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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 ($<5\times10^6$/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 (*CSPGL4Y*, *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 ($3'\leftarrow5'$ :: $5'\rightarrow3'$) (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* microdeleted 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.

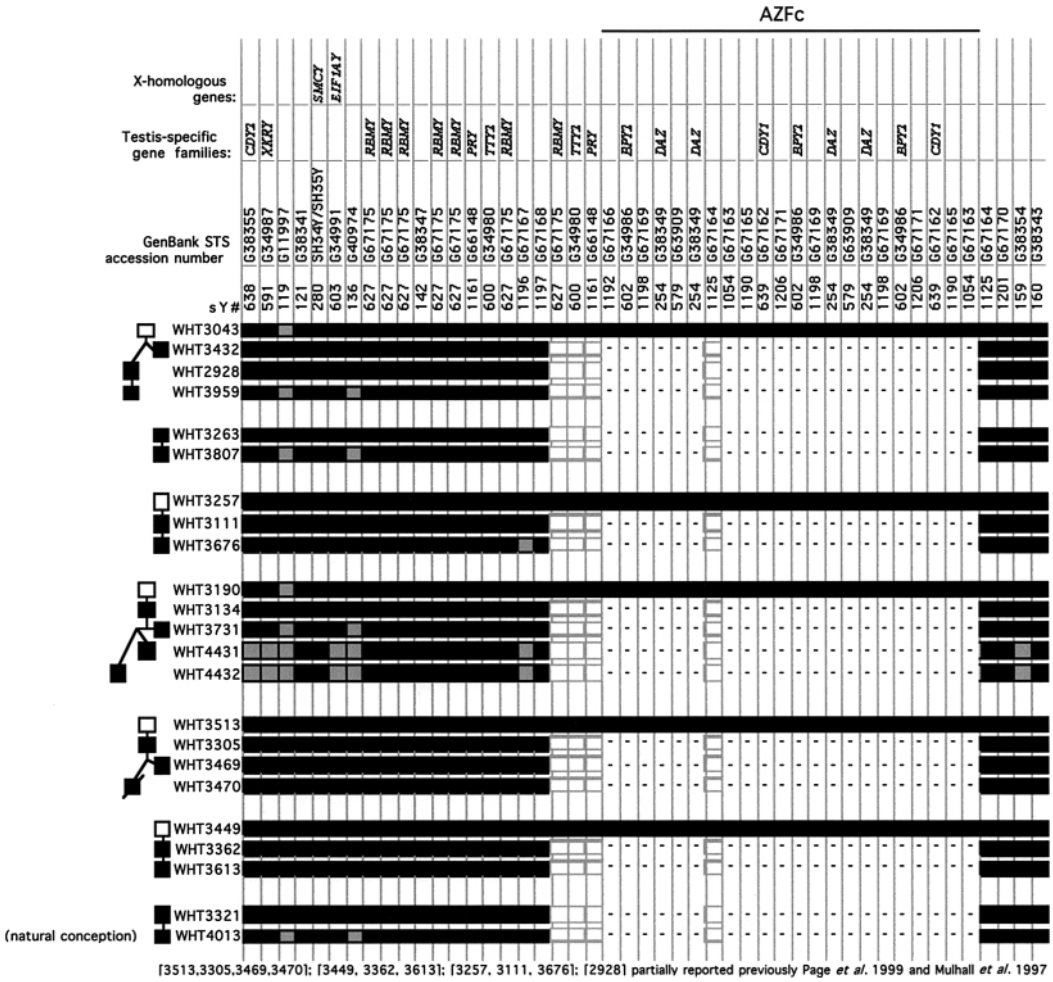

Figure 1. *AZFc* deletion map of eight infertile men, their 10 conceived sons and their fathers, when available. Solid black boxes indicate sequence tagged sites (STSs) that were present; minus signs indicate experimentally absent STSs (deleted STSs); solid grey boxes represent the inferred presence 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

Patient code	Age at diagnosis (years)	Medical history	Karyotype (mIU/ml)	FSH (mIU/ml)	LH (mIU/ml)	Testosterone (ng/dl)	Histology at TESE/Tbx	Father's age at birth of patient (years)
Severely oligospermic: $<5\times10^6$/ml (n = 16)								
WHT 3016	42		46,XY	4	5	238	–	32
WHT 3134	35		46,XY	13	6	565	–	39
WHT 3421	30		46,XY	3	3	219	MA	–
WHT 3706	38		46,XY	3	1	378	–	33
WHT 3628	27		46,XY	12	3	360	–	–
WHT 3185	34		46,XY	12	18	317	–	37
WHT 3254	31		46,XY	17	5	549	SCO/MA	35
WHT 3305	34		46,XY	2	6	413	MA	26
WHT 3321	32		46,XY	10	8	–	MA	33
WHT 3583	48		46,XY	28	–	550	–	30
WHT 3362	32		46,XY prominent 22p	6	4	320	MA	30
WHT 3985	40		46,XY	8	3	328	–	–
WHT 3722	30		46,XY	6	4	500	–	35
WHT 4489	35		46,XY	17	3	330	–	33
WHT 4550	37		–	12	3	282	–	–
WHT 4404	33		46,XY	6	6	135	–	–
Azoospermia: sperm detected in testis tissue (n=14)								
WHT 3263	33	Fraternal twin – 47,XXY	46,XY	15	–	266	SCO	30
WHT 3622	28		46,XY	12	6	476	MA	–
WHT 3279	34		46,XY	22	10	268	SCO	22
WHT 2928	28	Fraternal twin of WHT 3432	46,XY	17	11	349	SCO	30
WHT 2810	29		46,XY	11	–	–	MA	33
WHT 3111	32		46,XY	15	6	393	MA	21
WHT 2929	27		46,XY	16	10	223	SCO	25
WHT 2948	30		46,XY	6	7	482	MA	20
WHT 3330	53		46,XY	–	–	–	SCO/MA	–
WHT 2840	46		46,XY	14	9	310	MA	–
WHT 3329	31		46,XY	14	8	–	MA	–
WHT 4405	34		46,XY	9	7	–	SCO/MA	–
WHT 2381[a]	38		46,XY	28	2	327	SCO	45
WHT 2430[a]	39		46,XY	–	–	–	SCO	34
Azoospermia: no sperm detected in testis tissue (n = 7)								
WHT 4019[a]	24		Mosaic	9	3	309	SCO/MA	20
WHT 3444	28	Unilateral cryptorchidism	46,XY	13	8	423	SCO	–
WHT 3437	31		46,XY	11	–	377	–	26
WHT 3060	42		46,XY	17	11	438	SCO	25
WHT 3125	34		46,XY	35	6	362	SCO	28
WHT 3087	28		46,XY	6	1	263	MA	20
WHT 2820	37		46,XY	22	–	249	SCO/MA	26
Azoospermia: no TESE (n = 5)								
WHT 3432	28	Fraternal twin of WHT2928	46,XY	–	–	–	–	30
WHT 3452	30		46,XY	20	9	219	–	35
WHT 3630	40		46,XY	44	4	481	MA	–
WHT 3428	38		46,XY	35	12	286	–	30
WHT 3116	31		46,XY	6	6	451	–	36

[a]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 3.5×10^6 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

Table II. Mean FSH, LH, testosterone, patient age and paternal age values for each subgroup

	Mean (range)			
	Entire group	Oligospermia	Azoospermia: sperm detected	Azoospermia: no sperm detected
FSH mIU/ml	14.1 (2–44)	9.9 (2–28)	14.9 (6–28)	16.1 (6–35)
LH mIU/ml	6.3 (1–18)	5.2 (1–18)	7.6 (2–11)	5.8 (1–11)
Testosterone ng/dl	355 (135–565)	366 (135–565)	344 (223–482)	346 (249–438)
Age at diagnosis	34 (24–53)	35 (27–48)	34 (27–53)	32 (24–42)
Paternal age	30 (21–45)	33 (26–39)	29 (21–45)	24 (20–28)

completely evaluable exhibited some degree of spermatogenesis.

History 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 *AZFc*-deleted oligospermic group compared with 20 randomly selected Y-intact men: FSH, 9.9 versus 11 mIU/ml; LH, 5.2 versus 6 mIU/ml; testosterone, 366 versus 441 ng/dl respectively.

Paternal age (Tables I and II)

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 at the time of the patient's birth (*t*-test). The percentage distribution (in 5 year increments) of the fathers at the time of the birth of their *AZFc*-deleted sons was no different than a similar group of 60 men whose severely oligospermic or azoospermic sons were found to be Y-intact (χ^2-test) (Figure 2).

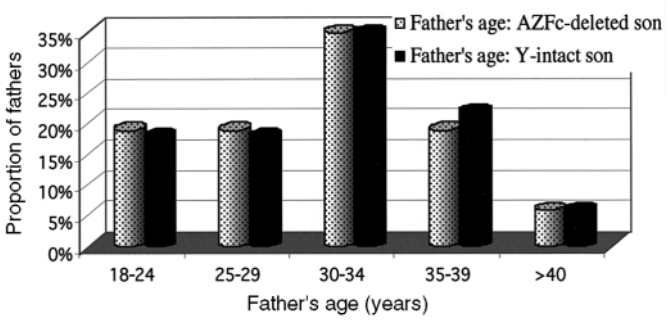

Figure 2. Distribution of fathers' ages at time of birth of *AZFc*-deleted or Y-intact sons demonstrating no effect of increasing paternal age on the frequency of having an *AZFc*-deleted son.

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

Patient	Sperm source	No. ICSI cycles total	No. oocytes inseminated	No. resultant embryos (2PN)	% fertilization	Outcome
WHT 3421	Ejaculate	1	16	5	31	Female singleton
WHT 3628	Ejaculate	1	8	7	87	No pregnancy
WHT 3722	Ejaculate	1	15	12	80	Female twins
WHT 3254	Ejaculate	1	14	9	64	No pregnancy
WHT 3321[a]	Ejaculate	1	25	19	76	Female singleton
WHT 3583	Ejaculate	1	3	3	100	No pregnancy
WHT 3305[b]	Ejaculate	1	15	9	60	Male twins WHT 3470[b], WHT 3469[b]
WHT 4404	Ejaculate	1	14	14	100	No pregnancy
WHT 3016	Ejaculate	1	22	12	55	No pregnancy
WHT 4489	Ejaculate	1	3	1	33	No pregnancy (poor oocyte quality)
WHT 3362[b]	Ejaculate	1	8	5	63	Twins : female and male WHT 3613[b]
WHT 3185	Ejaculate	2	30	21	70	No pregnancy (early SAB); Female singleton
WHT 3134	Ejaculate	3	37	24	65	No pregnancy; male singleton WHT 3731 (1 twin fetus lost early); male twins WHT 4431, 4432
WHT 3706	Ejaculate	3	24	18	75	No pregnancy ×2; male singleton
WHT 3329	Thawed testis	1	10	2	20	No pregnancy
WHT 2928[b]	Thawed testis	2	26	11	42	Female twins; male singleton WHT 3959 (1 twin fetus SAB)
WHT 2810[b]	Thawed testis	4	28	11	39	No pregnancy
WHT 3622	Thawed testis	4	73	25	34	No pregnancy ×4 (early SAB×1, poor oocyte quality noted)
WHT 3279	Thawed testis×1	3	5	0	0	No pregnancy
	Fresh testis×2		16	10	63	No pregnancy ×2
WHT 3623	Thawed testis×2	4	28	5	18	No pregnancy ×2
	Fresh testis×2		38	12	32	No pregnancy; male singleton WHT 3807
WHT 3330	Fresh testis	1	14	7	50	No pregnancy
WHT 2840[b]	Fresh testis	1	2	0	0	No pregnancy (extremely poor oocyte quality noted)
WHT 2948	Fresh testis	2	23	11	48	No pregnancy (early SAB)
WHT 2929	Fresh testis	1	20	6	30	No pregnancy
WHT 4405	Fresh testis	1	14	9	64	No pregnancy
WHT 3111[b]	Fresh testis	5	44	15	34	No pregnancy ×4; male singleton WHT 3676[b]
Subtotal	Ejaculated sperm	19	234	149	64	9 pregnancies, 13 babies (7 male, 6 female)
Subtotal	Testicular sperm	29	341	124	36	4 pregnancies, 5 babies (3 male, 2 female)
Overall total		48	575	273	47	13 pregnancies, 18 babies (10 male, 8 female)

[a]WHT 3321 also had a male (WHT 4013) conceived naturally.
[b]Reported previously (Mullhall *et al.*, 1997 and Page *et al.*, 1999).

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.

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

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 $>5\times10^6$/ml, but WHT 3706 had two analyses with counts slightly greater than this and total sperm density close to 10×10^6/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 WHT3444 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 spermatogonia 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 $1:16\times10^6$. 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

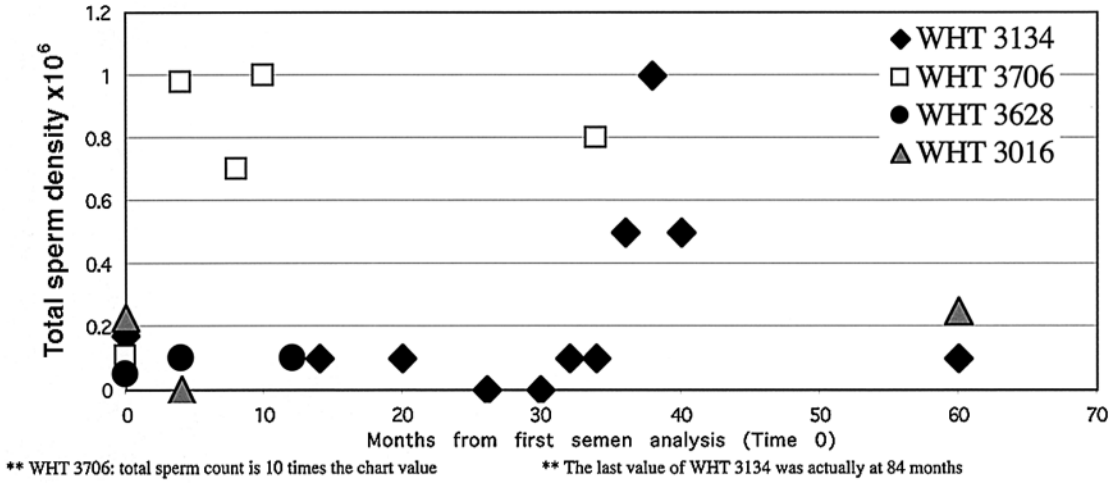

Figure 3. Fluctuation in sperm density over time in *AZFc*-deleted oligospermic men. Of note is WHT 3706 who had a total sperm count that reached 10×10^6 sperm/ejaculate (10 times the chart value). WHT 3016 and WHT 3134 both demonstrate sperm density fluctuation above and below a very low baseline, sometimes even to the azoospermic level. All pregnancies for WHT 3134 occurred with ejaculated sperm subsequent to his azoospermic samples.

of our *AZFc*-deleted men ranges from sterile (no sperm available, even in testis tissue) to severely oligospermic ($<5\times10^6$/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 $<1\times10^6$/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 anomalies 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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