A frequent Y chromosome b2/b3 subdeletion shows strong association with male infertility in Han-Chinese population

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BACKGROUND: Azoospermia factor c (AZFc) subdeletions were reported to be significant risk factors for spermatogenesis. In this study, we assessed the occurrence of classical AZF deletions and AZFc subdeletions and their impact on male infertility in a Han-Chinese population. METHODS: This study analysed a population of 699 subjects, including 451 idiopathic infertile patients with a range of fertility disorders and 248 fertile controls, using a retrospective design. Deletions were identified by multiplex PCR. RESULTS: The prevalence and phenotypes of the classical AZF deletions were similar to previous studies. Subdeletions of the AZFc region in patients showed similar overall frequencies in all sperm concentration categories of gr/gr (7.0%) and b2/b3 (8.9%). For controls, these subdeletions were also found with a prevalence of gr/gr (7.7%) and b2/b3 (3.2%). b1/b3 deletions were not found either in the patients or in the controls. CONCLUSIONS: Our data showed a higher frequency of deletion events in this Han-Chinese population than in populations elsewhere in the world. The classical AZF deletions were the primary genetic factors for spermatogenic failure, while no significant association was found for AZFc subdeletions with sperm concentration. However, the b2/b3 subdeletion was significantly associated with idiopathic male infertility (odds ratio, 2.93; 95% confidence interval 1.34–6.39) (P = 0.005), indicating a potential impairment of male fertility.

Key words: AZFc/b2/b3 subdeletion/gr/gr subdeletion/male infertility/Y chromosome microdeletion

Introduction

Y chromosome makes an essential contribution to the control of spermatogenesis. Azoospermia factors (AZF) (Tiepolo and Zerfardi, 1976) are located on the long arm of Y chromosome and has been divided into three regions (AZFa, AZFb, AZFc) (Vogt et al., 1996). With knowledge of the sequence in the AZF region (Skaletsky et al., 2003), the deletion events often occur between amplicons, which are long, and Y-specific repeats. These deletions, also called classical AZF deletions, usually cause spermatogenic failure (Simoni et al., 2004).

Now, the deletions have been thought to be derived from the homologous recombination of Y chromosome between amplicons. The AZFc region is particularly susceptible to deletions because its structure is completely composed of amplicons (Kuroda-Kawaguchi et al., 2001). For example, the b2/b4 deletion, which is thought to be the classical AZFc deletion, appears between the amplicon b2 and amplicon b4. It eliminates the whole region of AZFc, and is the most commonly known genetic cause of spermatogenic failure (Kuroda-Kawaguchi et al., 2001). After the ampliconic structure of AZFc was identified, more attention was paid to the study of the AZFc region and identification of the underlying molecular mechanisms leading to deletions. To date, some deletions resulting in partial loss of AZFc region have also been reported (Vogt, 2004). Two classes of subdeletion have been discriminated in the previous studies. The gr/gr type, which spans 1.6 Mb and occupies more than half of the AZFc region, is thought to be a risk factor for spermatogenic failure. This class of subdeletion is found to be caused by the direct homologous recombination which also accounts for another type, b1/b3 deletion (1.6 Mb) (Fernandes et al., 2002; Repping et al., 2003). Two other types belong to the second class and are preceded by an inversion event. The gr/rg inversion is followed by a b2/b3 deletion (1.8 Mb), while the b2/b3 inversion is followed by a gr/gr deletion (1.8 Mb) (Repping et al., 2004) or g1/g3 deletion (2.2 Mb) (Fernandes et al., 2004; Ferlin et al., 2005).

However, the frequencies of these deletions reported in different studies are not consistent with each other and the relationship between these subdeletions and spermatogenesis...
is still controversial (Machev et al., 2004; Repping et al., 2004; de Llanos et al., 2005; Giachini et al., 2005; Huckenbroich et al., 2005; Lynch et al., 2005; Zhang et al., 2005). All of the above indicated that geographical and ethnic differences might affect these deletions in the AZFc region, possibly in both the deletion patterns and the phenotypic expression. 

The aim of this study is to clarify better the prevalence and characteristics of all classical AZF deletions and AZFc subdeletions and examine their association with male infertility in a Han-Chinese population. These deletions in azoo–oligozoospermic patients and normozoospermic men are analysed using a combination of specific sequence-tagged site (STS) markers (Repping et al., 2003; Simoni et al., 2004).

Materials and methods

Study subjects
This study initially included 634 infertile men and 248 normozoospermic fertile men. All subjects were genetically unrelated ethnic Han-Chinese from eastern China. The patients with infertility were candidates seeking treatment in the Center of Clinical Reproductive Medicine from April 2004 to April 2006, (NJMU Infertile Study) on the basis of a retrospective design. The controls were healthy young men who volunteered for a study to examine the distribution of reproductive hormone levels and serum metabolites during the same time period as those of the cases recruited in the same hospital. The controls and the cases were matched by the age (±5 years) and ethnicity. A scheduled interview was arranged for each subject to collect demographic data, past medical and surgical history. After the interview, approximately 5 ml of venous blood sample was collected. All biological specimens were obtained from 634 patients and 248 controls. The study was approved by the Institutional Ethics Committee of Nanjing Medical University and Institutional Review Board. In addition, we obtained an informed consent from each subject.

Clinical patients
In total, 634 infertility patients were assessed. All patients went through physical examination, semen analysis, check of childbearing history, checks of FSH, LH, and testosterone and karyotype status. These tests excluded 183 individuals: three obstructive azoospermic cases, seven with cryptorchidism and 157 secondary sterility cases. Finally, we included 451 patients, whom aged from 25 to 38 years, with idiopathic infertility problem. On the basis of repeated semen analyses and World Health Organization criteria (WHO, 1999), idiopathic infertile patients were divided into three groups: 164 with non-obstructive azoospermia (no sperm in the ejaculate even after centrifugation), 78 with oligozoospermia (sperm count from 0.1 to 20 × 10⁶/ml) and 209 with normal spermatogenesis (sperm count ≥20 × 10⁶/ml).

Control subjects
Overall, 248 control subjects, whom aged from 26 to 40 years, were included. They were healthy young men with a normal reproductive history and normal physical examination. Because of the Family Planning in China, all controls have only one child. Moreover, all the children were newly born within one year. Of these controls, 86 took semen tests, according to WHO criteria, with an average sperm concentration of 53.6 ± 18.7 million/ml. An informed consent was obtained from each of them.

Y chromosome microdeletion analysis
Genomic DNA was extracted from peripheral leukocytes by means of proteinase K digestion followed by phenol–chloroform extraction and ethanol precipitation. Primer sequences and GenBank accession numbers were as described (Repping et al., 2003; Simoni et al., 2004 and GenBank).

Genotyping was performed without knowing the status of cases and controls in advance. Approximately equal numbers of the cases and the controls were assayed in each 96-well PCR plate with a positive control of a DNA sample which does not have these deletions. Two research assistants independently kept performing the repeated assays until an agreement was obtained on the tested genotype. In addition, ~10% of the samples (40 cases and 20 controls) were randomly selected for repeated assays and consistent results were obtained. The samples, in which the deletions were detected by the following multiplex systems, were confirmed by repeating the single primer (simplex) reactions.

Searching of Yq classical deletions
All patients with spermatogenesis impairment underwent the assessment for detecting classical AZFa, AZFb and AZFc (b2/b4) deletions. In order to confirm the specific relationship with spermatogenesis, we analysed the classical AZFc deletion in all patients and controls. Two multiplex PCR systems (A and B) were carried out according to the European Academy of Andrology/European Molecular Genetics Quality Network (EAA/EMQN) guidelines (Simoni et al., 2004).

Mapping of AZFc subdeletions
Patients without classical AZFa, AZFb and AZFc deletions and all control subjects underwent this assessment for AZFc subdeletions. The presence or absence of the AZFc subdeletions in all subjects was tested by multiplex PCR using genomic DNA, amplifying STS markers as described by Repping et al., 2003. The STSs were sY1191, sY1291, sY1206, sY1201, sY142, sY1258, sY1197, sY1054 and sY1161. The absence of amplification of the STS markers sY1291, sY1206, sY1191 and sY1054 as well as the presence of other STSs indicated a full AZFc deletion (b2/b4). The absence of markers sY1291, sY1191 and sY1161 combined with the presence of others indicated the b1/b3 deletion. Only the absence of amplification of sY1191 represented a b2/b3 (g1/g3) deletion, while the unique absence of sY1291 product showed a gr/gr deletion. Multiplex PCR of these STSs was performed in three groups in order to ease the size separation of multiplex PCR products on agarose gels. They were group 1: sY1191, sY1291 and sY142; group 2: sY1206, sY1201 and sY1161; and group 3: sY1258, sY1054 and sY1197. Multiplex PCR was performed using similar conditions for these three groups: 50 ng genomic DNA template using 2–8 pmol of each primer in 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl₂, 0.1% Triton-X100, 200 μM of each dNTP and 1 unit of Taq polymerase (Promega, USA) in a final volume of 20 μl. Amplification cycles consisted of an initial denaturation step at 94°C for 4 min, plus 35 cycles at 94°C for 30 s, annealing at 57°C, 61°C, 58°C (each for 45 s) and 72°C for 45 s, and a final extension of 72°C for 5 min. DNA amplification products were separated on 2% agarose gels in 1 × Tris–acetate–EDTA buffer and stained with ethidium bromide. PCR profiles identifying the different AZFc subdeletions are shown in Figure 1. The “Blank” bands (using pure water instead of genomic DNA) were used as Negative control.

Typing gr/gr and b2/b3 subdeletions
Qualitative and quantitative analysis for loss of deleted in azoospermia (DAZ) copy and Chromodomain of Y (CDY1a/CDY1b) was performed...
and their 95% confidence intervals (CI) with Woolf test. Male infertility were estimated by computing the odds ratios (ORs) from groups of sequence-tagged site markers (top). The control lane shows the PCR result when no concentration of $10^{10.66}$ was observed. Differences in frequencies of deletion types between cases and controls were calculated with the $\chi^2$ test according to Machev et al., 2004. For DAZ, we chose the sequence family variant (SFV) at STSs sY587, which distinguishes DAZ1/2 from DAZ3/4, and sY581, which distinguishes DAZ1/4 from DAZ3/4. For CDY1 we used an SFV situated 7750 bp 5’ of the CDY1 translation start codon (CDY7750), which distinguishes CDY1a from CDY1b.

### Statistical analysis

Differences in frequencies of deletion types between cases and controls were calculated with the $\chi^2$ test using Intercooled Stata 7.0. Probability ($P$) values $<0.05$ were regarded as statistically significant. The associations between gr/gr and b2/b3 subdeletions and the risk of male infertility were estimated by computing the odds ratios (ORs) and their 95% confidence intervals (CI) with Woolf test.

### Results

#### Screening for classical AZF deletions

Totally, 24 out of the 242 infertile men (9.9%) with spermatogenesis impairment were found to have classical AZF deletions in the AZF region using plus/minus STS analysis of genomic DNA (Figure 1) recommended by EAA/EMQN. These 24 cases included five AZFa deletions, one AZFb deletions, two AZFc deletions (AZFb + AZFc deletion) and four AZFabc deletions (AZFa + AZFb + AZFc deletion) found only in azoospermia patients, and 12 AZFc deletions, which included eight azoospermia, three with sperm concentration $<5 \times 10^9$/ml and one oligospermia with sperm concentration of $10.66 \times 10^9$/ml (Table I). Through a further screening of AZFc deletions in all patients and controls, we found that AZFc deletion did not exist in subjects with sperm concentration $>20 \times 10^9$/ml (Table II) and the maximum sperm concentration of AZFc deleted patients was $10.66 \times 10^9$/ml. Serum hormone level analysis (Table I) showed one patient with high serum FSH level and two patients with decreased serum testosterone level in all men with microdeletions. Other serum hormone levels were tested to be normal.

#### Screening for subdeletions of AZFc region

The gr/gr, b2/b3 and b1/b3 subdeletions in all subjects were determined by multiplex PCR analysis of genomic DNA (Figure 1). After having eliminated 24 classical AZF deletions from 242 infertile men with sperm concentration $<20 \times 10^9$/ml, we found 35 (16.1%) subdeletions in the AZFc region, including 15 (6.9%) gr/gr subdeletions, 20 (9.2%) b2/b3 subdeletions and 0 (0%) b1/b3 subdeletions (Table II). In contrast, screening of 248 control men revealed 19 (7.7%) gr/gr subdeletions, eight b2/b3 (3.2%) subdeletions and 0 (0%) b1/b3 subdeletions. There was a statistically significant difference in the frequency of b2/b3 deletions (OR, 3.03; 95% CI 1.31–7.03) ($P = 0.007$) between control and infertile men with spermatogenesis impairment. However, there was no significant difference in the frequency of the gr/gr or b1/b3 subdeletions between the patients and controls. In order to make sure of the correlation between b2/b3 subdeletions and spermatogenesis impairment, the subdeletion analyses was performed in 209 infertile men with normal sperm concentration. Fifteen (7.2%) gr/gr subdeletions, 18 (8.6%) b2/b3

### Table I. Clinical data of patients with classical azoospermia factor (AZF) deletions

<table>
<thead>
<tr>
<th>Deleted region</th>
<th>Number of cases</th>
<th>Clinical situation</th>
<th>Hormone level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>LH(IU/l)$^a$</td>
</tr>
<tr>
<td>AZFa</td>
<td>5</td>
<td>Azoospermia</td>
<td>8.6 ± 2.4</td>
</tr>
<tr>
<td>AZFb</td>
<td>1</td>
<td>Azoospermia</td>
<td>5.4</td>
</tr>
<tr>
<td>AZFc</td>
<td>8</td>
<td>Azoospermia</td>
<td>7.1 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Azoospermia</td>
<td>7.8 ± 1.8</td>
</tr>
<tr>
<td>AZFbc</td>
<td>2</td>
<td>Azoospermia</td>
<td>6.0/11.3</td>
</tr>
<tr>
<td>AZFabc</td>
<td>4</td>
<td>Azoospermia</td>
<td>9.6 ± 5.7</td>
</tr>
</tbody>
</table>

$^a$Data are shown as mean ± SD.
subdeletions and 0 (0%) b1/b3 subdeletions were found in this population. Interestingly, there was still no significant difference in the frequency of the gr/gr or b1/b3 subdeletions between controls and infertile men with normal sperm concentration. However, the statistically significant difference in the frequency of b2/b3 deletions still existed between controls and this population (OR, 2.93; 95% CI 1.34–6.39) (\(P = 0.005\)). The hormone levels (serum FSH, LH, testosterone) of the b2/b3 deleted men were similar to those in the whole database population.

**Typing gr/gr and b2/b3 subdeletions**

All subjects with gr/gr or b2/b3 deletions were typed for DAZ and CDY1 status. There were four types of deletion patterns in both gr/gr and b2/b3 subdeletions: DAZ1/2 + CDY1a, DAZ1/2 + CDY1b, DAZ3/4 + CDY1a, DAZ3/4 + CDY1b. In all the deletion cases, we found a reduction of gene dosage for DAZ and CDY1. The distribution of these four types is shown in Table III.

In the b2/b3 subdeletion group, 43 out of the 46 deleted cases showed the deletion of DAZ3/4. Along with the deletion of CDY1a, significance (OR, 2.78; 95% CI 1.13–6.78) was found between the 451 infertile patients and 248 fertile controls. Interestingly, the DAZ3/4 + CDY1b deletion type did not occur in the control group, while eight cases were found in the infertile group (Table III).

In the gr/gr subdeletion group, the distribution of the four types was more equilibration than b2/b3. No significant difference of these types in gr/gr subdeletion group was found among the populations (Table III).

**Haplogroup analysis**

The work of Y chromosome haplogroups was going on. From the preliminary data, we found something interestingly. The 8 cases carrying a b2/b3 deletion of the DAZ3/4 + CDY1b type were belong to HgO, not HgN, and the 51 cases with DAZ3/4 + CDY1a deletion were mainly belong to M9 (47 in M9 and 4 in HgC). Considering the bias of population, the relationship needs to be further confirmed. We will expand the hyplogroupe analysis to the whole population we studied, and type the Y chromosome hyplogroup more detailed to provide more proofs.

### Table II. Frequencies of classical AZFc deletions and AZFc subdeletions in the study population

<table>
<thead>
<tr>
<th>Population</th>
<th>Number of subjects</th>
<th>Classical AZFc deletion (%)</th>
<th>Number (%)</th>
<th>OR (95% CI)</th>
<th>Number (%)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fertility/normal spermatogenesis</td>
<td>248</td>
<td>0</td>
<td>19 (7.7%)</td>
<td>1.00</td>
<td>8 (3.2%)</td>
<td>1.00</td>
</tr>
<tr>
<td>Infertility/abnormal spermatogenesis</td>
<td>242</td>
<td>12 (5.0)</td>
<td>15 (6.9%)</td>
<td>0.89 (0.44–1.80)</td>
<td>20 (9.2%)</td>
<td>3.03 (1.31–7.03)</td>
</tr>
<tr>
<td>Oligozoospermia</td>
<td>164</td>
<td>8 (4.8)</td>
<td>10 (6.9)</td>
<td>0.90 (0.41–2.00)</td>
<td>13 (9.0)</td>
<td>2.98 (1.20–7.37)</td>
</tr>
<tr>
<td>Infertility/normal spermatogenesis</td>
<td>209</td>
<td>0</td>
<td>15 (7.2%)</td>
<td>0.93 (0.46–1.88)</td>
<td>18 (8.6)</td>
<td>2.83 (1.20–6.64)</td>
</tr>
<tr>
<td>Subtotal</td>
<td>451</td>
<td>12 (2.7)</td>
<td>30 (7.0)</td>
<td>0.86 (0.47–1.56)</td>
<td>38 (8.9)</td>
<td>2.93 (1.34–6.39)</td>
</tr>
</tbody>
</table>

*The population of infertility/abnormal spermatogenesis is the sum of azoospermia and oligozoospermia.

**Table III. The distribution of the deletion types of DAZ and CDY genes in gr/gr and b2/b3 subdeletions**

<table>
<thead>
<tr>
<th>Population</th>
<th>Number of subjects</th>
<th>DAZ1/2 + CDY1a</th>
<th>DAZ1/2 + CDY1b</th>
<th>DAZ3/4 + CDY1a</th>
<th>DAZ3/4 + CDY1b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>gr/gr</td>
<td>b2/b3</td>
<td>gr/gr</td>
<td>b2/b3</td>
</tr>
<tr>
<td>Fertility/normal spermatogenesis</td>
<td>248</td>
<td>4</td>
<td>1</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>With semen parameters</td>
<td>86</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Without semen parameter</td>
<td>162</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Infertility/abnormal spermatogenesis</td>
<td>242</td>
<td>3</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Oligozoospermia</td>
<td>164</td>
<td>3</td>
<td>0</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Infertility/normal spermatogenesis</td>
<td>209</td>
<td>3</td>
<td>1</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Subtotal</td>
<td>451</td>
<td>6</td>
<td>1</td>
<td>15</td>
<td>0</td>
</tr>
</tbody>
</table>

*The population of infertility/normal spermatogenesis is the sum of infertility/abnormal spermatogenesis and infertility/normal spermatogenesis subjects.

**Table III.** The distribution of the deletion types of DAZ and CDY genes in gr/gr and b2/b3 subdeletions
Discussion

Classical AZF deletions, defined by a few well-designed markers (Simoni et al., 2004), are clinically relevant because a clear cause–effect relationship between these deletions and spermatogenic failure has been established (Sun et al., 2000; van Golde et al., 2001; Ferlin et al., 2003; Fernando et al., 2006). The frequency of Y chromosome deletions in infertile men ranges from 5 to 20% in worldwide surveys (Vogt, 1998; Krausz et al., 1999, 2003), reflecting the differing composition of the study population such as race (Simoni et al., 2004). In this study, we determined the frequency of classical AZF deletions in a well-defined Han-Chinese population, and 24 (9.9%) cases with classical AZF deletions out of the 242 infertile men with spermatogenesis impairment were found. Almost all patients with such deletions were azospermic, except four AZFc deleted cases (three cases with sperm concentration $<5 \times 10^6$/ml and one case with a sperm concentration of $10.66 \times 10^6$/ml). Through a further screening of AZFc deletions in all patients and controls, we found that AZFc deletion did not exist in subjects with sperm concentration $>20 \times 10^6$/ml and the maximum sperm concentration of AZFc deleted patients was $10.66 \times 10^6$/ml. Therefore, in this Han-Chinese population, the frequency of Y chromosome deletions was similar to that observed in different regions of the world and no such deletions were found in normospermic men, which confirmed the specific relationship between Y chromosome deletions and spermatogenic failure (Krausz et al., 2003).

In contrast to the classical AZF deletions, genotype–phenotype correlations for the AZFc subdeletions seem to be more complicated. Using an STS plus/minus approach (Repping et al., 2003), we analysed three types of AZFc subdeletions in our population: gr/gr, b2/b3 and b1/b3. Strikingly, the b1/b3 deletion was totally absent in this Han-Chinese population, while the gr/gr subdeletion and b2/b3 subdeletion displayed higher frequencies than other populations.

The frequency of the gr/gr deletion in 451 infertile men of Han-Chinese population (7.0%) was much higher than that observed in a United States population (2.2%), and even higher than in patients with spermatogenic impairment from US and European populations (from 3.7 to 5.1%) (Repping et al., 2003; Machev et al., 2004; de Llanos et al., 2005; Hucklenbroich et al., 2005). This may be due to the different genetic background in these studies, and a recent study of East Asians which overviewed a gr/gr deletion prevalence of 10.3% in patients and 10.1% in controls provides such evidence (Zhang et al., 2005). Unlike some results of association studies in Europeans (Repping et al., 2003; de Llanos et al., 2005; Ferlin et al., 2005), no difference in the frequency of the gr/gr deletions was found between the patients (7.0%) and proven controls (7.7%). According to the sperm concentrations, we categorized the patients into three groups: non-obstructive azoospermia, oligo/azoospermia and normal spermatogenesis. The gr/gr deletion frequencies in these groups were 6.9, 7.7 and 7.2%, respectively, which did not correlate with the severity of the spermatogenesis impairment. Moreover, the similar frequency of normal spermatogenic infertile men (7.2%) and proven fertile men (7.7%) suggests that even the gr/gr deletion has no significant association with male infertility in a Han-Chinese population (Lynch et al., 2005). Considering the different populations we studied, the genetic background may play its role again.

The b2/b3 subdeletion which used to be ignored, was first described by Sjoerd Repping’s group. They screened 1563 men and found 25 who lacked sY1191 but possessed all flanking STSs. After confirmation by fluorescence in situ hybridization, they found that this special STS pattern occurred between b2 and b3 amplicons after a ‘gr/rg’ inversion (Repping et al., 2004). Because of the low frequency, the role of b2/b3 deletion in male infertility remained unknown (Repping et al., 2004; Ferlin et al., 2005; Hucklenbroich et al., 2005; Fernando et al., 2006). We detected a significant difference ($P=0.007$) between infertile men with spermatogenesis impairment (9.2%) and proven fertile men (3.2%), which gave us a hint that b2/b3 subdeletion may have some effect on spermatogenesis. Through the analysis of 209 infertile men with normal spermatogenesis, the 8.6% deleted cases rectified our viewpoint and gave us a conclusion that the b2/b3 subdeletion was significantly associated with idiopathic male infertility ($P = 0.005$), and not spermatogenesis impairment. The frequency of b2/b3 subdeletion in this study was much higher than that of the related studies (Repping et al., 2004; Ferlin et al., 2005; Hucklenbroich et al., 2005; Fernando et al., 2006). However, none of these recent studies found a significant association of b2/b3 deletion with spermatogenic failure when the infertile groups were compared with their respective control groups. Taking all of them into consideration, among a total group of 1852 infertile men studied so far with a control group of 668 men having proven or presumed fertility, 11 subjects had b2/b3 subdeletions in the patient samples when compared with seven men in the control groups. This comparison showed no significant association of b2/b3 deletions with infertility. This may reflect specific population differences or the low frequency in these populations which confused the association of b2/b3 subdeletion with male infertility (Repping et al., 2004).

After the DAZ and CDY1 gene copy definition, no significant association was found in the gr/gr deletion group, or DAZ1/2 deleted cases; while in the b2/b3 deletion group, the DAZ3/4 seems to play a role in the infertile outcome. In the DAZ3/4 + CDY1a type, significance (OR, 2.78, 95% CI 1.13–6.78) was found between the 451 infertile patients and 248 fertile controls. Interestingly, no DAZ3/4 + CDY1b type was found in the control group, whereas eight cases were found in the infertile group. However, the contribution of DAZ3/4 to male infertility may be limited in b2/b3 subdeletions because the DAZ3/4 deletion in gr/gr subdeletions showed no significance.

According to our experience, many reasons may contribute to this association. First, the b2/b3 deletion removes 1.8 Mb of AZFc, a region essential for normal spermatogenesis, including 12 testes specific genes or transcripts. Compared with gr/gr deletion, which removes 1.6 Mb of AZFc, three more gene
copies are deleted in b2/b3 subdeletion. They are one copy of BPY2, one copy of TTTY4 and one copy of TTTY17 (Repping et al., 2004). Considering the different associations of these two subdeletions with male infertility, the deletion of different genes might be one possible reason. Second, in addition to different genes, the different members deleted in the same gene family may have different effects (11 of these 12 genes are multicyclic genes). The most supportive evidence comes from the DAZ gene family. Different members of the DAZ gene family are known to have various intragenic duplications and might not be functionally equivalent as a consequence (Skaletsky et al., 2003; Ferlin et al., 2005). In Ferlin’s study, a different association between DAZ1/DAZ2 deletion and DAZ3/DAZ4 deletion with spermatogenesis impairment was found. Furthermore, the near sequence identity of other genes does not preclude expression differences that might be due to, for example, different organizations between the two deletions in long-range sequences (Repping et al., 2004).

In this case–control study, several limitations need to be addressed. First, as we enrolled the patients from hospitals and random controls from the population, inherent selection bias cannot be completely excluded. However, we applied a rigorous epidemiological design in selecting the study subjects to minimize potential biases. Second, the moderate sample size limited the statistical power of our study and large well-designed studies are necessary to confirm our findings, particularly the gene–gene and gene–environment interactions. Finally, our study does not have the related phenotypic and functional assays of spermatozoa, which limited our inquiry into the functional consequence of these variants. However, such association studies with significant findings may lead to further functional studies that will elucidate the underlying mechanisms of male infertility associated with these genetic variants. Further study needs a larger and better selected population to prove this association, and molecular characterization of b2/b3 subdeletion together with deleted gene copies should be carried out to explain the variable spermatogenic phenotypes.

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