No AZF deletion in 160 patients with testicular germ cell neoplasia

Lone Frydelund-Larsen1, Peter H.Vogt2, Henrik Leffers1, Alexandra Schadwinkel2, Gedske Daugaard3, Niels E.Skakkebaek1 and Ewa Rajpert-De Meyts1,4

1University Department of Growth and Reproduction, Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen, Denmark, 2Section of Molecular Genetics and Infertility, Department of Gynaecological Endocrinology and Reproductive Medicine, University of Heidelberg, D-69115 Heidelberg, Germany and 3Department of Oncology, Rigshospitalet, DK-2100 Copenhagen, Denmark
4To whom correspondence should be addressed. E-mail: erm@rh.dk

Testicular germ cell cancer is aetiologically linked to genital malformations and male infertility and is most probably caused by a disruption of embryonic programming and gonadal development during fetal life. In some cases, germ cell neoplasia is associated with a relative reduction of Y chromosomal material (e.g. 45,X/46,XY) or other abnormalities of the Y chromosome. The euchromatic long arm of the human Y chromosome (Yq11) contains three azoospermia factors (AZFa, AZFb, AZFc) functionally important in human spermatogenesis. Microdeletions encompassing one of these three AZF loci result in the deletion of multiple genes normally expressed in testis tissue and are associated with spermatogenic failure. The aim of our study was to investigate whether AZF microdeletions, in addition to causing infertility, predispose also to germ cell neoplasia, since subjects with poor spermatogenesis have an increased risk of testicular cancer. We screened for putative deletions of AZF loci on the Y chromosome in DNA isolated from white blood cells of 160 Danish patients with testicular germ cell neoplasia. Interestingly, although AZF microdeletions are found in all patients with idiopathic infertility, in all cases studied of testicular germ cell cancer the Yq region was found to be intact. We conclude that the molecular aetiology of testicular germ cell neoplasia of the young adult type most likely does not involve the same pathways as male infertility caused by AZF deletions. Malignant transformation of germ cells is thus caused by the dysfunction of some other genes that still need to be identified.

Key words: AZF/male infertility/testicular germ cell neoplasia/testicular dysgenesis syndrome/Yq microdeletions

Introduction

Testicular germ cell cancer primarily affects young men and is the most common type of cancer in this age group. The incidence of this cancer has been increasing in recent decades in many countries worldwide, particularly in north-western Europe, including Denmark (Adami et al., 1994). The incidence of other reproductive disorders such as cryptorchidism, hypospadias and decreased spermatogenesis has also shown a rise during the same period, and all these conditions increase the risk of testicular cancer (Batata et al., 1982; Giwercman et al., 1989; Carlsen et al., 1992; Toppari et al., 2001). Men with testicular cancer also have significantly reduced fertility with a lower proportion of male offspring and abnormal semen characteristics already prior to development of their tumour (Møller and Skakkebaek, 1999; Fosså and Kravdal, 2000; Jacobsen et al., 2000). Thus, we have suggested that testicular cancer and other male reproductive disorders, including infertility, may share some common aetiological factors, and that all these conditions may be manifestations of the testicular dysgenesis syndrome (Skakkebaek et al., 1998, 2001).

Testicular germ cell cancer is believed to originate from carcinoma in situ (CIS) cells, which closely resemble gonocytes with respect to morphology and expression of immunochemical markers (Skakkebaek et al., 1987; Jørgensen et al., 1995) and therefore presumably originate from gonocytes or primordial germ cells that escaped the normal differentiation pathway (Skakkebaek et al., 1987; Rajpert-De Meyts et al., 1998). This is best illustrated by a very high risk of germ cell neoplasia in individuals with severe abnormalities of gonadal development associated with the intersex syndrome (Scully, 1981; Savage and Lowe, 1990). Among those, a relative reduction of the Y chromosome genetic material is not uncommon e.g. mixed gonadal dysgenesis, 45,X/46,XY (Scully, 1981; Peltomäki et al., 1991). The commonest structural abnormalities of the Y chromosome are microdeletions of the long arm (Yq) in one or more of the three AZF (Azoospermic Factor) regions, AZFa, b, or c (Vogt et al., 1996). Microdeletions in the AZF regions are associated with testicular failure and male infertility due to removal of one or more of several genes essential for maintenance of spermatogenesis (Reijo et al., 1995; Vogt et al., 1996, 1997; Lahn and Page, 1997; Krausz et al., 2000). AZF deletions are found in ~2.5% of unselected Danish men presenting with all forms of infertility, but the frequency increases to 13.4% among patients with idiopathic azoospermia or very severe oligozoospermia with sperm concentration <0.2×10⁹/ml (Krausz et al., 2001; Frydelund-Larsen et al., 2002).

A number of genes in the AZF regions are expressed already in prenatal germ cells (Elliott et al., 1997; Xu et al., 2001). We therefore hypothesized that a deletion of one or more of these genes may also affect early development of the testis and differentiation of fetal germ cells, thus causing testicular dysgenesis and impaired spermatogenesis or germ cell neoplasia later in life. If this holds true, AZF deletions should be found in patients with testicular cancer with a frequency similar or perhaps even higher than in infertile men. To address this
hypothesis we performed a sequence-tagged site (STS) deletion analysis of the three AZF loci in a relatively large group of Danish patients with testicular germ cell neoplasia of the adolescent and young adult type.

Materials and methods

Subjects and clinical tests
A total of 160 patients diagnosed with unilateral or bilateral testicular germ cell neoplasia were included in the study. Patients with other types of testicular neoplasia, e.g. Leydig cell-derived tumours or malignant lymphomas were excluded. Testicular biopsies from the contralateral testes were available in 116 of the patients and 32 of these were diagnosed with carcinoma in-situ (CIS). In approximately half of the patients the primary tumour was classical seminoma whilst the other half had non-seminomas or mixed germ cell tumours (Table I). Fifteen patients (9%) had preinvasive CIS only. Those were men referred to our andrological clinic because of infertility, and CIS was found in their testicular biopsies performed because of severe oligozoospermia and/or an irregular picture on the scrotal ultrasound examination. Finally, one patient had an extragonadal germ cell tumour. We included this patient, because extragonadal tumours are frequently associated with the presence of CIS in the testis (Daugaard et al., 1997), and his two brothers, who were included in the study, were diagnosed with testicular germ cell tumours.

As a control group, we included 100 healthy recent fathers, who were recruited via their pregnant partners from an obstetric clinic for a study of reproductive health of European populations (Jørgensen et al., 2001). All patients and control subjects underwent a routine andrological examination, a comprehensive analysis of reproductive hormones (FSH, LH, SHBG, testosterone, oestradiol and inhibit B), and semen analysis according to the World Health Organization protocol, as previously described (Jørgensen et al., 2001). During the same visit, an additional small blood sample was drawn for comprehensive analysis of reproductive hormones (FSH, LH, SHBG, testosterone, oestradiol and inhibit B, and semen analysis according to the World Health Organization protocol, as previously described (Jørgensen et al., 2001). During the same visit, an additional small blood sample was drawn for comprehensive analysis of reproductive hormones (FSH, LH, SHBG, testosterone, oestradiol and inhibit B). and semen analysis according to the World Health Organization protocol, as previously described (Jørgensen et al., 2001). During the same visit, an additional small blood sample was drawn for comprehensive analysis of reproductive hormones (FSH, LH, SHBG, testosterone, oestradiol and inhibit B).

Statistical analysis
Relative incidences and their confidence intervals were calculated using the SPSS for Windows 11.0 (USA). Differences between groups were quantified using odds ratios and compared using Fisher’s exact test.

Results
The analysis of the structural integrity of the q11 region of the Y chromosome was performed in a homogeneous group of 160 patients with germ cell tumours of the adolescent and young adult type, which are preceded by the preinvasive CIS (Table I). The patients differed with respect to their spermatogenesis, as was established based on the pre-treatment semen parameters, which were available from 70 patients. The mean sperm concentration was 12×10⁶/ml (±6×10⁶/ml). The median value was 3.70×10⁶/ml ranging from 0 to 88×10⁶/ml. We stratified the patients into four groups: azoospermia, very severe oligozoospermia (<0.2×10⁶/ml), severe oligozoospermia (<5×10⁶/ml), moderate oligozoospermia (5–20×10⁶/ml), and normozoospermia (>20×10⁶/ml). The distribution of patients in these groups is shown in Table II.

Molecular deletion analysis of the AZF-Yq11 region was performed in the genomic DNA samples of 160 patients, screening first for nine loci spanning the AZFa, AZFb, and AZFc regions. This region was subsequently used for the detection of deletions in these patients. In all individuals where enough genomic DNA was still available (57 patients), we therefore extended our deletion analyses with a series of eight novel AZF gene specific STS markers (for TSPY-Yq11, USP9Y, DBY, EIF1AY, RBMY, SMCY, BYY2, CDY1) to identify also putative partial AZF gene deletions respectively, with a deletion STS marker for the VCY genes located between AZFa and AZFb, and the TPT1Y gene mapped distal to AZFz (P.H.Vogt et al., unpublished data). With this marker set, we also did not detect any deletions in AZF-Yq11 DNA.

Discussion
In this study, we performed a detailed deletion analysis of the AZF-Yq11 region in genomic DNA isolated from blood cells of 160 patients with a homogeneous phenotype of testicular germ cell neoplasia of the adolescent and young adult type. We did not identify any AZF deletions in these 160 DNA samples, neither did we find any deletions after the extension of the AZF gene deletion analysis by seven novel loci in 57 individuals. This finding suggests that most
cases of testicular germ cell cancer have a different molecular aetiology than male infertility caused by AZF deletions. It was estimated that AZF microdeletions occur in ~1:10 000 males (Reijo et al., 1995; Vogt et al., 1996). In 1990, the incidence of testicular cancer in Denmark was 9.2 per 100 000 (1:10 100) males (Parkin et al., 1997), which roughly corresponds to the prevalence of AZF deletions. However, the age-specific incidence of testicular cancer among 30–35 year old men, which is the age of most men seeking treatment for infertility, is currently ~28–30 per 100 000 (~1:3500) males (Møller, 2001), which is close to the prevalence of AZFc deletions, estimated as 1:4000 males (Krausz et al., 2000). When we compared the risk of having a deletion in the Danish patients with idiopathic infertility, 3.8% of whom have Yq microdeletions (Frydelund-Larsen et al., 2002) to those with testicular germ cell neoplasia, the odds ratio was 0 (95% confidence interval = 0 to 1.90). Based on our sample of 160 patients with testicular cancer, we can therefore conclude that there is no increased risk for an association with AZF deletions among men with germ cell neoplasia. To the contrary, the risk appears lower than predicted from the analysis of the infertile men ($P = 0.019$). However, although fertility in patients with germ cell neoplasia is decreased, most have retained spermatogenesis. In our series only 20 patients out of 70, who were tested before treatment, had sperm concentrations <0.2 × 10^6/ml, which was the threshold value in our previous study of infertile men with AZFc microdeletions (Frydelund-Larsen et al., 2002). Despite the prevalence of AZFc microdeletions in the infertile group being higher than that in the subgroup of testis cancer patients with equally low sperm counts (<0.2 × 10^6/ml), 13.4% versus 0% respectively, this difference was not statistically significant (Fisher’s exact test, $P = 0.127$) because of the small numbers of subjects in the latter group. Thus, we cannot yet exclude the possibility that AZF gene deletions may play a pathogenic role in a small fraction of patients with testicular cancer and severe testicular failure.

Our hypothesis of a decreased risk of germ cell cancer in men with a constitutive deletion in the AZF region may be explained if the AZF genes are necessary for the development and maintenance of the male germ cell phenotype, which is a feature of CIS cells (Rajpert-De Meyts et al., 2003). No CIS cells have been reported in testicular biopsies from infertile men with constitutive Yq microdeletions. Morphologically normal spermatogonia are often seen in these patients, but the histological picture frequently shows a partial or complete Sertoli cell-only pattern (Reijo et al., 1995; Vogt et al., 1996; Krausz et al., 2000; Frydelund-Larsen et al., 2002; Luetjens et al., 2002). AZF deletions might therefore also cause an early depletion of germ cells and effectively decrease the chance of overt germ cell tumours to develop.

However, we need to learn more about the expression of the candidate AZF genes in CIS cells and during germ cell development and differentiation. Both, the RBMY and DAZ gene family in AZFb and AZFc, respectively, encode testis-specific putative RNA binding proteins (Cooke, 1999). Gene products of the RBMY and DAZ genes are localized in human primordial germ cells (Elliott et al., 1997; Xu et al., 2001). RBMY expression was not detected in CIS cells, either in adult patients with testicular tumours (Lifschitz-Mercer et al., 2000) or in intersex children with dysgenetic gonads (Schreiber et al., 2003). The expression of DAZ in germ cell neoplasia has not been reported, but the autosomal DAZ homologue DAZL was recently analysed in testicular germ cell neoplasia by immunohistochemistry (Lifschitz-Mercer et al., 2002). The DAZL protein was identified in CIS cells, in pure seminomas and in the seminomatous components of mixed germ cell tumours, but not in teratomas, thus providing evidence that DAZL is only expressed in cells retaining the germ cell phenotype. DAZ and DAZL are similarly expressed in testes during development but it is not known whether or not structural or functional removal of the DAZ family in AZFc would stimulate or inhibit neoplastic transformation of germ cells.

Furthermore, we cannot exclude the possibility of a point mutation in one of the AZF genes in the patients screened in this study. Current AZF gene deletion analyses are only able to identify complete or partial gene deletions, but point mutations in AZF genes are expected to be more rare than deletions. Most cases of Yq microdeletions are probably the result of homologous recombination between repetitive sequence blocks removing several AZF genes at once (Blanco et al., 2000). Since most of the confirmed Yq11-STS deletions were found in tumour tissues, we think that these deletions might be a result of the genetic instability of aneuploid tumours and subsequent frequent loss of the Y chromosome in tumour cell subpopulations. This view is supported by an earlier report of one patient with CIS and a microdeletion in Yq11 (Papadimas et al., 2001). That patient also had sex chromosome mosaicism, which in itself predisposes strongly to genital dysgenesis and germ cell neoplasia.

In conclusion, AZF microdeletions are not frequent in patients with germ cell neoplasia, thus the high incidence of testicular cancer in Denmark is not associated with this Y chromosomal abnormality. The molecular aetiology of germ cell neoplasia of the adolescent and the young adult type most likely does not involve the same pathways as a subset of male infertility caused by AZF deletions. Thus most cases of testicular germ cell cancer are probably a consequence of a dysfunction of some other genes that still need to be identified.
Acknowledgements

The authors thank Drs E.Carlson, M.Holm, N.Jørgensen and A.G.Ander sen for help with collecting blood samples from patients and control individuals, J.Holm Petersen for help with the statistical analysis, and I.D.Garn, L.G.Petersen and K.Huellen for skilful technical assistance. The study was supported by grants from the Svend Andersen Foundation, the Danish Cancer Society, the Danish Medical Research Council, the Council of Copenhagen University Hospitals (H:S), the Willumsen Foundation, the Vissing Foundation, and the European Union.

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Submitted on April 8, 2003; accepted on May 15, 2003