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Linkage between androgen receptor gene CAG trinucleotide repeat length and testicular germ cell cancer histological type and clinical stage ☆

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Abstract

Sex hormones and/or gonadotropins may play a crucial role in the development of testicular germ cell cancer (TGCC). A direct link between this malignancy and endocrine factors has not been confirmed. We tested whether *CAG* and *GGN* repeats of the androgen receptor gene (*AR*) play a role in the aetiology or pathogenesis of TGCC. Eighty-three TGCC patients and 220 controls were included. Mean *CAG* or *GGN* lengths did not differ between the TGCC cases and controls. The proportion of males with *CAG* lengths above 25, indicative of reduced androgen sensitivity, was significantly lower among patients with pure seminomas and in the combined group of seminomas and mixed tumours compared with non-seminomas and controls. The median *CAG* length was higher if the tumour was metastasing at diagnosis. This is the first study showing an association between the *AR* polymorphism and histological type as well as the progression rate of TGCC.

Keywords: Testicular germ cell cancer; Androgen receptor; CAG repeat

1. Introduction

Testicular germ cell cancer (TGCC) is the most common malignancy of young men [1] and for unknown reasons, the incidence of this tumour has increased by a factor 3–4 during the last 50 years [2]. This trend has been noticed worldwide, at least in populations of Caucasian origin, although there are significant geographical differences in the magnitude of a lifetime risk. In Denmark, the risk of TGCC is 5 times higher than in Finland, whereas Sweden has an intermediate risk, despite the three countries being geographically and socially closely related [2].

TGCC is histologically divided into two main groups – non-seminomas and seminomas [3], the former group also including tumours of a mixed histological type. Non-seminomas have an earlier age peak of incidence than seminomas, are more aggressive and prone to metastasise. Both non-seminomas and seminomas are believed to originate from a common precursor – carcinoma-*in situ* (CIS) of the testis [4]. CIS cells are intratubular malignant germ cells, having the potential to progress into an invasive tumour stage. The factors responsible for this progression are presently unknown [4–6]. The major peak of incidence of these tumours occurs shortly after puberty. This period is characterised by a sharp rise in the levels of gonadotropins, luteinising hormone (LH) and follicle stimulating hormone (FSH),

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as well as of the sex steroids, and it has therefore been suggested that either one, or a combination of these endocrine factors might stimulate the progression of CIS cells to an invasive stage [4]. Clarification of this process is crucial for an understanding of the pathogenesis of testicular malignancy and, subsequently, for future preventive possibilities.

It has been speculated that TGCC could be causally related to other abnormalities of male reproductive organs, i.e. cryptorchidism, hypospadias and impairment of sperm production, given the common name Testicular Dysgenesis Syndrome (TDS) [7]. Environmentally-derived components, so-called endocrine disrupters, with oestrogenic and/or anti-androgenic activity were suggested as potential causes of the increasing incidence of TDS. It has also been hypothesised that TDS, including TGCC, is a result of an imbalance in sex steroid action in favour of oestrogens during the foetal period. Hence, based on epidemiological data, as well as the biological features of the CIS cells, the early foetal period was suggested as the time of origin of TGCC [4,5].

Individuals with the Androgen Insensitivity Syndrome (AIS), due to point mutations in the *androgen receptor* (*AR*) gene, are known to be at a high risk of developing testicular malignancy [8]. These subjects, not only present with high androgen levels, but also extremely high levels of LH and oestrogens in the circulation, the latter due to the aromatisation of testosterone to oestradiol [9]. The bio-chemical profile of AIS patients strengthens the hypothesis of an androgen/oestrogen imbalance as the cause of TGCC.

Mutations in the *AR* gene imply a dramatic loss of receptor activity, but are extremely rare [10], and thus not likely to be involved in most TGCC cases. In recent years, evidence for subtle genetic variants, modifying protein action or metabolism has been put forward. The *AR* gene comprises two polymorphic regions in the transactivating domain of the receptor - one glutamine stretch (*CAG*), encoded by (*CAG*)*nCAA* and one glycine segment (*GGN*) encoded by $(GGT)_3GGG(GGT)_2(GGC)_n$. The lengths of these repeats have been shown to play a role for the fine-tuning of AR activity [11,12]. For *CAG* numbers between 15 and 31, both *in vitro* and *in vivo* studies have shown inverse correlations between the length of the repeat and the transactivating capability of the receptor [13–15].

Epidemiological studies have indicated that *CAG* length might also play a role in the risk of TGCC. Afro-Americans were found to have shorter *CAG* repeats, on average, than Caucasians [16–18], which parallels the low risk of TGCC in the former group of men [19].

Although it seems unlikely that the rapid increase in the incidence of TGCC is due to genetic factors, but is rather environmental or life-style-related, the huge geographical and ethnic differences in the incidence of this cancer, indicate that some sub-populations could be genetically predisposed to the disease. It was therefore the aim of this study to elucidate whether variations in the *CAG* and *GGN* segments of the *AR* have any impact on the aetiology and pathogenesis of TGCC.

2. Patients and methods

2.1. TGCC patients

From March 2001 to August 2002, all patients with a diagnosis of TGCC passing through the outpatient clinic of the Department of Oncology, Lund University Hospital were invited to participate in a study of reproductive function. Seventy nine percent of them accepted the invitation. Blood samples for DNA analysis were drawn from 83 men of Swedish origin. The histological type of tumour and date at orchidectomy was noted. Age at diagnosis was considered equal to age at orchidectomy. Clinical staging was preformed according to the Royal Marsden staging system [20]: Stage I, disease limited to the testis; Stage II, spread to the retroperitoneal lymph nodes; Stage III, spread to supradiaphragmatic lymph nodes; Stage IV, metastases into the visceral organs.

2.2. Background characteristics of the TGCC patients

Among the 83 men included in the study, 41 presented with the diagnosis non-seminoma, 15 with mixed types and 27 with seminoma. Usually, the ratio between the seminomas and non-seminomas/mixed types is approximately 1:1. The under representation of seminoma patients in our group is due to the fact that some of the patients with Stage I seminoma, who did not wish to receive adjuvant irradiation of the retroperitoneal lymph glands, were followed at their local hospital and were not referred for centralised management at the Department of Oncology, Lund University Hospital.

The median age (range) at the time of diagnosis was: 29 years (15–47 years) for the whole group of men with TGCC, 27 years (15–42 years) in the non-seminoma group, 33 years (23–38 years) in the mixed type men and 35 years (17–47 years) in the seminoma patients (Table 1). The distribution of histological tumour types and clinical stages in those who refused to take part in the study was the same as among the participants.

2.3. Controls

Blood samples were obtained from 220 subjects, with Swedish mothers, presenting for the compulsory medical board prior to military service [18]. This group can be considered as representative for the general male population in southern Sweden. The men were aged 18–21 years (median: 18 years). Prior to inclusion, all men underwent andrological examination including testicular

Table 1
Main background characteristics of the 83 testicular germ cell cancer (TGCC) patients included in present study: pure non-seminomas, mixed non-seminomas–seminomas and pure seminomas

	Non-seminomas	Mixed non-seminomas–seminomas	Seminomas	All TGCC
N	41	15	27	83
Median age at diagnosis – in years (range)	27 (15–42)	33 (23–38)	35 (17–47)	29 (15–47)
Stage I[a]	27	11	20	58
Stage II[a]	9	3	5	17
Stage III[a]	1	1	2	4
Stage IV[a]	4	0	0	4

[a] According to the Royal Marsden staging system [20].

ultrasound. Testicular microlithiasis [21] or tumour was not found in any of them.

Both patients and controls were recruited after giving a written informed consent according to protocols approved by the ethical review board of Lund University.

2.4. Analysis of CAG and GGN repeat lengths of the androgen receptor gene

Genomic DNA was prepared from peripheral leucocytes and the *CAG* and *GGN* repeats were amplified for 40 cycles in a 25 μL polymerace chain reaction (PCR) reaction containing 10 ng DNA, 0.3 μM of flanking primers, 2.5 mM $MgCl_2$, 200 μM of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxythymidine triphosphate (dTTP) and deoxyguanidine triphosphate (dGTP) (Roche Diagnostics, Bromma, Sweden) 45 mM KCl, 10 mM Tris–HCl (pH 8.4 at 70 °C) and 0.5 units of Dynazyme DNA polymerase (Finnzymes Oy, Espoo, Finland). Instead of 200 μM dGTP, a mixture of 100 μM dGTP and 100 μM 7-deaza-dGTP (Roche Diagnostics, Bromma, Sweden) was used for the *GGN* analysis. Each amplification cycle included denaturation at 96 °C for 1 min, primer annealing at 56 °C for 1 min and primer extension at 72 °C for 3 min, with an initial denaturation step at 96 °C for 3 min and a final extension step at 72 °C for 7 min.

One μL of each PCR product was used for the subsequent nested amplification. Nested PCR-products were purified using the JETPURE PCR purification kit (Genomed GmbH, Bad Oeynhausen, Germany) according to the protocol provided by the manufacturer. Approximately 30 ng of the purified products were submitted to a 20 μL sequencing reaction with the CEQ Quickstart kit (Beckman Coulter, Bromma, Sweden). Samples were analysed externally on a Beckman Coulter CEQ 2000XL (Beckman Coulter, Bromma, Sweden) sequencing gear [18].

2.5. Statistical analysis

The statistical analysis was performed using the statistical package for the social sciences (SPSS) 11.0 software (SPSS Inc., Chicago, USA). The inter-group comparisons of the *CAG* and *GGN* lengths were performed by use of the Mann–Whitney test. The histological type of the tumour plays a role for its metastasising properties. Therefore, when assessing whether the CAG length has any impact on whether the tumour has metastasised at the time of diagnosis or not, a binary logistic regression model was applied with the the tumour type (non-seminomas; mixed non-seminomas–seminomas; seminomas) and CAG length (less than or equal to 21 (= median) and more than 21) as discrete variables and +/− metastases as the dependent variable. Subsequently, the proportion of subjects with long *CAG* (>25) was determined for the four groups (non-seminomas, mixed non-seminomas–seminomas, seminomas and controls) and compared pairwise by use of the Fisher's exact test. Finally, bivariate Spearman's correlation coefficients were calculated for the association between the age at diagnosis and the *CAG* or *GGN* length. All statistical tests were two-sided. *P*-values of $\leqslant 0.05$ were considered statistically significant.

3. Results

The means and standard deviations for lengths of *CAG* and *GGN* repeats in the controls and TGCC men are given in Table 2.

Comparison of the controls and TGCC patients by means of the Mann–Whitney test did not reveal any statistically significant difference for any of the two repeats, neither for the total group of cancer patients, nor for any of the three main diagnostic subgroups: seminomas, mixed non-seminomas–seminomas and non-seminomas.

The proportion of males with *CAG* lengths above 25 indicative of a reduced androgen sensitivity was 0% (95% confidence interval (CI): 0–13% and 0–22%, respectively) in men with seminomas or mixed types, 20% (95% CI: 8.8–35%) in patients with pure non-seminomas and 13% (95% CI: 8.8–35%) in the controls. This proportion was significantly lower among patients with seminomas ($P = 0.05$) and in the combined group of seminomas and mixed seminomas–non-seminomas ($P = 0.007$) compared with the controls. In comparison

Table 2
CAG and *GGN* repeat lengths in the control group and the TGCC patients, pure non-seminomas; mixed non-seminomas–seminomas and pure seminomas

N	Controls	Non-seminomas	Mixed non-seminomas–seminomas	Seminomas	All TGCC	$P < 0.05$[a]
	220	41	15	27	83	
Median *CAG* length (range)	21.0 (12.0–30.0)	21.0 (13.0–28.0)	22.0 (19.0–25.0)	21.0 (18.0–25.0)	21.0 (13.0–28.0)	–
Median *GGN* length (range)	23.0 (10.0–25.0)	23.0 (16.0–24.0)	23.0 (17.0–24.0)	23.0 (19.0–24.0)	24.0 (16.0–24.0)	–
N with *CAG* > 25 (percentage)	29 (13%)	8 (20%)	0 (0%)	0 (0%)	8 (10%)	Seminomas vs controls, $P = 0.05$; seminomas vs non-seminomas, $P = 0.02$; seminomas + mixed non-seminomas–seminomas vs controls, $P = 0.007$; seminomas + mixed non-seminomas–seminomas vs non-seminomas, 0.002

[a] Only inter-group differences with $P \leqslant 0.05$ are specified.

to non-seminomas, both the group of pure seminomas ($P = 0.02$) and the combined groups of seminomas and mixed types ($P = 0.002$) exhibited a lower percentage of long CAG trinucleotides. However, the non-seminomas group did not differ from the controls ($P = 0.33$) (Fig. 1).

There was a statistically significant difference in *CAG* ($P = 0.04$), but not *GGN* ($P = 0.51$) length between the three groups defined according to whether the tumour has metastasised at the time of diagnosis (Stages II–IV) or not (Stage I), with the longest repeats found among those with the most advanced disease at the time of diagnosis (Table 3 and Fig. 2). This trend was obvious for all three histological categories of tumours, seminomas, non-seminomas and mixed non-seminomas–seminomas (Table 3). However, probably due to the limited sample sizes, these comparisons did not reach the level of statistical significance. However, the results of the binary logistic regression analysis, confirmed that the CAG length is associated with the presence of metastases, irrespective of the histological type of the tumour. Thus, an Odds Ratio of 4.1 (95% CI: 1.5–11.5; $P = 0.007$) for the presence of metastases, independent of the histological type, was found for a CAG length of more than 21 compared with those with a CAG length of 21 or less.

The bivariate correlation between the *CAG* as well as *GGN* length and the age at diagnosis was not statistically significant.

4. Discussion

The development of TGCC is at least a two-step process; the first event probably taking place during early foetal life and implying transformation of primordial germ cells into a common precursor of this cancer – CIS [4]. The next step is the progression of CIS into manifest tumour. It is generally believed that both processes are under endocrine control [22]. Since we did not find any differences in the distribution of the *CAG* and *GGN* repeats between the controls and the total group of TGCCs, our data indicate that these polymorphisms of the *AR* gene do not constitute a risk for developing testicular cancer *per se*. By contrast, long *CAG* tracts, associated with decreased androgen sensitivity, were exclusively found in patients with tumours without seminoma components. This might indicate that higher androgen sensitivity predisposes to the transformation of CIS into seminoma, while relatively low androgen sensitivity facilitates the transformation into non-seminoma. CAG repeat lengths above the median value of 21 were found to be associated with a higher Odds Ratio for the presence of metastases, independent of the histological tumour type. To our knowledge, this

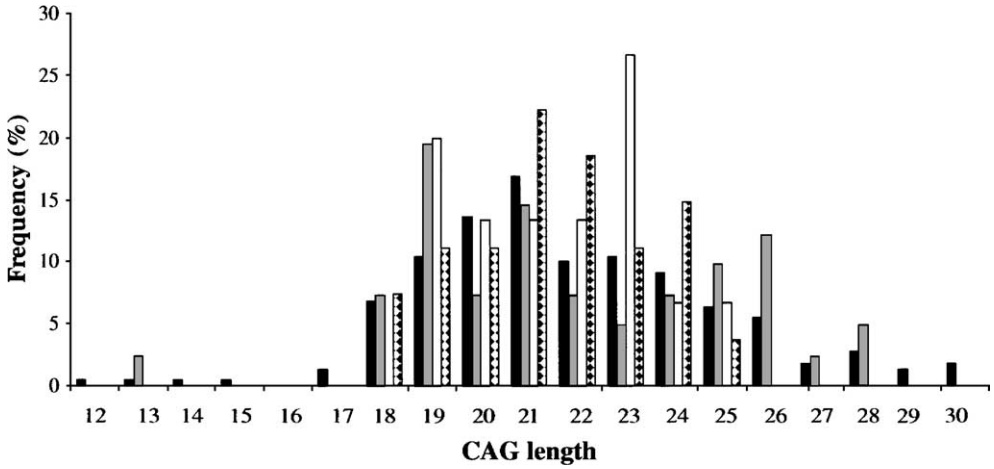

Fig. 1. Histogram showing frequencies of different CAG repeat lengths among controls (black), pure non-seminomas (grey), mixed seminomas and non-seminomas (white) and pure seminomas (dotted).

Table 3
Androgen receptor CAG repeat lengths in relation to whether the tumour has metastasised or not at the time of diagnosis

Tumour type	*N* Median (range)		*P* value
	−Metastases	+Metastases	
All	58 21.0 (13–28)	25 23.0 (19–27)	0.04
Pure non-seminoma	27 21.0 (13–28)	14 23.0 (19–27)	0.27
Mixed seminoma–non-seminoma	11 21.0 (19–24.0)	4 23.0 (19–25)	0.57
Pure seminoma	20 21.0 (18–24)	7 22.0 (20–25)	0.15

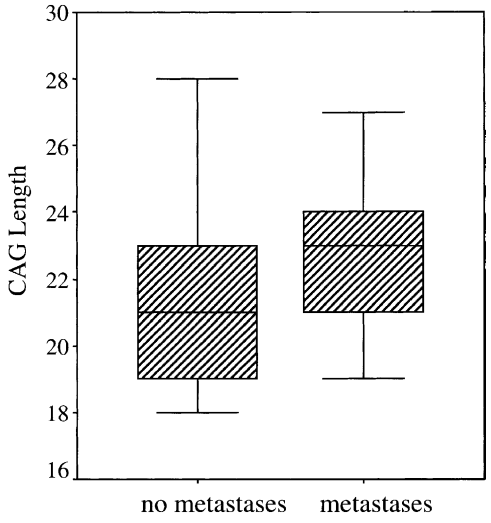

Fig. 2. Box and whisker plot illustrating *CAG* lengths in the two patient groups defined according to whether the tumour is metastasing at the time of diagnosis or not. The figure shows the median values (horizontal line), inter-quartile intervals (boxes) and 95% confidence intervals (CI) (whiskers).

is the first study demonstrating an association between *AR* gene polymorphisms and the pathogenesis of TGCC.

The assumption that gonadotropin and/or sex steroid action is necessary for the progression from the pre-invasive CIS stage to invasive tumour [4,22] has been based on observations showing a main peak of TGCC incidence shortly after puberty [1]. However, to date, a direct link between hormonal activity and testicular cancer development has not been confirmed.

There are data showing an inverse relationship between *CAG* length and androgen receptor transactivating capability [13]. This association was shown *in vitro* and confirmed in human studies by finding a negative correlation between the numbers of *CAG* repeats and sperm counts and higher LH levels in those with long CAG's [14,23]. Hence, men with *CAG* segments above 25 were less androgen-sensitive than those with shorter segments.

One can speculate which main hormonal factors might be responsible for the regulation of the progression from CIS to tumour. Possible candidates could be androgens, LH and oestrogens. Unlike other male germ cells, CIS germ cells, as well as seminomas, have been shown to express the androgen receptor [24]. However, LH might also be directly involved in the pathogenesis of TGCC. Increasing *CAG* length is accompanied by rising LH levels [23] and previous studies have shown that testicular malignancy is asso-

ciated with Leydig cell dysfunction and increased LH levels [25,26], an effect which might be further amplified in men presenting with long *CAG* repeats. Although the presence of LH receptors in malignant male germ cells has not yet been demonstrated, the presence of an increased immunoreactivity of the LH receptor has been shown in the basal lamina of tubules, which contain CIS cells [22]. Furthermore, patients with Kallmann's syndrome having hypogonadotropic hypogonadism, resulting in low LH levels as well as low concentrations of androgens, never develop testicular cancer, despite frequent cryptorchidism. Apart from exerting a direct effect on CIS germ cells, LH might also act by stimulating the intratesticular aromatisation of testosterone to oestradiol [4]. Recently, TGCC was reported to express oestrogen receptor-beta (ERβ) [27]. Interestingly, ERβ expression was mostly pronounced in non-seminomatous tumour elements, such as yolk sac tumours and teratomas, whereas it was markedly diminished in seminomas and mixed types, at both the transcriptional and translational levels. Thus, assuming that intratesticular oestradiol levels increase with the CAG repeat length, due to higher levels of both LH and testosterone, our findings fit with the ERβ expression pattern.

Hormone levels were not measured in our study. The pre-treatment levels of the gonadotropins and sex steroids might be influenced by the fact that the patients were harbouring tumours and are not representative for long-term exposure to gonadotropins and/or sex steroids. In post-treatment samples, the hormone levels can be affected by the therapy given, i.e. orchidectomy, cytotoxic drugs or irradiation. Thus, none of these hormonal markers would mirror the endocrine milieu of the CIS cells at the time of proliferation and progression to tumour. Ideally, serum samples obtained several years prior to the tumour diagnosis should be analysed, but such material was not available.

Our results are partly in agreement with the only report on the association between germ-line *CAG* length and TGCC published so far [28]. In a group of 102 Danish men with TGCC, no difference in *CAG* length was found between patients and controls. The authors found the more advanced tumour stages were associated with longer *CAG* repeats. However, this result did not reach the level of statistical significance, possibly because of a too low statistical power. In contrast to our findings, the proportion of men with long *CAG* stretches was identical for the two histological tumour types in the Danish study [28]. This discrepancy between the two reports might partly be due to different patient selection. Rajpert De-Meyts and colleagues were recruiting their patients in an andrological clinic, whereas our patients represent a consecutive cohort approached directly in an oncological outpatient clinic. Since long *CAG* repeats were found to be associated with decreased sperm counts [14], it cannot be excluded that men with long repeats are over-represented among patients seeking andrological advice. Moreover, more than 30% of the patients in the Danish study presented with bilateral disease, which, compared with the 5–6% among Danish TGCC patients in general, might not be representative for the whole group of TGCC patients [29]. Even our patient sample was not completely representative for the whole group of TGCC. Patients with seminomas were underrepresented due to the fact that a proportion of TGCC patients with Stage I disease are not referred for centralised follow-up if they refused to undergo adjuvant irradiation of the retroperitoneal lymph nodes. However, this selection bias can hardly explain the differences between the seminoma and non-seminoma groups that we found. Our data indicate that men with Stage I disease tend to have short CAGs and we have found a decreased proportion of long CAGs among our subgroup of patients with a seminoma component in their tumour.

One should also bear in mind that despite a close genetic relationship between the Danish and Swedish populations, the risk of TGCC is twice as high in Denmark compared with Sweden [2], which might be due to different exposures to environment or life-style-related factors, with adverse effects on the male reproductive system. Due to this different level of exposure to factors trigging the development of testicular cancer, one cannot exclude that the genetic background of patients who have developed a tumour might also be different in the two countries.

As controls, we used men aged 18–21 years. Among Swedish males, the life-time risk of developing TGCC is below 0.5%, and the presence of such patient(s) among the 220 controls can hardly influence the distribution of *CAG* and *GGN* lengths in this cohort. Furthermore, ultrasound revealed no tumour or testicular microlithiasis [21] in any of the controls.

In conclusion, our study demonstrated that *CAG* and *GGN* polymorphisms in the *AR* gene are not associated with the risk of developing TGCC, but CAG numbers exceeding 25 are more common in patients having tumours without any seminoma component. The length of this trinucleotide also seems to correlate with the presence or absence of metastases at diagnosis. Our results indicate an impact of gonadotrophins and/or sex steroids on the development from CIS to tumour, as well as on the growth and metastasising capacity of TGCC. Further clarification of these endocrine factors may add to our understanding of the biology of this cancer and help in hormonally-mediated prevention of the malignancy in high-risk groups, such as men with a history of cryptorchidism, individuals with partial androgen insensitivity and those having unilateral testicular cancer and harbouring CIS in the contralateral testis.

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