

Variants in *KITLG* predispose to testicular germ cell cancer independently from spermatogenic function

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Abstract

Epidemiological data suggest an association and a common pathogenetic link between male infertility and testicular germ cell tumor (TGCT) development. Genome-wide studies identified that TGCT susceptibility is associated with *KITLG* (c-KIT ligand), which regulates the formation of primordial germ cells, from which TGCT is believed to arise and spermatogenesis develops. In this study, we analyzed the link between *KITLG*, TGCT, and spermatogenic disruption by performing an association study between the *KITLG* markers rs995030 and rs4471514 and 426 TGCT cases and 614 controls with normal and abnormal sperm count. We found that TGCT risk was increased more than twofold per copy of the major G allele and A allele in *KITLG* rs995030 and rs4471514 (odds ratio (OR)=2.38, 95% confidence interval (95% CI)=1.81–3.12; OR=2.43, 95% CI=1.86–3.17 respectively), and homozygotes for the risk allele had a sevenfold increased risk of TGCT. *KITLG* markers were strongly associated with seminoma subtype (per allele risk increased more than threefold, homozygote risk increased by 13- to 16-fold) and weakly with nonseminoma. *KITLG* markers were not associated with sperm production, as no difference was observed in men with normozoospermia and azo-oligozoospermia, both in controls and in TGCT cases. In conclusion, this study provides evidence that *KITLG* variants are involved in TGCT development and they represent an independent and strong specific risk factor for TGCT independently from spermatogenic function. A shared genetic cause and a common pathogenetic link between TGCT development and impairment of spermatogenesis are not evident from this study.

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Introduction

Testicular cancer (TC) is the most common cancer in males aged 20–40 years, with a worldwide incidence of 7.5 per 100 000, but the rates vary considerably between countries and ethnic groups (Horwich *et al.* 2006). About 95% of all TCs are represented by testicular germ cell tumors (TGCTs), which include seminoma and nonseminoma histological types.

Although environmental factors clearly contribute to TGCT development, the genetic component is also strong and the proportion of TGCT susceptibility accounted for by the genetic effects is estimated at 25% (Czene *et al.* 2002). TGCT has high familial risks compared with most other cancer types that are generally no more than twofold (Dong & Hemminki

2001): brothers of individuals with TGCT have an 8- to 12-fold increased risk of disease, and sons of affected individuals have a four- to six-fold increased risk (Swerdlow *et al.* 1997, Hemminki & Li 2004). Despite this strong familial relative risk, early results from linkage studies identified a limited relationship with genetic factors (Crockford *et al.* 2006), suggesting that TGCT is a genetically complex trait.

The gr/gr deletion on the Y chromosome, which seems to increase TGCT risk twofold to threefold and is also associated with spermatogenic impairment (Ferlin *et al.* 2005, Stouffs *et al.* 2011), might account only for a limited number of cases because carrier frequency of this variant is low (2–3%; Nathanson *et al.* 2005). More recently, four genome-wide

association studies (GWAS) from UK and USA have reported association of TGCTs with six new loci (*KITLG*, *SPRY4*, *BAK1*, *DMRT1*, *TERT*, and *ATF7IP*; Kanetsky et al. 2009, 2011, Rapley et al. 2009, Turnbull et al. 2010). The strongest association for TGCT susceptibility was found for SNPs in *KITLG* (ligand for the membrane-bound receptor tyrosine kinase KIT) gene with a >2.5-fold increased risk of disease per major allele (Kanetsky et al. 2009, Rapley et al. 2009), which is the highest reported for any cancer to date (Chanock 2009). The *KITLG*–KIT system regulates the survival, proliferation, and migration of primordial germ cells (PGCs; Runyan et al. 2006, Boldajipour & Raz 2007), the cells from which spermatogonia and spermatocytes develop and from which TGCT is believed to arise. Accordingly, altered differentiation of PGCs is associated with the development of TGCT and infertility in mice (Heaney et al. 2008) and probably also in humans (Galan et al. 2006, Rajpert-De Meyts & Hoei-Hansen 2007).

The relationship between different aspects of PGC development, TGCT susceptibility, and spermatogenic disruption, and the well-known epidemiological data suggesting an association and a common pathogenetic link between male infertility and TGCT development (Skakkebaek et al. 2001, Horwich et al. 2006, Rajpert-De Meyts 2006), led us to perform the current association study to clarify the possible link between *KITLG*, TGCT, and spermatogenic disruption. In fact, analysis of *KITLG* variation in TGCT men with a history of infertility, even if suggested, was not tested in GWAS (Kanetsky et al. 2009, Rapley et al. 2009). We therefore analyzed the association between previously identified SNPs in *KITLG* (Kanetsky et al. 2009, Rapley et al. 2009) and TGCT cases and controls with normal and disrupted spermatogenesis.

Materials and methods

Cases and controls

TGCT cases ($n=426$) were recruited over the last 10 years among patients referred to our center for semen analysis. As a standard procedure, semen analysis is performed in every TGCT case just after TGCT diagnosis, before orchiectomy. To avoid confounding factors, selected TGCT cases had no history of cryptorchidism and familial history of TGCT and were affected by classic seminoma ($n=251$) or nonseminoma (including yolk sac, choriocarcinoma, and embryonal carcinoma; $n=175$), excluding mixed phenotypes. Extragenital TGCTs were also excluded and all TGCT cases were unrelated.

Controls ($n=614$) were recruited among men referred to our center for standard semen analysis, and TC was excluded by andrological examination and testicular ultrasound. Standard semen analysis was performed in cases and controls according to World Health Organization protocol and subjects were considered as having a normal spermatogenesis when total sperm count was ≥ 40 million/ejaculate (World Health Organization 1999). Oligozoospermic subjects (sperm count < 40 million/ejaculate) were further analyzed to exclude possible causes of spermatogenic impairment (seminal infection, sperm autoantibodies, varicocele, history of cryptorchidism and orchitis, Y chromosome microdeletions (Ferlin et al. 2007), karyotype anomalies, medication, fever in the previous month, systemic diseases, and endocrine disorders), so that oligozoospermia might be considered idiopathic. All cases and controls were from the northeast of Italy.

The study has been approved by the Local Institutional Review Board, and informed consent was obtained from each subject after full explanation of the purpose and nature of all the procedures used. The study has been conducted in accordance with the principles expressed in the Declaration of Helsinki.

KITLG variant analysis

KITLG polymorphisms were analyzed by direct sequencing (ABI PRISM 3730XL DNA Sequencer, Applied Biosystems, Monza, Italy). Genomic DNA was extracted from peripheral blood leukocytes using QIAamp DNA Blood Midi Kit according to the manufacturer's protocol (Qiagen). The oligonucleotide primers used for the analysis were the following: 5'-GCAAATTTGTTACTCATTCTGTGG-3' and 5'-CAGTACTGGCTCCCTTGCAT-3' for rs995030; 5'-TGGGGAGGCAGAGGATAAAT-3' and 5'-TCATTGACATGCCTGAGACAC-3' for rs4474514.

Table 1 Characteristics of testicular germ cell tumor cases and controls

	Cases ($n=426$)		Controls ($n=614$)	
	No.	Percentage (%)	No.	Percentage (%)
Total				
Age (mean \pm s.d.)		29.4 \pm 6.2		35.2 \pm 5.8
Tumor type				
Seminoma	251	58.9	–	–
Nonseminoma	175	41.1	–	–
Sperm count				
≥ 40 million/ejaculate	219	51.4	414	67.4
< 40 million/ejaculate	207	48.6	200	32.6

Reagents and conditions for the PCR were 14.3 μ l H₂O, 2.5 μ l 10 \times Gold Buffer, 1.5 μ l 25 mM MgCl₂ solution (from Applied Biosystems, Branchburg, NJ, USA), 2.5 μ l dNTPs (2 mM; from Amersham Biosciences), 1 μ l each primer (10 μ M; from Sigma–Aldrich), 0.2 μ l 5 U/ μ l AmpliTaq Gold (from Applied Biosystems), and 2 μ l template (100 ng/ μ l). The PCR program on a thermal cycler (GeneAMP PCR System 2700, Applied Biosystems, Monza, Italy) was first-step denaturation at 94 °C for 10 min, followed by 37 cycles of 94 °C for 60 s, 58 °C for 60 s, 72 °C for 60 s, and a final extension step of 4 min at 72 °C.

Statistical analysis

Test for Hardy–Weinberg equilibrium (HWE) was performed with the use of the χ^2 goodness-of-fit test. The extent of linkage disequilibrium (LD) among SNPs was quantified using Lewontin’s D' value and the correlation coefficient r^2 . Differences in allele and genotype distribution between cases and controls were tested with the use of Cochran–Armitage trend test. Odds ratios (ORs) and 95% confidence interval (95% CI) were estimated to assess the effect of each SNP on cancer risk using a logistic regression model. Allelic ORs and 95% CIs were estimated on the basis of a multiplicative model. P values were corrected to account for multiple comparisons using the false discovery rate Benjamini–Hochberg method. Two-sided raw and adjusted P values are reported, and adjusted $P < 0.05$ was considered to indicate statistical significance. Statistical analyses were conducted using SAS version 9.2 (SAS Institute, Inc., Cary, NC, USA).

Results

We studied 426 TGCT cases (251 classic seminoma and 175 nonseminoma) and 614 controls, and both groups were considered as having a normal spermatogenesis when total sperm count was ≥ 40 million/ejaculate (Table 1). Genotyping was performed for two SNPs in the *KITLG* gene, rs995030 and rs4471514. The two markers were in strong LD ($D' > 0.95$ and $r^2 > 0.90$) and in HWE both in controls and in cases.

We observed association of TGCT with both markers (corrected $P_{\text{trend}} = 1.06 \times 10^{-9}$ and 5.48×10^{-10} for rs995030 and rs4471514 respectively; Table 2). TGCT risk was increased more than twofold per copy of the major G allele and A allele in *KITLG* rs995030 and rs4471514 (OR = 2.38, 95% CI = 1.81–3.12; OR = 2.43, 95% CI = 1.86–3.17 respectively). Homozygotes for the risk allele had a sevenfold increased risk of TGCT (OR = 7.04, 95% CI = 2.10–

Table 2 Association of *KITLG* SNPs with testicular germ cell tumor (TGCT)

Marker ^b	Risk allele	Genotype count ^a			OR (95% CI)			Corrected P
		Controls (n=614)	TGCT cases (n=426)	Per allele	Heterozygote ^c	Homozygote ^d	Raw P trend ^e	
rs995030 G/A	G	398 (0.65)/192 (0.31)/24 (0.04)	350 (0.82)/73 (0.17)/3 (0.01)	2.38 (1.81–3.12)	3.04 (0.89–10.41)	7.04 (2.10–23.56)	2.39×10^{-10}	1.06×10^{-9}
rs4471514 A/G	A	388 (0.63)/197 (0.32)/29 (0.05)	347 (0.81)/75 (0.18)/4 (0.01)	2.43 (1.86–3.17)	2.76 (0.94–8.10)	6.48 (2.25–18.60)	4.88×10^{-11}	5.48×10^{-10}

^aNumber of individuals genotyped as homozygous for the risk allele/heterozygous for the risk allele/homozygous for the nonrisk allele.

^bdbSNP number and major/minor alleles.

^cOR for heterozygotes for the risk allele compared with homozygotes for the nonrisk allele.

^dOR for homozygotes for the risk allele compared with homozygotes for the nonrisk allele.

^eCochran–Armitage test for trend. Significant OR are in bold.

23.56; OR=6.48, 95% CI=2.25–18.60, for rs995030 and rs4471514 respectively) compared with homozygotes for the nonrisk minor allele. Interestingly, *KITLG* markers were strongly associated with seminoma and weakly with nonseminoma (Table 3): seminoma risk was increased more than threefold per copy of the major G allele and A allele in *KITLG* rs995030 and rs4471514 (OR=3.14, 95% CI=2.18–4.54; OR=3.20, 95% CI=2.24–4.58 respectively), and homozygotes for the risk allele had a extremely high increased risk of seminoma (OR=13.01, 95% CI=1.75–96.71; OR=16.00, 95% CI=2.16–118.24, for rs995030 and rs4471514 respectively) compared with homozygotes for the nonrisk minor allele. Nonseminoma risk was increased less than twofold per copy of the major G allele and A allele in *KITLG* rs995030 and rs4471514 (OR=1.73, 95% CI=1.22–2.46; OR=1.78, 95% CI=1.26–2.50 respectively), and only a trend for significant OR for homozygotes for the risk allele was observed.

KITLG markers were not associated with spermatogenic function. In fact, both in controls and in TGCT cases, allele and genotype distribution were not different between subjects with normal (≥ 40 million/ejaculate) and low sperm count (< 40 million/ejaculate; Table 4), and this behavior was maintained in seminoma/nonseminoma cases. More detailed analysis of genotype distribution with respect to the severity of spermatogenic impairment (azoospermia, severe oligozoospermia, moderate oligozoospermia, and normozoospermia) failed to find association between *KITLG* markers and sperm count, both in controls and in TGCT cases. Furthermore, the mean sperm number was not different among the different genotypes and alleles (data not shown). Confirming the lack of association between *KITLG* markers and spermatogenic function, the increased risk per copy of the major alleles in *KITLG* rs995030 and rs4471514 was similar in TGCT cases with low sperm count compared with controls with low sperm count (OR=2.18, 95% CI=1.53–3.11; OR=2.16, 95% CI=1.53–3.05, for rs995030 and rs4471514 respectively) and in TGCT cases with normal sperm count compared with controls with normal sperm count (OR=2.62, 95% CI=1.71–4.01; OR=2.75, 95% CI=1.80–4.20, for rs995030 and rs4471514 respectively; Table 5).

Discussion

Taken together, these data showed association of *KITLG* markers with TGCT, especially the seminoma subtype, independently from the spermatogenic function. By using one marker previously identified

Table 3 Association of *KITLG* SNPs with seminoma and nonseminoma testicular germ cell tumor

Marker	Risk allele	Seminoma OR (95% CI)				Nonseminoma OR (95% CI)				Raw P trend ^c	Corrected P
		Per allele	Heterozygote ^a	Homozygote ^b	Raw P trend	Corrected P	Per allele	Heterozygote ^a	Homozygote ^b		
rs995030 G/A	G	3.14 (2.18–4.54)	4.25 (0.56–32.39)	13.01 (1.75–96.71)	3.02×10^{-10}	1.06×10^{-9}	1.73 (1.22–2.46)	2.43 (0.55–10.72)	4.04 (0.94–17.29)	0.0018	0.0026
rs4471514 A/G	A	3.20 (2.24–4.58)	5.30 (0.70–40.14)	16.00 (2.16–118.24)	7.82×10^{-11}	5.48×10^{-10}	1.78 (1.26–2.50)	1.91 (0.56–6.59)	3.31 (0.99–11.05)	0.0010	0.0016

^aOR for heterozygotes for the risk allele compared with homozygotes for the nonrisk allele.

^bOR for homozygotes for the risk allele compared with homozygotes for the nonrisk allele.

^cCochran–Armitage test for trend. Significant OR are in bold.

in US cases (rs4471514; Kanetsky *et al.* 2009) and one marker previously identified in UK cases (rs995030; Rapley *et al.* 2009), we therefore replicated in Italians a similar increased risk of disease per major allele (2.5-fold). We also confirmed the risk of TGCT only for homozygotes for the risk allele (sevenfold increase) and not for the heterozygotes. However, association was found only for seminoma and not for nonseminoma. Different hypotheses might explain this different result, including ethnic differences, histological classification, or different number of cases (although the numbers were similar: 175 nonseminoma in our series, 180 and 141 in the discovery and replication phase of the US study, and 241 and 171 in the discovery and replication phase of the UK study). Studies dealing with genetic predisposition of TGCT frequently found differences between seminoma and nonseminoma. The *gr/gr* deletion of the Y chromosome is strongly associated with seminoma than with nonseminoma (Nathanson *et al.* 2005), longer CAG repeats on the androgen receptor gene are mainly associated with nonseminoma (Giwercman *et al.* 2004), and we also found higher association of FSH receptor (*FSHR*) polymorphisms only with nonseminoma (Ferlin *et al.* 2008), and on the contrary stronger association of hydroxysteroid (17-beta) dehydrogenase 4 (*HSD17B4*) markers with seminoma (Ferlin *et al.* 2010). Therefore, it might also be possible that the two classes of TGCTs might have different pathogenesis. Indeed, somatic KIT alterations are far more frequent in seminoma than in nonseminoma (Forbes *et al.* 2008).

Another possibility is related to the presence of possible confounding risk factors. In fact, both GWAS included subjects with familial history of TGCT and personal history of cryptorchidism (Kanetsky *et al.* 2009, Rapley *et al.* 2009), which represent to date the most important clinical risk factor for TGCT with a relative risk of 4.8 (Dieckmann & Pichlmeier 2004). On the contrary, they did not consider another important risk factor for TGCT, infertility, or low sperm count. Nevertheless, we did not observe association of *KITLG* markers with spermatogenic function, both in controls and in TGCT cases. Therefore, variations in *KITLG* seem to represent an independent and strong specific risk factor for TGCT.

KITLG–KIT pathway involvement in TGCTs is supported by numerous evidences: high expression of KIT, somatic activating mutations, and genomic amplification of *KIT* are frequently seen in TGCTs (especially seminomas; Izquierdo *et al.* 1995, Strohmeier *et al.* 1995, Bokemeyer *et al.* 1996, Rapley *et al.* 2004, McIntyre *et al.* 2005, Goddard *et al.* 2007); germline deletions of *Kitlg* increase susceptibility to

Table 4 Association of *KITLG* SNPs with sperm count in testicular germ cell tumor (TGCT) cases and controls

Marker	Controls ≥ 40 million total sperm (n = 414)	Controls (n = 200)	Controls < 40 million total sperm	TGCT ≥ 40 million total sperm (n = 219)	TGCT < 40 million total sperm (n = 207)
rs995030 G/A					
Allele (G/a)	666 (0.80)/162 (0.20)	322 (0.81)/78 (0.19)		394 (0.90)/44 (0.10)	379 (0.92)/35 (0.08)
Genotype (GG/Ga/aa)	267 (0.64)/132 (0.32)/15 (0.04)	131 (0.66)/60 (0.30)/9 (0.04)		177 (0.81)/40 (0.18)/2 (0.01)	173 (0.83)/33 (0.16)/1 (0.01)
P trend		0.9785			0.4191
rs4471514 A/G					
Allele (A/g)	654 (0.79)/174 (0.21)	319 (0.80)/81 (0.20)		390 (0.89)/48 (0.11)	379 (0.92)/35 (0.08)
Genotype (AA/Ag/gg)	258 (0.62)/138 (0.33)/18 (0.05)	130 (0.65)/59 (0.30)/11 (0.05)		174 (0.80)/42 (0.19)/3 (0.01)	173 (0.83)/33 (0.16)/1 (0.01)
P trend		0.7598			0.2176

Table 5 Association of *KITLG* SNPs with testicular germ cell tumor (TGCT) with low and normal sperm count

Marker	Risk allele	TGCT ≥ 40 million total sperm OR (95% CI)			TGCT < 40 million total sperm OR (95% CI)			Raw P trend ^e	Corrected P		
		Per allele	Heterozygote ^a	Homozygote ^b	Per allele	Heterozygote ^c	Homozygote ^d				
rs995030 G/A	G	2.18 (1.53–3.11)	2.27 (0.50–10.35)	4.97 (1.12–21.98)	1.31 × 10 ⁻⁵	2.29 × 10 ⁻⁵	2.62 (1.71–4.01)	4.95 (0.60–40.80)	11.89 (1.49–94.99)	8.24 × 10 ⁻⁶	1.81 × 10 ⁻⁵
rs4471514 A/G	A	2.16 (1.53–3.05)	1.83 (0.51–6.50)	4.05 (1.74–13.94)	9.06 × 10 ⁻⁶	1.81 × 10 ⁻⁵	2.75 (1.80–4.20)	6.15 (0.76–49.79)	14.64 (1.87–114.81)	3.45 × 10 ⁻⁶	9.67 × 10 ⁻⁶

^aOR for heterozygotes for the risk allele compared with homozygotes for the nonrisk allele in controls with ≥ 40 million sperms/ejaculate.

^bOR for homozygotes for the risk allele compared with homozygotes for the nonrisk allele in controls with ≥ 40 million sperms/ejaculate.

^cOR for heterozygotes for the risk allele compared with homozygotes for the nonrisk allele in controls with < 40 million sperms/ejaculate.

^dOR for homozygotes for the risk allele compared with homozygotes for the nonrisk allele in controls with < 40 million sperms/ejaculate.

^eCochran–Armitage test for trend. Significant OR are in bold.

TGCTs in mice (Heaney *et al.* 2008); other than *KITLG*, two of the loci identified by GWAS (*SPRY4* and *BAKI*; Kanetsky *et al.* 2009, Rapley *et al.* 2009) are involved in the *KITLG*–*KIT* pathway. The *KITLG*–*KIT* system is critical for the correct development of PGCs (Runyan *et al.* 2006, Boldajipour & Raz 2007), the cells from which TGCT is believed to arise. Delayed differentiation of PGCs has been associated with the development of TGCT in mice (Heaney *et al.* 2008) and humans (Rajpert-De Meyts & Hoei-Hansen 2007). Furthermore, germline homozygous-null mutations of either *Kit* or *Kitlg* lead to infertility in mice as a result of a failure of PGC development (Heaney *et al.* 2008). In spite of this postulated role of *KITLG*–*KIT* signaling pathway in male fertility (Blume-Jensen *et al.* 2000, Mahakali Zama *et al.* 2005, Runyan *et al.* 2006) and epidemiological data suggesting an association between TGCT and male infertility (Richiardi & Akre 2005), our data do not support a common genetic link between these two conditions, at least regarding variations in *KITLG* gene. Only one preliminary small study analyzing different *KIT* and *KITLG* markers in infertile males implicated this system in spermatogenic disruption also in humans (Galan *et al.* 2006). However, in accordance to our findings, that study failed to find association between *KITLG* rs995030 and male infertility. Larger studies are probably needed to correctly assess the possible association between *KITLG* markers and spermatogenesis, taking into account that, although epidemiological data suggest that male infertility confers a significantly increased risk of TGCT (standardized incidence ratio 2.8, 95% CI=1.5–4.8; Walsh *et al.* 2009), the prevalence of TGCT in men with infertility or azo-oligozoospermia is low (0.3%; Jacobsen *et al.* 2000, Walsh *et al.* 2009). Anyway, the phenotype ‘TC’ for which association with *KITLG* variants was found is definite and specific (germ cell tumor, especially the seminoma subtype), whereas male infertility is clearly a multifactor entity. Therefore, it is conceivable that a putative involvement of *KITLG*–*KIT* pathway disruption could be observed only in well-defined subtypes of ‘idiopathic’ spermatogenic impairment. While we tried to remove all other potential causes, male infertility is a heterogeneous condition and any effect of risk alleles might be subtle and difficult to detect because the risk alleles are at high frequency. Therefore, the power to detect a difference was limited and a much larger study is needed to conclude on a possible association between *KITLG* markers and male infertility.

In conclusion, our study not only further supports that *KITLG* variants are involved in TGCT development but also highlights that they represent an

independent and strong specific risk factor for TGCT, independently from spermatogenic function. Therefore, a shared genetic cause and a common pathogenic link between TGCT development and impairment of spermatogenesis still remain to be elucidated.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

A Ferlin and C Foresta participated in study design. A Ferlin, D Pizzol, U Carraro, and C Foresta enrolled patients. A C Frigo undertook statistical analysis. M Pengo made molecular analyses. A Ferlin, M Pengo, and A C Frigo contributed to data collection and generation of tables and figures. A Ferlin had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors had full access to the original data, reviewed the data analyses, contributed to data interpretation and to the writing of the report, made final decisions on all parts of the report, and approved the final version of the submitted report.

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