Clinical characterization of 42 oligospermic or azoospermic men with microdeletion of the AZFc region of the Y chromosome, and of 18 children conceived via ICSI

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BACKGROUND: Severe spermatogenic compromise may be the result of a Y-chromosomal deletion of the AZFc region. Prior studies are limited to relatively small numbers of AZFc-deleted men. In this study, we have fully characterized 42 infertile men with a Y chromosome microdeletion strictly confined to the AZFc region, and we report on 18 children conceived through the use of ICSI. METHODS: A total of 42 oligospermic or azoospermic men had AZFc deletions. History, physical examination, karyotype, FSH, LH, testosterone, testis histology and results of ICSI using ejaculated or testis sperm were retrospectively accumulated in two academic clinical practices. RESULTS: All men were somatically healthy. Karyotypes were 46,XY in all but two men. FSH, LH, testosterone and testis histology could not differentiate those with oligospermia or azoospermia, nor could they predict whether sperm could be found in harvested testis tissue. Paternal age was not increased. Sperm production appeared stable over time. The results of ICSI were not affected by the AZFc deletion. All but one of the offspring were healthy. The sons inherited the AZFc deletion with no increase in length. CONCLUSIONS: AZFc-deleted men are somatically healthy, will most likely have useable sperm, will have stable sperm production over time and will have a good chance to experience biological paternity, but their sons will also be AZFc-deleted.

Key words: AZFc/azoospermia/DAZ gene/Y chromosome

Introduction

Infertility management will be sought by ~15% of reproductive age couples, a sperm factor being present in at least 25% (Templeton, 1995; Abma et al., 1997). The most drastic of the male factors are non-obstructive azoospermia (NOA) and severe oligospermia (<5×10⁶/ml sperm) (Oates, 1999). There is usually no clinical abnormality other than infertility. Both NOA and severe oligospermia result from a defect(s) in the quantitative aspects of spermatogenesis, due in many cases to a genetic mishap underlying this complex biological process (Bhasin et al., 1997; Thielemans et al., 1998; Chiang et al., 2000).

The development of ICSI has allowed the effective treatment of men with severe oligospermia (Palermo et al., 1992; Van Steirteghem et al., 1993). It has recently been demonstrated that ~50% of the total group of NOA men will have a minute amount of ongoing spermatogenesis within their testicular parenchyma [azoospermia type 3 as suggested by Ezeh and Moore (Ezeh and Moore, 2001)] (Mulhall et al., 1997b; Silber et al., 1997; Amer et al., 1999; Mercan et al., 2000; Silber, 2000; Ezeh and Moore, 2001). Testicular sperm extraction (TESE) is surgically employed in NOA men in the hope of harvesting some of those individual sperm that might be sparsely scattered throughout the seminiferous epithelium, which can then be used with ICSI to achieve biological fatherhood (Silber et al., 1995; Schlegel et al., 1997; Gil-Salom et al., 1998). Therefore, even the most severe forms of spermatogenic compromise may be treatable with ICSI (Faddy et al., 2001).

In 1976 Tiepolo and Zuffardi, on the basis of simple karyotyping, discovered grossly apparent deletions of the long arm of the Y chromosome in ~0.6% of azoospermic men (Tiepolo and Zuffardi, 1976). They postulated the presence of a region on proximal Yq involved in spermatogenesis and termed it the azoospermia factor (AZF). Sprinkled throughout this stretch is a myriad of recently discovered transcriptional units with possible critical roles in the human male (Lahn and Page, 1997; Kuroda-Kawaguchi et al., 2001). Submicroscopic characterization of this region (Yq11) has led to its division into three subregions, termed AZFa, AZFb and AZFc (Vogt et al., 1996).

AZFc, the most frequently deleted region of the Y chromosome in infertile males, is a de-novo microdeletion found in ~1:4000 males (~13% of azoospermic men and ~6% of men
with severe oligospermia) (Reijo et al., 1995, 1996; Kremer et al., 1997; Foresta et al., 1998; Van Landuyt et al., 2000). A ‘microdeletion’ is not visually discernible on karyotypic analysis but is detected only with molecular methods. The complete nucleotide sequence of the AZFc expanse has now been entirely decoded and is composed of two major palindromes (mirror image repeats, the largest of which spans 3 Mb) constructed from six distinct families of amplicons (massive repeat units) (Kuroda-Kawaguchi et al., 2001). The size of the AZFc region is ~3.5 × 10^6 bp. These palindromes may have arisen during primate evolution via tandem duplication and inversion (Kuroda-Kawaguchi et al., 2001). It appears that homologous recombination between two direct repeat sequences on the perimeters of the AZFc region is the proximate cause of this recurrent deletion and the reason the deletion length is identical in so many men.

Embedded within AZFc are seven families of transcription units; three protein-coding gene families (DAZ, BPY2, CDY1); two transcription unit families with predicted open reading frames (CSPL4Y, GOLGA2LY); and two families of spliced but apparently non-coding transcription units (TTY3 and TTY4) (Kuroda-Kawaguchi et al., 2001). The most well-known is the DAZ gene family (deleted in azoospermia) which consists of four DAZ genes, comprised of two clusters of inverted pairs (3′←5′ : 5′→3′) (Saxena et al., 1996, 2000). DAZ mRNA has been detected in early germ cells (spermatogonia and spermatocytes), encoding an RNA-binding protein whose role in spermatogenesis has been speculated to be quantitative (Menke et al., 1997; Lee et al., 1998). It is expressed only in the testis. DAZ is homologous to the Drosophila gene boule which is essential for proper meiosis during fly spermatogenesis (Eberhart et al., 1996). Even though the DAZ gene cluster is likely to be critical for optimal spermatogenesis, deletion of other, potentially complementary genes in this region, e.g. CDY1, may also imperil the spermatogenic process (Kleiman et al., 2001). Most AZFc transcriptional units have functional homologues on autosomes, which may explain the presence of some degree of spermatogenesis, albeit diminished, in many of these men.

Numerous other laboratories have also detected AZFc microdeletions in variable percentages of azoospermic men depending upon the study design and specific patient population (Girardi et al., 1997; Pryor et al., 1997; Duell et al., 1998; Foresta et al., 1998; Grimaldi et al., 1998; Liow et al., 1998; Chang and Tsai, 1999; Kim et al., 1999) However, investigations describing the clinical characteristics of AZFc-deleted men are limited (Mulhall et al., 1997a; Silber et al., 1998; Kleiman et al., 1999; Page et al., 1999).

Critically important questions need to be answered and can only be done so by looking at a large group of men with identical AZFc microdeletions. How many of these men are oligospermic and how many are azoospermic? In those who are azoospermic, how often is sperm retrievable with TESE? Are there factors that predict whether sperm will or will not be found in testis tissue? Are there other health or testicular consequences that need to be addressed in these men? Is there a decline in sperm production over time and, if so, what is its pace? How well does the ejaculated and testicular sperm function when used in conjunction with ICSI? Most importantly, is there any harm to the male and female offspring that we help to create with our technologies?

Our study focused exclusively on 42 men with a Y-chromosomal microdeletion confined to the AZFc region. We sought to fully characterize these men in terms of age at diagnosis, the age of their father at the time of their birth, other illnesses/somatic defects, their androgenic axis and spermatogenic function (oligospermic, azoospermic with sperm present in testis tissue, azoospermic with sperm absent from the testis tissue), and whether or not their ability to produce sperm declined over time. For those who underwent TESE or prior diagnostic testis biopsy, we examined histopathology. In those with sperm available for ICSI, we calculated the rates of fertilization, pregnancy and delivery. Finally, we describe a cohort of 18 children resulting from ICSI using sperm from these AZFc-deleted men, including 10 males, and the transmission of identical Y-chromosomal microdeletions.

Materials and methods

Patient population

From an overall sample of 713 men who were diagnosed with severe oligospermia or NOA (283 and 430 respectively), 42 had microdeletions of the Y chromosome confined to the AZFc region [eliminating all sequence tagged sites (STSs) with GenBank accession numbers as shown in Figure 1]. These men constituted the study group (Table I). Institutional Review Board approval had been granted and all patients gave informed consent. A detailed history was taken with specific emphasis on risk factors for male reproductive dysfunction and testicular anomalies. The ages at which they were first diagnosed and the ages of their fathers at the time of their birth were recorded. A general physical examination with particular attention to scrotal contents was performed. Hormonal assays reflective of the spermatogenic axis (FSH) and the androgenic axis (LH, testosterone) were drawn. A karyotypic analysis was conducted via peripheral blood. Testicular histologic diagnosis was accomplished on 5/16 oligospermic and 21/26 azoospermic cases. The predominant pattern of spermatogenesis seen is listed in Table I; occasionally, distinctly different levels could be seen in roughly equal percentages of tubules and both are noted in Table I. ‘Sertoli cell-only’ (SCO) describes seminiferous tubules with a complete absence of all germ cells. ‘Maturation arrest’ (MA) refers to tubules with variable numbers of spermatogonia and spermatocytes, but a paucity or total lack of haploid spermatids.

For most men with sperm in the ejaculate or with sperm found in testis tissue during TESE (vide infra), ICSI was carried out. For the male children born, either cord or peripheral blood was used for Y-DNA analysis. Y-chromosome deletions in the sons were carefully compared with those in fathers. General paediatric examinations were carried out at birth to identify any obvious somatic abnormalities of both female and male offspring. Extracted from our database were the ages of the fathers of 60 randomly selected men with severe oligospermia or azoospermia who were shown to be Y-intact. This served as a comparison figure for the ages of the fathers of our AZFc microdeletected study population. Also extracted from our database were values of FSH, LH and testosterone from 20 randomly selected NOA, Y-intact men and 20 randomly selected oligospermic, Y-intact men for comparison with those values from our AZFc-deleted men.
Clinical characterization of 42 AZFc-deleted men

Figure 1. AZFc deletion map of eight infertile men, their 10 conceived sons and their fathers, when available. Solid black boxes indicate sequence tagged sites (STSs) that were present; minus signs indicate experimentally absent STSs (deleted STSs); solid grey boxes represent the inferred presence of a locus; open grey boxes represent positive PCR results that cannot be interpreted with confidence due to cross-amplifying loci elsewhere.

Y-chromosomal microdeletion assay
Informed consent was obtained from all patients after discussing with them the known possible genetic aberrations found in patients with spermatogenic deficiency. The methodology for Y-chromosomal STS deletion mapping has previously been well described (Kuroda-Kawaguchi et al., 2001).

Testis tissue extraction
There were slightly different clinical approaches employed to harvest testis tissue and sperm for ICSI. In Boston, TESE is typically carried out on a day remote from an ICSI cycle and, if sperm are present, the harvested testicular tissue is cryopreserved into multiple vials, each serving as the source of sperm for a later cycle of ICSI. In St Louis, all tissue extractions are microsurgical and co-ordinated with the day of oocyte retrieval during an ICSI cycle. The techniques and philosophy behind each approach have been described in detail previously (Silber et al., 1996; Mulhall et al., 1997a; Schlegel and Li, 1998; Silber, 2000). Most patients required little post-operative analgesia and no complications from TESE occurred. Whenever possible, a 3 mm portion of tissue was fixed for histological analysis in either Bouin’s or Zenker’s solution and subsequently underwent standard haematoxylin and eosin staining.

Ovarian stimulation, harvesting of oocytes and ICSI technique
The methodology for ovulation induction, transvaginal retrieval of oocytes and spermatozoal microinjection has been described previously (Van Steirteghem et al., 1993). In addition, when TESE was performed coincident with oocyte harvest, couples had been informed of the possibility that no useable sperm might be found and, consequently, donor sperm was chosen by some as a back-up sperm source. Sperm that exhibited any movement, even non-progressive or non-directional, were picked up in a micropipette and transferred to a ‘holding’ droplet of polyvinylpyrrolidone (10% w/v; Scandinavia IVF Science AG). If no motion could be detected in any sperm, ICSI was still performed, and in some cases, a micro-hypo-osmotic swelling analysis of each sperm was carried out until the requisite number was recovered.

Results
Of the 713 men with NOA or severe oligospermia who underwent Y-DNA analysis at the Page Laboratory, 42 (5.9%) had microdeletions strictly confined to the AZFc region and constituted the study group (Kuroda-Kawaguchi et al., 2001). These men represent a pure population with no patient having a deletion extending proximally into the AZFb region or
Table I. Clinical characteristics of AZFc-deleted men

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Age at diagnosis (years)</th>
<th>Medical history</th>
<th>Karyotype (mIU/ml)</th>
<th>FSH (mIU/ml)</th>
<th>LH (mIU/ml)</th>
<th>Testosterone (ng/dl)</th>
<th>Histology at TESE/Tbx</th>
<th>Father’s age at birth of patient (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severely oligospermic: &lt;5×10⁶/ml (n = 16)</td>
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<td></td>
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<tr>
<td>WHT 3016</td>
<td>42</td>
<td>46,XY</td>
<td>4</td>
<td>5</td>
<td>238</td>
<td>–</td>
<td>–</td>
<td>32</td>
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<tr>
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<td>46,XY</td>
<td>13</td>
<td>6</td>
<td>565</td>
<td>–</td>
<td>–</td>
<td>39</td>
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<td>WHT 3421</td>
<td>30</td>
<td>46,XY</td>
<td>3</td>
<td>3</td>
<td>219</td>
<td>MA</td>
<td>–</td>
<td>–</td>
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<td>38</td>
<td>46,XY</td>
<td>3</td>
<td>1</td>
<td>378</td>
<td>–</td>
<td>–</td>
<td>33</td>
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<td>27</td>
<td>46,XY</td>
<td>12</td>
<td>3</td>
<td>360</td>
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<td>31</td>
<td>46,XY</td>
<td>17</td>
<td>5</td>
<td>549</td>
<td>SCO/MA</td>
<td>35</td>
<td>–</td>
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<td>WHT 3305</td>
<td>34</td>
<td>46,XY</td>
<td>2</td>
<td>6</td>
<td>413</td>
<td>MA</td>
<td>26</td>
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<td>WHT 3321</td>
<td>32</td>
<td>46,XY</td>
<td>10</td>
<td>8</td>
<td>–</td>
<td>MA</td>
<td>33</td>
<td>–</td>
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<td>WHT 3583</td>
<td>48</td>
<td>46,XY</td>
<td>28</td>
<td>–</td>
<td>–</td>
<td>550</td>
<td>30</td>
<td>–</td>
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<td>32</td>
<td>46,XY</td>
<td>6</td>
<td>4</td>
<td>320</td>
<td>MA</td>
<td>30</td>
<td>–</td>
</tr>
<tr>
<td>Azoospermia: sperm detected in testis tissue (n=14)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WHT 3206</td>
<td>33</td>
<td>Fraternal twin – 47.XXY</td>
<td>46,XY</td>
<td>15</td>
<td>–</td>
<td>266</td>
<td>SCO</td>
<td>30</td>
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<td>WHT 3622</td>
<td>28</td>
<td>46,XY</td>
<td>12</td>
<td>6</td>
<td>476</td>
<td>MA</td>
<td>–</td>
<td>–</td>
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<td>WHT 3279</td>
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<td>46,XY</td>
<td>22</td>
<td>10</td>
<td>268</td>
<td>SCO</td>
<td>22</td>
<td>–</td>
</tr>
<tr>
<td>WHT 2928</td>
<td>28</td>
<td>Fraternal twin of WHT 3432</td>
<td>46,XY</td>
<td>17</td>
<td>11</td>
<td>349</td>
<td>SCO</td>
<td>30</td>
</tr>
<tr>
<td>Azoospermia: no sperm detected in testis tissue (n = 7)</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>WHT 4019</td>
<td>24</td>
<td>Mosaic</td>
<td>9</td>
<td>3</td>
<td>309</td>
<td>SCO/MA</td>
<td>20</td>
<td>–</td>
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<tr>
<td>WHT 3444</td>
<td>28</td>
<td>Unilateral cryptorchidism</td>
<td>46,XY</td>
<td>13</td>
<td>8</td>
<td>423</td>
<td>SCO</td>
<td>–</td>
</tr>
<tr>
<td>Azoospermia: no TESE (n = 5)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>WHT 3432</td>
<td>28</td>
<td>Fraternal twin of WHT 2928</td>
<td>46,XY</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>30</td>
</tr>
<tr>
<td>WHT 3452</td>
<td>30</td>
<td>46,XY</td>
<td>20</td>
<td>9</td>
<td>219</td>
<td>–</td>
<td>35</td>
<td>–</td>
</tr>
<tr>
<td>WHT 3630</td>
<td>40</td>
<td>46,XY</td>
<td>44</td>
<td>4</td>
<td>481</td>
<td>MA</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>WHT 3428</td>
<td>38</td>
<td>46,XY</td>
<td>35</td>
<td>12</td>
<td>286</td>
<td>–</td>
<td>30</td>
<td>–</td>
</tr>
<tr>
<td>WHT 3116</td>
<td>31</td>
<td>46,XY</td>
<td>6</td>
<td>6</td>
<td>451</td>
<td>–</td>
<td>36</td>
<td>–</td>
</tr>
</tbody>
</table>

*As assessed by quantitative histological evaluation of a previously obtained testis biopsy (Silber et al., 1997). MA = maturation arrest; SCO = Sertoli cell-only.

extending distally into the heterochromatin. All 42 men appeared to have identical or essentially identical deletions of the 3.5×10⁶ bp segment—the AZFc region—whose complete nucleotide sequence has been reported (Kuroda-Kawaguchi et al., 2001).

Spermatogenic potential (Table I)

The 42 men with identical AZFc microdeletions could be classified into four subgroups based upon spermatogenic capability: severe oligospermia (16 men); azoospermia with sperm detected on TESE or quantitative histological analysis (14 men); azoospermia with no sperm detected on TESE or quantitative histological analysis (seven men); azoospermia but no TESE or quantitative histological analysis was performed, leaving unanswered the question of whether testicular sperm might be present (five men). Therefore, 30 of the 42 men (71%) had some discernible spermatogenesis. Thirty of the 37 AZFc-deleted men (81%) who were
The average age of all fathers at the time of the birth of our patients was 30 years. There was no statistically significant difference between the groups in terms of the age of the fathers.

**Historical and Physical Examination (Table I)**

WHT 3444 had unilateral cryptorchidism; otherwise, medical history revealed no specific genito-urinary issues. WHT 3263 had a fraternal twin with 47,XXY Klinefelter Syndrome, while WHT 2928 and WHT 3432 were fraternal twins (both azoospermic) (vide infra). No other patient reported male infertility in his family. No patient had a history of testicular or other malignancy. No man exhibited a dysmorphic appearance. Penile length and width were normal in all cases. Testicular size ranged from slightly less than normal (~20 ml) to normal. No testicular masses or areas of induration suggestive of malignancy were detected.

**Age at Diagnosis (Tables I and II)**

There was no statistically significant difference in age at time of diagnosis between the four subgroups (t-test).

**Karyotypic Analysis (Table I)**

All but two patients tested were 46,XY with no identifiable karyotypic anomaly, consistent with the fact that these deletions are submicroscopic. One had a prominent 22p and another was weakly mosaic (2/50 cells: XXY and 1/50 cells: XO).

**Hormonal Parameters (Tables I and II)**

The mean FSH in the severely oligospermic men was significantly less than the mean of the entire cohort as well as both the azoospermic men with and without testis sperm (t-test). The mean LH and testosterone values did not differ significantly between the groups. In comparing the mean FSH, LH and testosterone values from the AZFc-deleted azoospermic group with those of 20 randomly selected Y-intact NOA men, no significant differences were seen: FSH, 15 versus 18 mIU/ml; LH, 7 versus 8 mIU/ml; testosterone, 348 versus 363 ng/dl respectively. No differences were seen in the distribution (in 5 year increments) of the fathers at the time of the birth of their sons (Figure 2).

**Histology of Testis Tissue (Table I)**

Histopathologic analysis of tissue sent at the time of TESE or during a prior diagnostic testis biopsy demonstrated variable results: pure SCO in nine men, pure MA in 12, and a combination of SCO and MA in five. No oligospermic man who underwent biopsy revealed a pattern of pure SCO. Of the 14 azoospermic men with sperm found in testis tissue, SCO was seen in six, MA in six and SCO/MA in two. A similar distribution was observed in six of the seven men in whom no sperm were detected during TESE or quantitative histological biopsy: SCO in three, MA in one and SCO/MA in two.

**ICSI (Table III)**

A total of 48 cycles of ICSI were performed in 26 couples. Sixteen couples had one cycle, three couples had two cycles, three couples had three cycles, three couples had four cycles, and one couple had five cycles of ICSI performed. Eleven of the 26 couples had at least one child, resulting in a per couple delivery rate of 42%. The overall 2PN fertilization rate was 47% (273 zygotes/575 oocytes injected) and the overall term pregnancy rate was 27% (13 pregnancies/48 cycles). The fertilization and term pregnancy rates for those cycles in which ejaculated sperm served as the gamete source were 64% (149 zygotes/234 oocytes injected) and 47% (nine pregnancies/19 cycles) respectively. Fertilization rate and term pregnancy rate

<table>
<thead>
<tr>
<th>Mean (range)</th>
<th>Entire group</th>
<th>Oligospermia</th>
<th>Azoospermia: sperm detected</th>
<th>Azoospermia: no sperm detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH mIU/ml</td>
<td>6.3 (1–18)</td>
<td>5.2 (1–18)</td>
<td>7.6 (2–11)</td>
<td>5.8 (1–11)</td>
</tr>
<tr>
<td>Testosterone ng/dl</td>
<td>355 (135–565)</td>
<td>366 (135–565)</td>
<td>344 (223–482)</td>
<td>346 (249–438)</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>34 (24–53)</td>
<td>35 (27–48)</td>
<td>34 (27–53)</td>
<td>32 (24–42)</td>
</tr>
<tr>
<td>Paternal age</td>
<td>30 (21–45)</td>
<td>33 (26–39)</td>
<td>29 (21–45)</td>
<td>24 (20–28)</td>
</tr>
</tbody>
</table>

**Figure 2.** Distribution of fathers’ ages at time of birth of AZFc-deleted or Y-intact sons demonstrating no effect of increasing paternal age on the frequency of having an AZFc-deleted son.
Discussion
Our study sought to clinically characterize, in a comprehensive fashion, the largest cohort to date of men whose spermatogenic failure is based on a Y-chromosomal microdeletion strictly confined to the AZFc region. What is the phenotype of these men? What are the chances of achieving a pregnancy? What risks do they assume for their offspring, both male and female? These are just a few of the many questions that need to be addressed in order to help patients intellectually understand their condition and allow them to knowledgeably formulate their therapeutic strategy (Liow et al., 2001). Based upon the extensive clinical data we have accumulated, we will systematically answer these queries.

Is the AZFc microdeletion the cause of spermatogenic deficiency?
Deletion of the AZFc region on the Y chromosome quantitatively reduces sperm density so severely that infertility and sterility are the rule and natural procreation the exception. Reijo et al. first documented that AZFc microdeletions were found in 13% (12/89) of their azoospermic men and were not detected in 90 fertile control males (Reijo et al., 1995). Additional confirmatory studies which have included fertile

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Table III. Results of ICSI using sperm from men with AZFc region microdeletions

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sperm source</th>
<th>No. ICSI cycles total</th>
<th>No. oocytes inseminated</th>
<th>No. resultant embryos (2PN)</th>
<th>% fertilization</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHT 3421</td>
<td>Ejaculate</td>
<td>1</td>
<td>16</td>
<td>5</td>
<td>31</td>
<td>Female singleton</td>
</tr>
<tr>
<td>WHT 3628</td>
<td>Ejaculate</td>
<td>1</td>
<td>8</td>
<td>7</td>
<td>87</td>
<td>No pregnancy</td>
</tr>
<tr>
<td>WHT 3722</td>
<td>Ejaculate</td>
<td>1</td>
<td>15</td>
<td>12</td>
<td>80</td>
<td>Female twins</td>
</tr>
<tr>
<td>WHT 3254</td>
<td>Ejaculate</td>
<td>1</td>
<td>14</td>
<td>9</td>
<td>64</td>
<td>No pregnancy</td>
</tr>
<tr>
<td>WHT 3321</td>
<td>Ejaculate</td>
<td>1</td>
<td>25</td>
<td>19</td>
<td>76</td>
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</tr>
<tr>
<td>WHT 3583</td>
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<td>1</td>
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<td>3</td>
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<td>WHT 3305b</td>
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<td>15</td>
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</tr>
<tr>
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<td>No pregnancy</td>
</tr>
<tr>
<td>WHT 3016</td>
<td>Ejaculate</td>
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<td>22</td>
<td>12</td>
<td>55</td>
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</tr>
<tr>
<td>WHT 4489</td>
<td>Ejaculate</td>
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<td>3</td>
<td>3</td>
<td>133</td>
<td>No pregnancy (poor oocyte quality)</td>
</tr>
<tr>
<td>WHT 2922</td>
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<tr>
<td>WHT 3722</td>
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<tr>
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<td>1</td>
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<td>7</td>
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</tr>
<tr>
<td>WHT 3134</td>
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<td>37</td>
<td>24</td>
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</tr>
<tr>
<td>WHT 3706</td>
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<td>24</td>
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</tr>
<tr>
<td>WHT 3321a</td>
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<td>WHT 3722</td>
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<td>24</td>
<td>65</td>
<td>No pregnancy</td>
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for the group who had testicular sperm employed were 36% (124 zygotes/341 oocytes injected) and 14% (four pregnancies/29 cycles) respectively. Comparing fertilization rates from the ejaculated sperm group with the testicular sperm group, there was a statistically significant difference (z-test, \( P < 0.0001 \)).

Thirteen of the 48 ICSI cycles led to the births of five sets of fraternal twins and eight singletons. Of the five sets of twins, two sets were girl-girl, two sets were boy-boy and one set was girl-boy. Of the singleton births, three were girls and five were boys. WHT 3321, who was severely oligospermic, conceived a son (WHT 4013) spontaneously through intercourse after a prior ICSI pregnancy resulted in the birth of a healthy girl.

**Phenotypic and Y-DNA assessment of the offspring**

All male and female babies were healthy with no obvious deficiencies or syndromic phenotypic findings except for WHT 3469 who was born with pulmonary atresia and a hypoplastic right ventricle [reported previously (Page et al., 1999)]. All 10 sons tested had inherited their fathers’ AZFc-deleted Y chromosome, with no detectable change in the size or extent of those deletions (Figure 1). The male singleton, conceived through ICSI by WHT 3706, has not yet had Y-DNA testing.
controls have found no AZFc microdeletions in a collective total of 368 men, while detecting microdeletions in 26/236 (11.3%) azoospermic men (Najmabadi et al., 1996; Stuppia et al., 1996; Simoni et al., 1997; Vereb et al., 1997; Liow et al., 1998). A smaller percentage of severely oligospermic men have also been shown to lack the AZFc region in ~6% of cases (Reijo et al., 1996; Krausz et al., 2001). It is rare to find an AZFc microdeletion in a patient with a sperm density >5×10⁶/ml, but WHT 3706 had two analyses with counts slightly greater than this and total sperm density close to 10×10⁶/ ejaculate. Except for WHT 3706, all of the men in our study population showed severely reduced sperm output, and seven had no available sperm in either the ejaculate or the testis tissue. Therefore, a Y-chromosomal microdeletion involving the AZFc region will lead to spermatogenic compromise, with the final level of spermatozoal generation variable but always very low.

Are there other health consequences related to an AZFc microdeletion?

The 42 men in this study were all healthy individuals with no major illnesses (Table I). Penile anatomy was normal in all men, circulating levels of testosterone were all biologically adequate, and no patient showed any overt signs of decreased virilization. Average testosterone and LH values did not differ significantly between the groups. Therefore, genes in the AZFc region do not appear to impair interstitial Leydig cell function. Tateno et al. found no Y-chromosomal microdeletions in a group of 44 males with hypospadias (Tateno et al., 2000). Although the aetiology of failure of labioscrotal fold fusion is unknown, it is unlikely to be related to the genes located in the AZFc region. Collectively, the data are reassuring, and suggest that the active genes populating this area of the Y chromosome are specifically and exclusively expressed in testis and only affect the quantitative production of sperm. Since our study cohort is young, the frequency of conditions that occur more commonly in the aged population cannot currently be commented on.

Are there other testis-specific consequences related to an AZFc microdeletion?

Our data do not support a possible aetiologic connection between cryptorchidism and AZFc region deletions (Simoni et al., 1997; Foresta et al., 1999; Krausz et al., 2001), as only WHT 3444 had a history of testicular maldescent, and are in agreement with Fagerli et al. who found no Y deletions in a cohort of 38 previously cryptorchid men (Fagerli et al., 1999). However, a history of maldescent in an oligospermic or azoospermic male should not sway the clinician away from Y-chromosomal microdeletion testing as the two conditions may occur coincidentally. No patient in our cohort developed a testicular malignancy, in agreement with Krausz et al. (Krausz et al., 2001). Therefore, the loss of genes residing in the AZFc region appears to have little or no effect on the likelihood of cryptorchidism or germ cell cancer.

Was the AZFc microdeletion inherited and is there a paternal age effect?

Twelve fathers of AZFc-deleted men were tested and none showed a Y-chromosomal microdeletion, adding to previous data suggesting that, for most affected men, the microdeletion is a de novo event. WHT 2928 and WHT 3432 are fraternal twins naturally conceived from an AZFc-intact father. Therefore, this brother pair is unique when contrasted to all other AZFc-deleted brothers reported whose fathers are also AZFc-deleted (Chang et al., 1999; Saut et al., 2000). Taken together, these different scenarios suggest three possibilities for the timing of an AZFc deletion. If AZFc region homologous recombination occurs during mitosis in one of the father’s spermatogonia, he may have a resultant derivative cohort of AZFc-deleted spermatogonia. The ultimate numbers present in the testis would depend upon how early in the spermatogonial lineage the event occurred (stem cell spermatogonial versus more differentiated forms such as type A and type B) and how severely the deletion restrained proliferation, mitosis or subsequent meiosis of those AZFc-deleted cells. In this circumstance, the frequency of AZFc-deleted sperm and AZFc-deleted offspring may be higher than the 1:4000 seen in the general population and may explain brothers WHT 2928 and WHT 3432, the chances of two AZFc-deleted brothers from an AZFc-intact father otherwise being 1:16×10⁹. If AZFc region homologous recombination occurs rarely and randomly during germ cell meiosis, only a small number of sperm would be produced that are AZFc-deleted. If AZFc region homologous recombination occurs shortly after fertilization, the 46,XY embryo will be AZFc-deleted. The latter two events may occur in ~1:4000 sperm or 1:4000 embryos, thus explaining the overall frequency noted. Any of the three mechanisms might account for a given individual.

We have detected no effect of paternal age on the likelihood of an AZFc microdeletion occurring in sperm. If there were such an effect, we would expect to see the average age of the fathers of our AZFc-deleted patients to be older than that in a similar group of infertile Y-intact men. At the time of the birth of our patients, the average age of the fathers (30 years) was no different from the average age of the fathers of a control group of 60 severely oligospermic or azoospermic men with no identifiable AZFc microdeletions. As Figure 2 demonstrates, there is no trend towards the upper increments of age in the fathers of our patients, and the distribution of the percentage of fathers in each age group is similar for fathers of both AZFc-deleted and Y-intact men.

Naturally conceived offspring have been reported from fathers who were determined to be AZFc-deleted themselves, indicating that not all AZFc deletions are the result of a de novo mishap, but some may indeed be inherited (Chang et al., 1999; Saut et al., 2000). This underscores that AZFc microdeletions reduce spermatogenesis dramatically, but do not prevent spontaneous conception.

What is the likelihood of an AZFc-deleted man having sperm in the ejaculate or in testis tissue?

Of all 42 AZFc-deleted men studied, 16 (38%) had sperm in their ejaculate (Table I). Of the 21 azoospermic men who had TESE or quantitative histological biopsy, 14 (67%) had some level of complete spermatogenesis. Therefore, of the 37 fully evaluated men, 30 (81%) produced sperm and were, or would be, candidates for ICSI. Although the spermatogenic spectrum
of our AZFc-deleted men ranges from sterile (no sperm available, even in testis tissue) to severely oligospermic (<5×10^6/ml), it is heavily weighted in favour of sperm availability, perhaps due to the existence of autosomal homologues for most of the transcriptional units that are found in the AZFc region (Kuroda-Kawaguchi et al., 2001). These data provide encouragement for infertile men with AZFc deletions who are azoospermic—it is likely that sperm can be harvested from testis tissue, thereby providing an opportunity for biological fatherhood.

If an AZFc-deleted man has sperm in his ejaculate or testis tissue, is this capacity evanescent or stable through time?

Prior reports on a few individual cases have been interpreted as evidence that an AZFc microdeletion constitutes a progressive and deteriorating insult to spermatogenesis leading to a steady and methodical decline in spermatogenic capability (Girardi et al., 1997; Simoni et al., 1997; Chang et al., 1999). In our oligospermic subgroup, four men had multiple semen analyses over time. The shortest course of study was 14 months and the longest 7 years. Fluctuations in sperm density for individual patients are plotted in Figure 3. In all four men, sperm production persisted over time, with no patient plummeting to azoospermic levels and remaining there. WHT 3016 and WHT 3134 are particularly instructive as they demonstrate what is commonly seen in clinical practice with severely oligospermic men, i.e. on any given day their sample may be azoospermic. WHT 3134 had three semen samples with counts of ~1×10^5/ml, followed by two azoospermic ejaculates. He then consistently showed sperm in his ejaculate (used for two cycles of ICSI) and still exhibited his original baseline sperm density 7 years after his first sample. These data provide no evidence that spermatogenesis declines progressively in the oligospermic, AZFc-deleted male, other than the usual moderate decline over decades that is seen in all males (Johnson, 1986).

In addition, if germ cell depletion in the testis were an ongoing and progressive event, we might expect the average age of the oligospermic men to be less than that of the azoospermic men. We might also anticipate that the ages of those azoospermic men with sperm present in their tissue would be less than that of the azoospermic men with no spermatozoal production. However, the average ages of the different subgroups do not provide evidence of any such progressive reduction in spermatogenesis.

What of the individuals shown to have sperm only within their testis tissue at the time of diagnosis? Are they on a steep slope of germinal epithelium degradation such that they will have no spermatogenic ability a short time later, as suggested by Calogero et al. (Calogero et al., 2001)? Our data do not support this contention as WHT 3279 and WHT 3111, who had multiple TESE procedures over the course of 3 and 4.5 years respectively, had spermatozoal retrieval each time. We suggest that the effect of an AZFc region deletion is stable, without any progressive decline. This issue is of profound importance for infertile men diagnosed with AZFc microdeletions who are contemplating delaying ICSI and/or TESE. Are they lowering their ultimate chances for success by waiting? Will they move from oligospermia to azoospermia, and then perhaps all the way to complete sperm absence in the testis tissue? Our data are encouraging for patients in this regard, suggesting that whatever level of spermatogenesis they have at the time of diagnosis will most likely be present in the foreseeable future. However, until more individual patients are followed through time, it is still possible that a rapid decline may occur. Our data do not support TESE with cryopreservation in the azoospermic male as a prophylactic manoeuvre to prevent possible future sterility. However, until this issue is clarified by observing the outcome of multiple, temporally separated TESE procedures in many more patients, we cannot make a definitive recommendation in this regard.

If an AZFc-deleted man is azoospermic, are there predictive factors as to whether sperm will or will not be found in his surgically retrieved testis tissue?

The age at diagnosis, paternal age at birth, FSH, LH and testosterone do not predict whether an individual azoospermic
man will or will not have sperm in his testis tissue. As noted in Table II, there is a statistically significant difference in FSH values between the oligospermic and azoospermic groups as a whole, but since the range is so wide there is no clinical significance.

The specific histologic diagnosis is not precisely predictive of the presence or absence of sperm in the testis tissue (Table I). For example, six of the 14 men (43%) with sperm in their testis tissue had a predominant pattern of SCO on histology. From a different perspective, of those in our study cohort with pure SCO on histology, 6/9 (67%) had sperm present. This is similar to the results of Mulhall et al. who looked at a population of azoospermic men with a variety of aetiologies, not just those with an AZFc microdeletion, and reported that sperm were found at TESE in 50% of those patients with a pattern of SCO (Mulhall et al., 1997b).

What are the chances of an AZFc-deleted infertile man having a child, either naturally or with ICSI?

As pointed out by Almagor et al. ~6% of couples in whom the male partner has severe oligospermia (defined by the authors as <1×10^6/ml, motility <30%) will have a spontaneous pregnancy (Almagor et al., 2001). This can also happen with severely oligospermic AZFc-deleted men, as evidenced by WHT 3321 in the present report and the exceptional families reported by Chang et al. and Saut et al. (Chang et al., 1999; Saut et al., 2000; Silber, 2001). As Krausz and McElreavey point out, ‘The pathogenetic significance of Y-chromosomal microdeletions is spermatogenic failure and not infertility’ (Krausz and McElreavey, 2001).

If natural pregnancy does not occur in the case of a severely oligospermic, AZFc-deleted man, ICSI can be used with great efficacy (Table III). Our data show a 64% fertilization rate and a 47% per cycle term pregnancy rate. van Golde et al. reported on eight men with AZFc microdeletions and oligospermia and their results during ICSI (van Golde et al., 2001). Compared with a control group of oligospermic men with intact Y chromosomes undergoing ICSI, the fertilization rate for AZFc-deleted men was statistically lower (55 versus 71%). In addition, the quality of the embryos was significantly poorer in the AZFc-deleted group. However, the implantation, pregnancy and take-home baby rates were the same. Our results were not compared with a control group, but certainly the ultimate pregnancy rate is excellent.

When harvested testicular sperm are used as the male gamete source for an ICSI cycle, we do see a reduction in fertilization (36%) and per cycle term pregnancy rates (14%). The fertilization rates using testis sperm in cases of NOA have been found to be lower than those using ejaculated or testis sperm from obstructed patients (Palermo et al., 1999). Our results may reflect this and not be related to the presence of the AZFc microdeletion.

Are there any health consequences for the children, either conceived naturally or through ICSI?

Of the 18 babies born, all were healthy and well except for WHT 3469 who died shortly after birth with pulmonary atresia and right ventricular hypoplasia [discussed in detail in Page et al. (Page et al., 1999)]. Congenital heart disease, seen in ~1% of newborns, is not increased in ICSI offspring and has not been reported in AZFc-deleted men or their naturally or technologically conceived children (Hoffman, 1995; Chang et al., 1999; Jiang et al., 1999; Kamischke et al., 1999; Saut et al., 2000). The male offspring, all of whom had the same deletion as their infertile fathers, were otherwise somatically normal, as were their fathers (Figure 1). The female children were also all normal. Genes within the AZFc region apparently do not affect overall body morphology, organogenesis, or general physiology and metabolism. The recent sequencing of the AZFc region has verified that its seven transcription unit families are all expressed exclusively in the testis (Kuroda-Kawaguchi et al., 2001). Aside from expected spermatogenic deficiency in male offspring, couples can be reassured that the children would be expected to be healthy and there may be no consequences at all in female offspring.

What will be the sperm production potential of the male children of AZFc-deleted men, either conceived naturally or through ICSI?

All Y-bearing sperm from AZFc-deleted men have been shown to also be AZFc-deleted (deVries et al., 2001). The data on our large number of male offspring as well as those collected from the literature confirm that an AZFc microdeletion is transmitted vertically to all male offspring (Chang et al., 1999; Jiang et al., 1999; Kamischke et al., 1999; Page et al., 1999; Saut et al., 2000). Most importantly, the deletion length was not increased in our male offspring (Figure 1). This is consistent with our proposed mechanism of deletion, i.e. illegitimate homologous recombination at breakpoints of sequence identity (Kuroda-Kawaguchi et al., 2001). Thus, an extension of the deleted segment would not be expected in the next generation. There is growing evidence that if a deletion involves both the AZFc and AZFb regions, the chance that sperm will be found either in the ejaculate or in testis tissue becomes highly unlikely (Brandell et al., 1998; Silber et al., 1998; Ferlin et al., 1999). Therefore, the fact that the deletion length does not expand allows us to predict that the sons will most likely display the same spermatogenic diversity as our patient population, but not necessarily as their own father. As we see from our 42 AZFc-deleted men, the spectrum of spermatogenesis is quite variable, but always clustered at the lowest end of spermatogenic output. We can only assume that the male offspring will also have spermatogenic deficiency, but cannot predict its severity. Since all of our men had exactly the same deletion length, there must be background modifying and modulating genetic and/or environmental factors that either augment or suppress the deleterious effects of an AZFc microdeletion. For example, most of the AZFc transcription units have autosomal homologues that may be sites of allelic variation. These allelic variants may differ between father and son. The son’s prenatal and pubertal internal and external environment may be different from his father. We will only know what their ultimate reproductive ability is when they are of appropriate age; but, as we have shown, many AZFc-deleted men have some small amount of spermatogenesis sufficient for ICSI.
Does an AZFc microdeletion lead to Y-chromosomal instability and loss during either meiosis or mitosis?

Siffroi et al. raised concern that a microdeletion of the Y chromosome may precipitate loss of that Y chromosome in a certain percentage of derivative cells during embryogenesis (Siffroi et al., 2000). This could lead to a 45,XO/46,XY (AZFc-deleted) karyotype in an offspring. Depending upon the percentage of cells that are 45,XO, somatic and genital defects (i.e. mixed gonadal dysgenesis) might then be encountered. These authors discussed five AZFc-deleted men, of whom four had a slight degree of XO mosaicism that could only be detected by FISH analysis and was above a baseline frequency found in 11 matched fertile controls. Their study raises the following questions: could germ cell mosaicism lead to the phenotypic variability we see in the spermatogenic spectrum of the AZFc-deleted male; will this mosaicism lead to 45,XO Ulrich–Turner syndrome in their daughters; and could a 45,XO/46,XY mosaicism occur early in embryogenesis resulting in a variable ratio 45,X/46,XY karyotype in their sons with clinical consequences of mixed gonadal dysgenesis or ambiguous genitalia (Hsu, 1994)?

However, none of the boys in our group had grossly recognizable abnormalities of the genitalia. None of the female children had features of Ulrich–Turner syndrome. In reported cases of offspring of AZFc-deleted men, no karyotypic abnormalities have been detected (Chang et al., 1999; Jiang et al., 1999; Lucas et al., 2000; Saut et al., 2000). One of our 42 AZFc-deleted infertile men (2.4%) was weakly mosaic for sex chromosomal abnormalities in his peripheral lymphocytes (47 of 50 cells were normal 46,XY; two were 47,XXY; one was 45,XO). It is not unexpected for non-Y-deleted infertile men to have a higher incidence of sex chromosomal karyotypic abnormalities (Van Assche et al., 1996). Therefore, the risk is more theoretical at this point than observed.

What is the fate of the Y chromosome and male fertility?

Of some concern is the future of the human Y chromosome vis-à-vis deletion of the AZFc region and its resultant detrimental impact on spermatogenesis (Kremer et al., 1998). Mathematical models based upon our current success rates with, and utilization of, ICSI do not predict a substantial increase in male infertility through transgenerational passage of genetically based defects in spermatogenesis, but do show a dramatic increase in male infertility in future generations if ICSI becomes more widely employed and the outcomes are improved (Faddy et al., 2001). Therefore, this is an issue which will need be watched carefully in the future.

In conclusion, 42 infertile men who have a Y-chromosomal microdeletion limited to the AZFc region are fully characterized in this report. The de-novo AZFc region microdeletion is the proximate cause of their spermatogenic deficiency. These men are phenotypically normal. The genes in this region do not appear to play a role in general physiological processes, organogenesis, testicular descent or germ cell oncogenesis. There may be three separate time points at which homologous recombination eliminating the AZFc region occurs: in the father’s spermatogonia, during paternal meiosis, or in the earliest stages of embryogenesis. There is no demonstrable paternal age effect. It is likely that an AZFc-deleted man will have sperm that can be used in conjunction with ICSI, either at low levels in the ejaculate or within harvested testis tissue. Our data do not support the concept that sperm production is rapidly deteriorating in these men, but rather that their baseline sperm production potential is stable over time. While most AZFc-deleted men will have functionally competent sperm in the ejaculate or in the testis tissue, there are some in whom no sperm are available and they are considered sterile, even in the era of ICSI. Testicular histology as well as levels of FSH, LH and testosterone do not distinguish individuals with regard to their spermatogenic potential. AZFc-deleted men have a good prognosis for ICSI, but the sons will inherit their father’s defect. The deletion lengths of the sons are not increased over that of the fathers and they would be expected to display the same range of spermatogenic capability as our cohort and not necessarily be an exact reflection of their own father. Both sons and daughters are somatically healthy. The threat of Y-chromosomal loss in the offspring has not been demonstrated to date. A small percentage of Y-microdeleted patients may choose not to undergo ICSI with their own sperm for fear of having infertile male offspring.

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References


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