Partial AZFc deletions and duplications: clinical correlates in the Italian population.

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ORIGINAL INVESTIGATION

Partial *AZFc* deletions and duplications: clinical correlates in the Italian population

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Abstract The role of partial *AZFc* deletions of the Y chromosome in spermatogenic impairment is currently debated. Recently, it was also reported that duplications of the same region are associated with oligozoospermia in Han-Chinese men. The aims of this study were (1) to evaluate the clinical significance of partial *AZFc* deletions in a large study population and (2) to define if partial *AZFc* duplications are a risk factor for spermatogenic failure also in a Caucasian population such as the Italian. We screened 556 infertile patients and 487 normozoospermic controls for partial *AZFc* deletions with a combined method based on STS+/− followed by *CDY1-DAZ* gene dosage and copy analysis. For the second aim, we performed *CDY1-DAZ* gene dosage in 229 infertile patients and 263 normozoospermic controls. The frequency of gr/gr deletions in patients was significantly different from the controls (3.2 vs. 0.4%, respectively; $P < 0.001$), with an OR = 7.9 (95% CI 1.8–33.8). b2/b3 deletions were rare in both groups (0.5% in patients, 0.2% in controls). Concerning gr/gr duplications, we observed no significant differences in their frequency between cases (2.6%) and controls (3.8%). This is the largest study population in the literature in which all potential methodological and selection biases were carefully avoided to detect the clinical significance of partial *AZFc* deletions and duplications. Our study provides strong evidence that gr/gr deletion is a risk factor for impaired spermatogenesis, whereas we did not detect a significant effect of b2/b3 deletions and partial *AZFc* duplications on spermatogenesis in this Caucasian ethnic group.

Introduction

The *AZFc* region of the Y chromosome consists almost entirely of repetitive sequence blocks called "amplicons," which are arranged in direct and inverted repeats including eight major palindromes (Skaletsky et al. 2003). Given its repetitive nature, the *AZFc* region is particularly susceptible to homologous intrachromosomal recombination events, which may lead to deletions. Different rearrangements at *AZFc* have been identified, and some of them have been reported to be either a direct cause or a risk factor for male infertility. It is now widely accepted that complete deletions of the *AZFc* region (b2/b4 deletion) is the most common known molecular genetic cause of spermatogenic failure. This deletion type was never found in normospermic men and thus shows a clear cut cause–effect relationship with spermatogenic impairment (Krausz and Degl'Innocenti 2006). Recently, new types of *AZFc* deletions, called "partial deletions," have been reported (Repping et al. 2003), which remove approximately half of the *AZFc* gene content—including two *DAZ*, one *CDY1* copies and one *BPY2* copy—and arise by the same molecular mechanism of the complete *AZF* microdeletions. Among them, gr/gr partial deletion is considered a genetic risk factor for spermatogenic impairment by a number of research groups, including ours (see Table 1), while b2/b3 and b1/b3

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Table 1 Summary of gr/gr deletion case–control studies available in the literature, with or without association with spermatogenic disturbances

	References	Population	Patients		Controls		
			Total *n*	gr/gr %	Total *n*	gr/gr %	Normozoospermic %
Association	Repping et al. (2003)[a]	Dutch	246	3.7	148	0.0	100
	de Llanos et al. (2005)	Spanish	283	4.2	232	0.0	14.6
	Ferlin et al. (2005)	Italian	337	4.7	263	0.4	100
	Giachini et al. (2005)[a]	Italian	150	5.3	189	0.5	100
	Lynch et al. (2005)	Australian	1,351	4.1	234	0.4	57.3
	Navarro-Costa et al. (2007)	Portuguese	300	5.3	300	1.0	0
	Yang et al. (2008)	Han-Chinese	414	10.6	262	5.3	100
No association	Machev et al. (2004)[a]	French (admixed)	300	6.0	399	3.5	1
	Hucklenbroich et al. (2005)	German	348	4.0	170	1.8	100
	Ravel et al. (2006)	Admixed	192	2.1	181	3.3	8.8
	de Carvalho et al. (2006a)	Brazilian	110	4.5	240	2.5	0
	de Carvalho et al. (2006b)	Japan	78	28.2	56	33.9	0
	Zhang et al. (2006)[a]	East Asian	87	10.3	89	10.1	100
	Fernando et al. (2006)	Sri Lanka	96	4.2	89	4.6	100
	Wu et al. (2007)	Han-Chinese	439	7.0	248	7.7	34.6
	Lardone et al. (2007)	Chilean	95	2.1	77	2.6	40.2
	Imken et al. (2007)	Moroccan	145	4.7	176	4.0	43.2
	Stouffs et al. (2008)	Mixed Caucasian	187	4.3	394	3.0	70.5

Ethnic/geographic origin and the size of the study populations are reported. Since the inclusion criteria for controls were different in different studies (general population or proven fertile men with unknown sperm count or normozoospermic men), the percentage of normozoospermic men in each control group is indicated

[a] Gene dosage was performed only in these studies (i.e., only in four of the 16 studies)

deletions seem not to have significant effects on male fertility (Giachini et al. 2005; Hucklenbroich et al. 2005; Lynch et al. 2005; Repping et al. 2004) with the exception of one study in the Han-Chinese population (Wu et al. 2007).

The first *AZFc* candidate gene isolated from the *AZFc* interval was *DAZ* (deleted in Azoospermia), which is expressed specifically in the testis (Reijo et al. 1995). The *DAZ* gene family has been transposed from chromosome 3 (3p25, *DAZL1* locus) on to the Y chromosome, where it is present in four copies on the reference Y chromosome sequence (*DAZ1*, *DAZ2*, *DAZ3* and *DAZ4*). It would suggest that *DAZ* genes are extending or improving the functional profile of their autosomal homologs, *DAZL* on chromosome 3 and *BOULE* on chromosome 2 (Xu et al. 2001). The *DAZ* gene family encodes different testis-specific RNA-binding proteins probably involved in the translational control of transcripts of other germ line genes (Yen 2004). Another *AZFc* testis-specific candidate gene is *CDY1*, present in two copies (*CDY1a* and *CDY1b*) and originated from a polyadenylated mRNA of the *CDYL* locus on chromosome 6, which has been then retrotransposed to the Y chromosome (Lahn and Page 1999). CDY proteins were identified as histone acetyltransferases with a strong preference for histone H4 and localized in the nuclei of maturing spermatids (Lahn et al. 2002). Histone hyperacetylation in late spermatids results in a more open chromatin structure, which facilitates not only the spermatogenic histone replacement but also provides an easier access of the transcriptional machinery to the postmeiotic sperm DNA. Another *AZFc* gene involved in micro- and partial deletion is *BPY2*, of which function is unknown, and five transcription units, *TTTY3*, *TTTY4*, *TTTY17*, *CSPG4LY* and *GOLGA2LY*.

In our previous study, using a combined method based on gene dosage and gene copy definition of two *AZFc* genes (*DAZ* and *CDY1*), we identified different subtypes of gr/gr deletions characterized by the loss of different gene copies (Giachini et al. 2005, 2007). A significantly higher frequency of *CDY1a* copy deletion and *DAZ1/DAZ2* deletion was found in the oligozoospermic group in respect to the normozoospermic group suggesting that certain deletion patterns may be more pathogenic than others (Giachini et al. 2005).

Homologous recombination between *AZFc* amplicons can generate, other than partial *AZFc* deletions, also partial *AZFc* duplications, which may occur among different amplicons. In a recent study on Taiwanese population, Yen and her group focused their interest on two types of partial *AZFc* duplications: (1) gr/gr duplication, which spans 1.6 Mb and involves nine genes, and (2) b2/b3 duplication,

which spans 1.8 Mb and involves 12 genes. The authors found an association between *AZFc* partial duplications and male infertility (Lin et al. 2007). The authors suggested that some *AZFc* gene would be dosage-sensitive and their increased expression may interfere with normal spermatogenesis.

Given that the reliability of case–control association studies are strongly dependent on the size of the study population, we aimed to verify if our previous finding on partial *AZFc* deletions in a relatively small study population ($n = 339$) can be replicated in a much larger study population ($n = 1043$). Moreover, since the majority of the published studies dealing with gr/gr deletions suffer from selection and methodological biases, there is an urgent need for large, unbiased studies able to provide reliable information about the clinical significance of gr/gr deletions. Our second aim was to define whether partial *AZFc* duplication is associated with an increased risk of spermatogenic failure also in a Caucasian population, such as the Italian, or it is restricted to the Taiwanese Han-Chinese population.

Materials and methods

Subjects

Infertile patients included in the study were seeking complete andrological work-up for couple infertility at the Andrology Unit and the Unit of Physiopathology of Reproduction of the University Hospital Careggi (Florence) and at the Endocrinology Unit of the University of Ancona. Infertile patients were selected on the basis of a comprehensive andrological examination including medical history, semen analysis, scrotal ultrasound, hormone analysis, karyotype and Y chromosome microdeletion screening. Patients with mono or bilateral cryptorchidism, varicocele of grades 2 and 3, obstructive azoospermia, recurrent infections, iatrogen infertility, hypogonadotrophic hypogonadism, karyotype anomalies, Y chromosome microdeletions and patients with no-Central Italian origin were excluded.

Concerning the study of partial *AZFc* duplications, we selected only subjects with the absence of partial *AZFc* deletions. Controls were selected on the basis of normal sperm parameters (sperm count, motility and morphology) defined according to the WHO criteria (World Health Organization 1999). The origin of controls were the following: (1) voluntaries (55%); (2) male partners of infertile couples with ascertained female factor (30%); (3) men attending at the andrology laboratory for semen analysis for secondary infertility (previous fertility with the same or an other partner) or for "andrological control" (15%). Forty percent of normozoospermic controls were also proven fertile men, since they fathered at least one child spontaneously or had normal fertilization after in vitro fertilization for pure tubal factor infertility.

To exclude recruitment bias, much care was taken for the ethnic and geographic matching of the patients and controls. All patients and controls were explicitly asked for their paternal and maternal origin (i.e., if the family of their mother and father were originally from Central Italy) and selected only those with Central Italian ancestry. In addition, we performed Y haplogroup (hgr) analysis in a proportion of patients ($n = 115$) and controls ($n = 171$), which showed a similar Y haplogroup distribution (with the highest frequency of hgr P, 40 and 45.6%, respectively) between oligo/azoospermic and normozoospermic men (personal communication of M. Mitchell).

Partial AZFc deletions

The study population consisted of 556 infertile patients and 487 normozoospermic controls. In the infertile group, 284 patients were "idiopathic" (without known abnormal andrological findings in their medical history and at the medical examination, normal hormone levels and normal genetic tests) and 272 presenting cofactors with potential mild negative effect on spermatogenesis [unilateral varicocele with grade < 2, previous (not recurrent) infections of the urogenital tract]. Although we divided our infertile patients into two groups on the basis of presence/absence of mild cofactors, it is worth to note that in the large majority of previous studies the exclusion criteria were much less strict, potentially including among "cases" also infertile men with well known causes of spermatogenic failure. Exclusion criteria from previous studies are reported in Supplementary Table 1.

According to the three major sperm parameters, the infertile group can be divided as follows: azoospermia in 72 patients; cryptozoospermia (<1 million spermatozoa/ml) in 26 patients; severe oligozoospermia (1–5 millions spermatozoa/ml) in 187 patients; moderate oligozoospermia (5–20 milions spermatozoa/ml) in 271 patients. One hundred and twenty-one infertile men and 189 controls were already analyzed in our previous study (Giachini et al. 2005).

Partial AZFc duplications

The study population consisted of a total of 492 subjects: (1) 229 infertile patients (120 "idiopathic" and 109 with "cofactors") and (2) 263 normozoospermic men. The patient group included 37 azoospermic, 15 cryptozoospermic, 80 severe oligozoospermic and 97 moderate oligozoospermic men. Samples were collected using approved protocols, and the informed consent of all individuals was obtained according to the local ethical committee policy.

STS+/− analysis

We detected gr/gr deletions and b2/b3 deletions by a polymerase chain reaction (PCR) amplification of Y chromosome sequence-tagged sites (STSs), originally described by Repping et al. (2003): sY1291, sY1191, sY1161, sY1206, sY142 and sY1197 (see GenBank accession numbers in the original article). We identified gr/gr deletions by the following STS results: sY1291 negative; sY1191, sY1161, sY1206, sY1201, sY142 and sY1197 all positive. The b2/b3 deletion was characterized by the absence of the STS sY1191 and the presence of the rest of the STSs. Positive (man with intact *AZFc*) and negative controls (woman) were screened with the samples to prevent false results. Suspected deletions were confirmed with subsequent PCR at less stringent conditions.

DAZ and *CDY1* gene dosage

To quantify the copy number of *DAZ* and *CDY1* genes, we performed a quantitative analysis, according to a previously reported method (Giachini et al. 2007; Machev et al. 2004). This method was validated against FISH analysis in the original paper by Machev et al. (2004). Samples with known copy number (previously analyzed with the original Machev method) were included in each batch of samples. The *DAZ* dosage method consists in the simultaneous amplification of a fragment of intron 10 from *AZFc DAZ* copies and from its homolog *DAZL* (localized outside the *AZFc* interval), using a single primer pair (o1130/o1313). This intron is present in one copy per *DAZ* or *DAZL* gene (according to the *AZFc* reference structure the number of *DAZ* copies is four, whereas there are two copies of *DAZL* in a normal 46,XY man). Thus, *DAZL* act as an internal standard with a known number of copies. The primers flank an insertion/deletion difference of 3 bp, which allowed the PCR products (*DAZ*: 214 bp; *DAZL*: 217 bp) to be separated by polyacrylamide gel electrophoresis. One of the primers (o1130) was labeled at its 5′ end with a fluorochrome (FAM).

The quantitative analysis for *CDY1* copies was analogous to *DAZ* dosage. In the reference sequence of the *AZFc* region, there are two copies of both *CDY1* and *CDY2*, which share 98% nucleotide identity. We amplified *CDY1* and *CDY2* across 3 bp indel difference in the coding region, to give fragments of 134 bp for *CDY1* and 137 bp for *CDY2* (primers: oMY953a/o1023). oMY953a was labeled at its 5′ end with a fluorochrome (FAM).

For both *DAZ* and *CDY1* dosage, the PCR reactions were performed in a maximum of 23 cycles (end point within the exponential phase). The PCR products were mixed with formamide, denatured at 95°C for 5 min and the different size loci separated on automatic sequencer (ABI PRISM 310 Genetic Analyzer PE). Quantification was performed comparing the peak area corresponding to the *DAZ* locus and to its homolog *DAZL* and *CDY1* to *CDY2*. Figure 1 reports examples of different *DAZ* and *CDY1* gene dosages. Some samples presented 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.

Gene copy type definition

Qualitative analysis for *CDY1* and *DAZ* 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 *DAZ3/4*. In addition we analyzed one SVF for each *DAZ* copy according to (Fernandes et al. 2002): (1) three single nucleotide variants (SNVs)—*DAZ*-SNV I (for *DAZ*4), *DAZ*-SNV II (for *DAZ*1), *DAZ*-SNV III (for *DAZ*2); (2) the STS Y-*DAZ*3 (for *DAZ*3).

For *CDY1*, we used a C/A SFV situated 7750 bp upstream of the *CDY1* translation start codon (*CDY*7750), which distinguishes *CDY1a* from *CDY1b*. SFVs were scored by PCR followed by enzyme digestion (except for the STS Y-*DAZ*3) using the following enzymes: *DraI* for *DAZ* sY587; *FspI DAZ*-SNV I; *MboI* for *DAZ*-SNV II; *TaqI* for *DAZ*-SNV III; *PvuII* for *CDY1*-7750. Digestion products were then analyzed by electrophoresis at 100 V on 4% agarose gels containing ethidium bromide and visualized under ultraviolet light.

Y haplogroup definition

Individuals with partial *AZFc* deletions were genotyped in the laboratory of C. Tyler Smith (Sanger Institut, Cambridge, UK) in the context of a multicenter study using the multiplexed primers previously described (Noordam and Repping 2006) adapted for SNaPshot single base extension (Applied Biosystems). The markers $RPS4Y_{711}$, M145, M96, M89, M9 and M45 were typed on all samples, and M123, M78, V6, M35 and M81 (hg E—derived for M96), M201, M170, M52 and 12f2 (hg F—derived for M89), M106, M61, M147, M214, M27, M76 and M70 (hg K—derived for M9), or M17, M343, M369 and M18 (hg P—derived for M45) on appropriate subsamples, but data were combined into the major hgs E, F*(×K), K*(×P) and P to provide numbers suitable for most statistical analyses.

Individuals with partial *AZFc* duplications (except C74, CS26, CS68, CS76, which were also haplotyped by the aforementioned method) were genotyped for six binary markers defining five haplogroups: E, J, K*(×N, P), N, and P, and one paragroup Y*(×A, D, E, J, K). Y chromosome haplotyping was performed as previously published for the YAP, M9, SRY1532, 92R7, LLy22 g and 12f2 polymorphisms (Rosser et al. 2000). Polymorphisms were visualized

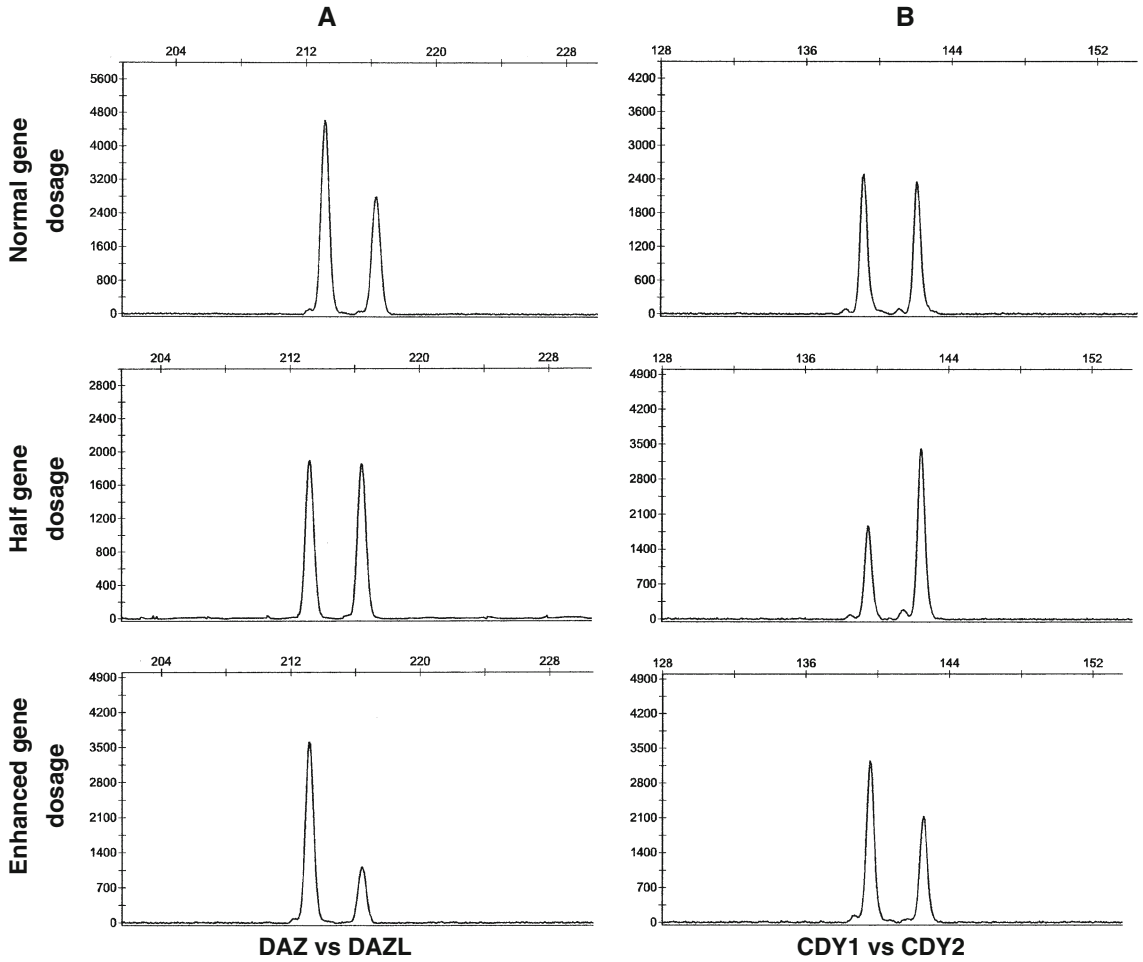

Fig. 1 Examples of electrophoretograms showing different gene dosages for the *DAZ/DAZL* and *CDY1/CDY2* genes. The *x*-axis shows length of PCR products in base pairs as determined by use of internal lane standard and the *y*-axis shows the fluorescent intensity in arbitrary units. **a** The peak area of *DAZ* is compared to that of *DAZL* (corresponding to two copies). A *DAZ/DAZL* pattern of 2:1 indicates four *DAZ* copies ("normal" *DAZ* gene dosage according to the reference sequence), 1:1 indicates two *DAZ* copies, 3:1 indicates six *DAZ* copies. **b** The peak area of *CDY1* is compared to that of *CDY2* (corresponding to two copies). A *CDY1/CDY2* pattern of 1:1 indicates two *CDY1* copies ("normal" *CDY1* gene dosage according to the reference sequence), 0.5:1 indicates one *CDY1* copy and 1.5:1 indicates three *CDY1* copies

by restriction enzyme digest for M9 (HinfI), SRY1532 (DraIII), 92R7 and LLy22 g (HindIII).

Statistical analysis

Statistical analysis was performed using the statistical package SPSS for Windows (version 12.0.1, Chicago, IL, USA). We tested the significance of the observed difference in the incidence of partial *AZFc* deletions and *DAZ* duplication between the two study groups using Fisher's exact test. Our null hypothesis was that incidence is the same in infertile patients and normospermic men. Median values between groups were compared using a nonparametric Mann–Whitney *U* test or Student's *t* test after normalization of the distribution by a log transformation, as shown by a one-sample Kolmogorov–Smirnov test; $P = 0.023$. A P value of 0.05 was considered statistically significant for each test.

Results

Partial *AZFc* deletions

Frequency and type of partial AZFc deletions in patients and normozoospermic men

Based on the STS+/− analysis, it was possible to identify different types of *AZFc* partial deletions: gr/gr deletions (20/1,043) and b2/b3 (4/1,043). We did not identify any b1/b3 deletion in our study population. In all gr/gr and b2/b3 deletion cases, we found half copy number of *DAZ* and *CDY1,* except for one sample (A624) with gr/gr pattern, in which *CDY1* and *DAZ* gene dosage was normal (for details see below).

The gr/gr deletions were found in both infertile (18/556; 3.2%)—including A624—and normozoospermic men (2/487;

0.4%), with frequencies significantly different between the two groups [$P < 0.001$; odds ratio (OR) = 7.9; 95% confidence interval (CI) 1.8–33.8]. When only pure idiopathic patients were considered ($n = 284$), the frequency was raised to 3.9% and the OR to 9.4 (95% CI 2.1–42.2). In contrast, the frequency of b2/b3 deletion was not different between patients and controls (3/556, 0.5% vs. 1/487, 0.2%; n.s.).

DAZ and CDY1 gene copy definition

To further characterize the deletions and to distinguish between "false" deletions and deletion–duplication, we defined the type of missing *DAZ* (*DAZ1/DAZ2* or *DAZ3/DAZ4*) and *CDY1* (*CDY1a/CDY1b*) gene copies. In patient A624, by using gene dosage analysis, we found four *DAZ* copies and two *CDY1* copies, which was compatible either with a "false" deletion or with a "gr/gr deletion–b2/b4 duplication" event. With the RFLP analysis, we were able to identify only one type of *CDY1* (*CDY1a*) and *DAZ* pair (*DAZ3/4*), which clearly suggested that gr/gr deletion has occurred and that was followed by a b2/b4 duplication. In case of false deletion, we would have found two different *DAZ* duplets and two types of *CDY1* copies.

On the basis of the RFLP analysis, we distinguished four different deletion "subtypes": deletion of *DAZ1/DAZ2* + *CDY1a* (subtype 1), *DAZ3/DAZ4* + *CDY1a* (subtype 2), *DAZ1/DAZ2* + *CDY1b* (subtype 3), *DAZ3/DAZ4* + *CDY1b* (subtype 4). The additional SFV assays for each *DAZ* copy have been performed in 23 of 24 cases of partial *AZFc* deletions (Table 2). In 18 cases, results were in concordance with the sY587 assay, i.e., showing the removal of either the *DAZ1/DAZ2* or the *DAZ3/DAZ4* duplets. In four cases in which the removal of *DAZ3/DAZ4* was predicted according to sY587, Y-*DAZ3* (specific for *DAZ3*) and *DAZ*-SNV I (specific for *DAZ4*) analysis, the *DAZ*-SNV III showed the absence of the *DAZ2* copy as well. Given that the gene dosage indicated the presence of two *DAZ* copies and the *DAZ1*-specific SNV resulted also positive, we considered these patients as *DAZ3/DAZ4* deleted. In patient A624, the single *DAZ* copy SNV analysis showed the presence of three different *DAZ* copies (*DAZ1*, *DAZ3* and *DAZ4*), whereas the gene dosage detected the presence of four *DAZ* copies. We considered this patient as *DAZ1/DAZ2* deleted on the basis of the combined sY587 and *DAZ*-SNV III (showing the absence of *DAZ2*) results. In conclusion, discrepant results were detected regarding gene copy loss when multiple SFVs were compared in 27% (4/15) of *DAZ3/DAZ4* deletions and 13% (1/8) in *DAZ1/DAZ2* deletions. These figures are concordant with other studies in which these effects have previously been reported as a consequence of gene conversion (Repping et al. 2003; Lin et al. 2007; Navarro-Costa et al. 2007; Stouffs et al. 2008). The observed discordances between gene dosage and SFV analysis may be related to the variability in *DAZ* sequences among individuals (polymorphisms) or gene conversion in the *DAZ*-SNVs.

Given the position of the *CDY1b* copy in the reference sequence, deletions involving *CDY1b* can be explained either by invoking an inversion polymorphism of the P1 palindrome or by gene conversion of *CDY1a* to *CDY1b* prior to deletion. Concerning the latter hypothesis, Machev et al. (2004) report a predicted frequency of this specific gene conversion event of <1%, whereas a relatively high conversion rates for the same region were previously detected in human and primates (Rozen et al. 2003). The high percentage of subjects with *CDY1b* deletion (30%) in our study and in that of Machev et al. (2004) suggests a more likely involvement of an inversion polymorphism of the P1 palindrome rather than a gene conversion event. Nevertheless, much care must be taken when single RFLP analysis is performed for the prediction of partial *AZFc* deletions, since the Machev et al. paper has also reported that CDY1 SFV-based deletion analysis may lead to high false deletion rate. Our data therefore indicate the importance of a combined gene dosage and multiple RFLP analysis to improve the reliability of the proposed deletion mechanisms.

In our 20 subjects bearing a gr/gr deletion, we found all deletion subtypes, although at different frequencies: subtype 1 (5/20; 25%), subtype 2 (9/20; 45%), subtype 3 (3/20; 15%), subtype 4 (3/20; 15%). Accordingly, *CDY1a* was missing in 70% of cases. The four subjects with b2/b3 deletion belong to subtypes 2 (1/4, 25%) and 4 (3/4, 75%) and, thus, in all b2/b3 deletions, the *DAZ3/4* gene pair was missing.

Genotype–phenotype correlation

gr/gr and b2/b3 deletions were associated with a wide range of sperm counts, from oligozoospermia to normozoospermia. We found no azoospermic men with partial *AZFc* deletions. The phenotype of gr/gr deletion carriers is reported in Table 3. The only subject with gr/gr deletion–b2/b4 duplication (A624) was moderate oligozoospermic (sperm concentration: 9.4 million spermatozoa/ml; progressive motility: 15%; normal morphology 19%). In our study population, *CDY1a* deletion was a specific feature of the patient group and the difference between the frequencies of gr/gr deletions with missing *CDY1a* in patients (14/18, 78%) and controls (0/2, 0%) was significant ($P = 0.026$). Interestingly, the majority of the patients belonged to subtypes 1 and 2 (*CDY1a* and *DAZ1/2* or *DAZ3/4* deleted), whereas both normozoospermic controls with gr/gr deletion belonged to subtype 4, in which *DAZ3/4* and *CDY1b* were deleted.

The Y chromosome haplogroup in patients and controls bearing partial *AZFc* deletions are reported in Table 3, for

Table 2 Summary of *DAZ* SFV analysis

Code	Deleted *DAZ* copies (sY587)	*DAZ*1 (*DAZ*-SNV II)	*DAZ2* (*DAZ*-SNV *III*)	*DAZ3* (*Y-DAZ3*)	*DAZ*4 (*DAZ*-SNV I)
Infertile men with gr/gr deletion					
A170[a]	3/4	+	−	−	−
A186	1/2	−	−	+	+
A202	1/2	−	−	+	+
A234	3/4	+	+	−	−
A239	1/2	−	−	+	+
A286[a]	3/4	+	−	−	−
A322	3/4	+	+	−	−
A500	3/4	+	+	−	−
A522	1/2	−	−	+	+
A624[a]	1/2	+	−	+	+
MMP55	1/2	−	−	+	+
MMP93	1/2	−	−	+	+
MMP109	3/4	+	+	−	−
MMP179	3/4	+	+	−	−
MMP259	3/4	n.d.	n.d.	n.d.	n.d.
MMP289	3/4	+	+	−	−
MMP345[a]	3/4	+	−	−	−
MMP551	1/2	−	−	+	+
Normozoospermic men with gr/gr deletion					
CS111	3/4	+	+	−	−
CSS47	3/4	+	+	−	−
Infertile men with b2/b3 deletion					
A49	3/4	+	+	−	−
A353[a]	3/4	+	−	−	−
A590	3/4	+	+	−	−
Normozoospermic men with b2/b3 deletion					
CS64	3/4	+	+	−	−

The sY587 analysis allows the detection of presence/absence of *DAZ* duplets: *DAZ1/DAZ2* and *DAZ3/DAZ4*. For each *DAZ* copy, a specific SFV was further analyzed

"+" presence of the corresponding *DAZ* copy; "−" absence of the corresponding *DAZ* copy; *n.d.* not determined

[a] Samples with discordant results

further comparison with other studies. The low number of controls precludes any speculation about the effect of Y background on the phenotypic expression of partial *AZFc* deletions.

Sperm phenotype

To verify whether the presence of gr/gr deletion influences sperm parameters in cases and controls, we compared the means of the three principal sperm parameters among the two groups. The means of the three principal sperm parameters were not significantly different between subjects with and without gr/gr deletions, neither among patients nor among controls (Table 4).

Since a gr/gr deletion with missing *CDY1a* was observed only in patients, it seems to be more deleterious for spermatogenesis. Nevertheless, the means of the three principal sperm parameters were not significantly different between patients with *CDY1a* and *CDY1b* deletions (Table 4).

Partial *AZFc* duplications

Frequency of partial AZFc duplications in patients and normozoospermic men

Based on the *DAZ* and *CDY1* gene dosage, it was possible to identify partial duplications of *AZFc* region, which is characterized by a higher number of both *DAZ* and *CDY1* gene copies than the reference *AZFc* sequence (>4 and >2 for *DAZ* and *CDY1*, respectively). In all "duplication" cases, we found three copies of *CDY1* and six copies of *DAZ*, indicating a partial duplication of the region containing

Table 3 Phenotype of patients and controls bearing gr/gr deletions, b2/b3 deletions and partial *AZFc* duplication with the indication of gene copy deletion pattern defined on the basis of the type of *CDY1* and *DAZ* copy loss and Y hgr

Code	Phenotype	Y hgr	Deleted gene copies		Sperm parameters		
			DAZ	*CDY1*	Concentration (no. of sp/ml × 10^6)	Motility A + B (%)	Normal morphology (%)
Infertile men with gr/gr deletion							
A170	Idiopathic	J	3/4	a	0.9	20	16
A186	Varicocele[a]	J	1/2	a	0.6	0	2
A202	Idiopathic	R1b1c	1/2	a	10	30	13
A234	Varicocele[a]	E	3/4	a	0.7	3	8
A239	Idiopathic	R1b1c	1/2	a	4.2	14	16
A286	Idiopathic	I	3/4	b	0.01	–	–
A322	Idiopathic	G	3/4	a	1	10	15
A500	Idiopathic	F[b] (×G, H, I, J)	3/4	a	2.2	10	3
A522	Prostatitis	R1b1c	1/2	b	4.3	20	21
A624[b]	Varicocele[a]	G	1/2	b	9.4	15	19
MMP55	Idiopathic	R1b1c	1/2	a	9.0	6	25
MMP93	Varicocele[a]	J	1/2	b	6.0	7	26
MMP109	Varicocele[a]	K2	3/4	a	7.0	9	23
MMP179	Idiopathic	K2	3/4	a	2.0	–	–
MMP259	Idiopathic	n.d.	3/4	a	7.0	6	20
MMP289	Idiopathic	R1b1c	3/4	a	3.0	–	–
MMP345	Varicocele[a]	J	3/4	a	12.0	3	27
MMP551	Idiopathic	J	1/2	a	11.0	4	21
Normozoospermic men with gr/gr deletion							
CS111		DE	3/4	b	153.0	63	30
CSS47		E	3/4	b	60.0	47	32
Infertile men with b2/b3 deletion							
A49	Idiopathic	E	3/4	b	0,4	10	13
A353	Idiopathic	F	3/4	a	10.0	15	22
A590	Varicocele[a]	E	3/4	b	3.9	46	8
Normozoospermic men with b2/b3 deletion							
CS64		N3	3/4	b	100.0	78	40
Infertile patients with partial AZFc duplication							
A395	Idiopathic	Y[b] (×A, D, E, J, K)			4.4	8	9
A413	Idiopathic	Y[b] (×A, D, E, J, K)			0.01	–	–
A429	Idiopathic	DE			0.0	0	0
A535	Varicocele[a]	K[b] (×N3, P)			0.5	1	16
A647	Prostatitis	P			2.5	19	12
MMP493	Idiopathic	P			10.0	7	19
Normozoospermic controls with partial AZFc duplication							
C74		F			110.0	55	31
C90		J			110.0	69	41
CS26		F			100.0	51	34
CS68		F			41.0	69	39
CS76		F			55.0	76	37
CS88		P			150.0	67	34
CS161		J			60.0	57	44

Table 3 continued

Code	Phenotype	Y hgr	Deleted gene copies		Sperm parameters		
			DAZ	*CDY1*	Concentration (no. of sp/ml × 10^6)	Motility A + B (%)	Normal morphology (%)
MM85		Y[b] (×A, D, E, J, K)			38.0	51	50
MM103		Y[b] (×A, D, E, J, K)			69.0	50	43
MM110		Y[b] (×A, D, E, J, K)			37.0	50	37

n.d. not determined

[a] In all cases, varicocele was unilateral and grade < 2

[b] gr/gr deletion–b2/b4 duplication

Table 4 Means ± SD of the three principal semen parameters (A) in patients with gr/gr and b2/b3 deletion and without partial *AZFc* deletions, and in controls with and without gr/gr deletion, and (B) in patients and controls with and without partial *AZFc* duplication

		Sperm concentration (no. of sp/ml × 10^6)	Motility A + B (%)	Morphology (%)
A	Patients			
	With gr/gr deletion (n = 18)	5.0 ± 4.0	9.9 ± 8.1	16.7 ± 7.9
	With b2/b3 deletion (n = 3)	4.8 ± 4.9	23.5 ± 19.2	14.3 ± 7.1
	Without partial *AZFc* deletions (n = 535)	5.4 ± 5.4	10.4 ± 11.6	17.5 ± 7.2
	Patients with gr/gr deletion[a]			
	CDY1a copy loss (n = 14)	5.0 ± 4.2	8.8 ± 8.4	15.4. ± 8.2
	CDY1b copy loss (n = 4)	4.9 ± 3.9	14 ± 6.5	22 ± 3.6
	Controls[b]			
	With gr/gr deletion (n = 2)	106.5 ± 65.8	55.0 ± 11.3	31.0 ± 1.4
	Without gr/gr deletion (n = 485)	91.2 ± 63.8	58.2 ± 10.8	38.7 ± 8.6
B	Patients[c]			
	With partial *AZFc* duplication (n = 6)	2.9 ± 3.9	11.3 ± 6.7	14.0 ± 4.4
	Without partial *AZFc* duplication (n = 223)	5.5 ± 6.9	13.6 ± 13.6	15.4 ± 6.4
	Controls[c]			
	With partial *AZFc* duplication (n = 10)	77.0 ± 38.4	59.5 ± 9.8	39.0 ± 5.7
	Without partial *AZFc* duplication (n = 253)	90.4 ± 57.9	59.8 ± 9.7	38.6 ± 7.7

[a] The mean values of three principal semen parameters in gr/gr deletion carriers with *CDY1a* and *CDY1b* copy loss are separately shown

[b] Means for b2/b3 deletions are not reported for controls, since only one carrier was found in this group

[c] No significant differences were found for any of the three sperm parameters between carriers of partial deletion or duplications and noncarriers

both gene families. The frequency of partial duplications in our study population was 16 of 492 (3.2%). Their frequency in patients (6/229; 2.6%) versus controls (10/263; 3.8%) was not significantly different (P = 0.461) even when the comparison was restricted to the group of "idiopathic" infertile patients (4/120; 3.3%) versus controls.

Genotype–phenotype correlation

Partial *AZFc* duplications were associated with a wide range of sperm count, from azoospermia to normozoospermia (Table 1). The means were not significantly different between subject with and without partial *AZFc* duplication, neither among patients nor among controls (Table 3).

Discussion

This study reinforces our previous finding and provides strong evidence that gr/gr deletion is a risk factor for impaired sperm production in a Caucasian population such as the Italian. It is the largest study population in the literature in which patients and controls were ethnically and geographically strictly matched (excluding recruitment bias) and in which the gene dosage combined to RFLP analysis confirmed in all cases the loss of genetic material (deletion) or the presence of a deletion followed by b2/b4 duplication (excluding false deletions). This data are in agreement with the recent meta-analysis (Tuttelmann et al. 2007), which, despite the multitude of biases of published studies,

were able to detect a significant association OR = 1.81 ($P < 0.001$). In our Central Italian study population, gr/gr deletion carriers have a 7.9-fold increased risk of having impaired spermatogenesis compared with men without such a deletion. According to Repping et al. (2003), if gr/gr deletions were selectively neutral, then population genetic theory suggests that more than 40% would be gr/gr-deleted. The combined frequency of gr/gr deletions in our study population is 1.9%. While the prevalence of the complete *AZFc* deletion—which specifically causes spermatogenic failure—is less than 0.03% (i.e. approximately the rate at which new deletions arise), the relatively high prevalence of gr/gr deletions reflect a combination of low penetrance and high mutability (the target of homologous recombination is three times the size of the target of the complete *AZFc* deletion) (Repping et al. 2003). In our study population, all gr/gr deletion carriers had spermatozoa in their ejaculates and 9 of 20 were moderate/mild oligozoospermic. This finding further supports the hypothesis that gr/gr deletions do not induce a drastic phenotypic effect. We attempted to define whether the molecular characterization of gr/gr deletions may lead to the distinction between "pathogenic" and "neutral" deletions. Although we found that our two normozoospermic controls clustered in subtype 4 (*DAZ3/4*- and *CDY1b*-deleted), which was shared only by 1 of 18 patients, and *CDY1a* deletion was a specific feature of the patient group, conclusions about the subtypes cannot be drawn due to the low number of deletion carriers. However, a similar prevalence of gr/gr deletions removing the *DAZ1/DAZ2* copies was reported in infertile men of Caucasian origin by other groups (Fernandes et al. 2002; Ferlin et al. 2005). Interestingly, a similar observation was made also in the Chinese Han population in which only the sY1291/*DAZ1/DAZ2* deletion (and not the sY1291/*DAZ3/DAZ4* deletion) is a significant risk factor for spermatogenic impairment (Yang et al. 2008). Recently, Navarro-Costa et al. (2007) have also attempted to further characterize partial *AZFc* deletion subtypes with amplicon-specific sequence markers. They found highly heterogeneous *AZFc* deletion products in terms of amplicon content; however, no specific subtype was identified for infertile or fertile men. It is worth to note that the sperm count of fertile controls were unknown; therefore, the conclusion of Costa et al. (2007) remains limited to the "fertility" status.

Our study is in contrast with a number of others (Table 1). Among the most plausible explanations are the following: (1) methodological differences (lack of confirmation of deletions by gene dosage); (2) inappropriate selection of controls (unknown sperm count); (3) lack of ethnic and/or geographic matching of the patients versus controls. This latter bias is especially relevant in populations with a high proportion of subjects with constitutive gr/gr deletions, in which the deletion may be compensated by other Y-related factors. Consequently, case–control studies from these countries and those based on admixed populations (de Llanos et al. 2005; Ravel et al. 2006; Stouffs et al. 2008) are especially susceptible to recruitment bias.

Different clinical inclusion criteria applied for the selection of patients and controls are also an important issue. The highest deletion frequency was found among oligozoospermic men and considering only this group of patients the OR in our study rises to 9.1 ($P < 0.001$; 95% CI 2.1–38.8). On the contrary, it is highly likely that idiopathic azoospermia is not caused by a risk factor but is rather related to causative mutation(s) in essential spermatogenic genes. Studies including a high proportion of azoospermic in respect to oligozoospermic men may therefore miss those subjects, which are the more likely carriers of gr/gr deletions. Apart from the heterogeneous semen phenotypes, patient exclusion criteria are also extremely different among different studies. It is clearly shown in Supplementary Table 1 that only a few studies provided evidence about the exclusion of all known causes of impaired spermatogenesis in their patient group. Therefore, the association between gr/gr deletion and spermatogenic failure may be weakened or lost by a relatively high proportion of patients with known causes of spermatogenic failure. This potential selection bias may not be crucial when patients with first grade varicocele or a history of previous (but not recurrent) infection are included, because these pathological conditions are not proven causes of impaired spermatogenesis and can be found frequently also in normospermic men. In fact, although our study population contained also patients with the aforementioned mild abnormal andrological findings, we still observed a significantly higher gr/gr deletion frequency in the whole patient group in respect to controls. Moreover, the frequency between the two patient subgroups were similar (3.9% in the "idiopathic" and 2.6% in the "cofactor" group) and significantly different in respect to the control group, with a P value < 0.001 in the "idiopathic" (OR 9.4; 95% CI 2.1–42.2) and $P < 0.01$ in the "cofactor" group (OR 6.3; 95% CI 1.3–29.9). Also the composition of the normoozoospermic group may influence the outcome of a case–control study, since even in populations in which gr/gr deletion is relatively frequent in normozoospermic men (Asian), the deletion frequency drastically decreases in subgroups with sperm counts >50 millions spermatozoa/ml (Yang et al. 2006).

In our study, we avoided all potential methodological and selection biases thereby providing highly reliable data about a significant association between gr/gr deletion and spermatogenic failure (OR = 7.9). Although we already found a significant association in our previous study on 339 subjects, obviously the clinical meaning of the same finding in >1,000 subjects is drastically different and represents a unique example in the field (Table 5). In fact, the lack of

Table 5 Comparison of gr/gr deletion frequencies between the following three different study populations: Giachini et al. (2005); replication of the previous study on an independent sample set; combination of the two study populations

	gr/gr deletion frequency		Chi-square test	
	Patients Number of gr/gr del/total number	Controls Number of gr/gr del/total number	OR (95% CI)	*P* values
Giachini et al. (2005)	8/150 (5.3%)	1/189 (0.5%)	10.2 (1.3–80.3)	0.012
New study population (2005–2008)	11/435 (2.5%)	1/298 (0.3%)	7.5 (1.0–58.1)	0.022
Combined study population	18/556[a] (3.2%)	2/487 (0.4%)	7.9 (1.8–33.8)	<0.001

[a] Twenty-nine subjects with a history of monolateral cryptorchidism originating from the Giachini et al. 2005 study population were not included in the combined study

replication of case–control association studies is a rather common phenomenon, and in general the first study tends to overestimate the disease protection or predisposition conferred by a genetic polymorphism (Ioannidis et al. 2001; Krausz and Giachini 2007). Our finding implies that gr/gr deletion screening, by identifying a cofactor for impaired sperm production, may have diagnostic value. Moreover, since this genetic risk factor will be obligatorily transmitted to the male offspring, it is also relevant for genetic counselling, i.e., to inform the couple about the transmission of a predisposition to impaired sperm production.

Concerning partial *AZFc* duplications, we were unable to detect a significant effect of an excess *AZFc* gene dosage on spermatogenesis. Ours is the first study in a Caucasian population and the discordance with the only study reporting such an association, based on a Han-Chinese population (Lin et al. 2007), may reflect genuine ethnic differences or related to different sample sizes.

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