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ORIGINAL ARTICLE

# Association of partial AZFc region deletions with spermatogenic impairment and male infertility

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**Background:** Complete deletions of the AZFc region in distal Yq are the most frequent molecular genetic cause of severe male infertility. They are caused by intrachromosomal homologous recombination between amplicons—large, nearly identical repeats—and are found in 5–10% of cases of azoospermia and severe oligozoospermia. Homologous recombination may also generate different partial deletions of AZFc, but their contribution to spermatogenic impairment has not been confirmed.
**Methods:** In this study we analysed the prevalence and characteristics of different partial AZFc deletions and their association with spermatogenic failure. We studied 337 infertile men with different spermatogenic impairment and 263 normozoospermic fertile men using AZFc specific sequence tagged site markers and DAZ specific single nucleotide variants.
**Results:** We identified 18 cases of partial AZFc deletions in the infertile group (5.3%) and one case in the control group (0.4%). Seventeen deletions had the ''gr/gr'' pattern, one the ''b2/b3'' pattern, and one represented a novel deletion with breakpoints in b3 and b4 amplicons. Partial AZFc deletions were associated with different spermatogenic phenotypes ranging from complete azoospermia to only moderate oligozoospermia.
**Conclusions:** Together with published data, our analysis of DAZ gene copy suggested that the contribution of the different deletions to male infertility varies: only partial AZFc deletions removing DAZ1/DAZ2 seem to be associated with spermatogenic impairment, whereas those removing DAZ3/DAZ4 may have no or little effect on fertility. These data show that, beside complete AZFc deletions, specific partial deletions represent a risk factor for male infertility, even if with different effect on spermatogenesis.

The AZFc region in the distal Y chromosome long arm is critical for male fertility as it contains many gene families required for normal spermatogenesis. Deletions in this region are the most frequent molecular genetic cause of severe infertility, observed with a prevalence of 5–10% in cases of azoospermia and severe oligozoospermia.[1] AZFc deletions are generated by intrachromosomal homologous recombination between repeated sequence blocks called ''amplicons'' organised into palindromic structures showing a nearly identical sequence.[2 3] The first described and best characterised deletion of AZFc results from recombination between b2 and b4 amplicons,[2 4] spans 3.5 Mb, and removes all of AZFc. The association of this b2/b4 complete AZFc deletion with spermatogenic failure is well established.[1 2 4–7] The b2/b4 deletion removes all members of the DAZ gene family, which consists of four nearly identical copies arranged in two head to head clusters in amplicons r1–4,[2 8] and represents the stronger candidate responsible for the AZFc phenotype.

Initial studies have reported that even partial DAZ deletions (loss of copies of the DAZ genes) might cause spermatogenic impairment.[9–15] Such analyses were performed by FISH and Southern blotting methods and more recently by SNV (single nucleotide variant) analysis able to distinguish DAZ copies. However, the precise nature and size of these deletions were not determined by these studies, and their actual role in determining spermatogenic damage was unclear. Subsequent identification of the ampliconic structure of AZFc allowed better study of such partial AZFc deletions and identification of the underlying molecular mechanisms leading to deletions. Direct homologous recombination was found to give rise to two different partial AZFc deletions, both spanning 1.6 Mb: the gr/gr deletions,[16] which includes the g1/g2 deletion,[12] and the b1/b3 deletion.[16] Two other partial AZFc deletions are preceded by an inversion event: the gr/rg inversion is followed by a b2/b3 deletion (1.8 Mb)[17] and the b2/b3 inversion is followed by a gr/gr deletion (1.8 Mb)[17] or g1/g3 deletion (2.2 Mb).[18] The impact of all such partial AZFc deletions on spermatogenesis is still under consideration because some of the deletions are rare and confirmation by specific association studies is lacking.

We therefore set out to better clarify the prevalence and characteristics of all such partial AZFc deletions and their association with spermatogenic failure, by analysing infertile azoo-oligozoospermic patients and fertile normozoospermic men using a combination of AZFc specific sequence tagged site (STS) markers and DAZ gene SNV analyses.

## METHODS

### Subjects

The study was approved by the Institutional Ethics Committee and informed consent was obtained from each subject. We selected 337 infertile and 263 normozoospermic fertile men. All patients and controls were of Caucasian origin and came from northern Italy. On the basis of repeated semen analysis and according to World Health Organisation criteria,[19] infertile men were divided into three groups: 73 with non-obstructive azoospermia (no sperm in ejaculate even after centrifugation), 193 with severe oligozoospermia (sperm count <5 million/ml), and 71 with moderate oligozoospermia (sperm count 5–20 million/ml). Non-obstructive azoospermia was diagnosed after bilateral testicular fine needle aspiration cytological analysis.[20] Normozoospermia was considered when sperm concentration was >20 million/ml with normal sperm motility and morphology. All patients

**Abbreviations:** SNV, single nucleotide variant; STS, sequence tagged site

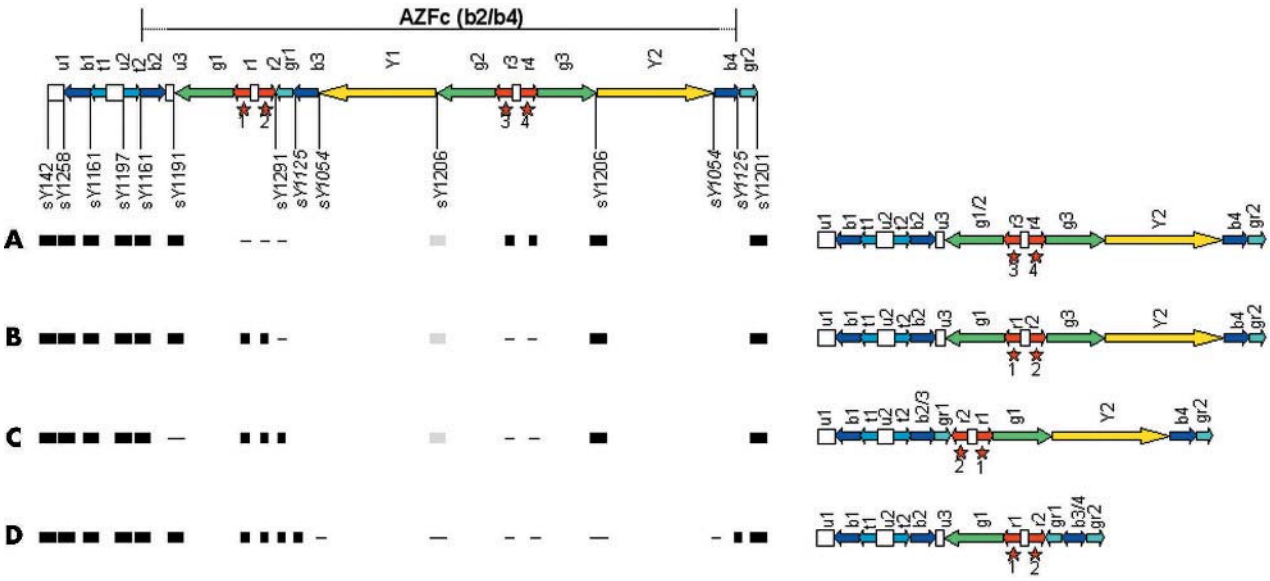


**Figure 1** Schematic representation of AZFc structure, and results of AZFc specific STS and DAZ specific SNV analyses. At the top the ampliconic structure of the AZFc region with the complete (b2/b4) deletion is shown. Red stars numbered 1 to 4 show the position of the four DAZ genes. Immediately below are indicated the STSs utilised to detect deletions, with italics indicating the STSs used to better define the novel deletion depicted in 'D' (see text). Below are the results for STS analysis (on the left) with the resulting organisation of AZFc amplicons (on the right). Black boxes: STS present; lines: STS absent; grey boxes: STS that normally amplifies by PCR but assumed to be absent in the context of the deletion pattern. (A and B) gr/gr deletions identified by the absence of sY1291. DAZ specific SNV analysis distinguishes between those with a DAZ1/DAZ2 deletion (A) and those with a DAZ3/DAZ4 deletion (B). (C) A b2/b3 deletion identified by the absence of sY1191. DAZ specific SNV analysis shows a DAZ3/DAZ4 deletion. (D) The novel deletion identified by the absence of sY1206 and named b3/b4 to indicate the amplicons where deletion breakpoints localise between sY1125 and sY1054. The corresponding organisation of AZFc palindromic structure is hypothetical because the specific deletion mechanism is unknown.

and controls exhibited a normal 46,XY karyotype and none had Y chromosome microdeletions in the AZF regions, as evaluated by multiplex PCR on genomic DNA extracted from peripheral leukocytes with the following STSs, as previously reported[15 21]: sY14-SRY on Yp as internal control; sY84, sY86, DF3.1 (for the USP9Y gene), and DBY1 (for the DBY gene) for the AZFa region; sY117, sY125, sY127, and F19/E355 (for the RBMY1 gene) for the AZFb region; and sY254 and sY255 (for the DAZ gene) for the AZFc region. This set of markers allows identification of deletions in AZFa and AZFb, and complete (b2/b4) AZFc deletion.

### STS analysis of AZFc

Analysis of AZFc specific STSs was performed on genomic DNA by PCR using as first step sY1291 (specific for the gr/gr-g1/g2 and b1/b3 deletions) and sY1191 (specific for the b2/b3 and rg/gr-g1/g3 deletions). Analysis was further confirmed with additional STSs: sY142, sY1258, sY1161, sY1197, sY1206, and sY1201 (fig 1). GenBank accession numbers and PCR conditions were as reported in the original reports.[2 16]

### SNV analysis of DAZ genes

Analysis of DAZ specific SNVs for determination of DAZ gene copy numbers was carried out for three SNVs (sY581, sY587, and SNVII) by a PCR amplification-restriction digestion assay and confirmed by direct sequencing, as previously reported.[15] sY581-Sau3A distinguishes DAZ2/DAZ3 (that have a T at position 125) from DAZ1/DAZ4 (that have a C at position 125), sY587-DraI distinguishes DAZ1/DAZ2 (that have a T at position 146) from DAZ3/DAZ4 (that have a C at position 146), and SNVII-MboI distinguishes DAZ1 (that has a G at position 121) from DAZ2/DAZ3/DAZ4 (that have a A at position 121).

### Statistical analysis

Differences among frequencies were calculated with both $\chi^2$ test and Fisher's exact test (two sided). Probability (p) values $<0.05$ were regarded as statistically significant.

## RESULTS

Using AZFc specific STS analysis we identified 18 cases of partial AZFc deletions among the infertile group (18/337, 5.3%) and one case among the normozoospermic fertile control groups (1/263, 0.4%) (table 1). This difference is statistically significant ($p<0.001$).

Seventeen deletions (including the one detected in the control group) resemble the gr/gr deletion with the absence of sY1291 and the presence of all the other STSs (fig 1A and B). In one case STS analysis identified a b2/b3 deletion with the absence of sY1191 and the presence of all the other markers (fig 1C). The last case showed a deletion pattern that differs from previously described deletions in AZFc, with the

**Table 1** Results of AZFc specific STS analysis in infertile and control groups

| | gr/gr del | b2/b3 del | b3/b4 del | Total partial AZFc deletions |
|---|---|---|---|---|
| Infertile azoo-oligospermic men | 16/337 (4.7%)* | 1/337 (0.3%) | 1/337 (0.3%) | 18/337 (5.3%)* |
| Fertile normospermic men | 1/263 (0.4%) | 0 | 0 | 1/263 (0.4%) |

*$p<0.001$ *v* fertile normospermic men.

**Table 2** Results of AZFc specific STS analysis in infertile azoo-oligospermic men

| | gr/gr del | b2/b3 del | b3/b4 del | Total partial AZFc deletions |
|---|---|---|---|---|
| Azoospermia | 3/73 (4.1%) | 0 | 0 | 3/73 (4.1%) |
| Severe oligospermia | 10/193 (5.2%) | 1/193 (0.5%) | 0 | 11/193 (5.7%) |
| Moderate oligospermia | 3/71 (4.2%) | 0 | 1/71 (1.4%) | 4/71 (5.6%) |

Azoospermia: no sperm in ejaculate; severe oligospermia: sperm count <5 million/ml; moderate oligozoospermia: sperm count 5–20 million/ml.

absence of sY1206 and the presence of all the other markers. Additional STS analysis in this patient showed the presence of sY1125 (which lies in b3 and b4 amplicons) and the absence of sY1054 (which lies at the b3/Y1 and Y2/b4 boundaries). The proximal breakpoint localised therefore in b3 and the distal breakpoint in b4, within the 229 kb region bounded by sY1054 and sY1125 (fig 1D). The distal breakpoint, therefore, is identical to that found in the classical b2/b4 deletion. Although no clear deletion mechanism was evident for this case observing the reference structure of AZFc, we refer to this deletion as ''b3/b4'' indicating the amplicons where deletion breakpoints localise. This deletion seems to remove the block Y1-g2-r3-r4-g3-Y2 and therefore spans approximately 2.2 Mb. No cases of b1/b3 deletion (identifiable by the absence of both sY1191 and sY1291) were found. The prevalence of gr/gr deletions is statistically different between infertile azoo-oligospermic men and fertile normospermic groups (4.7% *v* 0.4%, $p<0.001$), whereas the prevalence of the b2/b3 and b3/b4 deletions was not different (table 1). Subgrouping of infertile men according to sperm concentration showed no differences in the prevalence of partial AZFc deletions among groups (table 2).

Analysis of DAZ specific SNVs allowed us to characterise the DAZ gene copy number in men presenting partial AZFc deletions. Combined analysis of the three SNVs clearly showed a DAZ1/DAZ2 deletion in four men and a DAZ3/DAZ4 deletion in seven men (table 3). In the remaining eight cases, the results were less clearly interpretable and could only be inferred on the basis of current knowledge of partial AZFc deletions based on homologous recombination between amplicons. We therefore expected that DAZ genes could be removed only in doublets (DAZ1/DAZ2 or DAZ3/DAZ4). These inconsistent results were due to sY581 and are probably related to the variability in DAZ sequence among individuals or gene conversion events. Polymorphisms in DAZ SNVs have been previously reported.[8 14 18] Nevertheless, in all cases the results of sY587 and SNVII were unambiguous and consistent with each other. Six of these eight cases were considered as DAZ1/DAZ2 deleted even if sY581 apparently suggested the absence also of DAZ4, and two cases were considered as DAZ3/DAZ4 deleted, even if sY581 apparently suggested the absence also of DAZ2 (table 3). Taken together, of the 17 gr/gr deletions, 10 showed a pattern compatible with the loss of DAZ1/DAZ2 genes and could therefore be classified as the g1/g2 subtype (fig 1A), and seven a pattern compatible with the loss of DAZ3/DAZ4, including the one detected in the control groups (fig 1B). The man with a b2/b3 deletion showed absence of DAZ3/DAZ4 (fig 1C), as did the man with the b3/b4 deletion (fig 1D). The combined analysis of AZFc specific STSs and DAZ specific SNVs in the different groups of infertile men showed no differences in the prevalence of gr/gr deletions with loss of DAZ1/DAZ2 genes and with loss of DAZ3/DAZ4 (table 4). However, the prevalence of gr/gr-DAZ1/DAZ2 deletions in infertile men (10/377, 3.0%) was significantly different compared to that found in normozoospermic men (0/263, $p<0.05$), whereas there was no difference with regard to the gr/gr-DAZ3/DAZ4 deletion (6/337, 1.8% *v* 1/263, 0.4%) (table 4). No DAZ gene deletions were found with SNV analysis in men with normal AZFc specific STS analysis.

Three men with a gr/gr deletion with loss of DAZ3/DAZ4 were brothers who were referred independently to our centre for infertility problems (fig 2). They presented very different seminal patterns, ranging from azoospermia (V1), to severe oligozoospermia (V2), to moderate oligozoospermia (V3). Analysis of the father's DNA (V6) showed that he carried the same gr/gr deletion with loss of DAZ3/DAZ4. No semen analysis was available for the father due to his advanced age (76 years). Further family investigation showed the presence of additional two brothers, both carrying the same gr/gr-DAZ3/DAZ4 deletion. One of them had naturally fathered a child at 28 years of age (V5) and was therefore considered to be fertile even though no semen analysis was available. The fertility status of the other brother (V4) was undetermined and he gave no consent for semen analysis. Unfortunately, DNA for male relatives of the other 16 partial AZFc deleted patients could not be obtained. No other cases of relatives were present among patients.

## DISCUSSION

We found an increased prevalence of partial AZFc deletions in infertile men with spermatogenic impairment compared to normozoospermic fertile men, suggesting that such mutations represent a risk factor for male infertility. This prevalence is high (5.8%) considering that our screening was performed on unselected cases. No substantial difference was observed with respect to the degree of spermatogenic failure. In fact, association of partial AZFc deletions was found not only with azoospermia and severe oligozoospermia in a manner similar to complete AZFc deletions, but also with

**Table 3** Results of DAZ specific SNV analysis

| | n | DAZ1/DAZ2 deletion | n | DAZ3/DAZ4 deletion |
|---|---|---|---|---|
| sY587 | 10/10 | Absence of 122 and 73 bp fragments<br>Sequencing: C | 9/9 | Absence of 195 bp fragment<br>Sequencing: T |
| SNVII | 10/10 | Sequencing: A | 9/9 | Sequencing: A/G |
| sY581 | 4/10 | All fragments<br>Sequencing: T/C | 7/9 | All fragments<br>Sequencing: T/C |
| | 6/10 | Absence of 189 bp fragment*<br>Sequencing: T* | 2/9 | Absence of 130 bp fragment†<br>Sequencing: C† |

sY581 and sY587 were both subjected to fragment analysis on acrylamide gel and sequencing, whereas SNVII was only sequenced.
*This result conflicts with that of the sY587 and SNVII assay because it suggests the absence also of DAZ4; †this result conflicts with that of the sY587 and SNVII assay because it suggests the absence also of DAZ2.

**Table 4** Combined data of AZFc specific STS and DAZ specific SNV analyses in men with the gr/gr deletion

| | DAZ1/DAZ2 deletion | DAZ3/DAZ4 deletion | Total |
|---|---|---|---|
| Azoospermia | 2/73 (2.8%) | 1/73 (1.4%) | 3/73 (4.1%) |
| Severe oligospermia | 6/193 (3.1%) | 4/193 (2.1%) | 10/193 (5.2%) |
| Moderate oligospermia | 2/71 (2.8%) | 1/71 (1.4%) | 3/71 (4.2%) |
| Total | 10/337 (3.0%)* | 6/337 (1.8%) | 16/337 (4.7%) |
| Normozoospermia | 0/263 | 1/263 (0.4%) | 1/263 (0.4%) |

*$p<0.05$ *v* normozoospermia.

moderate oligozoospermia. Among partial AZFc deletions, the great majority were of the gr/gr subtype, and we found only one b2/b3 deletion and one novel deletion that we named b3/b4, indicating the amplicons where the deletion breakpoints localise. Clear association with spermatogenic impairment could therefore be drawn only for the gr/gr deletion. Analysis of DAZ gene copy number in these cases allowed us to identify two classes of gr/gr deletions, with possible different impacts on spermatogenesis. Deletions may remove DAZ1/DAZ2 genes or DAZ3/DAZ4 genes, but only the former are found exclusively in infertile men. Although with low frequency, the gr/gr deletion removing DAZ3/DAZ4 is found also in normozoospermic men, and is compatible with full fertility, as demonstrated by its finding in the father and one fertile brother of three infertile brothers. However, we cannot exclude a subtle influence of the gr/gr-DAZ3/DAZ4 deletion on spermatogenesis, because the frequency of this mutation in the fertile control group is much lower compared to the infertile group, and because at least three out of the five brothers with this type of deletion have spermatogenic damage and infertility.

Taken together, our findings are in agreement with and further support preliminary reports on partial AZFc deletions. These studies suggested that the gr/gr deletion[16] and the g1/g2,[12] which removes DAZ copies 1 and 2, indeed represented a risk factor for spermatogenic damage. In contrast, the b2/b3, rg/gr, and g1/g3 deletions, which remove DAZ copies 3 and 4, seemed to have no or little effect on fertility, probably representing common polymorphisms almost exclusively associated with the Y chromosome haplogroup N.[17 18] Therefore, if we assume that only partial AZFc deletions that remove the cluster DAZ1/DAZ2 are specifically associated with alterations in the spermatogenic process, the prevalence of clinically relevant deletions is 3.0% (10/337). This figure is significantly different with respect to controls, whereas the prevalence of the gr/gr-DAZ3/DAZ4 deletion is not different between patients and controls. Again, this figure is not different in men with azoospermia (2.8%), severe oligozoospermia (3.1%), or even moderate oligozoospermia (2.8%).

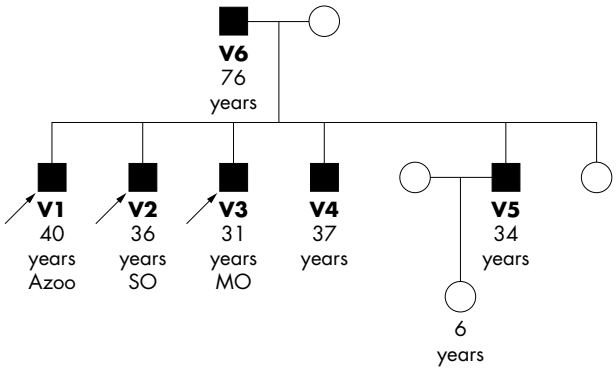


**Figure 2** Pedigree of the family with the gr/gr-DAZ3/DAZ4 deletion. Arrows indicate index patients. MO, moderate oligozoospermia; SO, severe oligozoospermia.

The reason for the different phenotypes observed in men with apparently identical deletions is not clear and probably reflects the contribution of other genetic or environmental factors. An age related contribution to this phenotypic difference may also be hypothesised. This possible effect has been previously supposed and also documented histologically in one case.[22] The familial case reported here does not add clear information on this topic. In fact, if we observe the five brothers, a possible effect of age on sperm production may be hypothesised, with the younger man fertile and the oldest completely azoospermic. However, their father naturally conceived for a period of 9 years from 36 to 45 years of age.

The AZFc region contains eight gene families (DAZ, BPY2, CDY1, CSPG4LY, GOLGA2LY, TTTY3, TTTY4, TTTY17) each with multiple copies, with a total of 21 copies (table 4). However, the functional contribution of such genes and transcription units to spermatogenesis is unknown and appears to be genetically redundant.[18] All partial AZFc deletions so far described reduce the copy number of such gene families, but a clear dosage effect on sperm production is not evident. They remove two copies of the DAZ genes and a variable number of the other genes: the gr/gr deletion subtype removes one copy of each of the other genes (a total of 12 genes left), and the b2/b3 deletion subtype also removes another copy of BPY2, TTTY4, and TTTY17 (a total of nine genes left) (table 5). The novel deletion described here (b3/b4) apparently removes additional genes with a total of only five genes left. Since this deletion was found in a man with moderate oligozoospermia, the contribution of genes such as CDY1, CSPG4LY, GOLGA2LY, and TTTY3 seems very limited. However, further studies are needed to ascertain the mechanism of deletion in this particular case and therefore to clarify these aspects. It should also be kept in mind that our screening is based on STS and SNV analyses that allow us to define deletions but not other mutations such as duplications, inversions, or other unexpected complex rearrangements. Such mutations and the resulting structures may be better defined by FISH[16] or Southern blotting.[12 18] For example, a combination of the deletion of DAZ1/DAZ2 or DAZ3/DAZ4 doublets with mutation in whichever of the DAZ gene family members is retained may also be hypothesised and not be identifiable by our screening. Such events may cause infertility and/or spermatogenic damage and may also explain some apparently ambiguous results obtained with SNV analysis.

In conclusion, besides complete AZFc deletions, partial deletions are also associated with impaired spermatogenesis. However, the contribution of the different partial AZFc deletions to male infertility varied. Deletions removing the DAZ1/DAZ2 cluster appear to represent an actual risk factor for spermatogenic impairment although the effect on final sperm production is not obvious, the phenotype ranging from complete azoospermia to moderate oligozoospermia. Although men with a partial AZFc deletion may naturally father children, in vitro fertilisation with sperm from these patients with deletions may transmit the mutation on to male children. The reintroduction of these partial deletions,

**Table 5** Number of genes present in the different partial AZFc deletions

| Genes | Reference Y chromosome | Partial AZFc deletion gr/gr with DAZ1/2 del | gr/gr with DAZ3/4 del | b2/b3 | b3/b4 |
|---|---|---|---|---|---|
| DAZ | 4 | 2 | 2 | 2 | 2 |
| BPY2 | 3 | 2 | 2 | 1 | 1 |
| CDY1 | 2 | 1 | 1 | 1 | 0 |
| CSPG4LY | 2 | 1 | 1 | 1 | 0 |
| GOLGA2LY | 2 | 1 | 1 | 1 | 0 |
| TTTY3 | 2 | 1 | 1 | 1 | 0 |
| TTTY4 | 3 | 2 | 2 | 1 | 1 |
| TTTY17 | 3 | 2 | 2 | 1 | 1 |
| Total | 21 | 12 | 12 | 9 | 5 |

and thus spermatogenic failure, into the population is of major concern. Clear conclusions as to whether screening for partial AZFc deletion (by AZFc specific STSs and DAZ specific SNVs) should be performed in the diagnostic workup of infertile male patients cannot be made, but accumulating evidence may suggest such analyses should be considered especially when men are candidates for assisted reproduction techniques.


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