



UNIVERSITÀ
DEGLI STUDI
FIRENZE

FLORE

Repository istituzionale dell'Università degli Studi di Firenze

Partial AZFc deletions in infertile men with cryptorchidism.

Questa è la Versione finale referata (Post print/Accepted manuscript) della seguente pubblicazione:

Original Citation:

Partial AZFc deletions in infertile men with cryptorchidism / C. GIACHINI ; F. NUTI ; E. MARINARI ; G. FORTI ; C. KRAUSZ. - In: HUMAN REPRODUCTION. - ISSN 0268-1161. - STAMPA. - 22:(2007), pp. 2398-2403.

Availability:

This version is available at: 2158/253216 since: 2019-07-23T17:15:39Z

Publisher:

Oxford University Press:Journals Department, Great Clarendon Street, Oxford OX2 6DP United Kingdom:

Terms of use:

Open Access

La pubblicazione è resa disponibile sotto le norme e i termini della licenza di deposito, secondo quanto stabilito dalla Policy per l'accesso aperto dell'Università degli Studi di Firenze (<https://www.sba.unifi.it/upload/policy-oa-2016-1.pdf>)

Publisher copyright claim:

(Article begins on next page)

Partial AZFc deletions in infertile men with cryptorchidism

C. Giachini, F. Nuti, E. Marinari, G. Forti and C. Krausz¹

Andrology Unit, Department of Clinical Physiopathology, Center for Research, Transfer and Higher Education on Chronic, Inflammatory, Degenerative and Neoplastic Disorders, University of Florence, 6 Viale Pieraccini, 50139 Firenze, Italy

¹Correspondence address. Tel: +39 055 4271485; Fax: +39 055 4271371; E-mail: c.krausz@dfc.unifi.it

BACKGROUND: A specific type of partial AZFc deletion, called ‘gr/gr’ deletion, was recently proposed as genetic risk factor for spermatogenic impairment and testis cancer. Since both pathologies can be part of the testicular dysgenesis syndrome (TDS), we aimed to define the role of ‘gr/gr’ deletion in the aetiopathogenesis of another component of the TDS: cryptorchidism. **METHODS:** A total of 146 cryptorchid and 140 infertile patients without a history of cryptorchidism were screened with a sequence tagged site plus/minus method and further confirmed and characterized by CDY1/DAZ gene dosage and copy analysis. **RESULTS:** The observed deletion frequency was 4.2% in cryptorchid and 5% in non-cryptorchid patients. Moreover, no differences in the CDY1/DAZ patterns were observed among the two groups. A significant difference in deletion frequency was present only when cryptorchid patients were compared with normospermic controls ($P < 0.03$). **CONCLUSIONS:** Our data show no relationship between ‘gr/gr’ deletion and cryptorchidism, however, provide further evidence of the deleterious effect of the ‘gr/gr’ deletion on spermatogenesis. The screening for ‘gr/gr’ deletion may therefore be proposed before ICSI to all patients with severe male factor infertility, without the exclusion of those with cryptorchidism, since this genetic risk factor for spermatogenic impairment will be transmitted to the male offspring.

Keywords: cryptorchidism; genetics; ‘gr/gr’ deletions; male infertility; Y chromosome

Introduction

The importance of AZF genes in spermatogenesis is undisputable since a clear cause-effect relationship between microdeletions of the AZF regions of the long arm of the Y chromosome (Yq) and severe spermatogenic disturbances has been ascertained by several reports (for review see Krausz *et al.*, 2004). Recently, a new type of Yq microdeletion—the so called ‘gr/gr’ deletion—which removes approximately half of the gene content of the AZFc region, has been reported by Repping *et al.* (2003). This specific type of partial AZFc deletion is considered a new genetic risk factor for spermatogenic impairment by a number of research groups (Repping *et al.*, 2003; de Llanos *et al.*, 2005; Ferlin *et al.*, 2005; Giachini *et al.*, 2005; Lynch *et al.*, 2005) but not by others (Machev *et al.*, 2004; Hucklenbroich *et al.*, 2005; Ravel *et al.*, 2006; de Carvalho *et al.*, 2006a; Lin *et al.*, 2007; Lardone *et al.*, 2007).

Depending on the aetiology, testicular dysfunction may manifest with impaired spermatogenesis alone or in association with other symptoms such as cryptorchidism (failure of the testis to descend into the scrotum), hypospadias and testicular cancer. According to the testicular dysgenesis syndrome (TDS) hypothesis (Skakkebeak *et al.*, 2001), reduced sperm count, cryptorchidism, hypospadias and testicular cancer may share common genetic and environmental factors acting on the Sertoli and Leydig cells. The question of whether Yq

deletions are responsible for the aetiopathogenesis of other components of this syndrome has been addressed only by a few studies. According to these studies, classical AZF deletions do not seem to induce cryptorchidism (Kunej *et al.*, 2003) nor testicular cancer (Frydelund-Larsen *et al.*, 2003), whereas the ‘gr/gr’ deletion has been recently reported as a significant genetic risk factor for testicular cancer (Nathanson *et al.*, 2005). The role of ‘gr/gr’ deletions in the aetiopathogenesis of cryptorchidism has not been evaluated so far since all of the earlier studies focused on the comparison of idiopathic infertile men (without abnormal andrological findings) versus fertile or normospermic controls or the general population. Cryptorchidism is the most frequent congenital abnormality of the male reproductive tract, affecting ~2–3% of full-term males. The aetiology of this pathological condition is multifactorial and can be due to endocrine disorders (e.g. hypogonadotropic hypogonadism, androgen resistance), anatomical abnormalities, or mutations of the INSL3 and LGR8 genes (Foresta and Ferlin, 2004; Bogatcheva and Agoulnik, 2005). Among genetic risk factors, polymorphisms in the ESR1 gene have been proposed, although with opposite effects in Japanese (Yoshida *et al.*, 2005) and Caucasian populations (Galan *et al.*, 2007).

Based on the TDS hypothesis and the recently proposed role of ‘gr/gr’ deletions in testis cancer, we now aimed to evaluate

whether cryptorchidism and 'gr/gr' deletions have a causal relationship. Considering the intimate intertalk between germ cells, Sertoli cells and Leydig cells, a genetic factor able to influence any of this component may lead to TDS including cryptorchidism. We previously found a significant association between 'gr/gr' deletions and reduced sperm count in the Italian population using a combined method based on sequence tagged sites +/- (STS +/-) deletion screening and further molecular characterization of the deletions (AZFc gene dosage and type of deleted gene copies) (Giachini *et al.*, 2005).

In the present study, we performed 'gr/gr' deletion screening in infertile cryptorchid men of Italian origin and data obtained was compared with our own published data (Giachini *et al.*, 2005) and with that published in the literature on idiopathic infertile men without a history of cryptorchidism. Beside the comparison of deletion frequencies, we also aimed to clarify whether specific deletion patterns are observed in this specific group of patients.

Materials and Methods

Subjects

The study population consisted of 146 Italian patients with a history of cryptorchidism. As a control group, we selected 140 men without cryptorchidism from our previously published 150 Italian infertile patients (Giachini *et al.*, 2005). The Y hgr distribution between oligo/azoospermic and normospermic men was similar (M. Mitchell, personal communication) which excludes recruitment bias. Since the same criteria (ethnic and geographic origin) was used for the recruitment of cyptorchid and non-cryptorchid men, significant differences in Y background distribution are not expected between groups. Table 1 reports the clinical characteristics of the study populations, including semen parameters evaluated according to the World Health Organization criteria. All patients underwent a complete andrological investigation for couple infertility at the Andrology Unit of the University Hospital Careggi in Florence (Italy). Cytogenetic analysis and Y chromosome microdeletion screening revealed 46,XY karyotype and the absence of AZF microdeletions in all included patients. Samples were collected using approved protocols and the informed consent of all individual was obtained.

Screening for 'gr/gr' deletions

We detected 'gr/gr' deletions by a polymerase chain reaction (PCR) amplification of Y chromosome STSs, originally described by Repping *et al.* (2003): sY1291, sY1191, sY1161, sY1206, sY1201, sY142, sY142 and sY1197 (see GenBank for PCR primers). We identified 'gr/gr' deletions by the following STS results: sY1291 negative; sY1191, sY1161, sY1206, sY1201, sY142, sY142 and

sY1197 all positive. A positive (normal men) and a negative control (women) were screened with the samples to prevent false results.

PCR was carried out in a total volume of 25 μ l. The reaction mixture included 100 ng of each DNA sample extracted from peripheral blood cells, 1X PCR buffer (containing 1,5 mM MgCl₂), 200 μ M of deoxy-nucleotidetriphosphates (dNTPs), 1 μ M of each primers and 1 U *Taq* polymerase (Promega Italia S.r.l.). After an initial denaturation step at 95°C for 5 min, amplification was performed for 35 sequential cycles, each including 1 min denaturation at 95°C, 1 min primer annealing at 55–62°C and 1 min extension at 72°C. The program was followed by a final extension step at 72°C for 7 min. PCR products were then analysed by electrophoresis on 2% agarose gels containing ethidium bromide and visualized under ultraviolet light.

Gene dosage

Quantitative analysis for CDY1 and DAZ, in order to quantify the copy number of these genes, was performed using a PCR-based method, according to Machev *et al.* (2004): we simultaneously amplified the AZFc locus to be quantified (CDY1 or 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 PCR reaction with a maximum of 28 cycles (end point into the exponential phase). The primers flank an insertion/deletion difference of 3–5 bp, which allowed the products amplified from the AZFc loci and the control loci to be separated by polyacrylamide gel electrophoresis. One of the primers was labelled at its 5' end with a fluorochrome (FAM). To overcome the problem of doublet bands—usually referred to as 'shadow bands'—caused by heterogeneous adenosine addition, PCR for DAZ/DAZL was followed by a treatment with polymerase T4 (1 U for 15 min at 37°C), which removed the extra base added by *Taq* polymerase at the end of the amplified fragments (Ginot *et al.*, 1996). Then, the reaction was mixed with formamide, denatured at 95°C for 5 min and the different size loci were separated on an automatic sequencer (ABI PRISM 310 Genetic Analyzer PE). Quantification was performed comparing the pick area of the AZFc locus and of its homologous.

CDY1 versus CDY2 (primers: oMY953a/o1023)

There are two identical copies of each CDY1 and CDY2, which share 98% nucleotide identity. We amplified CDY1 and CDY2 across 3 bp difference in the coding region, to give fragments of 134 bp for CDY1 and 137 bp for CDY2.

DAZ versus DAZL (primers: o1130/o1 313)

We co-amplified a fragment of intron 10 from DAZ (214 bp) and DAZL (217 bp). This intron is present in one copy per DAZ gene (in a normal 46,XY men there are four copies of DAZ and two copies of DAZL). Some samples present a 40 bp insertion polymorphism in the DAZL intron 10, resulting in an extra band at 260 bp, which could be in heterozygosis or, more rarely, in homozygosis.

Table 1: Seminal patterns of the two study populations

	Azoospermia	<5 × 10 ⁶ spermatozoa/ml	≥5 × 10 ⁶ spermatozoa/ml	Asteno and/or teratozoospermia	Normozoospermia	Not available
<i>Cryptorchid group</i> (n = 146)						
Unilateral (n = 77)	2	47	10	1	1	16
Bilateral (n = 66)	17	41	4	3	–	1
Total number	19	88	14	4	1	17
<i>Infertile group</i> (n = 140)	33	72	27	8	–	–

Gene copy type

Qualitative analysis for CDY and DAZ, in order to determine which copies of these genes have been removed by the 'gr/gr' deletion, was performed according to Machev *et al.* (2004). For DAZ, we chose the sequence family variant (SFV) at STS sY587 in intron 10, which discriminates DAZ1/2 from DAZ 3/4. For CDY1, we used a C/A SFV situated 7750 bp 5' of the CDY1 translation start codon (CDY7750), which distinguishes CDY1a from CDY1b. SFVs were scored by PCR followed by enzyme digestion (5 U for at least 4 h): dAZ sY587, DraI (DAZ1/2 cut); CDY1-7750, PvuII (CDY1b cut). Digestion products were then analysed by electrophoresis at 100 V on 4% agarose gels containing ethidium bromide and visualized under ultraviolet light. Primers pair: sY587, o912/o913; CDY1-7750, o1025/o1026 (Machev *et al.*, 2004).

Statistical analysis

Statistical analysis was performed using the statistical package SPSS for Windows (version 12.0.1; SPSS, Chicago, IL, USA). We tested the significance of the observed difference in the incidence of 'gr/gr' deletion between our two study groups using Fisher's exact test. Our null hypothesis was that incidence is the same in cryptorchid and infertile men without cryptorchidism.

All variables were checked for normal distribution by Kolmogorov–Smirnov one-sample test. For comparisons of means between groups of different genotypes, Student's *t*-test for independent samples, when normal distribution was observed, was applied. Sperm concentration is expressed as mean \pm standard deviation. Logarithmic transformation of data was performed in order to normalize the distribution when the presence of log normal distribution was checked. Finally, in case of non-normalized distribution, the non-parametric Mann–Whitney U test was applied to achieve the same objective. A *P*-value 0.05 was considered statistically significant for each test.

Results

Frequency and types of partial AZFc deletions in patients with and without cryptorchidism

Using the STS +/- analysis it is possible to distinguish between different types of partial AZFc deletions. We observed only the 'gr/gr' deletion (absence of the STS sY1291) in the cryptorchid group, whereas in the group of 140 infertile men we also found one subject with 'b2/b3' deletion (absence of the STS sY1191). The third possible deletion, called 'b1/b3', was absent in both study populations.

The frequency of 'gr/gr' deletions observed in the cryptorchid group was 4.2% (6/143), which is not significantly different from that observed in the re-analysed 140 infertile men without cryptorchidism (7/140; 5%).

Although a number of papers have been published on 'gr/gr' deletions in infertile and control men, only a minority of them confirmed the deletion with gene dosage analysis and ruled out deletion/duplication events. The 'gr/gr' deletion frequency in each study is reported in Table 2. All three studies were performed in Caucasian populations and the cumulative deletion frequency is 4.96%, which is not different from the deletion frequency of our cryptorchid (4.2%) and infertile (5%) groups.

Table 2: Frequency of 'gr/gr' deletion in infertile men of Caucasian origin, reported in studies in which gene dosage analysis was also performed

Reference	Total analysed, <i>n</i>	'gr/gr' deleted, <i>n</i>	'gr/gr' deletion, %
Repping <i>et al.</i> (2003)	246	9	3.7
Machev <i>et al.</i> (2004)	300	18	6
Giachini <i>et al.</i> (2005)	140	7	5
Total	686	34	4.96

Molecular analysis and clinical findings in cryptorchid men with 'gr/gr' deletions

Definition of 'gr/gr' deletion subtypes

The second step of molecular characterization is of particular importance since the currently used STS +/- PCR method is not able to exclude deletion/duplication events and other rearrangements in the AZFc region.

In order to confirm and to characterize 'gr/gr' deletions, we defined the gene dosage of CDY1 and DAZ and the type of missing DAZ (DAZ1/DAZ2/DAZ3/DAZ4) and CDY1 (CDY1a/CDY1b) copies. The number of gene copies remaining after the 'gr/gr' deletion events was the same in all subjects (one CDY1 copy and two DAZ copies), indicating the absence of false deletions and deletion/duplication events.

Based on the type of deleted DAZ and CDY1 copies, we found different subtypes of deletion patterns (Fig. 1) in the two study groups. Accordingly, 'gr/gr' deletion may be divided into at least four subtypes. The most frequent deletion subtypes (defined on the basis of the missing copies) both in cryptorchid and infertile groups was CDY1a+ DAZ1/2 followed by CDY1a+DAZ3/4. The deletion subtype CDY1b+ DAZ1/2 was present only in one cryptorchid patient, and CDY1b+DAZ3/4 was present only in one infertile man.

The frequency of 'gr/gr' deletions with missing CDY1a in the cryptorchid group was 83%, which is similar to that observed in the infertile men (86%). The loss of DAZ1/2 is more frequent, although not significantly different, in cryptorchid men compared with infertile men (67 and 43% respectively).

Genotype-phenotype association

The mean values of sperm concentration were similar in the infertile patients with and without cryptorchidism ($4.0 \pm 10.3 \times 10^6$ spermatozoa/ml and $4.3 \pm 8.6 \times 10^6$ spermatozoa/ml, respectively). Similarly, the comparison between 'gr/gr' deleted men with cryptorchidism ($2.1 \pm 3.3 \times 10^6$ spermatozoa/ml) and without cryptorchidism ($2.5 \pm 3.5 \times 10^6$ spermatozoa/ml) shows no differences. The clinical description of men with 'gr/gr' deletion is given in Table 3 along with the indication of the CDY1/DAZ gene patterns. The sperm concentration in the cryptorchid group ranges between azoospermia and 8×10^6 spermatozoa/ml, whereas in the non-cryptorchid group it was between 0.01 and 10×10^6 spermatozoa/ml. The 'gr/gr' deletion was observed both in men with unilateral and bilateral cryptorchidism.

In order to define if the presence of 'gr/gr' deletion in cryptorchid men has an additional negative effect on sperm concentration, we compared the mean values of sperm concentration

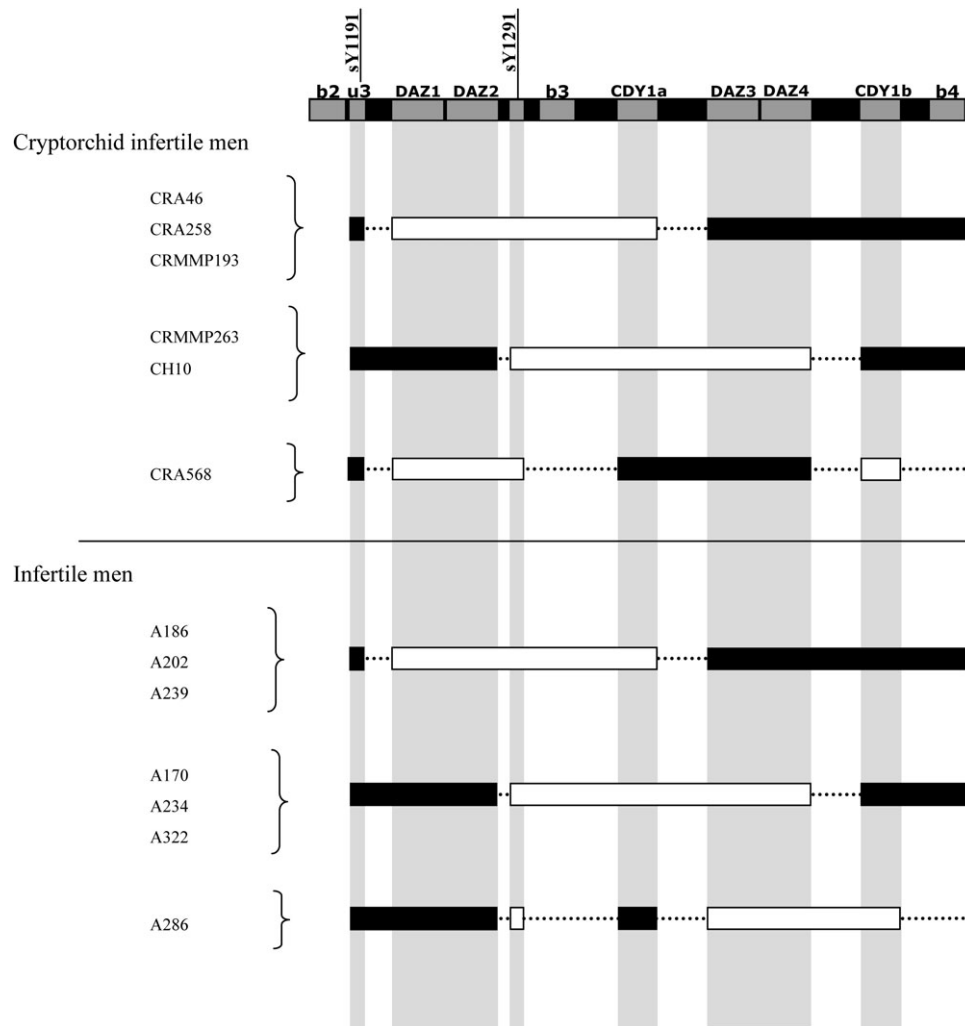


Figure 1: Schematic representation of the deletion patterns found in the cryptorchid and infertile subjects [from the paper by Giachini *et al.* (2005) with the exclusion of one monolateral cryptorchid man] with ‘gr/gr’ deletion, based on the type of CDY1 and DAZ copies deleted and on the analysis based on STS +/- described by Repping *et al.* (2003) Open and filled boxes indicate the absence or presence of a given marker or gene, respectively

between ‘gr/gr’ deleted and not deleted cryptorchid patients. Although the mean value was lower in cryptorchid men with ‘gr/gr’ deletion ($2.1 \pm 3.3 \times 10^6$ spermatozoa/ml) compared with those without ($4.1 \pm 10.5 \times 10^6$ spermatozoa/ml), the difference was not statistically significant. In our previous publication, we observed ‘gr/gr’ deletion with missing CDY1a copy only in the infertile group and not in normospermic control men. This observation leads us to propose a possible pathogenetic effect of these deletion subtypes on spermatogenesis. However, since all our cryptorchid patients with ‘gr/gr’ deletions were oligospermic and we found only one cryptorchid patient with CDY1b deletion, the comparison of the mean values of sperm concentration in the ‘gr/gr’ deleted cryptorchid men with loss of CDY1a versus CDY1b has no clinical relevance.

Discussion

The human Y chromosome has a unique structure, rich in very long near identical direct and indirect repeats which makes the

chromosome prone to rearrangements (Skaletsky *et al.*, 2003). The AZFc region, which is made up almost entirely of amplicons, is the most frequently deleted Yq region in infertile men (Kuroda-Kawaguchi *et al.*, 2001). Thanks to the presence of amplicons with the same orientation, deletion break points may occur at different sites leading to complete (‘b2/b4’) or a number of possible partial AZFc deletions. The most frequent partial AZFc deletions are the ‘gr/gr’ and the ‘b2/b3’, both removing approximately half of the AZFc region gene content. Despite a similar loss of gene number, ‘b2/b3’ deletions are not associated with spermatogenic impairment, whereas ‘gr/gr’ deletions have been reported as a risk factor for male infertility by the majority of the authors (Repping *et al.*, 2003; de Llanos *et al.*, 2005; Ferlin *et al.*, 2005; Giachini *et al.*, 2005; Lynch *et al.*, 2005). However, the pathogenic role of this genetic anomaly in male infertility is still under debate and unfortunately, it is impossible to perform a meta analysis of the published data due to methodological differences and different inclusion criteria applied for the selection of patients and controls (for review see Krausz and degl’Innocenti, 2006).

Table 3: Phenotype of cryptorchid and infertile patients bearing 'gr/gr' deletion with the indication of gene copy deletion pattern defined on the basis of the type of CDY1-DAZ gene copies loss

Code	Phenotype	Deleted gene copies		Semen parameters		
		DAZ	CDY1	Number (sperm/ml × 10 ⁶)	Progressive Motility (%)	Morphology (%)
'gr/gr' deleted cryptorchid men						
CRA46	Crypt. monolat.	1/2	a	0.01	–	–
CRA258	Crypt. bilat.	1/2	a	0	–	–
CRA568	Crypt. bilat.	1/2	b	1.40	11	8
CRMMP193	Crypt. monolat.	1/2	a	8.00	23	15
CRMMP263	Crypt. bilat.	3/4	a	1.00	–	–
CH10	Crypt. monolat.	3/4	a			
'gr/gr' deleted infertile men from Giachini <i>et al.</i> (2005) ^c						
A170	Idiopathic	3/4	a	0.90	30	16
A186	Varicocele sx	1/2	a	0.60	0	2
A202	Idiopathic	1/2	a	10	20	13
A234	Varicocele sx	3/4	a	0.70	3	8
A239	Idiopathic	1/2	a	4.10	5	12
A286	Idiopathic	3/4	b	0.01	–	–
A322	Idiopathic	3/4	a	1.00	10	15

^cexcluded one subject with monolateral cryptorchidism.

The appropriate selection of control group in association studies is of fundamental importance and should be represented by a group of subjects free of the disease under study. Therefore, in order to understand whether 'gr/gr' deletions are able to influence spermatogenesis, the controls should be normospermic subjects. In the literature, only a few studies used exclusively normospermic controls (Repping *et al.*, 2003; Ferlin *et al.*, 2005; Giachini *et al.*, 2005; Hucklenbroich *et al.*, 2005) and among them only two (Repping *et al.*, 2003; Giachini *et al.*, 2005) confirmed the deletions by dosage analysis. Recent East Asian studies, based on an STS +/– method, show a relatively high frequency of 'gr/gr' deletion in these populations (de Carvalho *et al.*, 2006b; Fernando *et al.*, 2006) and a lack of association with infertility. On the contrary, a study from Yen's group reports that partial duplication and not partial deletion of the AZFc region is a risk factor for impaired spermatogenesis in Han Chinese (Lin *et al.*, 2007). It is likely that ethnic background plays a determinant role in the phenotypic expression of this genetic risk factor. Therefore, the exclusion of recruitment bias (i.e. exclusion of significant differences in Y hgr distribution between controls and cases) is of fundamental importance especially in ethnically mixed populations.

In our previous study on the Italian population (with homogeneous inclusion criteria, normospermic controls, exclusion of recruitment bias, i.e. similar Y background in cases and controls), we observed that men with congenital 'gr/gr' deletions have a 10-fold increased risk of being oligospermic compared with men without such a deletion (Giachini *et al.*, 2005). A similar conclusion was made also by an other Italian study in which the selection criteria of the control group was based on normozoospermia (Ferlin *et al.*, 2005). With the aid of additional molecular characterization (gene dosage and type of deleted DAZ and CDY1 copies), we also showed that 'gr/gr' deletion is not a uniform entity, and it can be divided

into four major subtypes. We suggested that subtype 1, which is characterized by the loss of DAZ1/DAZ2 and CDY1a copies, may represent a more severe variant, since this subtype is absent in normospermic controls with 'gr/gr' deletions. The recently reported association between testis cancer and 'gr/gr' deletion (Nathanson *et al.*, 2005) opened the question about the role of this polymorphism in other TDS-related pathologies such as cryptorchidism.

In order to define the role of 'gr/gr' deletion in the aethiopathogenesis of cryptorchidism, we compared the deletion frequency in patients with and without undescended testis. The 'gr/gr' deletion frequency in 146 cryptorchid men (4.2%) compared with that of our control population based on 140 infertile men without cryptorchidism (5%) showed no significant differences. Moreover, we found a similar distribution of deletion patterns, defined on the basis of CDY1 and DAZ gene copy loss, in the two patient groups. In addition, the mean values of sperm concentration of 'gr/gr' deleted men, with and without cryptorchidism ($2.1 \pm 3.3 \times 10^6$ spermatozoa/ml and $2.5 \pm 3.5 \times 10^6$ spermatozoa/ml, respectively) were also overlapping, indicating a similar effect of 'gr/gr' deletion on spermatogenesis regardless of the presence or absence of cryptorchidism. Given that the composition of the study populations in terms of sperm concentration were similar ($4.0 \pm 10.3 \times 10^6$ spermatozoa/ml versus $4.3 \pm 8.6 \times 10^6$ spermatozoa/ml), we should have seen a higher percentage of deletion frequency in the cryptorchid group if 'gr/gr' deletion was also an independent risk factor for cryptorchidism and not only for oligospermia. We can therefore conclude that the deletion frequency is not influenced by the presence of cryptorchidism. According to this hypothesis, the deletion frequency in cryptorchids, which is similar to that of the infertile group, is significantly different ($P < 0.03$) from our previously published normospermic group (0.5%). Since both the normospermic and the infertile controls were not cryptorchid and a difference in deletion

frequency was found only when the comparison is made between normospermic and cryptorchid, but not between infertile and cryptorchid men, 'gr/gr' deletion is more likely to affect sperm parameters than testicular descent.

Although the majority of cryptorchid men (especially in case of bilateral undescended testes) will develop severe impairment of spermatogenesis in adulthood, seminal parameters in ex-cryptorchid men may range from azoospermia to normozoospermia. Since cryptorchidism is a multifactorial disease, the reason for such a heterogeneous phenotype can be related to specific aetiological factors which underlie cryptorchidism. Although on the basis of our results, we cannot consider 'gr/gr' deletion as a common aetiological risk factor for both spermatogenic failure and cryptorchidism, its presence in the cryptorchid group was associated with a lower sperm concentration ($2.1 \pm 3.3 \times 10^6$ spermatozoa/ml versus $4.1 \pm 10.5 \times 10^6$ spermatozoa/ml). This observation, needs to be confirmed, but may suggest an additional modulating effect of 'gr/gr' deletion on the final testicular phenotype of cryptorchid patients.

In conclusion, in analogy to classical AZF deletions (Kunej *et al.*, 2003), 'gr/gr' deletion is not associated with cryptorchidism but it is present at a similar frequency in infertile patients with and without history of maldescended testis. The comparison with normospermic men has further confirmed the deleterious effect of this type of AZFc deletion on spermatogenesis. Consequently, the screening for 'gr/gr' deletions can be extended to all severely oligospermic patients attending to assisted reproductive techniques, without the exclusion of those with cryptorchidism, since this test is able to provide the identification of a transmissible genetic risk factor for reduced sperm count.

Acknowledgements

We would like to thank the clinical staff of the Andrology Unit of the University Hospital Careggi of Florence for patient's recruitment (head of the service Professor Mario Maggi), Professor I. Noci from the Infertility Clinic of University of Florence and the members of the Androgenetic laboratory (Selene Degl'Innocenti, Elena Guarducci, Ilaria Laface) for technical assistance. This work was supported by a grant PRIN2005 (C.K.) from MIUR (Italy).

References

- Bogatcheva NV, AgoulNIK AI. INSL3/LGR8 role in testicular descent and cryptorchidism. *Reprod Biomed* 2005;**10**:49–54. Online.
- de Carvalho CM, Zuccherato LW, Bastos-Rodrigues L, Santos FR, Pena SD. No association found between 'gr/gr' deletions and infertility in Brazilian males. *Mol Hum Reprod* 2006a;**12**:269–273.
- de Carvalho CM, Zuccherato LW, Fujisawa M, Shirakawa T, Ribeiro-dos-Santos AK, Santos SE, Pena SD, Santos FR. Study of AZFc partial deletion gr/gr in fertile and infertile Japanese males. *J Hum Genet* 2006b;**1**:794–799.
- de Llanos M, Balleca JL, Gazquez C, Margarit E, Oliva R. High frequency of gr/gr chromosome Y deletions in consecutive oligospermic ICSI candidates. *Hum Reprod* 2005;**20**:216–220.
- Ferlin A, Tessari A, Ganz F, Marchina E, Barlati S, Garolla A, Engl B, Foresta C. Association of partial AZFc region deletions with spermatogenic impairment and male infertility. *J Med Genet* 2005;**42**:209–213.
- Fernando L, Gromoll J, Weerasooriya TR, Nieschlag E, Simoni M. Y-chromosomal microdeletions and partial deletions of the azoospermia

- factor c (AZFc) region in normozoospermic, severe oligozoospermic and azoospermic men in Sri Lanka. *Asian J Androl* 2006;**8**:39–44.
- Foresta C, Ferlin A. Role of INSL3 and LGR8 in cryptorchidism and testicular functions. *Reprod Biomed Online* 2004;**9**:294–298.
- Frydelund-Larsen L, Vogt PH, Leffers H, Schadwinkel A, Daugaard G, Skakkebaek NE, Rajpert-De Meyts E. No AZF deletion in 160 patients with testicular germ cell neoplasia. *Mol Hum Reprod* 2003;**9**:517–521.
- Galan JJ, Guarducci E, Nuti F, Gonzalez A, Ruiz M, Ruiz A, Krausz C. Molecular analysis of estrogen receptor alpha gene AGATA haplotype and SNP12 in European populations: potential protective effect for cryptorchidism and lack of association with male infertility. *Hum Reprod* 2007;**22**:444–449.
- Giachini C, Guarducci E, Longepied G, Degl'Innocenti S, Becherini L, Forti G, Mitchell MJ, Krausz C. The gr/gr deletion(s): a new genetic test in male infertility? *J Med Genet* 2005;**42**:497–502.
- Ginot F, Bordelais I, Nguyen S, Gyapay G. Correction of some genotyping errors in automated fluorescent microsatellite analysis by enzymatic removal of one base overhangs. *Nucleic Acids Res* 1996;**24**:540–541.
- Hucklenbroich K, Gromoll J, Heinrich M, Hohoff C, Nieschlag E, Simoni M. Partial deletions in the AZFc region of the Y chromosome occur in men with impaired as well as normal spermatogenesis. *Hum Reprod* 2005;**20**:191–197.
- Krausz C, Degl'Innocenti S. Y chromosome and male infertility: update. *Front Biosci* 2006;**11**:3049–3061.
- Krausz C, Quintana-Murci L, Forti G. Y chromosome polymorphisms in medicine. *Ann Med* 2004;**36**:573–583.
- Kunej T, Zorn B, Peterlin B. Y chromosome microdeletions in infertile men with cryptorchidism. *Fertil Steril* 2003;**3**:1559–1565.
- Kuroda-Kawaguchi T, Skaletsky H, Brown LG, Minx PJ, Cordum HS, Waterston RH, Wilson RK, Silber S, Oates R, Rozen S *et al.* The AZFc region of the Y chromosome features massive palindromes and uniform recurrent deletions in infertile men. *Nat Genet* 2001;**29**:279–286.
- Lardone MC, Parodi DA, Ebensperger M, Penalzoza P, Cornejo V, Valdevenito R, Pommer R, Castro A. AZFc partial deletions in Chilean men with severe spermatogenic failure. *Fertil Steril* 2007 (Epub ahead of print).
- Lin YW, Chia-Ling Hsu L, Kuo PL, Huang WJ, Chiang HS, Yeh SD, Hsu TY, Yu YH, Hsiao KN, Cantor RM *et al.* Partial duplication at AZFc on the Y chromosome is a risk factor for impaired spermatogenesis in Han Chinese in Taiwan. *Hum Mutat* 2007 (Epub ahead of print).
- Lynch M, Cram DS, Reilly A, O'Bryan MK, Baker HW, de Kretser DM, McLachlan RI. The Y chromosome gr/gr subdeletion is associated with male infertility. *Mol Hum Reprod* 2005;**11**:507–512.
- Machev N, Saut N, Longepied G, Terriou P, Navarro A, Levy N, Guichaoua M, Metzler-Guillemain C, Collignon P, Frances A-M *et al.* Sequence family variant loss from the AZFc interval of the human Y chromosome, but not gene copy loss, is strongly associated with male infertility. *J Med Genet* 2004;**41**:814–825.
- Nathanson KL, Kanetsky PA, Hawes R, Vaughn DJ, Letrero R, Tucker K, Friedlander M, Phillips KA, Hogg D, Jewett MA *et al.* The Y deletion gr/gr and susceptibility to testicular germ cell tumor. *Am J Hum Genet* 2005;**77**:1034–1043.
- Ravel C, Chantot-Bastaraud S, El Houate B, Mandelbaum J, Siffroy JP, McElreavey K. gr/gr deletions within the azoospermia factor c region on the Y chromosome might not be associated with spermatogenic failure. *Fertil Steril* 2006;**85**:229–231.
- Repping S, Skaletsky H, Brown L, van Daalen SK, Korver CM, Pyntikova T, Kuroda-Kawaguchi T, de Vries JW, Oates RD, Silber S *et al.* Polymorphism for a 1.6-Mb deletion of the human Y chromosome persists through balance between recurrent mutation and haploid selection. *Nat Genet* 2003;**35**:247–251.
- Skakkebaek NE, Rajpert-De Meyts E, Main KM. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Hum Reprod* 2001;**16**:972–978.
- Skaletsky H, Kuroda-Kawaguchi T, Minx PJ, Cordum HS, Hillier L, Brown LG, Repping S, Pyntikova T, Ali J, Bieri T *et al.* The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. *Nature* 2003;**423**:825–837.
- Yoshida R, Fukami M, Sasagawa I, Hasegawa T, Kamatani N, Ogata T. Association of cryptorchidism with a specific haplotype of the estrogen receptor alpha gene: implication for the susceptibility to estrogenic environmental endocrine disruptors. *J Clin Endocrinol Metab* 2005;**90**:4716–4721.

Submitted on April 5, 2007; resubmitted on May 23, 2007; accepted on May 29, 2007