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The Y chromosome-linked CNVs and male fertility

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Abstract

Since the first definition of the AZF regions, the Y chromosome has become an important target for studies aimed to identify genetic factors involved in male infertility. This chromosome is enriched with genes expressed exclusively or prevalently in the testis and their absence or reduction of their dosage is associated with spermatogenic impairment. Due to its peculiar structure, full of repeated homologous sequences, the Y chromosome is predisposed to structural rearrangements, especially deletions/duplications. This review discusses what is currently known about clinically relevant Y chromosome structural variations in male fertility, mainly focusing on Copy Number Variations (CNVs). These CNVs include classical AZF deletions, gr/gr deletion and *TSPY1* copy number variation. AZF deletions are in a clear-cut cause-effect relationship with spermatogenic failure and they also have a prognostic value for testis biopsy. gr/gr deletion represents the unique example in andrology of a proven genetic risk factor, providing an eight-fold increased risk for oligozoospermia in the Italian population. Studies on *TSPY1* copy number variation have opened new perspectives on the role of this gene in spermatogenic efficiency. Although studies on the Y chromosome have importantly contributed to the identification of new genetic causes and thus to the improvement of the diagnostic workup for severe male factor infertility, there is still about 50% of infertile men in whom the etiology remains unknown. While searching for new genetic factors on other chromosomes, our work on the Y chromosome still needs to be completed, with special focus on the biological function of the Y genes.

Introduction

It has been known for many decades that the Y chromosome harbours the master gene for testis determination (*SRY*) and the so called AZoospermia Factor (AZF) regions, which contain genes involved in spermatogenesis. Structural anomalies, such as deletions and duplications of the AZF regions have been reported in association with male infertility and the screening for Y chromosome microdeletions became part of the routine diagnostic workup of men with severe spermatogenic impairment (1). Recently, a role in spermatogenesis for a multicopy gene family, the *TSPY1* array, has also been proposed and it has been demonstrated that *TSPY1* copy number influences spermatogenic efficiency.

In this review, we will discuss what is currently known about clinically relevant Y chromosome structural variations in male fertility, with special attention to Copy Number Variations (CNVs).

CNVs on the human Y chromosome: mechanism of formation

The Y chromosome is singular for its haploid nature which precludes recombination with the X-chromosome for most of its length. This has led to the consequent accumulation of a high proportion of segmental duplications which provide the structural basis for the generation of CNVs (2, 3). The presence of such duplicated sequences allows two mechanisms to occur: i) gene conversion; ii) non-allelic homologous recombination (NAHR). The first is an unidirectional conversion–based system of gene copy "correction" which permits the preservation of a certain number of Y genes from the gradual accumulation of deleterious mutations ensuring their continuity in time; on the other hand, NAHR produces recurrent deletions/duplications affecting the dosage of different Y genes (2, 4) (Fig.1).

CNVs on the human Y chromosome: who are they?

1. Y- chromosomal microdeletions: the AZF deletions

Microdeletions of the Y chromosome are the most frequent known genetic cause of spermatogenic failure in infertile men, second only to the Klinefelter syndrome (5). The first association between azoospermia (absence of spermatozoa in the ejaculate) and microscopically detectable deletions of the long arm of the Y chromosome (Yq) has been demonstrated by Tiepolo and Zuffardi, in 1976 (6). They proposed the existence of a spermatogenesis factor, the AZoospermia factor (AZF), encoded by a gene on distal Yq. With the development of molecular genetic tools it became possible to circumscribe the AZF region, in which microdeletions arise, and to highlight a certain deletion pattern with three recurrently deleted sub-regions in proximal, middle and distal Yq11, designated AZFa, AZFb and AZFc, respectively (7, 8) (Fig. 2).

Type of clinically relevant AZF deletions

The *AZFa* region is 792 Kb long and contains two single copy genes *USP9Y* and DDX3Y (former *DBY)* which are ubiquitously expressed. Complete *AZF*a deletions occurs after homologous recombination between identical sequence blocks within the retroviral sequences in the same orientation HERVyq1 and HERVyq2 (9-11).

The complete deletion of AZFb is caused by homologous recombination between the palindromes P5/proximal P1 which removes also part of the AZFc region belonging to P1 (8). This deletion removes 6.2 Mb (including 32 copies of genes and transcription units). The AZFc region includes 12 genes and transcription units, each present in a variable number of copies making a total of 32 copies. The complete deletion of AZFc removes 3.5 Mb, originates from the homologous recombination between amplicons b2 and b4 in palindromes P3 and P1, respectively (12). Deletions

of both AZFb and AZFc together occur in two breakpoints between P4/distal P1 (7.0 Mb, 38 gene copies removed) or between P5/distal P1 (7.7 Mb and 42 gene copies removed).

Clinical correlations of AZF deletions

The vast majority of complete AZF deletions are "de novo", although exceptional cases of transmission have been reported and pertain uniquely the complete AZFc deletion. However, "fertility" simply reflects that natural fertilization may occur even with relatively low sperm counts depending on the female partner's fertility status. AZF deletions are specific for spermatogenic failure as no deletions have been reported in the genomic DNA (derived from lymphocytes) of normozoospermic men (13). AZF deletions are likely to occur in germ cells during meiosis, when NAHR between sister chromatids may take place (figure 1). In order to investigate whether a testicular mosaicism for AZF deletions exist, we estimated the meiotic rate of AZFa deletion in 15 normozoospermic men. We found a meiotic deletion rate varying between 0.4 and 4.7 x 10^{-5}, implying that in an ejaculate containing several millions of spermatozoa, those bearing AZF deletions are several thousands (unpublished data).

Indications for AZF deletion screening are based on sperm count since clinical parameters such as hormone levels, testicular volume, varicocoele, maldescended testis, infections, etc. do not have any predictive value (14-16). The test is currently performed in all infertile men with < 5 millions/ml spermatozoa during the routine diagnostic workup. The highest deletion frequency is found in idiopathic azoospermic men (10%) who are more likely to be carrier of genetic anomalies . AZF deletions are less frequent in severe oligozoospermic men (2-5%) and have been exceptionally reported in mild oligozoospermic men. Since AZF genes are mainly expressed in the testis, a number of studies have been undertaken in order to clarify if AZF deletions may cause testis related pathologies other than spermatogenic failure. No final evidence for a cause–effect relationship was observed with varicocele, cryptorchidism and testis cancer (for review see (5) and references therein).

Apart from the diagnostic value, Yq deletion screening provides additional prognostic information for testicular biopsy (TESE) in azoospermic men. In fact, deletions removing the entire AZFa or AZFb regions ("complete" deletions) are associated with Sertoli Cell Only Syndrome (SCOS) and spermatogenic arrest, respectively, resulting in absence of mature spermatozoa in the testis. Therefore, the presence of such deletions represents a negative predictive factor for TESE and carriers are discouraged from undergoing this invasive procedure (17, 18). Such a strict genotype/phenotype correlation is lacking for both partial deletions of these regions (exceptionally rare events) and AZFc deletions, which are associated with a semen phenotype varying from oligozoospermia to azoospermia with different testis histology. An azoospermic man with complete AZFc deletion has an average chance of 50% for successful testicular sperm retrieval. This variable phenotype might be due to a progressive regression of the germinal epithelium over time leading from oligozoospermia to azoospermia. An alternative explanation for this phenotypic variability could be the influence of the genetic background (i.e. compensation for the absence of Yq genes, by autosomal or X-linked factors), the presence of 45X lines (a more severe phenotype) and environmental factors in different individuals.
In case the deletion is found in a man undergoing ICSI or TESE/ICSI, genetic counselling is mandatory in order to provide information about the risk of giving birth to a son with impaired spermatogenesis.

Diagnostic testing for AZF deletions

The diagnostic testing of Yq deletions should follow the EAA/EMQN guidelines (1). The standard procedure is based on PCR amplification of AZF specific STS primers and control markers. Although alternative methods have been proposed (19), the use of the method described in the guidelines is highly advised for its high specificity and sensibility (detection of clinically relevant deletions is close to 100%). It is worth noting that the MSY sequence and the mechanism underlying microdeletions have definitely established that a fourth *AZFd* region postulated by Kent-First et al. (20) and considered in a popular commercial kit does not exist.

2. Partial AZFc deletions and duplications

The AZFc region consists almost entirely of repetitive sequence blocks called 'amplicons' which are arranged in direct and/or inverted repeats (12, 21). The region contains multicopy genes expressed specifically in the testis and their dosage may vary according to different types of rearrangements.

The first AZFc candidate gene isolated from the AZFc region on the long arm of the human Y chromosome was *DAZ* (Deleted in AZoospermia), which is specifically transcribed in the adult testis (22). The *DAZ* gene belongs to a gene family consisting of three members: *BOULE* on chromosome 2 , DAZ-Like (*DAZL*) on chromosome 3 and *DAZ*, on the Yq. Members of this gene family are expressed exclusively in germ cells and encode testis-specific RNA-binding proteins that contain a highly conserved RNA-Recognition Motif (RRM) and a unique *DAZ* repeat (23).With regard to the reference sequence (corresponding to a Y chromosome belonging to haplogroup R), DAZ is present on the Y chromosome in four copies (*DAZ1*, *DAZ2*, *DAZ3* and *DAZ4*). The AZFc region also harbours *CDY1*, present in two copies (*CDY1a* and *CDY1b*). CDY protein products have been identified as histone acetyltransferases with a strong preference for histone 4 (24), thus are likely to be involved in both spermatogenic histone replacement and DNA transcription. Other genes involved in AZFc deletions are *BPY2*, the function of which is still unknown, and five transcription units *TTTY3*, *TTTY4*, *TTTY17*, *CSPG4LY* and *GOLGA2LY*.

Due to its structure, the AZFc region is particularly susceptible to NAHR events which may cause the formation of both partial deletions or duplications and therefore alter the AZFc gene dosage (figure 2). Although a number of different partial AZFc deletions have been described, only one of them resulted to be clinically relevant. This is the "gr/gr" deletion, named after the fluorescent probes ("green" and "red") used when it was detected for the first time (21). It removes half of the AZFc gene content, including two *DAZ* copies, one *CDY1* copy and one *BPY2* copy. The clinical significance of the gr/gr deletion has been object of a long debate. Controversies are mainly related to a number of selection biases (lack of ethnic/geographic matching of cases and controls; inappropriate selection of infertile and control men) and methodological issues (lack of confirmation of gene loss) (25-27). Moreover, another potential confounding factor derives from the fact that the frequency and phenotypic expression may vary among different ethnic groups, on the basis of the Y chromosome background; for example, in specific Y haplogroups, such as D2b, Q3 and Q1, common in Japan and certain areas of China, the deletion is fixed and apparently does not have any negative effect on spermatogenesis (28, 29). The presence of gr/gr deletion in Caucasian normozoospermic controls (although at a significantly lower frequency) prompted us to evaluate whether Y background could influence the phenotypic variability in Caucasians, as well (30). It has been previously described that the loss of *DAZ1/DAZ2* and *CDY1* is prevalent (or even specific) in carriers with impaired sperm production (31-33) while it was hypothesized that the restoration of normal AZFc gene dosage in case of gr/gr deletion followed by b2/b4 duplication may explain the lack of effect on sperm count (22). Using a combined method based on gene dosage and gene copy definition of *DAZ* and *CDY1* genes (31), we could identify four different subtypes of gr/gr deletions characterized by the loss of different gene copies and could assess the presence of deletion followed by duplication. Notwithstanding the detailed characterization of subtypes of gr/gr deletions based

on the type of missing gene copies and the detection of secondary rearrangements (deletion followed by b2/b4 duplication) together with the definition of Y haplogroups, it was impossible to define a specific pattern which would be associated with either a "neutral" or a "pathogenic" effect (30). Moreover, we also demonstrated that the restoration of normal gene dosage after secondary duplication is not specific for normozoospermic men. However, it is undeniable that the gr/gr deletion has some sort of effect even within the normal range of sperm count. It was observed, indeed, that normozoospermic carriers have a significantly lower sperm count, compared to men with intact Y chromosome (25). In addition, Yang et al. (34) reported that, in the Asian population, the deletion frequency drastically decreases in subgroups with sperm counts >50 millions spermatozoa/ml. More than 20 studies have been published during the last 7 years on this topic (35, 36). According to the largest study population published to date on Caucasians, gr/gr deletion is significantly more frequent among oligozoospermic men (3,4%) compared to normozoospermic men (0.4%) and gr/gr deletion carriers are at a 7.9-fold increased risk for spermatogenic impairment (OR= 7.9 (95% CI 1.8–33.8). As stated above, the heterogeneity of the study populations available in the literature thwarts the fulfillment of a reliable meta-analysis. Nevertheless, despite multiple biases three meta-analyses have been attempted on this topic all achieving significant Odd Ratios reporting on average a 2-2,5 fold increase of risk (25, 35, 36). The gr/gr deletion represents a unique example of a significant risk factor for impaired sperm production.

The screening for gr/gr deletion is based on a PCR method described by Repping et al. (21). However, given a 5% false deletion rate detected in a our multicenter study (30), deletions should be confirmed by gene dosage analysis.

The reasons why infertile men should undergo gr/gr deletion testing are mainly two: i) the deletion contributes to the etiopathogenesis of impaired sperm production since it is able to influence significantly the spermatogenic potential of the carrier; ii) the couple should be aware that the deletion (i.e. a genetic risk factor for impaired sperm production) will be obligatorily transmitted to their male offspring and may become a complete AZFc deletion (i.e. a clear-cut causative factor for spermatogenic impairment) in the next generations (37, 38).

Since a detailed characterization of Y chromosomes belonging to different lineages found limited variation in the copy number of Y-linked genes, it raised the possibility of selective constraints (39). At this regard, about 90% of men carries four DAZ copies implying that a normal spermatogenesis requires an optimal copy number and therefore both a reduction and an increase of AZFc gene dosage may have a negative effect. This observation prompted two research groups to study the clinical consequences of partial AZFc duplications (26, 40), but they reached to different conclusions, reporting an association between increased AZFc gene dosage and male infertility in the Han Chinese study and a lack of effect in our Italian study population. Since this discordance may reflect genuine ethnic differences, such as those observed for the corresponding partial AZFc deletions, if increased AZFc gene content is to play a role in spermatogenic impairment, the effect will probably be modulated by population specific factors. Further studies are needed to give proof for this hypothesis.

3. AZF gene-specific deletions

Despite the efforts of many laboratories, only five cases of confirmed (after sequencing the breakpoints) isolated Yq gene mutation have been reported to date (41). The rarity of single AZF gene-specific mutations or deletions is in sharp contrast with the relatively high frequency of AZF deletions (described above) and this might be a consequence of the peculiar organization of the Y chromosome, which makes it more prone to the loss of large portions - such as the AZF region - rather than single genes. The only reported isolated mutation occurs in the AZFa region which contains two widely expressed genes, *USP9Y* and *DDX3Y* (2). In the first place, sequencing of the

two genes in 576 patients brought to believe that the loss of *USP9Y* had a direct effect on spermatogenesis, causing azoospermia, whereas no mutation was found in the DDX3Y gene (42). However, following findings revealed that what seemed to be a definitive result turned out to be just one of the possible phenotypes related to *USP9Y* deletion. In fact, Luddi et al. (43) reported that this deletion has no effect on spermatogenesis and is thus compatible with fertility. On the other hand, previous studies (42, 44, 45) irrefutably demonstrate that the loss of the gene disturbs spermatogenesis to different degrees and that natural transmission is possible in case of a mild phenotype. Therefore, *USP9Y* has been proposed as a fine spermatogenic modulator (44), the absence of which is compatible with a highly variable phenotype probably linked to the genetic or other background of the carrier (42). Given the extreme rarity of AZF gene specific deletions and the heterogeneous phenotype of the *USPY9* deletion, the routine screening for AZF gene specific deletions is not advised.

4. *TSPY1* array

During the last years, Y-linked copy number variation analyses have been extended to the short arm of the Y chromosome which contains a *TSPY1* gene array with variable number of *TSPY1* copies (46, 47). The *TSPY1* belongs to a protein superfamily comprising SET and NAP, which are activating factors of the replication process. Indeed, *TSPY1* is abundantly expressed in early stages of tumorigenesis in gonadoblastoma and could be potentially involved in other human cancers (48). Expression analysis in the testis indicates the involvement of the TSPY1 in spermatogenesis as a pro-proliferative factor (48). In fact, TSPY1 is mainly expressed in gonocytes/ pre-spermatogonia of embryonic testis and in spermatogonia and spermatocytes at meiotic prophase I in adult testis. A role in early fetal germ cells development has also been addressed by Schoner et al. (49) who provided evidence of *TSPY1* ability to partially rescue spermatogenesis and fertility in transgenic Kit$^{w\text{-}v}$/Kit$^{w\text{-}v}$ mice.

TSPY1 is unusual in being arranged in a tandem array of 20.4 Kb of repeated units, bearing a single active *TSPY1* copy each (Fig. 2). Although copy number varies among individuals within a range of 11 to 76 (26, 50, 51), the majority of men (about 65 % of the Italian population) remain within a restricted interval (21 to 35 copies) (47). The evolutionary conservation of multiple *TSPY1* copies on the Y chromosome of other mammals as well as the above mentioned limited variation in copy number in humans suggest that a minimum *TSPY1* copy number is likely to be maintained through selection (52). Only few studies have focused on the eventual *TSPY1* influence on spermatogenesis and frustratingly they all reached three different conclusions, probably due to study design biases (26, 50, 51). Indeed, crucial for a reliable analysis is the *TSPY1* copy number variation susceptibility to stratification biases. As a matter of fact, significantly different means of *TSPY1* copy number were found among different Y haplogroups (46, 53), highlighting the importance of Y haplogroups-matching between cases and controls. The only available study to date in which cases and controls were matched for Y hgr distribution has been performed in the Italian population by our group. The method used for the detection of *TSPY1* copy number was validated against pulsed-field gel electrophoresis (the gold standard method) (46). The initially published study population has been recently enlarged and previous results confirmed i.e. a significantly lower *TSPY1* copy number in 212 infertile men with abnormal sperm parameters compared to 168 normozoospermic subjects (28.5 ± 7.9 vs. 32.6 ± 10.1, respectively; $p<0.001$) has been found. The relevance of *TSPY1* copy number variation in spermatogenesis is also attested by the positive correlation observed with sperm count both in infertile and normozoospermic subjects (fig. 3). In the light of these findings, low *TSPY1* copy number can be regarded as a new genetic risk factor for male infertility with potential clinical consequences and should be taken into consideration in the context of a multigenic approach to idiopathic infertility.

Conclusive remarks

The pivotal role of the Y chromosome in spermatogenesis is supported by the presence of Y-linked genes specifically expressed in germ cells and by pathological phenotypes deriving from the deletion of regions containing the genes mentioned above. Although Y studies have importantly contributed to the identification of new genetic factors in male infertility and thus to the improvement of the diagnostic work-up of severe male factor infertility, there are still many unsolved issues. Among them the most relevant are: i) the lack of knowledge about the exact function of AZF gene products; ii) the correlation, if existing, between *TSPY1* copy number and its level of expression; iii) lack of information about the consequences of AZFc gene dosage variation on mRNAs involved in spermatogenesis and eventually in embryogenesis. It is also unknown whether the "fragility" of the Y chromosome is a marker for general "genomic instability" potentially affecting the general health status of the Y deletion carrier. At this regard, gr/gr deletion has been reported as a risk factor also for testicular germ cell tumors but data needs further confirmation (54). Beside the Y chromosome-linked genes, several thousands autosomal and X-linked genes are predicted to play a role in the complex process of spermatogenesis. The two sex chromosomes share common features, in particular the peculiar repeated structure containing a number of multicopy gene families with testis specific expression. Therefore, we expect that similarly to the Y chromosome, also X-linked CNVs would affect gene dosage and thus be responsible for a portion of severe male factor infertility. Then, it seems high time to stop focusing only on the Y chromosome rearrangements and to start shifting our attention also on its partner, the X chromosome.

Figure legend:

Figure 1. *A*) Representation of two sister chromatids on which the four arrows represent homologous sequences at the border of recurrently deleted regions on the Yq (i.e. AZFa, AZFb, AZFc and "gr/gr" region). *B*) Schematic representation of the molecular mechanism responsible for deletions/duplications involving the AZF regions: interchromatidic Non-Allelic Homologous Recombination (NAHR), occurring between homologous sequences orientated in the same direction. *C*) Meiotic division of a spermatocyte gives origin to four spermatozoa: i) two bearing the X-chromosome (X); ii) two bearing the Y chromosome. If NAHR between sister chromatids occurs (see B), it will lead to a spermatozoon bearing: i) a Y chromosome with a duplication (Y+); ii) a Y chromosome with a deletion (Y-).

Figure 2. Schematic representation of the Y chromosome showing different regions/genes involved in spermatogenesis and Y-linked CNVs. A) AZFa, AZFb and AZFc regions are located on the long arm of the Y chromosome (Yq) with an overlap between AZFb and AZFc; the *TSPY1* gene is present on the short arm of the Y chromosome (Yp) arranged in a tandemly repeated array. B) AZFc region showing the location of multicopy genes and transcription units in the reference sequence (Y hapogroup R). The arrows with the same motifs represent repeated homologous sequences, which may undergo NAHR. C) The "b2/b4" deletion (complete AZFc deletion) removing all AZFc genes is depicted. Three alternative breakpoints for gr/gr deletion(s) are shown which all remove half of the AZFc gene content. An example of partial AZFc duplication (gr/gr) is shown (similarly to the gr/gr deletion, different breakpoints may give origin to different types of gr/gr duplications).

Figure 3**.** Scatter plots between *TSPY1* copy number and total sperm count in normozoospermic controls. Spearman's correlation coefficient (Rho) = 0,179; p= 0.021.

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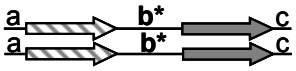
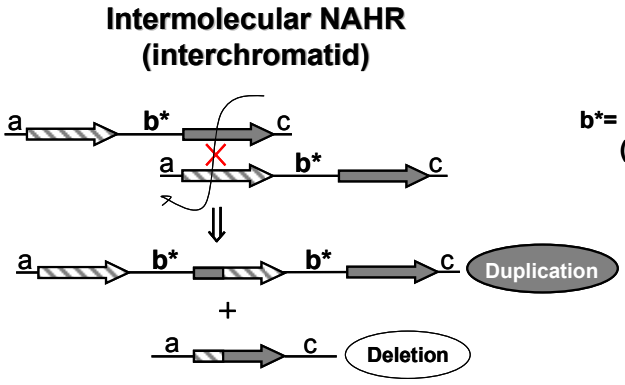
A)



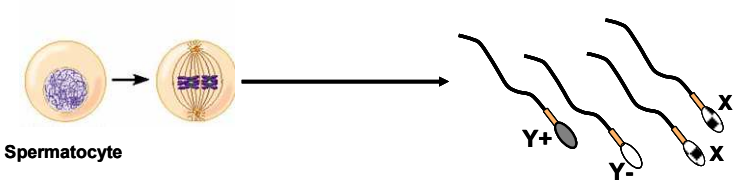
SISTER CHROMATIDS

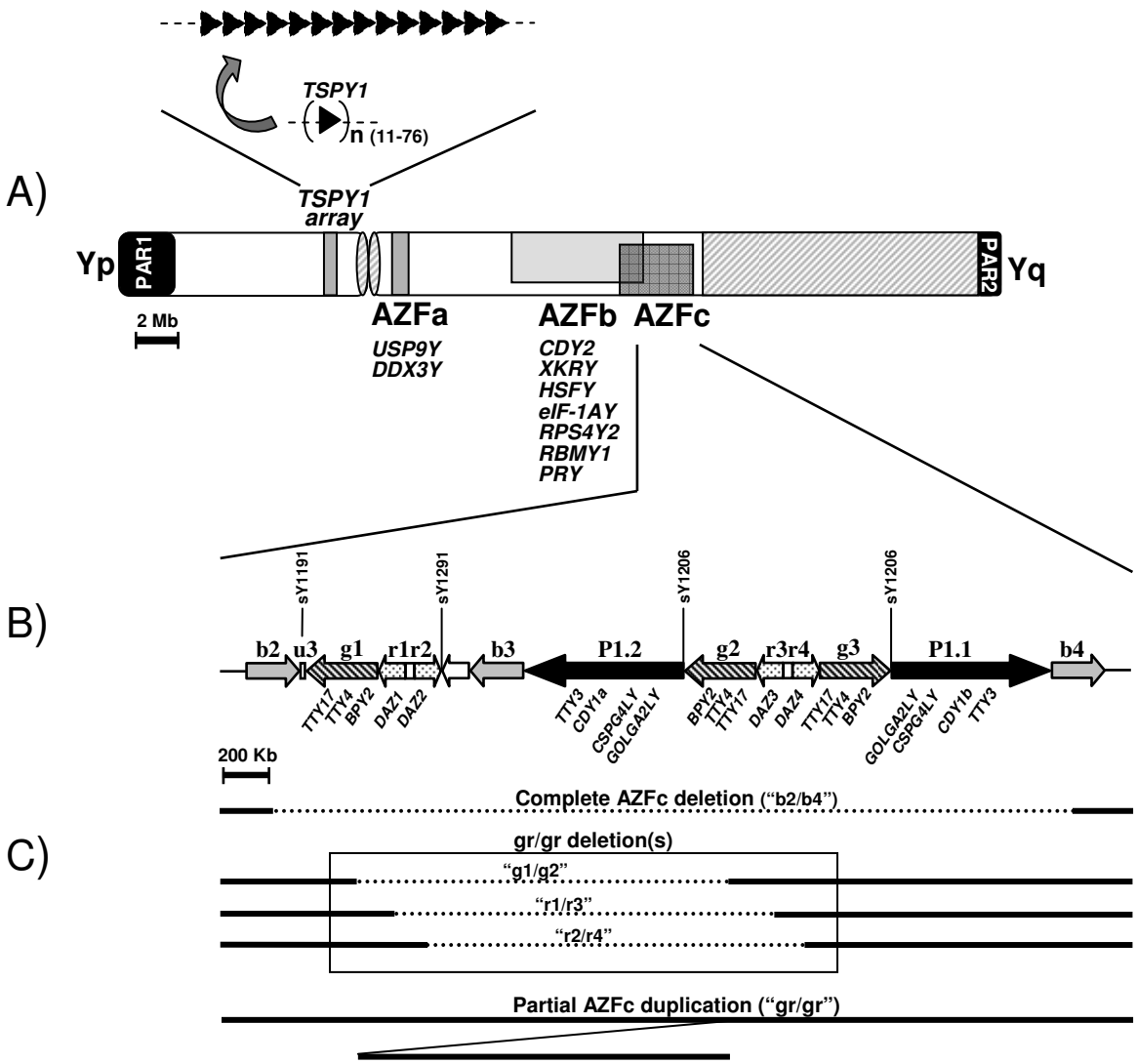
B)

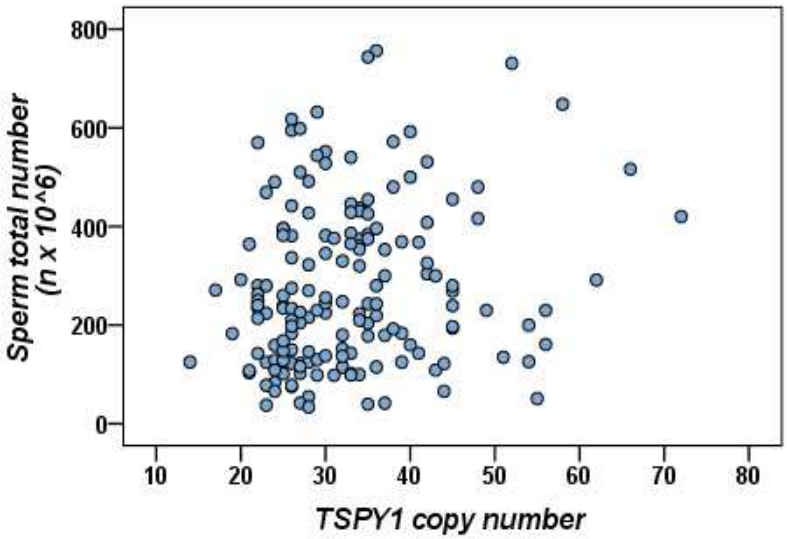


b*= recurrently deleted regions
(AZF and "gr/gr" regions)

C)




A)
TSPY1
n (11-76)
TSPY1 array
Yp
PAR1
PAR2
Yq
2 Mb
AZFa
AZFb
AZFc
USP9Y
DDX3Y
CDY2
XKRY
HSFY
eIF-1AY
RPS4Y2
RBMY1
PRY
B)
sY1191
sY1291
sY1206
sY1206
b2 u3 g1 r1r2 b3 P1.2 g2 r3r4 g3 P1.1 b4
TTY17 TTY4 BPY2 DAZ1 DAZ2
TTY3 CDY1a CSPG4LY GOLGA2LY
BPY2 TTY4 TTY17 DAZ3 DAZ4
TTY17 TTY4 BPY2 GOLGA2LY CSPG4LY CDY1b TTY3
200 Kb
C)
Complete AZFc deletion ("b2/b4")
gr/gr deletion(s)
"g1/g2"
"r1/r3"
"r2/r4"
Partial AZFc duplication ("gr/gr")


Sperm total number
(n x 10^6)
800
600
400
200
0
10
20
30
40
50
60
70
80
TSPY1 copy number