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ARTICLE

gr/gr deletion predisposes to testicular germ cell tumour independently from altered spermatogenesis: results from the largest European study

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Abstract

The association between impaired spermatogenesis and TGCT has stimulated research on shared genetic factors. Y chromosome-linked partial AZFc deletions predispose to oligozoospermia and were also studied in TGCT patients with controversial results. In the largest study reporting the association between *gr/gr* deletion and TGCT, sperm parameters were unknown. Hence, it remains to be established whether this genetic defect truly represents a common genetic link between TGCT and impaired sperm production. Our aim was to explore the role of the following Y chromosome-linked factors in the predisposition to TGCT: (i) *gr/gr* deletion in subjects with known sperm parameters; (ii) other partial AZFc deletions and, for the first time, the role of partial AZFc duplications; (iii) *DAZ* gene dosage variation. 497 TGCT patients and 2030 controls from two Mediterranean populations with full semen/andrological characterization were analyzed through a series of molecular genetic techniques. Our most interesting finding concerns the *gr/gr* deletion and *DAZ* gene dosage variation (i.e., *DAZ* copy number is different from the reference sequence), both conferring TGCT susceptibility. In particular, the highest risk was observed when normozoospermic TGCT and normozoospermic controls were compared (OR = 3.7; 95% CI = 1.5–9.1; $p = 0.006$ for gr/gr deletion and OR = 1.8; 95% CI = 1.1–3.0; $p = 0.013$ for *DAZ* gene dosage alteration). We report in the largest European study population the predisposing effect of *gr/gr* deletion to TGCT as an independent risk factor from impaired spermatogenesis. Our finding implies regular tumour screening/follow-up in male family members of TGCT patients with *gr/gr* deletion and in infertile *gr/gr* deletion carriers.

Introduction

Testicular germ cell tumour (TGCT) is the most common malignant neoplasia in reproductive age [1]. The worldwide incidence of TGCTs shows significant variation between different geographical regions and ethnic groups. In Europe it ranges from 1.6 to 9.9 cases per 100,000 individuals [1]. These tumours are derived from germ cell neoplasia in situ

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(GCNIS) cells, which are arrested and transformed fetal gonocytes that failed to mature to spermatogonia [2]. Data in the literature indicate that both environmental and genetic factors acting on the primordial germ cells/gonocytes are implicated in the etiopathogenesis of this tumour [2–5].

For instance, familiarity and ethnic differences in TGCT incidence indicate a strong genetic component. Studies in families demonstrated that the familial risk of TGCT is 8-fold to 10-fold elevated for brothers of affected men and 4-fold to 6-fold elevated for fathers/sons of cases, compared with the general male population [6, 7]. In addition, it is well known that Asian and African populations have a low risk of TGCTs and men with African or Asian descent maintains their low risk even living in a geographic area of high risk [4, 8]. Based on population and genomic data Litchfield et al. [9] estimated the heritability of TGCT to be in a consistent range of 37–49%.

Besides genetic factors, a number of clinical predisposing factors are known. Among them, the presence of the components of the "testicular dysgenesis syndrome" [10], such as cryptorchidism, testicular hypotrophy/infertility, hypospadias and microlithiasis, confer higher risk for TGCT [11, 12].

Given the association between infertility and TGCT, genetic anomalies related to infertility has been studied as potential predisposing factors to TGCT.

The human Y chromosome harbors genes essential for testis development and function, such as *SRY*, which is indispensable for testis determination, and the genes residing in the azoospermia factor regions (AZFa, AZFb, and AZFc), which are involved in spermatogenesis [13]. AZF microdeletions are a well-established genetic cause of severe spermatogenic failure (5–10% of azoospermic and 2–5% of severe oligozoospermic men) [14]. Among the partial AZFc deletions, the clinically most relevant one is called *gr/gr*, discovered by Repping et al. [15]. This deletion removes half of the gene content and represents a significant risk factor for spermatogenic impairment. However, the entity of the risk for a *gr/gr* deletion carrier to be affected by oligozoospermia varies between different ethnicities and shows the highest values in the Mediterranean area: 5.8 (CI 2.0–16.9, $p = 0.001$) and 2.9 (1.0–8.0) in the Italian and Spanish ethnicity, respectively [13].

Three studies focused on the role of complete AZF deletions in TGCT, but a relationship with this disease could not be proven [16–18]. No association was found between increased risk of TGCT and Y haplogroups neither [19–21]. Concerning the partial AZFc deletions, the role of *gr/gr* deletions in TGCTs is still debated. In fact, only one out of three published studies observed an increased risk. Indeed, a large multicenter study by Nathanson and collaborators [22] show a 2-fold and 3-fold increased risk for sporadic and familiar TGCT in subjects carrying the *gr/gr* deletion, respectively. The two subsequent studies based on relatively small cohorts from England and Italy did not confirm the relationship between *gr/gr* deletions and TGCT [21, 23]. Worth noting that only one of the three studies considered sperm parameters in TGCT patients [21]. Due to the lack of information on the semen phenotype in cancer patients carrying the *gr/gr* deletion, it remains to be established whether this genetic defect truly represents a common genetic link between TGCT and impaired sperm production.

Only a single manuscript addressed the role of other partial AZFc deletions and in this study no correlation was found with TGCT [23].

We aimed at providing a comprehensive characterization of partial AZFc rearrangements in the largest European case–control study population available to date. In addition, we have addressed an important clinical question concerning the role of *gr/gr* deletion in TGCT in relationship with the semen phenotype.

Materials

Subjects

We prospectively evaluated 497 patients with TGCT and 2030 control subjects without TGCT who were referred to the University Hospital of Careggi (Italy), Fundació Puigvert (Spain) and University of Rome "La Sapienza" (Italy). The control group included 1068 normozoospermic and 962 non-normozoospermic men with no history of TGCT, no family history of TGCT, no history of cryptorchidism or other abnormal andrological findings and no genetic abnormalities (karyotype alterations and complete AZF deletions). The subjects belonging to these two control groups have been studied previously in relationship with male infertility (for review see Krausz and Casamonti [13]); for this study 24 novel normozoospermic and 18 non-normozoospermic controls were added. TGCT patients came to visit for semen cryopreservation before chemo/radiotherapy. Semen samples were collected by masturbation after 3–5 days of sexual abstinence and semen parameters were investigated according to the WHO guidelines (World Health Organization) [24]. A complete clinical history and andrological exams (including physical examination, scrotal ultrasound and hormonal analysis) were performed. Among TGCT patients, 353 (71%) were affected by seminoma, while 144 (29%) were affected by non-seminoma. Fifty-seven TGCT patients had history of cryptorchidism, 3 had hypospadias and 4 had family history of TGCT. Overall, 268 (54%) TGCT subjects were normozoospermic (total sperm count $\geq 39 \times 10^6$) and 229 (46%) were non-normozoospermic (total sperm count $< 39 \times 10^6$). Among the latter, 35 were affected by azoospermia. This study was approved by local ethics committees and all participants provided written informed consent.

Methods

Molecular analysis

We used standard salting-out method to extract genomic DNA from peripheral blood samples [25]. The analysis for Y chromosome AZF microdeletions was performed by multiplex PCR (using markers from AZFa, AZFb, and AZFc regions according to the EAA/EMQN [14]). The screening for partial AZFc rearrangements was performed in Florence and Barcelona centers by using a multistep method described by Giachini et al. [26]. The first step consisted in multiplex PCR plus/minus based on the amplification of Specific sequence-tagged sites (STSs): sY1291 and sY1191. We identified the *gr/gr* deletion by the absence of sY1291. The *b2/b3* deletion is characterized by the absence of sY1191 and the *b1/b3* deletion by the absence of sY1291 and of sY1191. To confirm the multiplex PCR results, we repeated the analysis in all samples with suspected deletion by using a simplex PCR for sY1291 and sY1191. In addition, two subsequent confirmatory steps have been performed in each deletion carrier (as described in Giachini et al. [26].): (i) gene dosage analysis; (ii) RFLP analysis. All the partial AZFc rearrangements identified in the present study were submitted in the LOVD v.3.0 database, https://databases.lovd.nl/shared/variants (variant IDs 461227–461232).

AZFc gene dosage

CDY1 and *DAZ* gene dosage was performed in all TGCT patients and in 1424 control subjects in order to confirm gene-copy loss in deletion carriers and to define gene duplications in the AZFc region. In brief, we simultaneously amplified the AZFc locus to be quantified (*CDY1* and *DAZ*) and a homologous locus outside the AZFc interval (*CDY2* and *DAZL*, respectively) as an internal standard with a known number of copies, using a single primer pair in a Quantitative Fluorescence-PCR (QF-PCR) reaction with the maximum of 24 cycles (end point of exponential phase). The fluorescence PCR products were separated on automatic sequencer (ABI PRISM ® 3730 XL DNA Sequencer, Applied Biosystem, Milan, Italy). For copy number quantification we compared the peak area corresponding to the *CDY1* and *DAZ* loci to their homologs *CDY2* and *DAZL*, respectively.

RFLP analysis

For *DAZ* qualitative analysis, we chose the sequence family variant (SFV) at STS sY587 in intron 10, which discriminates *DAZ1/2* from *DAZ3/4*. For *CDY1*, we used a SFV situated 7750 bp upstream of the *CDY1* translation start codon (CDY7750), which distinguishes *CDY1A* from *CDY1B*. SFVs were scored by PCR followed by enzyme digestion using the following enzymes: DraI for *DAZ* sY587 and PvuII for *CDY1*–7750.

Y chromosome haplogroups definition

Subjects with partial AZFc deletions were genotyped for six binary markers (M168, M145, M9, M45, M96, LLy22g). In brief, multiplex PCR of the regions spanning the six binary makers was followed by enzymatic clean-up of PCR products and multiplex single-base primer extension (SNaPshot Multiplex System, Applied Biosystems) [27]. DNA purification and analysis of primer extension products by ABI PRISM ® 3730 XL DNA Sequencer. The combination of genotypes allowed us defining specific clades or haplogroup clusters: (i) AB; (ii) DE*DE; (iii) CFGHI or J; (iv) E; (v) KLMNO*OST; (vi) N1; (vii) P*QR (see Karafet et al. [28]). A single PCR of the 12f2 marker was performed to differentiate the J haplogroup from the CFGHI cluster.

Statistical analysis

SPSS software (version 25.0 Chicago, IL, USA) was used. We tested the significance of the observed differences in the incidence of the AZFc rearrangements between the TGCT patients and the control group using Fisher´s exact test: our null hypothesis was that the incidence is the same in the TGCT patients and in the control group. For comparisons of medians between groups of different genotypes, the non-parametric Mann–Whitney *U*-test for independent samples was applied. After Bonferroni correction considering two independent variables, (i.e., deletions and duplications), a p-value ≤ 0.025 was considered statistically significant for each test.

Results

Analysis of complete AZF deletions and partial AZFc rearrangements in TGCT patients

In this study we aimed at: (i) defining the frequency of complete AZF deletions, partial AZFc deletions, partial AZFc duplications and *DAZ* dosage alteration; (ii) providing a detailed molecular and phenotypical characterization of the rearrangements carriers.

Complete AZF deletions

None were found in the entire cohort of TGCT patients ($n = 497$).

Partial AZFc deletions

14/497 patients (2.8%) carried the *gr/gr* deletion (chrY:g.(24876071_25505070)_(25505734_25316178)del) (hg19) (LOVD3 DB-ID:chrY_000067, ID Variant:0000461229), whereas *b2/b3* (chrY: g.(24524070_24872541)_(24876071_25505070)del) (hg19) (LOVD3 DB-ID: chrY_000068, ID Variant: 0000461230), *b1/b3* (chrY: g.(23978074_24354700)_(25316178_25505734)del (hg19) (LOVD3 DB-ID: chrY_000135, ID Variant: 0000461228) and atypical (chrY: g.(25505596_26191376)_(26194116_26979967) del) (hg19) (LOVD3 DB-ID: chrY_000134, ID Variant: 0000461227) deletions were found in only 5 subjects (1.0%) with a frequency of 0.6%, 0.2%, and 0.2%, respectively (Table 1).

1) *gr/gr deletion*: this deletion type was more frequent in normozoospermic compared to non-normozoospermic TGCT patients (3.4% vs. 2.2%), and in seminoma *vs.* non-seminoma patients (3.1% vs. 2.1%) without reaching statistical significance ($p = 0.305$ and $p = 0.385$, respectively). The highest frequency was observed in normozoospermic seminoma patients (4.1%) (Table 1).

The histology and semen phenotypes together with the *gr/gr* deletion subtypes for the 14 *gr/gr* deletion carriers are reported in Table 2. As for histology, 78.6% were seminomas, whereas only 21.4% were non-seminomas. Only 2 patients (14.3%) were azoospermic, 3 (21.4%) were oligozoospermic and the large majority were normozoospermic (64.3%). The combined gene dosage and RFLP analysis allowed a more detailed molecular characterization of the deletion subtypes based on the number and type of *CDY1* and *DAZ* copies. All patients, except one, showed a reduced *CDY1* and *DAZ* gene copy number in respect to the reference sequence, i.e., 1 copy of *CDY1* and 2 copies of *DAZ*. The increased *CDY1* and *DAZ* copy number in one patient was due to deletion followed by multiple duplications. In fact, RFLP analysis confirmed the presence of 3 *CDY1A* and 6 *DAZ3/4* copies. This subject was affected by non-seminoma and the semen phenotype was severe oligozoospermia.

Based on the type of the deleted *CDY1* and *DAZ* copies, four different subtypes were identified: *CDY1A+DAZ3/4* (7/14, 50%), *CDY1A+DAZ1/2* (4/14, 28.6%), *CDY1B+DAZ1/2* (2/14, 14.3%), and *CDY1B+DAZ3/4* (1/14, 7.1%). Summing frequencies up, *CDY1A* was missing in 11 (78.6%), while *CDY1B* was missing only in 3 (21.4%) patients. Concerning *DAZ* copy number, *DAZ3/4* was deleted in 8 (57.1%) and *DAZ1/2* in 6 (42.9%) subjects. All oligo/azoospermic patients affected by seminoma were lacking *CDY1A* whereas those with normozoospermia had *CDY1A* copy deletion in 66.7%. Altogether, no relationship was found between the type of deleted gene copy and the cancer histology or the semen parameters.

The analysis of the Y chromosome haplogroups in the deletion carriers did not reveal a specific haplogroup associated with *gr/gr* deletion. The most frequent haplogroup in our cohort was P*QR (36%). No differences were observed in haplogroup distribution in relationship with the semen phenotype (Table 2).

2) *b2/b3, b1/b3 and atypical deletions*: similar to *gr/gr*, these deletions were slightly more frequent in

Table 1 Frequency of AZFc rearrangements and *DAZ* dosage alterations in TGCT patients (reported in subgroups based on histotypes and total sperm count) and in controls (reported in subgroups based on total sperm count)

		gr/gr deletion	Other partial AZFc deletions[a]	Partial AZFc duplications	*DAZ* dosage alteration
TGCT	Total	14/497 (2.8%)	5/497 (1.0%)	22/478 (4.6%)	51/497 (10.3%)
	NZ	9/268 (3.4%)	3/268 (1.1%)	9/256 (3.5%)	28/268 (10.4%)
	NNZ	5/229 (2.2%)	2/229 (0.9%)	13/222 (5.9%)	23/229 (10.0%)
	Seminoma	11/353 (3.1%)	4/353 (1.1%)	16/338 (4.7%)	38/353 (10.8%)
	Non-seminoma	3/144 (2.1%)	1/144 (0.7%)	6/140 (4.3%)	13/144 (9.0%)
	Seminoma NZ	8/196 (4.1%)	2/196 (1.0%)	7/186 (3.8%)	23/196 (11.7%)
	Seminoma NNZ	3/157 (1.9%)	2/157 (1.3%)	9/152 (5.9%)	15/157 (9.5%)
	Non-Seminoma NZ	1/72 (1.4%)	1/72 (1.4%)	2/70 (2.9%)	5/72 (6.9%)
	Non-Seminoma NNZ	2/72 (2.8%)	0/72 (0.0%)	4/70 (5.7%)	8/72 (11.1%)
Controls	Total	40/2030 (2.0%)	11/2030 (0.5%)	51/1373 (3.7%)	111/1424 (7.8%)
	NZ	10/1068 (0.9%)	2/1068 (0.2%)	27/756 (3.6%)	46/768 (6.0%)
	NNZ	30/962 (3.1%)	9/962 (0.9%)	24/617 (3.9%)	65/656 (9.9%)

The columns report the number of subject observed with rearrangements/total subject patients and the percentage in parenthesis

NZ normozoospermic, *NNZ* non-normozoospermic

[a]Other partial AZFc deletions refers to *b2/b3*, *b1/b3*, and atypical deletion

Table 2 Phenotype/genotype description of the 14 TGCT patients with *gr/gr* deletion and of the 5 TGCT patients with other partial AZFc deletions

Patient	Origin	Testis tumor histology	Total sperm count (10^6)	Y chromosome clade/ haplogroups cluster	PCR plus/minus		*CDY1/DAZ* dosage		RFLP	
					sY1191	sY1291	*CDY1* copy number	*DAZ* copy number	*CDY1* deleted copy	*DAZ* deleted copy
gr/gr deletion										
A818	Italian	Non-seminoma	0.00	J	Present	Absent	1	2	*CDY1A*	*DAZ3/4*
A1171	Italian	Seminoma	720.00	P*QR	Present	Absent	1	2	*CDY1A*	*DAZ1/2*
A1445	Italian	Seminoma	244.80	P*QR	Present	Absent	1	2	*CDY1A*	*DAZ3/4*
A1663	Italian	Non-seminoma	7.56	CFGHI	Present	Absent	3	6	*CDY1B*	*DAZ1/2*
A1713	Italian	Seminoma	109.20	P*QR	Present	Absent	1	2	*CDY1A*	*DAZ3/4*
A1734	Italian	Seminoma	0.36	CFGHI	Present	Absent	1	2	*CDY1A*	*DAZ3/4*
A1912	Italian	Seminoma	209.00	E	Present	Absent	1	2	*CDY1B*	*DAZ3/4*
A2140	Italian	Seminoma	240.00	P*QR	Present	Absent	1	2	*CDY1B*	*DAZ1/2*
KG8	Italian	Non-seminoma	152.00	KLO	Present	Absent	1	2	*CDY1A*	*DAZ3/4*
KG62	Italian	Seminoma	56.00	KLO	Present	Absent	1	2	*CDY1A*	*DAZ1/2*
KG162	Italian	Seminoma	35.00	J	Present	Absent	1	2	*CDY1A*	*DAZ1/2*
16–001	Spanish	Seminoma	98.70	E	Present	Absent	1	2	*CDY1A*	*DAZ3/4*
17–484	Spanish	Seminoma	89.60	J	Present	Absent	1	2	*CDY1A*	*DAZ3/4*
17–733	Spanish	Seminoma	0.00	P*QR	Present	Absent	1	2	*CDY1A*	*DAZ1/2*
Other partial AZFc deletions:										
b2/b3 deletion										
A1881	Italian	Seminoma	36.00	CFGHI	Absent	Present	2	4	*CDY1A*	*DAZ3/4*
KG117	Italian	Seminoma	38.40	E	Absent	Present	1	2	*CDY1B*	*DAZ3/4*
KG34	Italian	Seminoma	135.00	CFGHI	Absent	Present	1	2	*CDY1A*	*DAZ3/4*
b1/b3 deletion										
KG156	Italian	Seminoma	40.00	CFGHI	Absent	Absent	2	4	No deletion	*DAZ1/2*
Atypical deletion										
A1623	Italian	Non-seminoma	45.51	J	Present	Present	1	4	*CDY1B*	No deletion

normozoospermic compared to non-normozoospermic TGCT patients (1.1% *vs* 0.9%), and in seminoma (1.1%) than in non-seminoma patients (0.7%). The highest frequencies were observed in normozoospermic non-seminoma patients (1.4%) and non-normozoospermic seminoma (1.3%) (Table 1).

The molecular characterization and the associated semen and histology phenotypes of the 5 patients carrying the *b2/b3, b1/b3* and atypical deletions are reported in Table 2. All patients with *b2/b3* deletion had seminoma. Two patients (66.7%) were non-normozoospermic. Two patients showed reduced *CDY1* and *DAZ* gene copy number (1 *CDY1* copy and 2 *DAZ* copies), the other patient presented 2 *CDY1* and 4 *DAZ* copies as a consequence of partial deletion followed by duplication, i.e., RFLP analysis revealed the deletion of the *CDY1A* and *DAZ3/4* copies. The three *b2/b3* deletion carriers showed a heterogeneous situation concerning the type of deleted *CDY1* copy: *CDY1A* was deleted in 2 patients and *CDY1B* in 1 patient. Interestingly enough, all patients presented a deletion of *DAZ3/4* copy. The *b1/b3* deletion carrier had seminoma with normozoospermia and presented 2 *CDY1* copies and 4 *DAZ* copies (deletion of *DAZ1/2* followed by duplication). The patient with atypical deletion had non-seminoma and was normozoospermic and he showed *CDY1B* copy deletion without *DAZ* copy deletion, i.e., presenting *DAZ1/2* and *DAZ3/4* copies.

Concerning the Y haplogroup analysis, it revealed that 3 out of 5 patients belong to the CFGHI cluster (60%) whereas the remaining two presented haplogroup E and J, respectively (Table 2).

Partial AZFc duplications

(Chry:g.(24876071_25505070)_(25505734_ 25316178)dup) (hg19) (LOVD3 DB-ID: chrY_000086, ID Variant: 0000461232); (chrY:g.(24524070_24872541)_(25316578_ 28457316)dup) (hg19) (LOVD3 DB-ID: chrY_000074, ID Variant: 0000461231): these rearrangements are defined when both *DAZ* and *CDY1* present increased copy number and it was observed in 22 TGCT patients (4.6%). We

identified patients with 3 or 4 copies of *CDY1* and 6 or 8 copies of *DAZ*. Partial AZFc duplications were observed more frequently in non-normozoospermic compared to normozoospermic TGCT patients (5.9% *vs* 3.5%) and in seminoma (4.7%) vs. non-seminoma patients (4.3%), without reaching statistical significance ($p = 0.159$ and $p = 0.523$, respectively). The highest frequency was observed in non-normozoospermic seminoma patients (5.9%) (Table 1).

DAZ dosage alteration

We defined it as any rearrangement (partial AZFc deletion or duplications or isolated *DAZ* duplication) causing an alteration in *DAZ* copy number from the canonical 4 copies (greater or less). In the 497 patients with TGCT, 51 (10.3%) presented alterations in *DAZ* dosage. *DAZ* dosage alteration was similar in normozoospermic and non-normozoospermic TGCT patients (10.4% vs. 10.0%) and in seminoma (10.8%) vs. non-seminoma patients (9.0%). The highest frequency was observed in normozoospermic seminoma patients (11.7%) (Table 1).

Comparison of the phenotypic characteristics between TGCT patients with and without gr/gr deletion, partial AZFc duplications, and DAZ dosage alteration

Genotype/phenotype correlation is reported in Table 3. For all AZFc rearrangements the most frequent histology was seminoma both in carriers and non-carriers ranging between 70 and 78%. The normozoospermic phenotype was more frequently encountered in *gr/gr* deletion carriers (64.3%) than in non-carriers (53.6%). On the contrary, partial AZFc duplication carriers were more frequently non-normozoospermic than non-carriers (59.1% vs. 45.8%, respectively). No statistical differences were found between carriers and non-carriers of the distinct rearrangements as far as testis volume, hormonal dosage values and routine sperm parameters are concerned (Table 3 and Supplementary Table 1). History of cryptorchidism was more frequent in TGCT patients with *gr/gr* deletion than in those without deletion (27.3% vs. 15%; $p = 0.27$). History of cryptorchidism was absent in partial AZFc duplications. No familial cases of TGCT and history of hypospadias were reported in carriers of the distinct rearrangements.

Comparison of the frequency of AZFc rearrangements between TGCT patients and controls

Partial AZFc deletions

The frequency of all partial AZFc deletions carriers was higher in our TGCT cohort than in the control group (3.8% vs. 2.5%), without reaching statistical significance (Table 4). Partial AZFc deletions confer significant TGCT risk in the following comparisons performed against the normozoospermic controls: i) the entire TGCT group (OR = 3.5, 95% CI = 1.7–7.3, $p = 0.001$); ii) normozoospermic TGCT (OR = 4.1, 95% CI = 1.8–9.3, $p = 0.001$). In addition, partial AZFc deletions were observed more frequently in non-normozoospermic TGCT compared to normozoospermic controls (3.1% vs. 1.1%), although not reaching statistical significance (Table 4).

1) gr/gr deletion: although the frequency of this deletion type was higher in TGCT patients than in the control group (2.8% vs. 2.0%), this difference was not statistically significant (Table 4). It is worth noticing that, in the control cohort, non-normozoospermic patients presented a significantly higher frequency (3.1%) compared to normozoospermic subjects (0.9%) (OR = 3.4; 95% CI = 1.7–7.0; $p = 0.000$). The comparison of the deletion frequency between the entire TGCT group (2.8%) and the normozoospermic control group (0.9%) shows a 3-fold increased risk for TGCT in the deletion carriers (OR = 3.1; 95% CI = 1.4–7.0; $p = 0.006$) (Table 4). An even higher risk was observed when normozoospermic TGCT patients (3.4%) were compared to normozoospermic controls (0.9%) (OR = 3.7; 95% CI = 1.5–9.1; $p = 0.006$) (Table 4).

2) b2/b3, b1/b3, and atypical deletions: the frequency of these deletions was higher in the TGCT group (1%) in respect to the controls (0.5%) but without reaching statistical significance, $p = 0.191$.

Partial AZFc duplications

These rearrangements were observed in 22 TGCT patients (4.6%), while they were detected in 51/1373 controls (3.7%). We did not found significant differences between the TGCT group and the control group, even when the comparison was done in subgroups based on seminal parameters (Table 4).

DAZ dosage alteration

The frequency was higher in TGCT patients than in the control group (10.3% vs. 7.8% $p = 0.056$). It is worth noticing that in the control cohort, non-normozoospermic patients presented a significantly higher frequency (9.9%) compared to normozoospermic subjects (6.0%) (OR = 1.7; 95% CI = 1.2–2.6; $p = 0.004$). The dosage alteration frequency between the entire TGCT group (10.3%) and the normozoospermic control group was statistically significantly higher (OR = 1.8; 95% CI = 1.2–2.7; $p = 0.004$) (Table 4). The comparison of normozoospermic controls vs. normozoospermic TGCT patients showed that *DAZ* dosage alteration confers a significant TGCT risk (OR = 1.8; 95% CI = 1.1–3.0; $p = 0.013$) (Table 4). Concerning the

Table 3 Comparison of the phenotypic characteristics between TGCT patients with and without *gr/gr* deletion, partial AZFc duplications and *DAZ* dosage alteration. a) Histology and semen phenotype; b) testis volume, hormonal dosage and clinical risk factors. The frequency between subgroups did not result significantly different for any of the analyzed parameters

(a)	*gr/gr* deletion		Partial AZFc duplications		*DAZ* dosage alteration	
	No ($n = 483$)	Yes ($n = 14$)	No ($n = 456$)	Yes ($n = 22$)	No ($n = 446$)	Yes ($n = 51$)
Histology						
Seminoma	342 (70.8%)	11 (78.6%)	322 (70.6%)	16 (72.7%)	315 (70.6%)	38 (74.5%)
Non-seminoma	141 (29.2%)	3 (21.4%)	134 (29.4%)	6 (27.3%)	131 (29.4%)	13 (25.5%)
Semen parameters. Median value (minimum-maximum)						
Volume (ml)	3.2 (0.3–10.5)	3.6 (1.8–5.9)	3.2 (0.3–10.5)	3.0 (1.0–5.9)	3.2 (0.3–10.5)	3.2 (1.0–5.9)
Sperm Concentration (10^6/ml)	14.9 (0–336.0)	30.0 (0–100.0)	15.0 (0–336.0)	7.0 (0–125.0)	15.0 (0–336.0)	21.0 (0–125.0)
Total sperm count (10^6)	47.3 (0–915.9)	94.2 (0–720.0)	48.3 (0–915.9)	30.0 (0–375.0)	46.5 (0–915.9)	63.0 (0–720.0)
Progressive motility (%)	47.0 (0–81.0)	44.0 (0–84.0)	47.0 (0–81.0)	41.0 (0–69.0)	47.0 (0–81.0)	40.5 (0–84.0)
Total motile sperm count (10^6)	19.6 (0–650.3)	41.2 (0–316.8)	19.6 (0–650.3)	10.3 (0–258.8)	19.4 (0–650.3)	37.4 (0–316.8)
Normal morphology (%)	8.0 (0–47.0)	7.0 (0–35.0)	8.0 (0–47.0)	6.0 (0–33.0)	8.0 (0–47.0)	6.0 (0–35.0)
Semen phenotype						
Normozoospermic	259 (53.6%)	9 (64.3%)	247 (54.2%)	9 (40.9%)	240 (53.8%)	28 (54.9%)
Non-normozoospermic	224 (46.4%)	5 (35.7%)	209 (45.8%)	13 (59.1%)	206 (46.2%)	23 (45.1%)

(b)	*gr/gr* deletion		Partial AZFc duplications		*DAZ* dosage alteration	
	No ($n = 361$)	Yes ($n = 11$)	No ($n = 348$)	Yes ($n = 11$)	No ($n = 338$)	Yes ($n = 34$)
Testis volume (ml), Median value (minimum-maximum)						
Right testis (ml)	20.0 (8.0–30.0)	22.0 (18.0–30.0)	20.0 (8.0–30.0)	17.5 (8.0–25.0)	20.0 (8.0–30.0)	18.0 (8.0–30.0)
Left testis (ml)	20.0 (7.0–30.0)	19.0 (8.0–25.0)	19.0 (7.0–30.0)	20.0 (12.0–25.0)	20.0 (7.0–30.0)	19.5 (8.0–25.0)
Hormonal analysis. Median value (minimum-maximum)[a]						
LH (mU/ml)	5.9 (1.9–25.5)	5.7 (1.5–11.2)	6.1 (1.9–25.5)	5.0 (2.4–8.0)	5.9 (1.9–22.5)	5.5 (1.5–25.5)
FSH (mU/ml)	9.7 (1.4–45.7)	6.7 (3.7–28.6)	9.9 (1.4–45.7)	7.2 (2.3–12.9)	9.9 (1.4–45.7)	7.2 (1.5–42.2)
Testosterone (nmol/l)	14.1 (4.4–33.6)	18.8 (15.8–24.8)	14.1 (4.4–33.6)	13.1 (5.9–23.1)	14.3 (4.4–33.6)	14.8 (5.9–24.8)
Family history of TGCT	4 (1.1%)	0 (0.0%)	4 (1.2%)	0 (0%)	4 (1.2%)	0 (0%)
Cryptorchidism	54 (15.0%)	3 (27.3%)	53 (15.2%)	0 (0%)	53 (15.7%)	4 (11.8%)
Hypospadias	3 (0.8%)	0 (0.0%)	3 (0.9%)	0 (0%)	3 (0.9%)	0 (0%)

[a]Patients with a choriocarcinoma component in their testis histology were excluded ($n = 29$)

non-normozoospermic TGCT patients, a higher frequency of the *DAZ* dosage alteration in this subgroup of patient vs. normozoospermic control group was found, with a p-value close to significance ($p = 0.027$) (Table 4).

Discussion

Testicular germ cell tumors (TGCTs) derive from a pre-invasive precursor lesion, called germ cell neoplasia in situ (GCNIS) and show an incidence that peaks in young adulthood [1, 2]. Basic research studies and epidemiological trends support the hypothesis that TGCT is of fetal origin and it is due to failure of normal fetal programming of the differentiation of primordial germ cells through a gonocyte stage into spermatogonia [2]. The differentiation process of primordial germ cells/gonocytes is regulated through several germ cell-specific genes including Y chromosome-linked ones. The alteration of germ cell differentiation might set the basis both for the development of testicular tumour and for impaired spermatogenesis.

The AZF genes on the long arm of the Y chromosome are important regulators of spermatogenesis and genes belonging to the AZFc region are specifically expressed in male germ cells. The most frequent, known molecular genetic cause of oligo/azoospermia are complete AZF deletions [14] and their role in TGCT has been ruled out by previous studies [16–18, 21]. On the contrary, the role of *gr/gr* deletion (removing half of the AZFc gene content) is still debated due to contrasting results in the literature [21–23]. The largest multiethnic study by Nathanson et al. [22] tested for the *gr/gr* deletion in 1842 TGCT cases and 2599

Table 4 Comparison of the frequency of *gr/gr* deletion, partial AZFc duplications and *DAZ* dosage alteration between TGCT patients and control subjects (total and subgroups based on total sperm count: normozoospermia vs. non-normozoospermia)

AZFc region rearrangements	TGCT ($n = 497$)			Controls ($n = 2030$[a])			*p*-value (OR, 95% CI)								
	Total (%)	NZ (%)	NNZ (%)	Total (%)	NZ (%)	NNZ (%)	p1	p2	p3	p4	p5	p6	p7	p8	p9
Partial AZFc deletions	3.8	4.5	3.1	2.5	1.1	4.1	0.078	**0.001 (3.5, 1.7–7.3)**	0.477	0.056	**0.001 (4.1, 1.8–9.3)**	0.435	0.373	0.036 (2.8, 1.1–7.1)	0.314
gr/gr deletion	2.8	3.4	2.2	2.0	0.9	3.1	0.159	**0.006 (3.1, 1.4–7.0)**	0.444	0.109	**0.006 (3.7, 1.5–9.1)**	0.486	0.486	0.108	0.308
Partial AZFc duplications	4.6	3.5	5.9	3.7	3.6	3.9	0.232	0.224	0.332	0.526	0.572	0.483	0.097	0.097	0.151
DAZ dosage alteration	10.3	10.4	10.0	7.8	6.0	9.9	0.056	**0.004 (1.8, 1.2–2.7)**	0.459	0.094	**0.013 (1.8, 1.1–3.0)**	0.444	0.152	0.027 (1.7, 1.0–3.0)	0.521

NZ normozoospermic, *NNZ* non-normozoospermic

p1 = total TGCT vs. total controls; p2 = total TGCT vs. NZ controls; p3 = total TGCT vs. NNZ controls; p4 = NZ TGCT vs. total controls; p5 = NZ TGCT vs. NZ controls; p6 = NZ TGCT vs. NNZ controls; p7 = NNZ TGCT vs. total controls; p8 = NNZ TGCT vs. NZ control; p9 = NNZ TGCT vs. NNZ control

p-value of 0.025 was considered statistically significant, after Bonferroni correction, significant values are reported in bold

[a]Analysis for partial AZFc duplications were performed in 1424 subjects

unaffected men. TGCT cases were significantly more likely to carry the *gr/gr* deletion compared to unaffected males (OR = 2.1; 95% CI = 1.3–3.6; $p = 0.005$) and this association was stronger in men with seminoma than for those with non-seminoma. In a subsequent study [23], which evaluated 263 TGCT cases (167 with family history and 96 sporadic cases), the authors were not able to demonstrate a significant association neither for *gr/gr* deletion nor for other rare partial AZFc deletions. In these two studies the fertility status and/or sperm parameter of the patients were not available. The only study in which semen parameters were reported is based on a small Italian cohort ($n = 118$) in which no *gr/gr* deletion carriers were found among TGCT patients [21]. Hence, it remains to be established whether the association between *gr/gr* deletion and TGCT is restricted to a specific seminal subgroup of patients (subjects with reduced sperm count).

Our study has been designed to evaluate the role of partial AZFc deletions/duplications and specifically the *DAZ* gene dosage variation in TGCT in the largest European study population available to date.

Our first objective was to elucidate the role of *gr/gr* deletion as genetic risk factor for TGCT in two Mediterranean populations with full semen/andrological characterization. When dealing with Y chromosome-related risk factors, matching for ethnicity and geographic origin is fundamental in order to avoid population stratification bias [29]. Consequently, much care was taken for geographic and ethnic matching in our study. Semen parameters were known both for cases and controls, allowing us to evaluate the role of *gr/gr* deletion as TGCT genetic risk factor in combination with spermatogenic efficiency. The similar frequency of *gr/gr* deletion in the TGCT group (2.8%) and the entire control group (2.0%) is due to the relatively high frequency of *gr/gr* deletion in patients with idiopathic oligo/azoospermia (3.1%). In fact, in our previous studies we demonstrated that *gr/gr* deletion is significantly associated with oligozoospermia in the Spanish and Italian populations [13, 27, 30]. Surprisingly enough, our most important finding is related to the normozoospermic group: while only 0.9% of controls were carrier of *gr/gr* deletion, this percentage was higher in normozoospermic TGCT patients (3.4%) and even higher in normozoospermic seminoma patients (4.1%). *gr/gr* deletion in normozoospermic subjects confers almost 4-fold increased risk for developing testis tumor.

The specific genes removed can vary between *gr/gr* deletions, and this has been suggested as the most direct modulating factor for the associated semen phenotype [13]. In this regard, mainly the *DAZ* and *CDY1* copies, which lie within the deleted AZFc region, have been examined as possible predictors for pathogenicity. The loss of *DAZ1*/*DAZ2* and *CDY1*A has been proposed as more deleterious

than the removal of *DAZ3/DAZ4* and *CDY1B* [26, 31, 32], however this was not fully proved by a large multicenter study [33]. Concerning *DAZ* gene copies, different members of the *DAZ* gene family have different number of RNA recognition motifs (RRMs) and *DAZ* repeats, which may confer different functional activity to the four *DAZ* copies [33]. Hence, we were interested in investigating on the deletion "haplotypes" (based on different combinations of *DAZ* and *CDY1* copies) in TGCT patients with and without altered spermatogenesis and with different tumor histology. Moreover, we questioned whether the deletion haplotypes in TGCT patients were different from that of the controls especially concerning the deleted *DAZ* copies. 11 (78.6%) patients presented a *CDY1A* deletion, and data break-down showed that this type of deletion was the most frequent in all semen and histology phenotypes. A similarly high frequency of *CDY1A* deletion was found in the control group (77.8%). Concerning the subtype of *DAZ* gene copy deletion, there was no predominant copy loss in specific sperm count-based subgroups or histology types. The same finding was observed in the control group. This data indicates that the type of deleted *CDY1* or *DAZ* copies are irrelevant to sperm output or histology type in TGCT patients carrying the *gr/gr* deletion. Most importantly, the deletion "haplotype" was not different between TGCT cases and tumour-free controls.

Beside *gr/gr* deletions, three other types of deletions were identified in our cohorts (*b2/b3*, *b1/b3* and atypical deletions). The frequency of these deletions was extremely rare both in patients and controls (0.6% vs. 0.4%, 0.2% vs. 0.0%, 0.2% vs. 0.2%, respectively), hence their role, if any, in TGCT remains to be established.

Given that partial AZFc deletions perturb AZFc gene dosage, we were interested in analyzing the role of an increased gene dosage (>4 for *DAZ* and >2 for *CDY1*, respectively) due to partial duplications. Although case/control association studies failed to reach to a univocal conclusion in respect to the hypothetical "optimal" AZFc gene dosage needed for normal spermatogenesis [27, 30, 34, 35], it seems that a positive selection for an "optimal" gene dosage does exist (four *DAZ* and two *CDY1* copies) during evolution [36]. Hence, our working hypothesis was a possible predisposing effect of AZFc dosage variation (not only deficit but also an excess) to TGCT development. Partial AZFc duplications frequency was 4.6% in TGCT patients while it was 3.7% in controls. Therefore, on the contrary of *gr/gr* deletion, we were unable to detect a significant effect of partial AZFc duplications on TGCT development.

We have separately evaluated the *DAZ* gene dosage variation (as a consequence of partial AZFc deletions, duplications or isolated *DAZ* duplication event). We found that patients with TGCT have a higher frequency of alterations of the *DAZ* dosage with respect to the entire control group (10.3% vs. 7.8%) (Table 4). The comparison of the entire TGCT group vs. normozoospermic controls showed a significant difference (10.3% vs. 6.0%, $p = 0.004$) indicating that *DAZ* dosage alteration in normozoospermic subjects confers almost 2-fold increased risk for developing TGCT (OR = 1.8, 95% CI = 1.2–2.7).

The biological link between AZFc gene dosage variation (especially its reduction due to deletions) and tumourigenesis remains elusive. One hypothesis could be that *DAZ* gene dosage may influence the differentiation of the gonocytes. In fact, it has been demonstrated that *DAZ* is present in both the nuclei and cytoplasm of fetal gonocytes and in spermatogonial nuclei [37]. Hence, based on its expression profile, *DAZ* gene dosage variation may play a role in TGCT etiology. Alternatively, we can also speculate that the "fragility" expressed as deletions/duplications of the Y chromosome is a marker for general "genomic instability" potentially leading to tumors, including TGCT.

In conclusion, our data provide evidence that the *gr/gr* deletion is a risk factor for TGCT also in the Italian and Spanish populations and that normozoospermic *gr/gr* deletion carriers have almost 4-fold increased risk to develop TGCT. The observed significant association between *gr/gr* deletion and normozoospermic TGCT, suggests that this deletion is an independent risk factor from impaired spermatogenesis. In line with this finding, a recent epidemiological study reported an increased risk for TGCT in first-degree relatives of subfertile normozoospermic men [38]. Diagnosing *gr/gr* deletion in a TGCT patient has a potential clinical relevance to his male relatives, especially to the brothers in whom preventive measures such as regular autopalpation or ultrasound scan of the testis can be proposed. In some countries, *gr/gr* deletion screening is already part of the routine genetic diagnosis of oligozoospermic men [14]. This implies that similarly to brothers of *gr/gr* deletion carriers, also infertile patients diagnosed with this type of deletion should receive careful long-term andrological follow-up.

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Author contributions Conceived and designed the experiments: C.K. Patient recruitment and their clinical characterization: D.M.M., D.P., A.M., J.S.C., F.L., A.L. Performed the experiments D.M.M., E.C., C.C., A.R.E., C.G., M.G.F., S.V., F.C. Analyzed the data: D.M.M., E.C., C.K. Contributed reagents/materials/analysis tools: C.K., D.P., F.L., E.R.C., E.A. Wrote the paper: C.K., D.M.M., E.C.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All patients were consented for collection of data. The local ethical committees of the University Hospital Careggi, Fundació Puigvert and "Sapienza" University of Rome approved the study. The study was performed in accordance with the Declaration of Helsinki.

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