Association of the polymorphism of the CAG repeat in the mitochondrial DNA polymerase gamma gene (POLG) with testicular germ-cell cancer

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Background: A possible association between the polymorphic CAG repeat in the DNA polymerase gamma (POLG) gene and the risk of testicular germ-cell tumours (TGCT) was investigated in this study. The hypothesis was prompted by an earlier preliminary study proposing an association of the absence of the common 10-CAG-long POLG allele with testicular cancer as well as previously reported in some European populations’ association with male subfertility, which is a condition carrying an increased risk of TGCT.

Patients and methods: The number of CAG repeats in both POLG alleles was established in 243 patients with TGCT and in 869 controls by the analysis of the genomic DNA fragment.

Results: A significantly higher proportion of men homozygous allele of other than the common 10 CAG repeats was found among the patients with TGCT in comparison to the controls (4.9% versus 1.3%, respectively, \( P = 0.001 \)). The vast majority of the homozygous patients had a seminoma (11 of 12; 97%), despite that only about half (55%) of the studied patients had this tumour type.

Conclusions: The findings indicate that the POLG polymorphism may be a contributing factor in the pathogenesis of TGCT particularly in seminoma, but the mechanisms remain to be elucidated.

Key words: CAG repeat, mitochondria, POLG gene, seminoma, testicular dysgenesis syndrome, testicular neoplasm

introduction

The incidence of testicular germ-cell tumours (TGCT) has more than doubled in the last 30 years and has become the most common malignant neoplasm among men aged 25–40 years [1]. The vast majority of TGCTs, except infantile germ-cell tumour and spermatocytic seminoma of the elderly men, are derived from carcinoma in situ testis (CIS-T) [2] and that includes both main types of overt TGCTs, seminoma (so-called classical, to distinguish from spermatocytic seminoma) and non-seminoma [3, 4].

In addition to the observed increase in TGCT, there has also been a rise in congenital malformations of male genitalia, such as cryptorchidism or hypospadias, and an apparent decline in sperm counts [5, 6]. The latter—even though is first observed in young adults—may in many cases be linked to prenatal development, as recently shown in men exposed in utero to maternal smoking [7]. According to our hypothesis, TGCT and the above-mentioned reproductive disorders are manifestations of the testicular dysgenesis syndrome (TDS). We proposed that the aetiology of TDS is linked to adverse influence of environmental/lifestyle factors during early foetal development, most probably combined with genetic predisposition [8, 9]. It has been shown in animal models that prenatal exposure to hormones or endocrine disrupters may target specific genes and lead to different forms of TDS, for example, in the case of oestrogens that target InsI3 and lead to cryptorchidism in rodents [10]. The observed effects are, however, highly variable, probably depending upon differences in genetic background of the animals [11]. Genetic predisposition is also an obvious explanation for the ethnic differences in incidence of TGCT in humans [12, 13]. We believe that multiple genes may be responsible for predisposition to TGCT and other forms of TDS, but the genes in question remain elusive.

One of the genes that have recently attracted our attention is DNA polymerase gamma gene (POLG), which encodes the catalytic subunit of DNA polymerase gamma (POLG), the key nuclear enzyme responsible for replicating, elongation and repair of the mitochondrial DNA (mtDNA). The POLG gene...
contains a polymorphic CAG repeat region [14, 15] with a high frequency of repeat lengths of 10 codons (75%–80%), which indicates that it is maintained by selection [16]. A new study supported the idea that the length of the POLG microsatellite region, rather than its exact nucleotide or amino acid sequence, is what is maintained in animals and humans [17]. The repeat located in the mature polypeptide is not found in the orthologous genes in mouse and yeasts, but a shorter repeat is found in African great apes. Deletion of the POLG CAG repeat was shown not to affect enzymatic properties, but modestly up-regulate expression [17, 18].

Absence of the 10-codon repeat on both alleles (named here the homozygous variant) has been associated with male subfertility in Finland and Denmark, where the frequency of this polymorphism among fertile men is ~1%–1.5% [15], while it was 2.8% in the Danish subfertile patients [19]. Studies in other countries, however, did not confirm this association [20–23]. Despite that the expression of POLG was found in human sperm [24], the previous studies taken together indicated that the POLG polymorphism had no consistent influence on semen concentration, sperm motility or morphology, thus limiting its predictive clinical value in fertility clinics [25]. In our study, however, we observed one case of TGCT among the few subfertile men homozygous for the lack of the common 10-codon repeat [19]. Moreover, a small preliminary study of 49 polish patients reported that the POLG polymorphism may be a genetic risk for TGCT [26].

Another reason for our interest in the CAG–POLG polymorphism was a previously reported CAG repeat instability, which was first observed in tumour genome and also described in germline DNA of some families with testicular cancer [27], although this was disputed in another study [28]. We hypothesized that—whether or not linked to a general CAG repeat instability in the genome—CAG repeat polymorphism in the POLG gene could perhaps subtly impair its function.

To investigate whether or not there is a link between the POLG polymorphism and TGCT, we analysed a large group of patients with testicular germ-cell neoplasia and well-characterized controls and report here the results indicating that this polymorphism may indeed be associated with an increased risk of testicular cancer.

**Materials and methods**

**Subjects and clinical analysis**

The study group included 243 Danish patients diagnosed with testicular germ-cell neoplasia and referred to our hospital for oncological treatment, semen banking or post-therapy fertility assessment. The average age of patients at the time of the diagnosis was 33 years. Patients with testicular tumours derived from other cell types (e.g. lymphoma or Leydig tumour) were excluded. In the TGCT group, 55% of the patients had a seminoma, while nearly 42% had a non-seminoma or mixed germ-cell tumours. The remaining nine patients (3.7%) harboured pre-invasive CIS-T, without an overt tumour. Diagnosis was based on histological evaluation of the orchidectomy preparation by pathologists.

The control group included 869 young healthy men, without any signs of malignancy, 495 were young military conscripts and 374 proven fertile men. All subjects were Danish. The same control groups were used in our previous study of the POLG gene polymorphism in subfertile men [19]. A subset of TGCT patients (those referred for semen banking or fertility treatment) and all control subjects underwent an andrological examination including analysis of semen and reproductive hormones in serum. The study was conducted in accordance with the law concerning the protection of personal information and approved by the regional Medical Research Ethics Committee.

**Molecular analysis**

DNA was isolated from peripheral blood samples using a kit (Roche Diagnostics GmbH, Mannheim, Germany). Two primers (GGTCCCGTCACCAACCATGA and CTGGCCGAAAGATTGGCTG), matching positions 267–286 and 533–553, respectively, of the POLG transcript (ENSEMBL gene ENSG00000140521), were used to amplify a 286-bp DNA fragment, using the Pfu DNA polymerase (Stratagene, San Diego, CA). PCR was carried out in 30 μl of (final concentrations) 12 mM Tris–HCl, pH 8.3; 50 mM KCl; 1.9 mM MgCl₂; 0.1% Triton X-100; 0.005% gelatine; 250 μM dNTP; 30 pmol of each primer and 2 μl Pfu polymerase. PCR conditions were 98°C for 5 min, 40 cycles of 98°C for 30 s, 63°C for 1 min, 72°C for 1 min. After an additional 15 min and one cycle of 72°C for 5 min.

Eight per cent of the samples failed the amplification and were excluded. Earlier investigations showed a success rate ~90%, and our rate was slightly higher after amplification and determining the CAG repeat length. DNA fragments were purified from 1% agarose gels and analysed first by applying a Cy5-labelled sequencing primer (C5S-CTGGATGTCCAATGGGTTGT, positions 494–513) under standard PCR conditions, resulting in a DNA fragment, which was run on an ALF express sequencer and analysed by the Fragment Analyser software (Amersham-Pharmacia Biotech, Uppsala, Sweden).

The reproducibility of the assay was tested in the beginning of our first study [19]. It included 1298 men and the first 300 were analysed using two different methods by direct sequencing and by fragment length on the ALF express sequencer, with no difference in outcome. All bands that differed from a normal CAG 10/10 band (putative x/x) were analysed twice.

**Statistical analysis**

Differences between allele frequencies were assessed using Fisher’s exact test, which correctly takes into account the low number of polymorphic homozygotes in both cancer patients and controls.

**Results**

**Frequency and distribution of the POLG genotypes**

All the frequencies of the alleles in the studied groups are shown in Table 1. In concert with the previous report [19], homozygotes with 10 repeats on both alleles (10/10) were the most common (76.6% of all studied subjects). The frequency of the common POLG variant among the TGCT patients was 75.7%, which was close to that in the combined control groups (76.6%), $P = 0.80$. The frequency of the absence of the common 10 repeats on both alleles among the TGCT patients was significantly higher compared with the controls (4.9% versus 1.3%, respectively, $P = 0.001$).

CAG repeat lengths ranging from 4 to 12 triplets were detected. This range and distribution are comparable to what was shown earlier [25], where the majority of alleles that deviate from 10 are close to 10 in length, with a small tendency to be >10, the majority being 11. The proportion of repeats <10 was comparable to the Finnish study [16] but...
was much higher than that in the Italian study [21] (data not shown).

**Phenotype versus genotype**

The distribution of homozygous patients among tumour types was highly non-random ($P = 0.035$). Nearly all homozygous patients had a seminoma (11 of 12, 96.7%, Tables 1 and 2). Except this finding there were no obvious phenotypic differences between the TGCT patients with and without the POLG polymorphism. Low serum inhibin B concentrations in two patients homozygous for the POLG polymorphism indicate that they probably had impaired spermatogenesis, but their semen characteristics were unknown (Table 2). There was only one known TGCT patient with severe oligozoospermia. There was no significant trend or difference in the average sperm concentration stratified on the three different haplotypes (data not shown). As illustrated in Table 3, in patients with heterozygosity we found no association of the numbers of CAG repeats in the POLG gene with tumour histology, clinical history or aggressiveness of TGCT.

**Discussion**

In this study, we found a significantly higher frequency of the homozygous absence of the common POLG haplotype among patients with testis cancer (TGCT) compared with young Danish men without TGCT and frequencies of this polymorphism in the European populations [29]. The frequency of the homozygous POLG polymorphism (4.9%) was even higher than that reported among infertile men, a condition linked epidemiologically to testicular cancer [9]. We found a clear predominance of one tumour type (seminoma) among the patients with the homozygous absence of the common allele, but did not find any correlation of the POLG polymorphism with other clinical parameters.

We are aware of only one previous study of POLG genotypes in patients with testicular cancer; a very small preliminary study which found a higher frequency of polymorphic genotypes; 26% versus 11% (13 heterozygotes and one homozygous combined) in a group of 49 Polish TGCT patients versus 55 controls [26]. That study, however, showed a large difference in the frequency of the POLG haplotypes among the controls.

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**Table 1. Distribution of the DNA polymerase gamma (POLG) alleles in patients with testis cancer (testicular germ-cell tumour (TGCT)) and controls**

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>N and frequency (with 95% confidence intervals) of the three CAG repeat genotype patterns in both alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGCT total</td>
<td>243</td>
<td>184 (75.7%, 70.0% to 80.7%) 47 (19.3%, 14.9% to 24.8%) 12 (4.9%, 2.8% to 8.4%)*</td>
</tr>
<tr>
<td>CIS-T alone</td>
<td>9</td>
<td>6 (66.7%, 35.4% to 87.9%) 3 (33.3%, 12.1% to 64.6%) 0 (0%, 0% to 29.9%)</td>
</tr>
<tr>
<td>Seminoma</td>
<td>133</td>
<td>100 (75.2%, 67.2% to 81.8%) 22 (16.5%, 11.2% to 23.8%) 11 (8.3%, 4.7% to 14.2%)**</td>
</tr>
<tr>
<td>Non-seminoma</td>
<td>101</td>
<td>78 (77.2%, 68.1% to 84.3%) 22 (21.8%, 14.8% to 30.8%) 1 (1.0%, 0.2% to 5.4%)</td>
</tr>
<tr>
<td>Controls</td>
<td>869</td>
<td>666 (76.6%, 73.7% to 79.3) 192 (22.1%, 19.5% to 25.0%) 11 (1.3%, 0.7% to 2.3%)</td>
</tr>
</tbody>
</table>

The distribution of the wild-type 10/10, heterozygotes 10/x and the homozygous x/x variants among patients and controls. The patients with TGCT are divided according to the histological pattern. CIS-T, carcinoma in situ testis.

* $P = 0.001$ in comparison to the control group.
** $P < 0.001$ in comparison to the control group.

**Table 2. Characteristics of the testicular germ-cell tumour (TGCT) patients homozygous for the DNA polymerase gamma (POLG) polymorphism**

<table>
<thead>
<tr>
<th>N</th>
<th>POLG genotype</th>
<th>Histology</th>
<th>Stage and spread of disease</th>
<th>Relapse</th>
<th>Sperm concentration ($\times 10^6/ml$)</th>
<th>Inhibin B serum concentration (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9/12</td>
<td>S</td>
<td>Stage 1</td>
<td>No</td>
<td>4.84</td>
<td>148</td>
</tr>
<tr>
<td>2</td>
<td>9/9</td>
<td>S</td>
<td>Stage 1</td>
<td>No</td>
<td>26</td>
<td>73</td>
</tr>
<tr>
<td>3</td>
<td>7/12</td>
<td>NS (EC)</td>
<td>Advanced (good prognosis*)</td>
<td>No</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>4</td>
<td>11/12</td>
<td>S</td>
<td>Stage 1</td>
<td>No</td>
<td>0.002</td>
<td>250**</td>
</tr>
<tr>
<td>5</td>
<td>11/12</td>
<td>S</td>
<td>Stage 1</td>
<td>No</td>
<td>15</td>
<td>&lt;20</td>
</tr>
<tr>
<td>6</td>
<td>11/11</td>
<td>S</td>
<td>Stage 1</td>
<td>Yes</td>
<td>n.a.</td>
<td>&lt;20</td>
</tr>
<tr>
<td>7</td>
<td>9/11</td>
<td>S</td>
<td>Stage 1</td>
<td>No</td>
<td>7.2</td>
<td>94</td>
</tr>
<tr>
<td>8</td>
<td>11/11</td>
<td>S</td>
<td>Advanced (good prognosis*)</td>
<td>No</td>
<td>n.a.</td>
<td>32</td>
</tr>
<tr>
<td>9</td>
<td>11/11</td>
<td>S</td>
<td>Stage 1</td>
<td>No</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>10</td>
<td>9/11</td>
<td>S</td>
<td>n.a.</td>
<td>n.a.</td>
<td>30</td>
<td>68</td>
</tr>
<tr>
<td>11</td>
<td>11/12</td>
<td>S</td>
<td>n.a.</td>
<td>n.a.</td>
<td>&lt;20</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>11/11</td>
<td>S</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

Note that it was not possible to gather information on all the patients because they were treated in different hospitals and some records were no longer available. S, seminoma; NS, non-seminoma; EC, embryonal carcinoma; n.a., not available.

*According to International Germ Cell Cancer Collaborative Group classification.
**With a timespan of 3 years, he harboured in the contralat. testis a Seminoma and a focal EC in the frozen section, EC not found in paraffin after orchiectomy.
compared with the study of Eurasian populations that included also 102 Poles; 19.6% of whom had the polymorphic alleles [29]. The inconsistency of the distribution of the genotypes between the two studies raises the question of a possible chance finding in the small study of Nowak et al. [26]. Nevertheless, our current results obtained in much larger groups of patients and controls support the existence of a true association of the POLG polymorphism with TGCT. In the Danish population, however, the difference was only found in the distribution of the homozygous variant genotype (with the lack of 10 CAG repeats on both alleles) and there was no difference in the frequency of heterozygotes.

The association of the POLG polymorphism with testicular cancer is consistent with the previously reported link to male subfertility, although—except for two studies [16, 24]—the latter link was not confirmed by several studies mentioned in the introduction, gathered recently by Westerveld et al. [30]. Also in the current study, no association was found between the POLG polymorphism and any phenotypic parameter linked to sperm quality, such as sperm motility or morphology. Although fertility is impaired in the majority of patients with TGCT [9], the sperm concentration is often retained within the normal range.

An association of the POLG polymorphism with both subfertility and testicular cancer in the Danish population supports our hypothesis that impaired spermatogenesis and TGCT are likely to be aetiologically linked in some patients. As our hypothesis stipulates that these two diseases are parts of TDS, the POLG polymorphism may be considered a predisposing genetic factor, perhaps increasing sensitivity to environmental factors such as endocrine disrupters. Higuchi et al. [31] showed that depletion of the mtDNA determines androgen dependence in prostate cancer cell lines, and this could be a possible explanation of this polymorphism’s effect on testicular germ cells. Still we do not know the effect of this genetic ‘defect’, but a new study indicated that the length of the POLG microsatellite region is maintained in all investigated species, including humans [17].

Surprisingly, we found a significant \( P = 0.035 \) association with the absence of the common CAG repeat length on both chromosomes and the histology of the tumour as 11 out of 12 tumours were seminomas. The one patient with a non-seminoma (embryonal carcinoma) had the genotype 7/12 CAG repeats, which included the shortest repeat of all the homozygous variants. Interestingly, we found one heterozygote patient with 7/10 genotype and the tumour was a non-seminoma as well. We did not find any correlation between the repeat length and histology among all the heterozygotes, where \( \sim 50\% \) had seminomas, while the remaining half had non-seminomas. We cannot explain the high frequency of seminomas among the subjects with homozygous polymorphism but we speculate that this genotype may, by an unknown mechanism, promote the development of seminomas, rather than non-seminomas. The progression of CIS-T to a seminoma is thought to involve mainly increased proliferation of tumour cells, which have essentially the same phenotype as CIS-T cells, whereas the progression to non-seminomas is associated with a profound reprogramming with loss of many germ-cell-specific features and up-regulation of the embryonic phenotype.

Concerning the mechanisms and the consequences of this polymorphism on the enzymatic function of POLG, there is no obvious explanation. Perhaps, the polymorphism affects the function of POLG, thus increasing probability of errors in the replication or proofreading of the mtDNA. We know that point mutations in the coding regions of the POLG gene accelerate accumulation of mutations, deletions and frame shifts in the mitochondrial genome, and deletions in mtDNA have been associated with male infertility [18, 32]. However, any explanation remains speculative until an independent corroboration.

In conclusion, we found an association between the POLG gene polymorphism and TGCT, especially classical seminoma. The POLG polymorphism can be a contributing genetic risk factor for testicular cancer but the possible mechanisms remain elusive and require further studies.

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**references**