Y chromosome haplogroups and susceptibility to testicular cancer

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Although in the past decades much progress in testicular cancer (TC) management has been made, little is known about the possible genetic causes and molecular mechanisms involved in its aetiology. Some studies on possible contribution of the Y chromosome in TC development have been previously published, but data are not conclusive. In particular, ethnic influence and spermatogenic activity of patients with TC have not been adequately considered in previous studies, although they may represent important confounding factors. The objective of this study is to analyse the contribution of the Y chromosome in testicular germ cell cancer subjects who are well defined at the microgeographical, clinical and seminological level. We analysed Y chromosome classic azoospermia factor (AZF) deletions, partial AZFc deletions and Y haplogroups in 118 sporadic cases of testicular germ cell cancer and 93 microgeographically matched controls. Y chromosome screening failed to identify Y chromosome microdeletions in either cases or controls. Y chromosome haplogroup distribution and frequencies did not differ between cases and controls. Furthermore, no difference was observed when comparing patients with seminoma and non-seminoma, nor when comparing patients with TC with normozoospermia and azoo-oligozoospermia. Our findings combined with data reported so far suggest that classic AZF deletions and partial AZFc deletions are not a frequent cause or risk factor for TC and that different Y haplogroup distribution does not contribute to susceptibility to this tumour.

Keywords: AZF; haplogroups; testicular cancer; Y chromosome

Introduction

Testicular cancer (TC, MIM 273300) is the most common cancer in white males aged 20–40 years. The worldwide incidence is 7.5 per 100,000, but rates vary between countries. About 95% of all TCs are represented by germ cell tumours (TGCTs) and seminoma accounts for ∼50% of them.

TGCT has some epidemiological hallmarks, including peak incidence at a very young adult age, a markedly increasing incidence worldwide but with striking geographic and ethnic differences, and association with other reproductive conditions. The incidence of TGCT has doubled in the past 40 years (Huyghe et al., 2003; Jacobsen et al., 2006), with an annual increase of 3–6% in Caucasian populations (Giwercman et al., 1993; Swerdlow et al., 1993; Rajpert-De Meyts, 2006). More recently, smaller palindromes were discovered (Kawaguchi et al., 2001). These regions are especially prone to interstitial deletions originating from non-homologous recombination and are associated with variable grades of testicular failure and impaired spermatogenesis. Microdeletions of the AZF region are the most commonly known molecular causes of spermatogenic failure, with a prevalence of 5–10% in severely oligospermic and non-obstructive azoospermic men (Foresta et al., 2001; Ferlin et al., 2006a, 2007). More recently, smaller palindromes were discovered within the large amplicons in AZFc region. Deletions of these sequences cause the so-called partial AZFc deletions and are associated with variable clinical and histological phenotypes, possibly representing risk factors for decreased spermatogenesis (Repping et al., 2007).
Given the important function of the genes in AZF regions, and the link between spermatogenic impairment and TGCT, it has been postulated that deletions in AZF may be associated with TGCT. Classical, complete AZF deletions has not been associated with increased risk of TC in previous studies (Frydelund-Larsen et al., 2003; Latke Holzik et al., 2005; Bor et al., 2006), whereas a large multicentre report suggested that partial AZFc deletions of the gr/gr subtype might confer susceptibility to TGCT (Nathanson et al., 2005). However, more recently these results have not been clearly confirmed in a study from the UK (Linger et al., 2007). Therefore, final conclusions cannot be drawn and proper case-control studies from well-defined ethnic populations are needed. In fact, these association studies require particular attention with regard to the geographical structure of the Y chromosome variations in the population under investigation, because the Y chromosome genetic variability is highly geographically structured and the Y haplogroup distribution changes over different geographical areas. Some partial AZFc deletions have been found associated with particular Y chromosome haplogroups (Ferlin et al., 2004; Repping et al., 2004), whereas gr/gr deletions found in infertile men (Repping et al., 2003; Carvalho et al., 2006) or in men with TC (Nathanson et al., 2005) have been observed to be in association with different Y haplogroups. However, only two studies on small numbers of TGCTs have looked for a possible association between Y haplogroups and TC, failing to find any association (Quintana-Murci et al., 2003; Ewis et al., 2006). Nevertheless, we have recently described an association between certain Y haplogroups and AZFc deletions in North Italy (Arredi et al., 2007), evidencing that studies on microgeographically controlled populations are desirable and more informative. Furthermore, previous studies on TGCT patients and Y chromosome deletions/haplogroups have not considered the spermatogenic status, which may represent a confounding factor.

Therefore, in this study we evaluated the possible contribution of classic AZF and partial AZFc deletions and Y chromosome haplogroups to susceptibility to TGCT in men from North Italy, who are well characterized at the geographical, clinical and seminological level.

Materials and Methods

Subjects

Patients and controls from North Italy were prospectively recruited for this study with the approval of the Hospital Ethical Committee and informed consent was obtained from each subject after full explanation of the purpose and nature of all procedures used. We evaluated 118 consecutive subjects (mean age 29.6 ± 7.1 years) from North Italy, who were orchiectomized for TGCT, and who consulted our Centre for semen cryobanking before initiating chemo- and/or radiotherapy. All patients had a tumour in stage I at the time of our evaluation. A complete medical history and physical examination were undertaken. All patients provided samples for complete semen analysis following WHO recommendations (World Health Organization, 1999) and reproductive hormone (FSH, LH, testosterone, estradiol and prolactin) plasma concentrations. We had excluded patients with karyotype abnormalities (1 subject with 47,XXY Klinefelter syndrome) and androgen receptor (AR) gene mutations (3 subjects) (Ferlin et al., 2006b). We had also excluded patients with history of cryptorchidism (18 subjects) and patients with family history of TC (2 subjects). Controls were represented by 93 North Italian age-matched (mean age 30.1 ± 6.5 years) healthy normozoospermic (sperm concentration >20×10^9/ml) fertile men (fathers of at least one child) without previous TC or familial history of TC (Arredi et al., 2007). We paid particular attention to the geographical origin of the patient and control groups, to exclude the geographic factor as cause of possible different haplogroup frequencies. All the individuals were unrelated and of North Italian origin up to the grand father. Among the TGCT patients, 68 were affected by seminoma and 50 by non-seminoma (Table 1). There were 73 TGCT subjects who had normozoospermia and 45 who had azoospermia (absence of sperm in the ejaculate) or oligozoospermia (sperm concentration <20×10^9/ml).

\[ \text{Table 1: Histotypes of the 118 subjects with TGCT} \]

<table>
<thead>
<tr>
<th>Histotype</th>
<th>n (%)</th>
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<tbody>
<tr>
<td>Seminoma</td>
<td>68 (57.6)</td>
</tr>
<tr>
<td>Non-seminoma</td>
<td>50 (42.4)</td>
</tr>
<tr>
<td>Mixed germ cells</td>
<td>16 (13.6)</td>
</tr>
<tr>
<td>Embryonal carcinoma</td>
<td>14 (11.9)</td>
</tr>
<tr>
<td>Teratoma</td>
<td>12 (10.2)</td>
</tr>
<tr>
<td>Choriocarcinoma</td>
<td>4 (3.4)</td>
</tr>
<tr>
<td>Yolk sac</td>
<td>4 (3.4)</td>
</tr>
</tbody>
</table>

Y chromosome AZF microdeletion analysis

Patients and controls were analysed by routine diagnostic Y home-made multiplex PCR screening using markers from AZFa, AZFb and AZFc regions, as previously described (Ferlin et al., 2007), for identification of classic AZF deletions. Partial AZFc deletions were analysed with AZFc specific sequence-tagged sites (STSs) sY1291 (specific for the gr/gr and b1/b3 deletions) and sY1191 (specific for the b1/b3 and b2/b3 deletions), as previously described (Ferlin et al., 2005; Arredi et al., 2007). STSs position and the most common partial AZFc deletions are shown in Fig. 1.

Y chromosome haplogroup analysis

Each subject was screened for seven Y chromosome binary markers (12f2, M89, M99, M45, M96, M145, TAT) defining eight Y haplogroups, as previously described (Arredi et al., 2007). All the biallelic markers, except 12f2 and M89, have been typed through denaturing high-performance liquid chromatography on a 2100 WAVE DNA fragment analysis system (Transgenomics, Omaha, NE, USA). The 12f2 insertion/deletion polymorphism was typed by co-amplification of a fragment within the deleted region and a PCR control. M89 was sequenced (ABI PRISM 3730 XL DNA Sequencer, Applied Biosystem, Milan, Italy).

Statistical analysis

The difference in Y haplogroup distribution between the control and TGCT samples was statistically tested through Fisher exact probability test on contingency table for single haplogroup after Bonferroni correction and through the exact test of population differentiation (Raymond and Rousset, 1995). P-values (two sided) of <0.05 were considered to indicate statistical significance.

Results

Y chromosome multiplex PCR screening using markers from AZFa, AZFb and AZFc regions did not identify AZF deletions either in controls or in TGCT patients. Similarly, no partial AZFc deletions were found in either group of subjects by analysis of sY1191 and sY1291 STSs. These data therefore showed the absence of classic AZF and partial AZFc deletions in TGCT patients, as well as in normozoospermic fertile men.

Y chromosome haplogroup analysis showed the same haplogroups both in the control population and in the TGCT group, with haplogroups P, J and F*(xKJ) being the most frequent (Table 2). The most frequently observed haplogroup, P, is the same in the control (55%) and TGCT group (50%). Haplogroups F*(xKJ) and J are observed at frequencies of 15% in controls and 22 and 18%, respectively, in TGCT subjects. We did not observe Y*(xF,DE), N3 and D haplogroups. Haplogroup distribution and frequencies were not different between the control and TGCT groups.
To better analyse the possible contribution of Y chromosome haplogroups to TGCT susceptibility, we divided TGCT patients according to seminoma (68 subjects) and non-seminoma (50 subjects), but no differences in haplogroup distribution and frequencies were observed with respect to controls and between the two groups of TGCT (Table 2). To exclude the possible contribution of spermatogenic impairment as a confounding factor, we then compared Y haplogroups distribution and frequencies in TGCT patients with normozoospermia (73 subjects) and TGCT patients with azoo-oligozoospermia (45 subjects). Also in this case, no differences were observed (Table 3).

**Discussion**

Although in the past decades much progress in TGCT management and treatment has been made, little is known about the possible genetic causes and molecular mechanisms involved in the aetiopathogenesis of these tumours (Horwich et al., 2006). Recent research suggests that TGCT in adults initiate during fetal development and involve changes to primordial germ cells either during migration to the embryonic genital ridges or after cells have arrived at the gonads (reviewed in Rajpert-De Meyts, 2006). This early origin and the unusual epidemiological aspects (peak incidence in young adult age) are unique features of TGCTs. Other clinical features include the strong association with other male reproductive tract abnormalities, such as spermatogenic impairment, cryptorchidism and hypospadias, collectively included in the so-called TDS (Skakkebaek et al., 2001). This association was the basis for postulating two major mechanisms involved in TGCT development, its increase in recent decades and the geographical and ethnic differences: environmental factors and genetic predisposition, including genetic susceptibility to endocrine disruption. In this light, some studies on possible genetic aspects acting as risk factors for TGCT have been

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**Table 2:** Haplogroup frequencies in TGCT patients divided on the basis of cancer histology (seminoma versus non-seminoma) and controls

<table>
<thead>
<tr>
<th>Y haplogroups (%)</th>
<th>Y* (xF,DE)</th>
<th>F* (xK,J)</th>
<th>P</th>
<th>K* (xP)</th>
<th>N3</th>
<th>J</th>
<th>D</th>
<th>E</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>0 (0.0)</td>
<td>14 (15.0)</td>
<td>51 (54.8)</td>
<td>5 (5.4)</td>
<td>0 (0.0)</td>
<td>14 (15.0)</td>
<td>0 (0.0)</td>
<td>9 (9.7)</td>
<td>93</td>
</tr>
<tr>
<td>TGCTs seminoma</td>
<td>0 (0.0)</td>
<td>16 (23.5)</td>
<td>34 (50.0)</td>
<td>2 (2.9)</td>
<td>0 (0.0)</td>
<td>10 (14.8)</td>
<td>0 (0.0)</td>
<td>6 (8.8)</td>
<td>68</td>
</tr>
<tr>
<td>TGCTs non-seminoma</td>
<td>0 (0.0)</td>
<td>10 (20.0)</td>
<td>25 (50.0)</td>
<td>3 (6.0)</td>
<td>0 (0.0)</td>
<td>11 (22.0)</td>
<td>0 (0.0)</td>
<td>1 (2.0)</td>
<td>50</td>
</tr>
<tr>
<td>TGCTs total</td>
<td>0 (0.0)</td>
<td>26 (22.0)</td>
<td>59 (50.0)</td>
<td>5 (4.2)</td>
<td>0 (0.0)</td>
<td>21 (17.8)</td>
<td>0 (0.0)</td>
<td>7 (5.9)</td>
<td>118</td>
</tr>
</tbody>
</table>
performed, e.g. on the contribution of polymorphisms on the AR gene (Rajpert-De Meyts et al., 2002; Giwercamn et al., 2004; Garolla et al., 2005), mutations in INSL3-RXFP2 genes regulating testicular descent (Ferlin et al., 2006c) and deletions, polymorphisms and haplogroups of the Y chromosome (Frydelund-Larsen et al., 2003; Quintana-Murci et al., 2003; Lutke Holzik et al., 2005; Nathanson et al., 2005; Bor et al., 2006; Ewis et al., 2006; Linger et al., 2007). A genome-wide linkage search on 237 TGCT pedigrees identified several regions of interest but highlighted that susceptibility to TGCT is more likely due to several genes with modest or small effects on risk (Crockford et al., 2006).

Previous studies failed to identify an association between increased risk of TC with classic AZF deletions (Frydelund-Larsen et al., 2003; Lutke Holzik et al., 2005; Bor et al., 2006) or Y haplogroups (Quintana-Murci et al., 2003; Ewis et al., 2006). On the contrary Nathanson et al. (2005) showed that partial AZFc deletions of the gr/gr subtype are associated with a two-fold increased risk of TGCT (three-fold in TGCT patients with a positive family history) in a large multicentre study involving 1807 affected subjects. However, this association has not been confirmed in a more detailed study from UK involving 96 sporadic TGCTs and 167 TGCTs with positive family history (Linger et al., 2007). These studies therefore highlighted that only the largest studies combining different populations might unveil genetic predispositions acting at low penetrance. It is noteworthy that also in the study of Nathanson et al. (2005) the distribution of gr/gr deletions did not differ between TGCTs and controls in the two study centres contributing both cases and controls (Philadelphia and Washington State, 266 cases and 953 controls).

Ethnic influence and genetic background are therefore important factors when evaluating the possible contribution of Y chromosome deletions and haplogroups in TGCT susceptibility. This is exemplified by our recent study reporting an association between AZFc deletions and a certain Y haplogroup in a microgeographically controlled population from North Italy (Arredi et al., 2007). Other reports failed to find such association when studying less homogenous populations (Paracchini et al., 2000; Quintana-Murci et al., 2001; Carvalho et al., 2003). Furthermore, although histotype, family history and undescended testis have been considered in TC patients in previous studies, the spermatogenic activity has not been taken into account. However, given the association of spermatogenic damage with AZF deletions and TC, this element could represent a substantial confounding element.

In the present study, we performed Y chromosome microdeletion analysis including classic AZF and partial AZFc deletions, and Y haplogroup analyses in a well-defined group of TGCT patients. In particular, we selected cases and controls of North Italian origin up to the grand father, and TGCT patients were classified also on the basis of their spermatogenic activity. Notably, other possible causes of testicular damage, such as cryptorchidism, karyotype anomalies and AR gene mutations were excluded in TGCT patients. Furthermore, patients with non-germ cell tumours and positive family history were excluded. Therefore, the study was conducted in well-defined sporadic and idiopathic cases of TGCT compared with a microgeographically matched control population.

We failed to identify classic AZF and partial AZFc deletions in either TGCT cases or controls. Classic AZF deletions are specifically associated with severe spermatogenic impairment (Ferlin et al., 2007) and their absence in TGCTs confirmed previous studies from Denmark and The Netherlands (Frydelund-Larsen et al., 2003; Lutke Holzik et al., 2005; Bor et al., 2006). Although partial AZFc deletions, particularly of the gr/gr subtype, have been proposed as risk factors for decreased spermatogenesis, they can also be found at a lower frequency in fertile men with normal spermatogenesis (reviewed in Ferlin et al., 2006a). Therefore, it remains to be resolved whether they play a role alone or in combination with other genetic or environmental factors. Our study confirms the lack of association between sporadic TGCTs and gr/gr deletion in an English population (Linger et al., 2007). In this latter study 4/96 sporadic TGCTs were found to carry gr/gr deletion, whereas no case was found in our series. The spermatogenic activity in the study by Linger et al. (2007) was not reported and therefore it is unknown whether these deletions were present in subjects with azoo-oligozoospermia. The majority of our TGCT cases had normozoospermia (73/118) and this could justify the absence of partial AZFc deletions. The proportion of TGCT patients with gr/gr deletions with normozoospermia or azoo-oligozoospermia in the study of Nathanson et al. (2005) is not known.

Y chromosome haplogroup analysis showed no differences in distribution and frequency between TGCTs and controls. Therefore, we confirmed previous studies in English and Japanese populations (Quintana-Murci et al., 2003; Ewis et al., 2006).

In conclusion, our findings combined with data reported so far, suggest that classic AZF deletions and partial AZFc deletions are not a frequent cause or risk factor for TGCT and that different Y haplogroup distribution does not contribute to susceptibility to this tumour. The molecular aetiology of sporadic TGCT most likely does not involve the same pathways as male infertility caused by deletions of genes located in the AZF regions. Future studies on these aspects should consider possible confounding factors represented by the spermatogenic damage and other male tract abnormalities that frequently associate with TGCT, as well as the ethnic and geographical structure of the studied population.

Acknowledgements

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