ASSOCIATION OF PARTIAL AZFc REGION DELETIONS WITH SPERMATOGENIC IMPAIRMENT AND MALE INFERTILITY

A Ferlin, A Tessari, F Ganz, E Marchina, S Barlati, A Garolla, B Engl, C Foresta

ORIGINAL ARTICLE

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.

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, 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. 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
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\(^\text{15,21}\): 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 gr/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.\(^\text{21,6}\)

**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.\(^\text{15}\) 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

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

<table>
<thead>
<tr>
<th></th>
<th>gr/gr del</th>
<th>b2/b3 del</th>
<th>b3/b4 del</th>
<th>Total partial AZFc deletions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infertile azoo-oligospermic men</td>
<td>16/337 (4.7%)(^*)</td>
<td>1/337 (0.3%)</td>
<td>1/337 (0.3%)</td>
<td>18/337 (5.3%)(^*)</td>
</tr>
<tr>
<td>Fertile normospermic men</td>
<td>1/263 (0.4%)</td>
<td>0</td>
<td>0</td>
<td>1/263 (0.4%)</td>
</tr>
</tbody>
</table>

\(^*\)p<0.001 v fertile normospermic men.
DAZ SNVs have been previously reported.81 Nevertheless, individuals or gene conversion events. Polymorphisms in probably related to the variability in DAZ sequence among these inconsistent results were due to sY581 and are removed only in doublets (DAZ1/DAZ2 or DAZ3/DAZ4). AZFc deletions based on homologous recombination between only be inferred on the basis of current knowledge of partial cases, the results were less clearly interpretable and could showed a DAZ1/DAZ2 deletion in four men and a DAZ3/DAZ4 deletion in seven men (table 3). In the remaining eight showed a DAZ1/DAZ2 deletion absence of sY1191 and sY1291) were 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 15 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 2 Results of AZFc specific STS analysis in infertile azoo-oligospermic men

<table>
<thead>
<tr>
<th>AZFc status</th>
<th>Total partial AZFc deletions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gr/gr del</td>
</tr>
<tr>
<td>Azoospermia</td>
<td>3/73 (4.1%)</td>
</tr>
<tr>
<td>Severe oligospermia</td>
<td>10/193 (5.2%)</td>
</tr>
<tr>
<td>Moderate oligospermia</td>
<td>3/71 (4.2%)</td>
</tr>
</tbody>
</table>

Table 3 Results of DAZ specific SNV analysis

<table>
<thead>
<tr>
<th>SNV</th>
<th>n</th>
<th>DAZ1/DAZ2 deletion</th>
<th>n</th>
<th>DAZ3/DAZ4 deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>sY587</td>
<td>10/10</td>
<td>Absence of 122 and 73 bp fragments</td>
<td>9/9</td>
<td>Absence of 195 bp fragment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sequencing: C</td>
<td></td>
<td>Sequencing: T</td>
</tr>
<tr>
<td>SNVII</td>
<td>10/10</td>
<td>Sequencing: A</td>
<td>9/9</td>
<td>Sequencing: A/G</td>
</tr>
<tr>
<td>sY581</td>
<td>4/10</td>
<td>All fragments</td>
<td>7/9</td>
<td>All fragments</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sequencing: T/C</td>
<td></td>
<td>Sequencing: T/C</td>
</tr>
<tr>
<td>6/10</td>
<td></td>
<td>Absence of 189 bp fragment*</td>
<td>2/9</td>
<td>Absence of 130 bp fragment*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sequencing: T</td>
<td></td>
<td>Sequencing: C†</td>
</tr>
</tbody>
</table>

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.
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 and the g1/g2, 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 haplotype N.\textsuperscript{7,18} 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%).

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 historically in one case.\textsuperscript{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.\textsuperscript{16} 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\textsuperscript{14} or Southern blotting.\textsuperscript{22} 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,
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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Competing interests: none declared

REFERENCES

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

<table>
<thead>
<tr>
<th>Genes</th>
<th>Reference Y chromosome</th>
<th>Partial AZFc deletion</th>
<th>gr/gr with</th>
<th>gr/gr with</th>
<th>b2/b3</th>
<th>b3/b4</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAZ</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>BPY2</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CDY1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CSPG4L</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GOLGA2LY</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TTY3</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TTY4</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TTY17</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>21</td>
<td>12</td>
<td>12</td>
<td>9</td>
<td>5</td>
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</table>

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