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ISSN: 2047-2919
ANDROLOGY

ORIGINAL ARTICLE

Correspondence:
Loredana Gandini, Laboratory of Seminology-Sperm Bank, Department of Experimental Medicine, University of Rome "La Sapienza", Rome, Italy, Viale del Policlinico 155, 00161 Roma, Italy.
E-mail: loredana.gandini@uniroma1.it

Keywords:
androgen receptor, gene polymorphism, testis cancer

Received: 13-Dec-2013
Revised: 30-Jun-2014
Accepted: 5-Jul-2014

doi: 10.1111/j.2047-2927.2014.00252.x

Androgen receptor polymorphisms and testicular cancer risk

D. Grassetti, F. Giannandrea, D. Paoli, P. Masciandaro, V. Figura, T. Carlini, F. Rizzo, F. Lombardo, A. Lenzi and L. Gandini

Laboratory of Seminology-Sperm Bank, Department of Experimental Medicine, University of Rome "La Sapienza", Rome, Italy

SUMMARY

Testicular cancer (TC) is currently the most common malignant solid tumour in Caucasian males aged 15–39 years. Epidemiological evidence suggests that its onset may be due to an imbalance in the action of steroidal sex hormones and their receptors. A faulty androgen receptor signalling pathway can, in fact, cause various male reproductive disorders. The androgen receptor (AR) gene has two polymorphic segments consisting of CAG and GGC repeats. The length of CAG repeats has been shown to affect the regulation of AR activity. In our study, we used fragment analysis to evaluate the AR gene repeats of 302 TC patients and 322 controls, to establish if there is any association between repeat number and TC. This study of the largest Italian caseload investigated to date highlighted three particularly significant aspects. First, a CAG repeat number of ≥25 may be considered a risk factor for the onset of TC, given its greater frequency in patients in comparison with controls. This difference became significant for the non-seminoma group. Second, men with CAG repeats below 21 or above 24 were found to have a, respectively, 50 and 76% higher risk of TC than those with CAG 21–24, suggesting that these too can be considered a risk factor for TC. Finally, stage II patients were more likely to have a CAG repeat number <21 or >24 than stage I patients.

INTRODUCTION

Testicular cancer is currently the most common malignant solid tumour in Caucasian males aged 15–39 years. Its incidence varies by geographical area and may be up to three or four times higher in northern Europe and New Zealand than in the global population as a whole (Adami *et al.*, 1994; Hemminki & Li, 2004; Richiardi *et al.*, 2004). In Scandinavian countries, the epidemiological trends for testicular germ cell tumours (TGCTs) differ greatly, despite their geographical proximity and social similarities. In Denmark, the risk of TGCT is, in fact, five times higher than in Finland, with Sweden having an intermediate risk.

Among TGCTs, seminoma alone accounts for about 50% of all TC (Huyge *et al.*, 2003). Various risk factors correlated with TC have been known for many years, even though the main causes remain obscure. The most important risk factors include familial history of TC, previous TGCT and cryptorchidism (Forman *et al.*, 1992; Schnack *et al.*, 2009). It has also been suggested that TGCT associated with impaired spermatogenesis and other male reproductive system abnormalities may form part of a larger condition known as testicular dysgenesis syndrome (TDS) (Skakkebæk *et al.*, 2001). Epidemiological evidence suggests that the onset of TDS, including TGCT, may be due to an imbalance in the action of steroidal sex hormones. Reduced androgen activity as a function of oestrogen activity during male foetal development would thus lead to the various reproductive abnormalities typical of TDS (Martin *et al.*, 2008). Because of androgen receptor (AR) gene mutations, such patients have a high risk of developing malignant testicular tumours, as this gene is essential for the correct development of the male phenotype and of spermatogenesis. The AR gene is located on the X chromosome at Xq11-12 and has two polymorphic regions on exon 1, (CAG_n) CAA and $(GGT)_3GGG(GGT)_2(GGC)_n$, known, respectively, as CAG and GGN, where the first includes CAG repeats and the second GGC repeats. The extreme variability of these repeats determines the different lengths of the polyglutamine and polyglycine segments in the N-terminal transactivation domain. This seems to

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have a fundamental role in modulating AR function and thus sensitivity. The number of CAG repeats has been shown to affect the regulation of AR activity (Gao *et al.*, 1996). In vitro and in vivo studies have demonstrated that the more CAG repeats, the weaker and looser the bond and thus the weaker the receptor's transactivating capacity (Chamberlain *et al.*, 1994; Choong *et al.*, 1996; Buchanan *et al.*, 2004). The two factors thus show an inverse correlation, according to many authors, although a 2010 study (Nenonen *et al.*, 2010) seems to contradict this, finding instead that receptor activity is lower in both short and long repeats than in those of an average length. In men, the number of CAG repeats can vary from 8 to 37, with a mean of 20–22 depending on ethnic origin. African Americans have a lower number of repeats than Caucasians, and a reduced risk of TGCT (Schottenfeld *et al.*, 1980; Edwards *et al.*, 1992; Tut *et al.*, 1997; Kuhlenbaumer *et al.*, 2001). In the last decade, some studies have tried to establish if there is any association between CAG and GGC repeat number and testicular tumours, but the results appear contradictory (Rajpert-De Meyts *et al.*, 2002; Giwercman *et al.*, 2004; Davis-Dao *et al.*, 2011; Västermark *et al.*, 2011; Kristiansen *et al.*, 2012). Some of these studies also found other AR mutations, such as single-nucleotide polymorphism (SNP), only in those patients with TC (Garolla *et al.*, 2005; Västermark *et al.*, 2011).

In our study, we analysed CAG and GGC repeats in a large caseload of TC patients to attempt to establish if there is any association between the two. We were particularly interested in investigating any correlation with the histotype and stage, which despite its potential importance has been generally neglected in the literature. This approach could help clarify the role played by AR in various cancer types and stages, as well as facilitating understanding of and simplifying a topic which is still highly controversial, often because of the use of caseloads too small to have any clinical significance.

MATERIALS AND METHODS

Subjects

The study was approved by our University Hospital Ethics Committee. This case-controlled study recruited 302 TC patients (seminoma and non-seminoma) attending the Seminology laboratory sperm bank at the University of Rome "La Sapienza" Department of Experimental Medicine - Medical Pathophysiology Section for cryobanking of semen. All patients were studied after removal of the affected testicle and before the start of chemo- or radiotherapy. The control group consisted of 322 cancer-free men, recruited in the same Department, who were undergoing a preventive andrological investigation comprising medical history, andrological examination, semen analysis, hormone study and testis ultrasound. These subjects were chosen on the basis of the absence of any clinical signs resulting from this investigation, namely: no family history of TC, normal scrotal palpation and ultrasound, normozoospermia and hormone profile in the normal range.

Genotyping

A sample of peripheral venous blood was taken from all patients and controls for molecular evaluation of the length of the polyglutamine and polyglycine segments of the AR gene (CAG and GGC repeats).

Genomic DNA was extracted from peripheral blood leucocytes using the Wizard Genomic extraction kit (Promega Corporation Madison, WI, USA). Concentration and purity were evaluated by Nanodrop ND 1000 (Thermo Fisher Scientific, Waltham, MA, USA).

The length of the polymorphic fragments and thus the number of CAG and GGC repeats were analysed by primers flanking the triplet repeat regions.

The amplification reactions for both CAG and GGC repeats were carried out in 25 μL containing 0.5 ng of genomic DNA, 0.8 μM of each primer and 12.5 μL of Ampli Aaq Gold 360 Master Mix (Applied Biosystems, Carlsbad, CA, USA). The CAG amplification protocol consisted of 10 min at 95 °C followed by 30 cycles of 45 sec at 94 °C, 30 sec at 59 °C and 1 min at 72 °C. GGC amplification involved 5 min at 95 °C followed by 30 cycles of 40 sec at 95 °C, 30 sec at 55 °C and 30 sec at 72 °C, with a final extension step at 72 °C for 15 min.

Capillary electrophoresis was carried out using 10 μL of a reaction mixture consisting of 2.5 μL of PCR product (for both CAG and GGC), 0.3 μL of Genescan LIZ 600 (Applied Biosystems) and 7.7 μL of formamide. Samples were first denatured for 5 min at 95 °C and then loaded into a 3500 Genetic Analyser (Applied Biosystems). The forward primer for CAG and GGC had been fluorescently labelled with FAM at 5′ to enable the fragment to be seen during electrophoresis (fragment analysis). The following primers were used:

CAG – forward: FAM-TCCAGAATCTGTTCCAGAGCGTGC, reverse: GCTGTGAAGGTTGCTGTTCCTCAT; GGC – forward: FAM-GTGTGTAGTCCACGCCACTTCAGCGAAAGG, reverse: GGA AAGCGACTTCACCGCACCTGATGTGTG.

Raw data from the capillary electrophoresis were analysed by Gene Mapper Analysis (Applied Biosystems).

Statistical analysis

The statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA). The inter-group comparisons of the CAG and GGC repeats were performed using the Mann–Whitney test. All statistical tests were two-sided. A p-values of <0.05 were considered statistically significant. Logistic regression models were used to derive the odds ratio (OR) of TC according to the number of CAG and GGC repeats.

The CAG repeat numbers were divided into CAG = 21–24, set as the reference, CAG <21 and CAG >24. The reference corresponded to the median quartiles ranging from the lower 25th (CAG = 21) to the upper 75th (CAG = 24) percentile of the control group, i.e. the cut-points delimiting the shortest and longest stretches of our control sample.

We identified the cut-points on the basis of CAG number distribution in the control group (322 patients), taking inspiration from Nenonen's in vitro study. This study showed that extremely long and short CAG repeats (16, 28 CAG) have lower AR activity than does the median CAG length (22 CAG) (Nenonen *et al.*, 2010).

We also conducted a sensitivity analysis based on different cut-points and excluding the variable stage I, to evaluate any change to the magnitude of the association between CAG repeat length and TC.

Binary logistic regression was applied with presence of TC (yes/no) as the dependent variable. The proportion of subjects with long CAG ($\geq$25) was determined for the two groups

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(seminomas, non-seminomas) and controls and compared by the use of Fisher's exact test. Analyses were also conducted with respect to stage of the disease, excluding advanced stage because of the low number of subjects (n = 24) affected.

RESULTS

Of the 302 TC patients included in the study, 166 had been diagnosed with seminoma and 136 non-seminoma. Patients were also classified on the basis of clinical stage (TNM classification), with 153 patients in stage I, 125 in stage II and 24 in advanced stage.

The mean age ±SD at the time of diagnosis was 30.85 ± 6.59 years (range 15–55 years) for the TC group as a whole and 32.08 ± 6.27 (15–48) for the control group. This difference was statistically significant (p = 0.018). There was no appreciable difference in the age at diagnosis for seminoma cases and controls, whereas non-seminoma patients were significantly younger than the controls (p <0.05). The CAG and GGC repeat number distribution is shown in Fig. 1.

The means and standard deviations for CAG and GGC repeat number in the TC patients and controls are given in Table 1. The mean number of CAG repeats was 22.25 ± 3.12 and 22.26 ± 2.75 for patients and controls respectively; this difference was not significant. The median was 22 for both patients and controls. There was no appreciable difference in the mean number of GGC repeats between patients (17.06 ± 2.02) and controls (17.08 ± 1.68). The median number of GGC repeats was 17 for both patients and controls.

Comparison of the TC patients and controls by the Mann–Whitney test did not reveal any statistically significant difference in either of the repeats for the TC group as a whole or for the various histotypes (seminomas, non-seminomas) compared against each other and against the control group. The proportion of males with CAG repeat number 25 or over was significantly higher in TC patients (26.2%) than in controls (18.6%) (p = 0.027) (Fig. 2). There was no significant difference in mean CAG repeat number between the TC patients and the controls (equal variance, p >0.05). However, when the CAG repeat number was divided into three subgroups (<21, 21–24 and >24) for the stratified analysis, the OR for TC patients was 50% higher [95% confidence interval (CI): 1.02–2.20] for CAG repeats <21 and 76% higher (95% CI: 1.18–2.63) for CAG repeats >24 with respect to the reference group of controls. This difference was more significant in subjects with stage II disease at the time of diagnosis (Table 2). The OR for the presence of disease stage II, regardless of the histotype, was thus 2.00 (95% CI: 1.22–3.26) for CAG repeats <21 and 1.92 (95% CI: 1.13–3.26) for CAG repeats >24, compared with a CAG repeat number 21–24 (Table 2). Advanced stage was excluded from the analysis because of the low number of subjects (n = 24) concerned. These results were confirmed by a sensitivity analysis excluding stage I patients, which showed that the direction and magnitude of the association between CAG repeat length and TC were amplified at stage II (data not shown).

We also examined the joint association of CAG and GGC repeats in relation to the risk of TC in these two categories. The OR value was significantly increased (OR: 2.20; 95%

Table 1 Means and standard deviations of CAG and GGC repeat number in testicular cancer patients and controls

	Seminomas	Non-seminomas	All testicular cancer	Controls
No. of patients	166	136	302	322
CAG repeat number	22.17 ± 3.27	22.36 ± 2.93	22.25 ± 3.12	22.26 ± 2.75
GGC repeat number	17.10 ± 2.00	17.01 ± 2.05	17.06 ± 2.02	17.08 ± 1.68

Figure 1 Bar charts displaying distributions of the CAG and GGC repeat number in testicular cancer patients (light bars) and controls (dark bars).

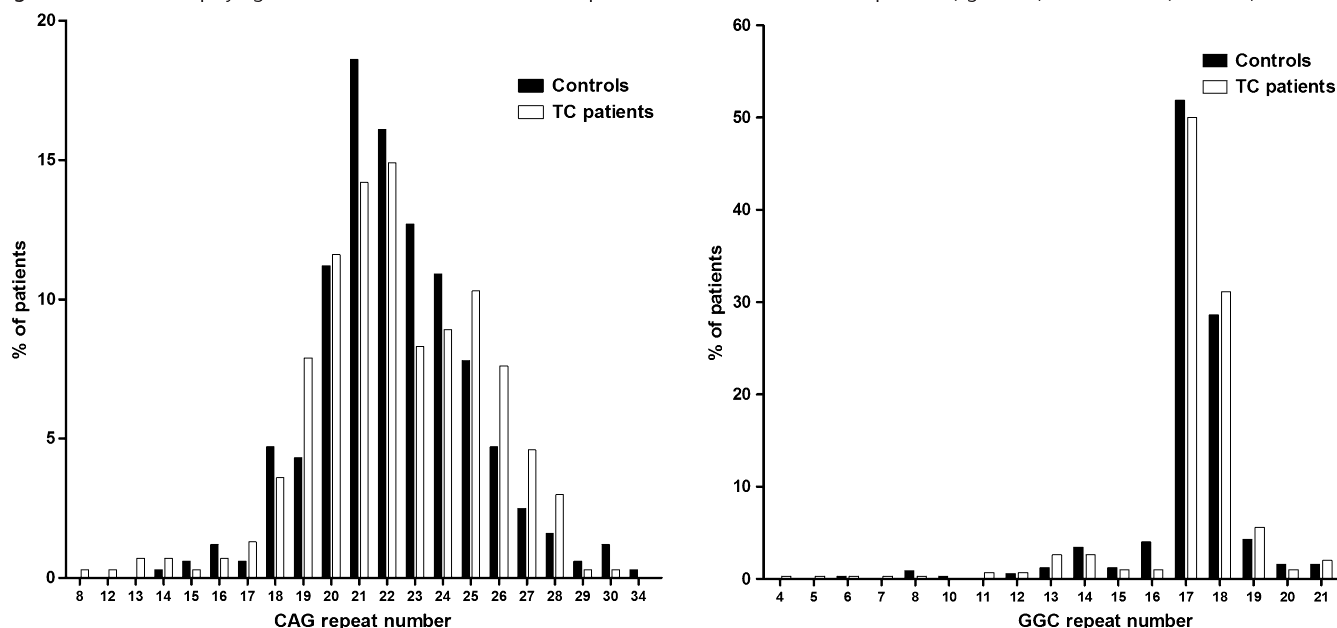

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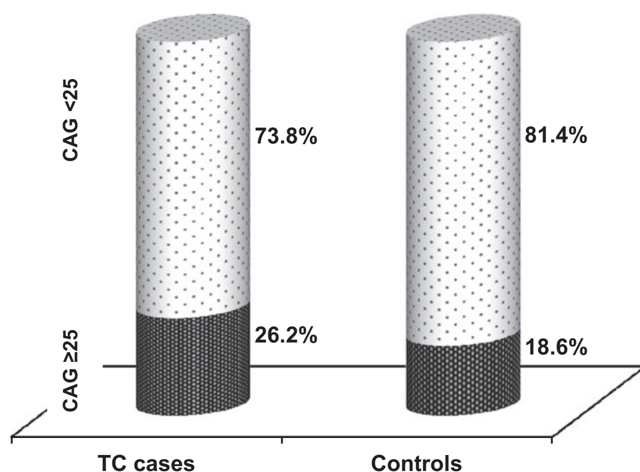
Table 2 Risk of testicular cancer in relation to CAG and GGC repeat number

	No. of CAG repeats			No. of GGC repeats	
	<21	21–24	>24	≤17	>17
Controls (322 pts)	74	188	60	206	116
All TC (302 pts)	83	140	79	182	120
OR	1.50	1.0	1.76	1.0	1.17
95% CI	1.02–2.20	Reference	1.18–2.63	Reference	0.84–1.61
p value	0.036		0.005		0.34
Stage I (153 pts)	35	76	42	91	62
OR	1.17	1.0	1.73	1.0	1.21
95% CI	0.72–1.89	Reference	1.07–2.78	Reference	0.81–1.79
p value	0.52		0.024		0.34
Stage II (125 pts)	41	52	32	77	48
OR	2.00	1.0	1.92	1.0	1.10
95% CI	1.22–3.26	Reference	1.13–3.26	Reference	0.72–1.69
p value	0.005		0.015		0.64

Figure 2 Percentage of males with CAG lengths ≥25 or <25 in TC patients and controls.



CI: 1.13–4.28) in the haplotype, in which both CAG and GGC repeat numbers alleles were high (CAG >24; GGC >18) compared to the reference group (CAG 21–24; GGC ≤ 17) (Table 3). When analysing cancer stage II only, the OR for TC was 165% higher [95% confidence interval (CI): 1.12–6.30] for men in which both the alleles were long (CAG >24; GGC >18) and 139% higher (95% CI: 1.28–4.46) for men with short repeats (CAG <21, GGC ≤17) than for the reference group (Table 3).

DISCUSSION

Testicular cancer is a very common disease whose incidence worldwide has been rising, especially in recent decades. Testicular germ cell tumours make up 95% of all TCs and are the most common solid tumour in men aged 15–39 years (Devesa *et al.*, 1995; Huyge *et al.*, 2003). Although there has been enormous progress in the clinical treatment of TC and preservation of fertility through sperm banking in recent years, the main causes of this disease remain still unclear. However, important risk factors include familial history, lifestyle, diet, environmental conditions and genetic susceptibility (Skakkebæk *et al.*, 2001; Krausz & Looijenga, 2008; Giannandrea *et al.*, 2011, 2013). The development of TC is postulated to be due to endocrine disruption, particularly abnormalities in the action of gonadotropins and steroidal sex hormones (Rajpert-De Meyts & Skakkebæk, 1993). Men with androgen insensitivity syndrome caused by AR gene mutations have a higher risk of developing TC. There is some evidence of an inverse correlation between AR gene CAG repeat number variability and the receptor's transactivation efficiency. Irvine suggested that a longer CAG repeat region might reduce the receptor's transactivation activity (Irvine *et al.*, 2000).

Androgen receptor gene abnormalities are also common in other disorders, such as cryptorchidism and impaired spermatogenesis. Analysis of the number of CAG repeats in infertile men has produced contrasting results (Giwercman *et al.*, 1998; Patrizio & Leonard, 2001; Ferlin *et al.*, 2004; Milatiner *et al.*, 2004). Many authors have worked to try to understand if reduced androgen sensitivity because of point mutations, or more often caused by an excessively long CAG repeat segment, might lead to the development of testicular dysgenesis and consequently increase susceptibility to TC. Rajpert-De-Meyts *et al.* (2002) analysed CAG repeats in a Danish population of 102 TC patients and 110 controls. No statistically significant differences were found in the distribution of CAG repeat number between the two groups, analysed by both histotype and stage. Giwercman *et al.* investigated the correlation between CAG and GGC repeats in a population from Malmo consisting of 83 TGCT patients and 220 controls. No statistically significant differences in CAG or GGC repeat number were seen between TGCT patients and the control group. However, it is interesting to note that the number of men with CAG repeat number >25 was significantly lower in seminoma patients and in seminoma + non-seminoma patients than in the controls. Longer CAG repeat numbers were found in patients with more advanced cancer at the time of diagnosis, although this was not statistically significant. This study seems to suggest, therefore, that CAG repeats may be correlated with the presence or absence of metastasis on diagnosis, where a longer repeat number would indicate a higher chance of metastasis. This was the first study that demonstrated a correlation between AR CAG repeats, TGCT histology and disease progression, albeit in a limited caseload (Giwercman *et al.*, 2004).

Garolla *et al.* (2005) analysed 123 TC patients, all at stage 1 at the time of study, against a control group of 300 fertile men studied for AR mutations, of whom 115 were selected for the study of CAG and GGC repeats. There were no differences in the number of CAG and GGC repeats between patients and controls. This study did not confirm the differences found between cancer histotypes or the greater frequency of CAG >25 in patients vs controls in Giwercman *et al.* (2004) study. Instead, it seemed to corroborate the results of Rajpert-De Meyts *et al.* (2002) Danish study. However, when Garolla *et al.* considered both CAG and GGC repeats together, they found that the distribution of CAG/GGC = 20/17 was significantly higher in TC patients (8.1%) than in controls (1.7) ($p < 0.05$). This study also found two single nucleotide point mutations involving amino acid substitution: proline to serine at position 390 P390S and alanine to threonine at position 297 (A297T), as well as a trinucleotide deletion of leucine at position 57 (Del L57). All three of these mutations were only found in seminoma patients, suggesting their involvement in this specific histotype.

A Scandinavian study examined 367 Danish and Swedish TGCT patients, with 214 Swedish men as the control group. CAG

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Table 3 Joint association of CAG and GGC repeats in relation to the risk of testicular cancer

Combined CAG and GGC repeats	<21, ≤17	>24, ≤17	21–24, ≤17	21–24, >18	<21, >18	>24, >18
Controls (322 pts)	48	43	115	73	26	17
TC cases (302 pts)	45	51	86	54	38	28
OR	1.25	1.58	1.0	0.98	1.95	2.20
95% CI	0.76–2.05	0.96–2.59	Reference	0.63–1.55	1.10–3.46	1.13–4.28
p value	0.36	0.06		0.96	0.02	0.02
Seminomas (166 pts)	25	22	50	30	22	17
OR	2.32	2.02	1.0	1.43	2.14	2.46
95% CI	1.28–4.19	1.08–3.79	Reference	0.80–2.54	1.03–4.46	1.06–5.66
p value	0.005	0.027		0.22	0.041	0.034
Non-seminomas (136 pts)	20	29	36	24	16	11
OR	1.33	2.15	1.0	1.05	1.96	2.06
95% CI	0.70–2.52	1.18–3.93	Reference	0.58–1.90	0.95–4.06	0.88–4.81
p value	0.38	0.012		0.87	0.06	0.092
Stage I (153 pts)	13	26	52	24	22	16
OR	0.59	1.33	1.0	0.72	1.87	2.08
95% CI	0.29–1.20	0.74–2.40	Reference	0.41–1.28	0.97–3.60	0.97–4.43
p value	0.14	0.33		0.26	0.061	0.05
Stage II (125 pts)	28	21	28	24	13	11
OR	2.39	2.00	1.0	1.35	2.05	2.65
95% CI	1.28–4.46	1.03–3.90	Reference	0.72–2.50	0.93–4.49	1.12–6.30
p value	0.006	0.040		0.34	0.072	0.027

and GGC repeat number were evaluated, along with 11 AR SNPs. One of these, genotype G of the non-coding SNP rs12014709, was found in 10% of TGCT cases and 5.1% of controls, and was thus associated with the TGCT group. None of the other SNPs or CAG repeats was significantly associated with an increased or reduced risk of developing TGCT. This study also found that a GGN repeat number <23, consisting of <17 GGC repeats, was correlated with an increased risk of developing non-seminomatous TGCT and with an increased risk of metastasis (Västermark *et al.*, 2011) .

In 2012, Kristiansen *et al.* investigated the correlation between CAG and GGN repeats and TC in a Norwegian population. The study involved 651 TC patients and 313 controls. No statistically significant differences were seen in the number of CAG and GGN repeats between patients and controls, even when analysed by histotype (Kristiansen *et al.*, 2012). In addition, they could not confirm Giwercman's finding that CAG >25 was more common in patients with non-seminomatous tumours (Giwercman *et al.*, 2004).

In our study, there was no statistically significant difference in average CAG and GGC repeat number between the TC patients and controls. There was a larger variability of CAG than GGC repeats in both patients and controls, especially among the rare alleles. When stratified, men with CAG repeats below 21 or above 24 were found to have a, respectively, 50 and 76% higher risk of TC than those with CAG 21–24. In other words, the risk of developing TC would seem to be lower for men with a CAG repeat number between 21 and 24.

This finding of a non-linear relationship between CAG repeat number and the risk of TC may be comparable with similar results showing a U-shaped correlation between CAG repeat number and total sperm number. Such an association was in fact shown in vitro and confirmed in human studies which found that men with CAG repeats below 22 or above 23 have an approximately 20% higher risk of infertility than those with CAG 22 or 23 (Nenonen *et al.*, 2011). It has been hypothesized that as 91–99% of the CAG alleles in the general population have a length between 16 and 29, repeats outside this critical range could be a more important mediator of reproductive diseases such as male infertility (Buchanan *et al.*, 2004). In this context, our results support the suggestion that normal AR function is sustained over a critical but limited range of CAG repeat numbers. A similar finding has also been reported in vitro in relation to GGC repeats. The most common GGN segment (17 GGC repeats) was shown to have the highest transactivating capacity (Lundin *et al.*, 2007) and was also associated with the lowest risk of male genital malformations (Aschim *et al.*, 2004) linked to reduced androgen activity (Lundin *et al.*, 2006).

Previous studies showed that men with CAG repeats above 25 were less androgen sensitive than those with shorter segments. In our study, the proportion of subjects with long CAG repeats (≥25) was higher in TC cases than controls. This difference became significant for the non-seminoma group with respect to controls. Similar results were found by Giwercman *et al.* (2004), suggesting that CAG repeats >25 were more common in patients with non-seminoma. However, other studies did not find such differences between the histological groups (Kristiansen *et al.*, 2012).

Previous studies have correlated CAG repeats with clinical stage of TC, with some finding that CAG repeat number was higher if the tumour was advanced at diagnosis (Giwercman *et al.*, 2004). In our study, there was a statistically significant difference in CAG but not GGC repeats according to the stage of the disease, with the longest or shortest repeats found among patients with stage II disease at the time of diagnosis. Analysing stage II, the OR of TC was higher for men in which the alleles were both long (CAG >24; GGC >18) or both short (CAG <21, GGC ≤17). This trend was obvious for both histotypes under study (seminomas, non-seminoma) vs controls.

In conclusion, this study of the largest Italian caseload investigated to date has highlighted three particularly significant aspects. First, a CAG repeat number of ≥25 may be considered a risk factor for the onset of TC, given its greater frequency in patients in comparison with the controls. This is of considerable scientific and oncological interest, although it is difficult to understand what biological mechanism might be responsible. It

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is possible that a greater CAG repeat number and consequent reduced efficiency of the transactivation domain leads to a diminished AR capacity to recognize and bind androgens, to such an extent as to make them incapable of functioning correctly and result in a higher concentration of free hormones. These two factors might thus play a part in the onset of TC.

Second, CAG repeats <21 and >24 are significantly associated with TC, suggesting that they can be considered a risk factor for the onset of this disease. The least risk is thus seen with CAG repeat numbers between 21 and 24, which are the most common in the general population, thus confirming in vitro findings.

Finally, stage II patients were more likely to have a CAG repeat number <21 or >24 than stage I patients. All these aspects lead us back to the crucial role played by the length of the polymorphic segment in androgen receptor function; a change in the number of repeats can lead to various disorders and, above all, is a risk factor for TC that should not be neglected. However, we believe that comparative studies of groups of single ethnic origins are in any case necessary, given the highly variable distribution of these polymorphisms in different populations worldwide. This will enable further understanding of the role of the AR gene and polymorphism frequency in the onset of TC in patients of different ethnic origins.

FUNDING

This work was supported by a grant from the Italian Ministry of Education and Research (MIUR-PRIN) and the University of Rome “La Sapienza” Faculty of Medicine.

ACKNOWLEDGEMENTS

The authors wish to thank Marie-Hélène Hayles for her assistance in the English translation of the manuscript.

CONFLICT OF INTEREST

The authors have no conflicts of interest.

AUTHOR CONTRIBUTIONS

L.G., D.P. and D.G. were responsible for the conception and design of the work; L.G., D.G. and F.G. drafted the article; L.G. has finally approved the version to be published; P.M., V.F., T.C., F.G. and F.R. acquired and analysed the data; F.L. and A.L critically revised the manuscript.

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