study design appeared to modify sRR estimated among case-control and cohort studies: time CO was recorded, and inclusion of spontaneous descent in CO definition. The RR estimate was notably greater, 9.9 (95% CI = 3.5–28.2), in the single study (Mills et al., 1984) that determined CO history from medical record entries made around the time of TGCT diagnosis, and substantially lower, 1.3 (95% CI = 0.7–2.5), in the single study (Sabroe and Olsen, 1998) in which CO had been recorded at birth. This latter study was likely the only one to have included in the CO group a large proportion of men whose testes would have descended in the first months of life. The same study appeared to be largely responsible for modification by whether definition of CO included spontaneous descent (pheterogeneity = 0.002 for analysis including this study, pheterogeneity = 0.57 for analysis excluding it, Table S2Cvi). Accordingly, this study was a clear outlier in the funnel plot (Figure 1B), while all others fell very near or within the 95% confidence limits.

MODIFICATION BY FEATURES OF CRYPTORCHIDISM
Several features of CO appeared to modify the CO-TGCT association (Figure 2; Table S3). Analyses stratified on laterality of undescended testes relative to TGCT revealed an apparent trend: sRR estimates increased steadily in progression from TGCT arising in testes contralateral to unilateral CO [sRR = 1.5 (95% CI = 0.9–2.6)], on unspecified side relative to unilateral CO [sRR = 3.5 (95% CI = 2.7–4.7)], ipsilateral to unilateral CO [sRR = 5.4 (95% CI = 3.7–7.8)], and in either testis following bilateral CO [sRR = 9.8 (95% CI = 6.2–15.7)] (Figure 2A; Table S3C). Data on anatomic location of undescended testes, provided for no case-control study and only one cohort study (Swerdlow et al., 1997), were insufficient to determine whether TGCT risk differs appreciably following abdominal vs. inguinal non-descent (Table S3Cii). Therefore, the meta-analysis did not confirm the report from a single TGCT case series (Stone et al., 1991) of far higher RR following abdominal non-descent (Table S3Dii).

In analyses stratified on means of resolving CO, estimates increased in the progression of testes that descended spontaneously [sRR = 1.3 (95% CI = 0.9–1.8)], testes repositioned by surgery or hormone therapy [sRR = 4.7 (95% CI = 3.7–6.1)], and testes remaining undescended [sRR = 6.2 (95% CI = 3.7–10.4)] (Figure 2B; Table S3Ciii). Analyses stratified on both means of resolving CO, estimates increased in progression of testes that descended spontaneously [sRR = 1.5 (95% CI = 1.0–2.2)], testes repositioned by surgery or hormone therapy [sRR = 4.7 (95% CI = 3.7–6.1)], and testes remaining undescended [sRR = 6.2 (95% CI = 3.7–10.4)] (Table S3Civ).

MODIFICATION BY FEATURES OF TGCT
Analyses relating CO to tumor histology were conducted first in a subset of data excluding three studies that reported “no difference” in RR of seminoma vs. non-seminoma without providing stratum-specific estimates. The results hinted that RR of seminoma may exceed that of non-seminoma (Table S4C). To minimize the possibility that systematic omission of data from studies reporting no difference had spuriously created this impression, we repeated this analysis using a fuller set of data that included values imputed for those studies; these results also suggested a stronger association of CO with seminoma (Figure 3A; Table S4Cii).

Analyses within strata defined by year of TGCT diagnosis suggest that magnitude of the CO-TGCT association diminished steadily from the 1950’s forward. Highest sRR, 6.8 (95% CI = 4.2–10.9), was estimated among studies with midpoint year of TGCT diagnosis in the 1950’s, and lowest, 3.6 (95% CI = 3.1–4.1), in studies with midpoint 1990 or later (ptrend < 0.001; Figure 3B; Table S4Ciii). Further stratification on histology, possible only for 1960’s forward, revealed that this decrease results largely from a dramatic decrease in the CO-seminoma association [sRR = 9.3 (95% CI = 4.0–21.8) for 1960–1969, sRR = 3.4 (95% CI = 2.5–4.5) for 1990+; ptrend = 0.009], in contrast to no clear trend for other histologies (ptrend = 0.320) (Figure 3C; Table S4Civ).

TRENDS IN OCCURRENCE OF INDIVIDUAL CONDITIONS
Analyses of frequency of CO among controls participating in the case-control studies, (Morrison, 1976; Henderson et al., 1979; Loughlin et al., 1980; Schottenfeld et al., 1980; Coldman et al., 1982; Depue et al., 1983; Mills et al., 1984; Pottern et al., 1985; Moss et al., 1986; Swerdlow et al., 1987; Gershman and Stolley, 1988; Strader et al., 1988; Haughey et al., 1989; United Kingdom Testicular Cancer Study Group (UK), 1994; Gallagher et al., 1995; Davies et al., 1996; Moller et al., 1996; Prener et al., 1996; Petridou et al., 1997; Sabroe and Olsen, 1998; Weir et al., 2000; Stang et al., 2001; Bonner et al., 2002; Herrinton et al., 2003; Dieckmann and Pichlmeier, 2004; Hardell et al., 2007; McGlynn et al., 2007; Walschaerts et al., 2007; Dusek et al., 2008; Lacson et al., 2012) for which midpoint TGCT diagnosis years ranged from 1960’s through 1990+; revealed no evidence that CO became more common among TGCT-free men during this period (ptrend = 0.295, data not shown).

Separate analyses of SEER 9 incidence data revealed that while annual age-standardized incidence of non-seminoma/mixed GCT increased over 28% (from 1.8 to 2.3 new diagnoses per 100,000) from 1973–1978 to 1994–1998, the increase in seminoma was far greater, 68% (from 2.2 to 3.7 per 100,000) during the same interval (Figure S2).

DISCUSSION
Meta-analysis of data from case-control and cohort studies suggests that young men with a history of CO experience approximately 4-fold increased risk of TGCT. Estimates from TGCT case-series whose frequency of CO was compared with separate populations were regarded as unreliable; this practice may cause severe bias (Rothman et al., 2008), which may account for the far larger sRR and between-study variation estimated from these reports.

Addressing more subtle differences in study design, we found that sRR estimates from case-control and cohort studies were notably modified by methods used to determine a man’s history of CO. The stronger association estimated in the single
case-control study in which history of CO was determined from medical record notes made at TGCT diagnosis may have arisen from better recall of CO among cases than controls, since CO was a recognized TGCT risk factor when these diagnoses were made. Such recall bias may have contributed, also, to large effects reported for case series, because history of CO was similarly determined in all of these studies. Undescended testes that descended spontaneously in infancy would rarely be recorded in medical records after the neonatal period, or recalled by study participants or their mothers. We therefore anticipate that only the single study in which CO was recorded at birth (Sabroe and Olsen, 1998) would have identified a high proportion of men with this history. Weak CO-TGCT association reported in this study may indicate that boys born with CO whose testes descend spontaneously in early months experience TGCT risk approaching that of the general population. This possibility has implications for both managing CO and understanding origins of TGCT, so we hope that it will be explored further in retrospective cohorts for which there are detailed neonatal records.

The apparent modification of sRR by features of CO and TGCT may provide insight regarding TGCT etiology. Two general explanations for the CO-TGCT association have long been offered. The common cause hypothesis attributes the association to one or more unidentified etiologic factors shared by CO and TGCT, whereas the position hypothesis asserts that suprascrotal environment increases malignant potential of undescended testes. Hussman suggested two testable predictions of the position hypothesis (Husmann, 2005): (1) in unilateral CO, fully descended contralateral testes should not experience elevated TGCT risk, and (2) early orchiopexy should decrease TGCT risk. Regarding the first, we estimated a lesser sRR for contralateral testes [1.5 (95% CI = 0.9–2.6)] than for ipsilateral testes [5.4 (95% CI = 3.7–7.8)], suggesting a deleterious effect of suprascrotal position. Also consistent with positional effects, sRR among those with bilateral non-descent [9.8 (95% CI = 6.2–15.7)] was, within statistical precision of the meta-analysis, indistinguishable from twice sRR of those with unilateral non-descent [2 x sRR_ipsilateral = 10.8 (7.4–15.6)]. However, published data are insufficient (24 cases in 7 studies) to rule out a small increase in risk to contralateral testes (e.g., 20%), as would be required to strictly affirm Hussman’s first prediction. We note, however, that some true increase in risk to contralateral testes would not necessarily rule out the position hypothesis, because central responses to a single testis in a suprascrotal position could, in theory, contribute to malignant potential of the contralateral testis. For example, in rodent models of unilateral CO created surgically, degenerative changes (Quinn, 1991; Zakaria et al., 1998) and altered gene expression (Iizuka et al., 1996) were demonstrated in contralateral, descended testes. A phenomenon observed in humans is also consistent with this possibility: among patients with unilateral TGCT who undergo biopsy of the contralateral testis, men with a history of CO are more often found to have the presumptive TGCT precursor carcinoma in situ testis/infra-tubular germ cell neoplasia than those without history of CO (Dieckmann and Loy, 1996). Regarding Hussman’s second prediction, we observed greater TGCT risk among those who experienced later resolution. However, we cannot confidently conclude that deleterious effects of suprascrotal position are responsible. An alternate explanation, which we cannot rule out, is that men with earlier resolution of CO experienced as a group inherently lower risk of TGCT. This might occur, for example, if this group included a higher proportion of boys destined to experience spontaneous descent if therapeutic intervention had been delayed. Because elevated risk was observed, regardless of age at orchiopexy, the clinical significance of available data is that patients undergoing orchiopexy at any age should be closely monitored; thus along with their parents or primary care givers and primary care physicians, they should be made aware of the increased risk.

Unfortunately, published data could not distinguish between risks experienced by men with histories of abdominal vs. inguinal non-descent. These distinct phenotypes may provide a means of determining relevance to human CO and TGCT of animal models of CO with high (Hsieh-Li et al., 1995; Rijli et al., 1995; Good et al., 1997; Nef and Parada, 1999; Overbeek et al., 2001) or low (Hutson, 1986; Lahoud et al., 2001) non-descent resulting from disruption of specific genes. If found to be relevant to human CO and TGCT, these models may become valuable tools in TGCT research, which has long suffered from absence of animal models of common forms of TGCT (Oosterhuis and Looijenga, 2005). Therefore, documenting position of undescended testes before orchiopexy may be useful for future research and in follow-up of men with a history of CO, particularly in the era of electronic medical records.

Modification by features of TGCT may suggest etiologic heterogeneity of these tumors. Morrison (1976) apparently first suggested that CO is more strongly associated with seminoma than non-seminoma, although subsequent reports were inconsistent. The meta-analysis suggested greater RR for seminoma than for non-seminoma, and an intermediate value for mixed GCT, by addressing a far larger set of data than any single study and treating mixed GCT as a distinct histologic type. Although mechanisms underlying this pattern remain unknown, this finding suggests that tumors of distinct histologic types may have separate etiologies and/or result from events at different developmental stages. This possibility accords with the far more dramatic decreases in the CO-seminoma association observed over time. This trend, together with the observation that incidence of seminoma has risen more rapidly than that of non-seminoma/mixed GCT (Figure S2), raises the intriguing possibility that increasing occurrence of seminoma may involve increasing exposure to unidentified environmental factors through processes unrelated to CO.

In conclusion, this meta-analysis provides a detailed quantitative summary of available high-quality observational data on the association between CO and TGCT, including observations that can no longer be made due to trends toward younger age at CO repair and increasing TGCT incidence. Results of subgroup analyses indicate possible future directions in understanding both stratification of TGCT risk among boys born with undescended testes and TGCT etiology. Meta analyses addressing features of CO suggest that while bilateral CO is associated with nearly twice the TGCT risk as unilateral CO, data are inadequate to assess the role of anatomic position of the undescended testis. Early repair is associated with lower TGCT risk, but published data do not provide a basis for recommending optimal time of repair or to determine whether optimal repair can reduce risk to baseline.
Therefore, all CO patients and their families should be counseled to be aware of future risk. Additional data are needed to confirm the possibility that TGCT risk is not elevated among boys whose testes descend spontaneously after birth. Results of subgroup analyses addressing features of TGCT suggest multiple pathways to malignancy and indicate considerable heterogeneity in risk of TGCT following CO. Both possibilities warrant mechanistic examination using contemporary tools of molecular biology.

DEDICATION

The authors dedicate this report in memory of the life and work of our esteemed colleague, Dr. Brian E. Hardy.

ACKNOWLEDGMENTS

This work was supported by grants California Cancer Research Program grants 99-00505V-10260 and 03-00174VRS-30021 and the National Cancer Institute grant CA102042 to Dr. Victoria K. Cortessis from the National Cancer Institute and a grant from the Whittier Foundation to the Norris Comprehensive Cancer Center.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Cancer_Endocrinology/10.3389/fendo.2012.00182/abstract

REFERENCES


Testicular cancer and cryptorchidism

Lydia Ferguson and Alexander I. Agoulnik *
Department of Human and Molecular Genetics, Herbert Wertheim College of Medicine, Florida International University, Miami, FL, USA

The failure of testicular descent or cryptorchidism is the most common defect in newborn boys. The descent of the testes during development is controlled by insulin-like 3 peptide and steroid hormones produced in testicular Leydig cells, as well as by various genetic and developmental factors. While in some cases the association with genetic abnormalities and environmental causes has been shown, the etiology of cryptorchidism remains uncertain. Cryptorchidism is an established risk factor for infertility and testicular germ cell tumors (TGCT). Experimental animal models suggest a causative role for an abnormal testicular position on the disruption of spermatogenesis however the link between cryptorchidism and TGCT is less clear. The most common type of TGCT in cryptorchid testes is seminoma, believed to be derived from pluripotent prenatal germ cells. Recent studies have shown that seminoma cells and their precursor carcinoma in situ cells express a number of spermatogonial stem cell (SSC) markers suggesting that TGCTs might originate from adult stem cells. We review here the data on changes in the SSC somatic cell niche observed in cryptorchid testes of mouse models and in human patients. We propose that the misregulation of growth factors’ expression may alter the balance between SSC self-renewal and differentiation and shift stem cells toward neoplastic transformation.

Keywords: testis, cryptorchidism, testicular cancer, spermatogonial stem cells, somatic cell niche

INTRODUCTION

Cryptorchidism, or maldescended testes, is a common clinical diagnosis in newborn boys and one of the strongest risk factors for infertility and testicular cancer (Hutson et al., 2010). The position of the cryptorchid testicle may vary and can be located in the abdominal cavity, inguinal canal, or subcutaneous cavity, which could determine the extent of the associated phenotype. While some populations are affected at a higher frequency than others, around 2–4% of boys are globally diagnosed with either unilateral or bilateral cryptorchidism (Barthold and Gonzalez, 2003).

About 10% of all cases of testicular germ cell tumors (TGCT) occur in men with a history of cryptorchidism (Manuel et al., 2012). Thus, a disruption of a common regulatory pathway, for example, androgen signaling, might be an underlying reason for the association of cryptorchidism and TGCTs. An alternative explanation is that the abnormal testis position itself is directly responsible for infertility and germ cell tumorigenesis. Indeed, the causative role of an abnormal testis position in infertility has been demonstrated in several animal experimental models. The elevated temperature of the undescended testis inhibits the differentiation of spermatogonia resulting in an arrest of spermatogenesis, reduced seminiferous tubule size, germ cell depletion, and fibrosis. The link between cryptorchidism and TGCT is however less clear. The most common type of TGCT in cryptorchidism is seminoma. It is commonly accepted that the precursor cancer cells are pluripotent germ cells. Whether such cells are derived from primordial germ cells (PGCs) also known as gonocytes, that continue to proliferate or undergo improper differentiation (Skakkebaek et al., 1987) or are a result of spermatogonial stem cell (SSC) transformation may be debatable. The abnormal testis position dramatically alters the function of somatic cells providing the niche for SSC self-renewal and differentiation. We review here the epidemiologic, genomic, and experimental data that might explain the higher incidence and the causes of TGCT in cryptorchidism. Clinical aspects of this disease are beyond the scope of this review and can be found elsewhere (Isidori and Lenzi, 2008).

TESTICULAR DESCENT

During embryonic development, the gonads differentiate from the genital ridges. After completion of sex determination, both ovary and testis remain in a high pararenal position attached to the body walls by a mesenterial ligamentous complex derived from the mesonephric mesenchyme (Hutson et al., 1997; Borteczko and Jacob, 2000). At this stage, the cranial mesonephric ligament and the caudal genitouinguinal ligament (or gubernaculum) connect the gonads to the abdominal wall. The development and reorganization of these two ligaments, along with the differentiation of the epididymis, growth, and orientation of the gonads and reproductive tracts and finally the intra-abdominal pressure, direct the movement of the testis to the scrotum.

The two-stage model of testicular descent (Hutson et al., 1997), distinguishes the transabdominal phase characterized by the descent of the testis into the lower abdominal position, and the inguinoscrotal phase during which the testis moves through the inguinal canal and into the scrotum. During the transabdominal phase, the gubernaculacord and bulb are formed followed by the differentiation of muscle layers around the bulb. In humans, the first stage of testicular descent occurs between 10–15 weeks of gestation (Hutson et al., 1997). Transgenic studies in mice have identified INSL3 as the major factor in transabdominal testicular descent (Nef and Parada, 1999; Zimmermann et al.,...
abdominal position (Adham et al., 2002). Combined with the leads to gubernaculum differentiation and ovary descent to a low abdominal position (Adham et al., 2002). Combined with the fact that an androgen deficiency does not affect transabdominal descent, one can assume that INSL3 is the primary peptide responsible for this process. Analysis of mutant gubernaculum development and the comparisons of gene expression in mutant and wild-type tissues performed in our laboratory, indicated that the NOTCH and WNT/beta-catenin cell signaling pathways might mediate the INSL3 effects at the cellular level (Kaftanovskaya et al., 2011). The effect of INSL3 deficiency is multifold; it causes suppression of myoblast differentiation in the muscle layers of the gubernaculum, apoptosis, and reduction of androgen receptor (AR)-positive cells within the base of the gubernaculum, and a failure of processus vaginals development (Kaftanovskaya et al., 2011).

The second inguinoscrotal stage of testicular descent is clearly androgen-dependent (Hutson et al., 2010; Kaftanovskaya et al., 2012). Suppression of androgen production or AR deficiency has been linked to cryptorchidism in humans and various other species (Bay et al., 2011). Interestingly, an increase in testosterone production in human embryos precedes inguinoscrotal testicular descent (Hutson et al., 2010). The level of serum testosterone peaks at 15–18 weeks of fetal life and declines thereafter, whereas the testis remains in the same position for 5–10 weeks after the completion of transabdominal descent. In addition, several other processes occur during this time. Starting at about 8–10 weeks the scrotum anlage is formed and the floor of the gubernaculum base inverts to become the tunica of the sac-like processus vaginals peritonei. The muscle layers at the rim of the gubernaculum further develop to become the wall of the cremasteric sac and the testes glide along the newly formed inguinal canal and into the scrotum (Van Der Schoot, 1996). Because the majority of these processes are androgen-sensitive, it is not surprising that many abnormalities that lead to a compromised differentiation or function of stereogenic Leydig cells as well as the failure of androgen signaling result in various degrees of cryptorchidism (Hutson et al., 1997).

A non-functional hypothalamo-pituitary-gonadal (HPG) axis results in hypogonadotrophic hypogonadism. The homozygous mutant mice for gonadotropin-releasing hormone (GNRH, hpg), GNRH-receptor (Gnrhr), and the LH receptor knockout mouse (LurKko) devoid of LH stimulation, all have impaired inguinoscrotal testicular descent (Klonisch et al., 2004; Pask et al., 2005; Feng et al., 2006). A number of human syndromes related to testicular feminization or genitourinary dysplasia have the hallmarks of androgen deficiency as well (Klonisch et al., 2004). Estrogen-like and anti-androgen compounds have been shown to affect testicular descent through the suppression of the functional activity of Leydig cells and decreased testosterone and INSL3 production (Klonisch et al., 2004). Little is known, however, about local cell signaling pathways activated by androgen signaling in the developing gubernaculum, scrotum, cranial ligament, and epididymis. The link between inguinoscrotal cryptorchidism detected in the transgenic mouse and in some cases human mutants for several transcriptional factors, such as homeobox genes HOXA10 or HOXA11; Wilms tumor 1 (WT1); ARID-domain-containing protein 5B (ARID5B), and androgen signaling in gubernaculum development still need to be determined (Klonisch et al., 2004; Kaftanovskaya et al., 2013).

**EPIDEMIOLOGY OF CRYPTORCHIDISM**

The prevalence of cryptorchidism varies somewhat from country to country with the highest incidence reported in Denmark (9.0%) compared to that of the lowest, found in Finland (2.4%) (Boisen et al., 2004). Global cryptorchidism rates of around 2–4% are generally accepted (Barthold and Gonzalez, 2003). Several studies reported an increase in incidence during the 1970s and 1980s including Lithuania and the USA (Paolozzi, 1999; Preiksa et al., 2005), however in England rates have been declining since the 1990s (Jones et al., 1998).

Variations in rates of cryptorchidism over the years and also between countries such as the difference between Finland and Denmark, may reflect the impact of many contributing environmental factors. Man-made environmentally used chemicals such as pesticides, phthalates, bisphenol A (BPA), and polychlorinated biphenyls (PCBs) are known endocrine disrupters (Acrini and Hughes, 2006). Pregnant women treated with DES gave birth to boys with a higher incidence of cryptorchidism than non-treated women (Stillman, 1982).

Consumption of liver or smoked products, which have been found to contain a higher concentration of PCB has been implicated in higher rates of cryptorchidism (Giordano et al., 2008). Likewise, maternal consumption of more than five alcoholic drinks a week has been linked to an increase in the risk of cryptorchidism in one study (Damgaard et al., 2007), however disputed by others (Moller and Skakkebaek, 1997; Biggs et al., 2002; Kurahashi et al., 2005).

Similar disputes are also a factor in the contribution of maternal smoking to cryptorchidism. One study found that smoking more than 10 cigarettes a day during pregnancy (Jensen et al., 2007), increased the risk, whereas other studies have found no viable link (Mongraw-Chaffin et al., 2008). The discrepancies in the results of studies looking at maternal dietary and lifestyle factors may be a result of inconsistent data collection methods or inaccuracies from maternal questionnaires.

Given that the inguinal stage of testicular descent is between weeks 26 and 35, premature birth and low birth weight often associated with prematurity are contributing factors to an increased risk of cryptorchidism (Boisen et al., 2004; Jensen et al., 2007). However spontaneous correction is seen more frequently in boys who reach average weight within 1 year after birth, than in those who remain smaller (Preiksa et al., 2005; Jensen et al., 2007). Lifestyle factors that contribute to reproductive birth defects such as cryptorchidism may therefore have an indirect contribution to the formation of testicular germ cells in affected individuals.
GERM CELL TUMORS IN CRYPTORCHIDISM

Testicular cancer afflicts 1% of the male population and is the most common solid tumor to affect young men between the ages of 15–34. The association between cryptorchidism and TGCT has been well documented since the 1940s. Cryptorchidism is an accepted risk factor with a relative risk of 3.7–7.5 times higher than the scrotal testis population (Thorup et al., 2010). Conversely, it has been shown that 5–10% of men who develop testicular cancer, were or are cryptorchid (Thorup et al., 2010). There is an increased cancer risk in bilateral as opposed to unilateral cryptorchidism. Some studies have indicated that there is a direct correlation between how long the testis was subjected to a cryptorchid position and TGCT incidence. This can be seen from the data on surgical correction of cryptorchidism and the reduction of the risk of testicular cancer. One particular study found 13 out of 14 uncorrected cryptorchid patients between 1934 and 1975 developed TGCT in their abdominal testes (Batata et al., 1980). A Swedish group studied almost 17,000 men treated for cryptorchidism between 1964 and 1999 with the average age of surgery being 8.6 years. In this group, 56 individuals developed testicular cancer. Individuals who had corrective surgery before the age of 13 had an incidence rate of 2.23%, whereas those who were treated after 13 had an incidence rate of 5.4% (Pettersson et al., 2007). Based on such data in recent years, the recommended age of surgical correction was reduced and now is usually performed before the age of 2 (Pettersson et al., 2007). It should be mentioned however that some other reports did not find correlation between the time of surgery and risk of TGCT (Hack et al., 2007). In any case, even after early surgical correction the risk of TGCT is somewhat higher in patients with cryptorchidism.

The other factor that appears to play a role in TGCT incidence is the relative position of the cryptorchid testes, and hence the degree of environmental insults on the gonads, such as heat. It was shown that an abdominal testis presents a greater risk for TGCT than an inguinal testis (Cortes et al., 2001).

Although corrective surgery has been found to reduce the risk from fivefold to twofold, in some cases the formerly cryptorchid testis becomes cancerous, indicative of permanent epigenetic changes in the cryptorchid testes (Hutson et al., 2010). Indeed, differences in promoter methylation and corresponding gene expression of several genes have been reported in TGCT. Apart from cell transformation such changes might be a result of environmental insults in cryptorchid testis. The other aspect that was extensively studied is the risk of TGCT in normally descended contralateral testes in men with unilateral cryptorchidism. Recent meta-analysis of such data indicated that the TGCT risk factor is much higher in affected testes than in scrotal one (6.33 vs. 1.74) (Akre et al., 2009).

It should be noted, that in many epidemiologic association studies the relative position of testes, age of surgical or spontaneous correction, presence of additional developmental abnormalities, or even variable definitions of cryptorchidism were not always taken into account. However a large majority of data indicates that age of surgery and the relative position of the cryptorchid testis are contributing factors to a greater risk of TGCT.

GENETIC FACTORS IN TGCT

The genetic factors and mutations involved in the potential predisposition to TGCT in the cryptorchid testes are not entirely clear as most studies do not differentiate between individuals with a history of cryptorchidism and those without. Genome-wide studies have identified six susceptibility loci, KITLG and ATF7IP on chromosome 12, SPRY4 on chromosome 5, BAK1 on chromosome 6, TERT-CLPTM1L on chromosome 5, and DMR1 on chromosome 9 (Rapley et al., 2009; Turnbull and Rahman, 2011). The KITLG-KIT pathway has been implicated in PGC development and is consistent with a role in contributing to TGCT (Kanetsky et al., 2009). SPRY4 a downstream target of the KITLG-KIT pathway and an inhibitor of the protein kinase pathway was linked to TGCT. Similarly, BAK1 was also associated with TGCT in the same GWAS study. BAK1 is repressed by the KITLG pathway and acts as a germ cell apoptosis promoting factor by binding to the apoptosis repressor BCL2 (Turnbull and Rahman, 2011).

Significantly, mice with mutations in Kit genes require a specific genetic background to develop testicular cancer. Mice harboring a mutation of the Steel locus, which deletes Kitl, bred on a 129 background have a higher incidence of testicular cancer than wild-type controls. These mice also exhibit PGC defects in proliferation, migration, and survival demonstrating a possible link between pluripotent cell differentiation and TGCT (Heaney et al., 2008).

The telomerase encoding TERT locus and its transcription factor regulator ATF7IP are often overexpressed in cancers. Both alleles were associated with a predisposition for TGCT in a UK based whole genome association study (Turnbull et al., 2010). A similar study conducted in the USA, identified two SNPs within the DMR1 allele – a zinc finger-like DNA-binding motif, significantly linked to TGCT. DMR1 is expressed in the male gonad during Sertoli cell maturation and the deletion of this gene is associated with gonadoblastoma (Kanetsky et al., 2011).

Other genetic abnormalities were found to be associated with TGCT, however the causative nature of such mutations is not clear. Among these, the alleles of AR gene with short GGN repeats were linked to an increased risk of metastatic disease (Vastermark et al., 2011).

SOMATIC MUTATIONS AND GENE MISEXPRESSION

The role of somatic mutations in p53, PTEN, and other classical tumor suppressor genes in TGCT remains contradictory, however TGCT response to cisplatin-based chemotherapy indicates cancer cell sensitivity to p53 activation (Gutekunst et al., 2011). In some studies, a downstream target of p53, DAPK-1 was hypermethylated in seminomas compared to normal testes and was found to be clinically useful for testicular germ cell tumor stage diagnosis (Christoph et al., 2007). In contrast, a study that looked at 31 primary germ cell tumors found no mutations in p53 however 9 of 14 tumors tested positive with a p53 antibody (Lothe et al., 1995).

Platelet derived growth factors (PDGFs) and their receptors expressed in the pre- and postnatal testis, have also been implicated in testicular tumor formation (Basciani et al., 2010). An aberrant 1.5 kb transcript of the PDGF α-receptor was detected in TGCT and testis parenchyma with carcinoma in situ and was completely absent in normal testicular tissue. Moreover, this same aberrant
The association between cryptorchidism and testicular cancer. It should be noted however that individuals presenting three or all four of the symptoms are extremely rare, and are usually associated with a 45X/46XY genotype or some forms of androgen insensitivity. Manifestations of one or two symptoms (such as cryptorchidism and infertility, or cryptorchidism and testicular cancer) are much more common.

In recent years, the common entity hypothesis of TDS has been the subject of debate. The critical evaluation of epidemiologic studies has brought into question the existence of widespread TDS due to the absence of non-casual associations between its different manifestations (Akre and Richardi, 2009; Thorup et al., 2010). Most affected individuals exhibit one or two features, bringing into question whether one factor may be accountable for the occurrence of the abnormalities with such vastly diverse developmental etiology. On the other hand, the experimental data indicate that causative relationships can explain some of the epidemiologic correlation.

Since the TDS concept was proposed multiple attempts have been undertaken to identify genetic factors responsible for such a syndrome. The most recent genome-wide study investigated genetic variants affecting Danish males with at least one symptom of TDS in an attempt to attribute symptoms to a specific region of the genome. The study identified an association of subsets of these symptoms to genetic factors, in particular between cryptorchidism and testicular cancer (Dalgaard et al., 2012). The analysis highlighted a single nucleotide polymorphism in TGFB3 mildly associated with all four symptoms of TDS. This gene encodes the TGFβ receptor type III found to be expressed in testicular Leydig cells and peritubular cells and when silenced in mouse, impedes Leydig cell function and normal cord formation (Sarraj et al., 2010). A member of the TGFβ superfamily, BMP7 was found to contain genomic variants in some patients with TDS symptoms, most notably those with cryptorchidism and testicular cancer. Likewise, mutations in the KITLG locus were mostly closely linked to an increased risk of testicular cancer and were not connected to any other TDS symptoms. Mutations in this gene have previously been associated with infertility as well as germ cell tumors (Galan et al., 2006). It should be pointed out however, that currently no mouse mutations in the genes encoding members of BMP/TGFβ signaling are known to cause isolated cryptorchidism.

The identification of a single or pairs of features of symptoms of TDS, as opposed to finding all four symptoms consistently running together, has further fueled speculation that the phenotypes of TDS are not due to a single cause. The occurrence of testicular cancer has increased over the last 40 years in Western countries along with rates of infertility, however rates of cryptorchidism remain unchanged. Hypospadias, most commonly associated with low birth weight, have shown a marginal increase in some studies since the 1950s but show wide epidemiological variation between countries. Interestingly, hypospadias are found isolated from cryptorchidism in 95% of cases and men with hypospadias alone do not suffer from reduced semen quality unless their symptom is accompanied with cryptorchidism. A much greater correlation between an undescended testis and a high risk of infertility or testicular cancer is supported by the alleviation offered by early orchiopexy. With 5% of germ cell tumors arising in previously cryptorchid testes, it...
is apparent that many genetic and pathologic factors interplay in each feature associated with TDS (Thorup et al., 2010).

**CRYPTORCHIDISM AS THE UNDERLYING CAUSE OF INFERTILITY AND GERM CELL APOPTOSIS**

An alternative explanation to TDS, at least, with regard to cryptorchidism, is that the undescended testis itself causes an increased disposition for infertility and spermatogonial arrest, which in some cases may lead to abnormal germ cell differentiation and the formation of TGCT. This notion is strongly supported by the beneficial effects of surgical correction of cryptorchidism on future infertility and cancer risk. Experimental animal data also support this possibility. Induced cryptorchidism in animal models has been shown to lead to a depletion of germ cells, eventually resulting in infertility (Agoulnik et al., 2012), whereas orchiopexy restored spermatogenesis in several cryptorchid mouse mutants (Bogatcheva and Agoulnik, 2005).

The cause of germ cell depletion is strongly linked to the elevated environmental temperature of 37°C within the body cavity compared to the optimal temperature of 32°C for germ cells in the scrotum, although the mechanism behind this cause is not clear (Kumagai et al., 2000; Yin et al., 2002; Izu et al., 2004). Later stage haploid germ cells have been shown to be the most susceptible germ cells when exposed to abdominal temperature.

Apoptosis in the testis is essential for the establishment and maintenance of germ cell populations. In the cryptorchid testis, temperature induced apoptosis is responsible for the depletion of germ cells, however the molecular mechanisms behind this have not been fully determined. In the p53 knockout model, apoptosis is delayed by 3 days, from day 7 to 10 in the cryptorchid induced mouse, compared with the wild-type cryptorchid group. Thus it can be deduced that p53 is responsible for the initial phase of apoptosis in the germ cells (Yin et al., 1998). However, the initiation of apoptosis from day 10 is indicative of a p53-independent pathway after the initial phase of germ cell loss. In p53−/− and lpr/lpr double mutant mice, apoptosis is further delayed compared to the cryptorchid control group, showing that Fas is responsible for the later stage germ cell loss (Yin et al., 2002). In addition, the occurrence of testicular apoptosis despite the delay, suggests that a third pathway is activated in the cryptorchid testis.

**SOMATIC CELL CHANGES IN CRYPTORCHIDISM**

Spermatogonial cells are less affected by higher temperatures than all other germ cells and in the cryptorchid testis they survive the longest, however with time they are also eventually depleted. The question then arises, why do the spermatogonia cells decrease in the cryptorchid testis? Can it be linked to the changes in the somatic cell niche in seminiferous tubules?

The abnormal effects of the intra-abdominal environment in cryptorchid testes are not limited only to germ cells; somatic cells have also been shown to display dramatic changes in morphology, function, and gene expression. Sertoli cell vacuolization and abnormal cell adhesion are some of the most common consequences of cryptorchidism. Sertoli cells in an induced cryptorchid testis of the Rhesus monkey were found to have significant changes in the cytoskeleton (Zhang et al., 2004). Reactivation of cytokeratin 18 in adult Sertoli cells and increased expression of vimentin with disorganized staining characterized the heat stressed Sertoli cells in these primates with similar findings in the Sertoli cells of experimentally induced cryptorchid testes in rats (Wang et al., 2002).

In another study, rats with induced cryptorchidism showed disrupted actin filaments in the basal junction regions of the Sertoli cells compared to the regular lattice structures seen in the control rats (Mackawa et al., 1995). The morphological changes in the somatic cells of cryptorchid testes were also accompanied by changes in multiple genes’ expression of these cells.

In mice, Ribosomal Binding Protein Motif 3 (Rbm3) expression in adult Sertoli cells was reduced within 12 h of induced cryptorchidism (Danno et al., 2000). Likewise, the expression of FSH and ARs was dramatically reduced in cryptorchid lambs (Monet-Kuntz et al., 1987). While surgical correction restored their expression, up to 50% of seminiferous tubules did not recover, indicative of cryptorchidism-induced permanent damage.

Hadziselimovic et al. (2011) looked at differentially expressed transcripts in testis RNA isolated from patients with cryptorchidism with or without type A4 spermatogonia (A dark spermatogonia are believed to be SSCs in human) and therefore at a low or a high risk of developing azoospermia later in life. In the latter group, a number of genes involved in A4 spermatogonia self-renewal were not expressed, but were expressed in patients with a low risk and in the control groups. These factors included FGF9 which is essential for SSC self-renewal and acts as an inhibitor of meiosis through regulation of pluripotent genes, and FGFR3, expressed in prepubertal spermatogonia in the control and completely absent in the high risk group (Hadziselimovic et al., 2011).

Disruption of the blood-testis-barrier (BTB) enforced by Sertoli cells has also been reported in cryptorchid mice by breakdown of inter-Sertoli cell tight junctions which may have repercussions on the later stage germ cells. It was recently shown that cystic fibrosis transmembrane conductance regulator (CFTR) which enhances Sertoli cell tight junctions is significantly downregulated in cryptorchid testes and may contribute to the disruption of the BTB (Chen et al., 2012). This was also supported by the discovery that incubation of primary Sertoli cell cultures at 37°C, results in a decrease of CFTR expression compared to those incubated at 32°C. Breakdown of the BTB in both cases was shown by diffusion of injected tracker dye into the interstitial space (Chen et al., 2012). The physiological separation between pre and post meiotic germ cells, maintained by Sertoli cells in the testis, ensures that regulatory products are targeted to specific germ cell populations. Disruption of their cellular morphology, BTB, and expression of growth factors may lead to the misregulation of germ cells and abnormal differentiation, potentially leading to the formation of TGCTs.

**ORIGIN OF TGCT**

The TGCT most commonly associated with cryptorchidism is seminoma. It is generally accepted that the classical seminomas develop from a precursor lesion, intratubular germ cell neoplasia (or carcinoma in situ, CIS). It is proposed to develop in utero from PGCs or early gonocytes (Skakkebaek et al., 1987). After remaining quiescent during infancy, the CIS is thought to proliferate at puberty and later progress to an invasive disease.
under the influence of factors such as gonadotrophins and/or testicular steroids (Skakkebaek et al., 1987). Contrary to the classical seminoma, the spermatocytic seminoma is believed to derive from differentiating spermatogonia. Recent data however showed that CIS and spermatocytic seminoma share a number of common markers with embryonic stem cells, for example, KIT, OCT3/4, SOX17, LIN28, NANOG, FGFR3, DMTR1, and others (Looijenga et al., 2003; Rajpert-De Meyts et al., 2003; Almstrup et al., 2004; Houldsworth et al., 2006; Gillis et al., 2011; Ryser et al., 2011). Thus, according to this hypothesis, expression of stem-related markers is a reflection of their origin from gonocytes. It should be pointed out however, that the expression of some of these genes is maintained and characteristic for adult SSCs (Waheeb and Hofmann, 2011).

The alternative theory of CIS transformation initiated during meiosis postulates that the common duplication of chromosome 12p in TGCT occurs due to abnormal recombination during meiosis (Chaganti and Houldsworth, 1998). The amplification of genes in this genomic region including NANOG, DPPA3, GDF3 may provide a selective proliferation advantage and subsequent reactivation of a stem-like phenotype (Houldsworth et al., 2006).

It is difficult to test experimentally which model is correct. One indirect approach is to evaluate the incidence of CIS as a precursor to TGCT in cryptorchid boys. If CIS is derived from embryonic gonocytes then the CIS should be detectable long before cancer development. In a study by Cortes et al. (2004), one invasive TGCT and six CISs were found in testicular biopsies of 182 cryptorchid boys. If CIS is derived from embryonic gonocytes, then CIS should be detectable long before cancer development. In a study by Cortes et al. (2004), one invasive TGCT and six CISs were found in testicular biopsies of 182 cryptorchid boys.

The alternative theory of CIS transformation initiated during meiosis postulates that the common duplication of chromosome 12p in TGCT occurs due to abnormal recombination during meiosis (Chaganti and Houldsworth, 1998). The amplification of genes in this genomic region including NANOG, DPPA3, GDF3 may provide a selective proliferation advantage and subsequent reactivation of a stem-like phenotype (Houldsworth et al., 2006).

It is difficult to test experimentally which model is correct. One indirect approach is to evaluate the incidence of CIS as a precursor to TGCT in cryptorchid boys. If CIS is derived from embryonic gonocytes then the CIS should be detectable long before cancer development. In a study by Cortes et al. (2004), one invasive TGCT and six CISs were found in testicular biopsies of 182 cryptorchid boys. If CIS is derived from embryonic gonocytes, then CIS should be detectable long before cancer development. In a study by Cortes et al. (2004), one invasive TGCT and six CISs were found in testicular biopsies of 182 cryptorchid boys. If CIS is derived from embryonic gonocytes, then CIS should be detectable long before cancer development. In a study by Cortes et al. (2004), one invasive TGCT and six CISs were found in testicular biopsies of 182 cryptorchid boys. If CIS is derived from embryonic gonocytes, then CIS should be detectable long before cancer development. In a study by Cortes et al. (2004), one invasive TGCT and six CISs were found in testicular biopsies of 182 cryptorchid boys. If CIS is derived from embryonic gonocytes, then CIS should be detectable long before cancer development. In a study by Cortes et al. (2004), one invasive TGCT and six CISs were found in testicular biopsies of 182 cryptorchid boys. If CIS is derived from embryonic gonocytes, then CIS should be detectable long before cancer development. In a study by Cortes et al. (2004), one invasive TGCT and six CISs were found in testicular biopsies of 182 cryptorchid boys.
cryptorchidism-induced somatic niche environment most of the differentiating germ cells will be eliminated due to arrest of spermatogenic differentiation, lack of proper cell signaling, abnormal cell junction, and increased apoptosis. The depletion of the SSC pool will eventually lead to the complete loss of germ cells in the cryptorchid testis, which would explain the infertility in patients with surgical correction performed later in life. Accumulation of additional mutations or chromosome rearrangements, including amplification of chromosome 12p, might provide a selective growth advantage for SSC or differentiating germ cells. Instead of the spermatogenetic pathway the cells can now escape into a pre-neoplastic state. Depending on the differentiation stage when such an event occurs, this might give rise either to CIS further progressing to a classical seminoma or the development of a spermatocytic seminoma, derived from more differentiated spermatogonial cells. This scenario does not rely on a common factor causing two seminoma phenotypes with dramatically different etiology, but rather defines cryptorchidism only as an independent causative risk factor in the development of TGCT.

**CONCLUSION**

Testicular cancer is accountable for 1% of all cancers in men and is the most common in men between the ages of 15 and 34. The risk of developing testicular cancer due to a cryptorchid testis is increased to 5–10 times that of the general male population. This increased risk for a cryptorchid or previously cryptorchid individual is indicative of long-term damage, despite early orchioxy in many cases. Very little is known about mechanisms of TGCT tumorigenesis and at present there is no animal model that develops testicular cancer as a result of an undescended testis phenotype. Gene expression studies on cryptorchid patients and animal models have indicated that growth factors known to be important for the balance of self-renewal and the proliferation of germ cells are deregulated. The aberrant testicular environment also has a detrimental effect on Sertoli and Leydig cells that may lead to an inability to support the stem cell population. Accumulation of mutations in the somatic cells may lead to misexpression of important growth factors and morphological breakdown. Consequently, in the cryptorchid testis, an alternative differentiation pathway for SSCs is proposed which can result in the formation of TGCT. Identification of new markers specific for fetal germ cells and SSCs in human patients will help to delineate the origin of TGCT. The detailed analysis of changes induced in the SSC somatic cell niche by cryptorchidism and the fate of SSCs will be crucial for our understanding of the link between cryptorchidism and TGCT.


Van Der Schoot, P. (1996). Towards a rational terminology in the study of the gubernaculum testis: arguments in support of the notion that the cremaster sac should be considered the gubernaculum in postnatal rats and other mammals. J. Anat. 189(Pt 1), 97–108.


Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 12 November 2012; accepted: 04 March 2013; published online: 20 March 2013.


This article was submitted to Frontiers in Cancer Endocrinology, a specialty of Frontiers in Endocrinology. Copyright © 2013 Ferguson and Agoulnik. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.
ADVANTAGES OF PUBLISHING IN FRONTIERS

FAST PUBLICATION
Average 90 days from submission to publication

COLLABORATIVE PEER-REVIEW
Designed to be rigorous – yet also collaborative, fair and constructive

RESEARCH NETWORK
Our network increases readership for your article

OPEN ACCESS
Articles are free to read, for greatest visibility

TRANSPARENT
Editors and reviewers acknowledged by name on published articles

GLOBAL SPREAD
Six million monthly page views worldwide

COPYRIGHT TO AUTHORS
No limit to article distribution and re-use

IMPACT METRICS
Advanced metrics track your article’s impact

SUPPORT
By our Swiss-based editorial team