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# Detection of sperm in men with Y chromosome microdeletions of the AZFa, AZFb and AZFc regions

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**BACKGROUND: Y chromosome microdeletions are associated with severe male factor infertility. In this study, the success rate of testicular sperm retrieval was determined for men with deletions of AZF regions a, b or c. METHODS: AZF deletions were detected by PCR of 30 sequence-tagged sites within Yq emphasizing the AZFa, b and c regions. Semen analysis and diagnostic testis biopsy or testicular sperm extraction (TESE) findings were correlated with the specific AZF region deleted. RESULTS: A total of 78 men with AZF deletions included three with AZFa deletion, 11 with AZFb, 42 with AZFc, 16 with AZFb+c and six with Yq (AZFa+b+c). All men with AZFa, AZFb, AZFb+c and Yq deletions were azoospermic and no sperm were found with TESE or biopsy. Of men with isolated AZFc deletion, sperm were found in 75% (9/12) by TESE and 45% (9/20) on biopsy (56% overall); 62% (26/42) were azoospermic and 38% (16/42) severely oligozoospermic. A total of 7 patients with deletion patterns that included the complete AZFa region and 23 that included the complete AZFb region who underwent TESE or biopsy did not have sperm detected by these surgical measures. CONCLUSIONS: Microdeletion of the entire AZFa or AZFb regions of the Y chromosome portends an exceptionally poor prognosis for sperm retrieval, whereas the majority of men with AZFc deletion have sperm within the semen or testes available for use in IVF/ICSI.**

*Key words:* AZF/genetics/male infertility/spermatogenesis/Y chromosome

## Introduction

Approximately 15% of couples attempting to conceive over a period of 1 year are unable to become pregnant and 20% of those cases are attributable to a male factor alone, while a male factor is contributory in nearly another 40% of cases (Thonneau *et al*., 1991). Genetic aetiologies play a prominent role in male infertility as up to 12% of men with non-obstructive azoospermia have karyotypic abnormalities (De Braekeleer *et al*., 1991), whereas Y chromosome microdeletions are identified in 6–18% of men with non-obstructive azoo- or oligozoospermia (Reijo *et al*., 1995; 1996; Vogt *et al*., 1996; Brandell *et al*., 1998; Martinez *et al*., 2000). A significant role for the Y chromosome in spermatogenesis was established over a quarter of a century ago when Tiepolo and Zuffardi (1976) detected, on karyotype analysis, large terminal deletions of the long arm of the Y chromosome (Yq) in six men with azoospermia, suggesting that factors (azoospermia factors) essential for spermatogenesis reside within the euchromatic region of Yq. Subsequent cytogenetic (Hartung *et al*., 1988; Chandley *et al*., 1989) and molecular (Andersson *et al*., 1988; Bardoni *et al*., 1991; Ma *et al*., 1992) studies resulted in elucidation of multiple terminal and interstitial deletions of the Y chromosome associated with abnormal spermatogenesis. Reijo *et al*. (1995) detected 12 de-novo microdeletions within the euchromatic portion of the Y chromosome in 89 azoospermic men by PCR amplification of 84 sequence-tagged sites (STS) along Yq. The region disrupted contained the gene DAZ (deleted in azoospermia) and was associated with variable degrees of spermatogenesis in different patients. Subsequently, two of 35 men with severe oligozoospermia were found to have de-novo microdeletions within the AZF region as well (Reijo *et al*., 1996). Not only was the absence of AZF associated with presence of a low number of sperm within the ejaculate, but the sperm were also shown to contain the same mutation. Three out of six azoospermic men with AZF/DAZ deletion had sperm identified within harvested testicular tissue, and when used for ICSI, fertilization occurred in 36% of injected oocytes (Mulhall *et al*., 1997a). One twin conception resulted, proving the functionality of sperm from an AZF/DAZ-deleted patient.

Further analysis of Yq in 370 men with idiopathic azoospermia or severe oligozoospermia utilizing STS analysis of 76 DNA loci revealed de-novo microdeletions in 12 men and an inherited microdeletion in one individual (Vogt *et al*., 1996). In this sentinel study, the deletions localized to three non-overlapping regions in Yq, AZFa, AZFb and AZFc. The AZFc region corresponded with the DAZ region. However, the recent discovery that a large portion of euchromatic Yq consists of massive, nearly identical ampliconic repeats arranged in palindromes has elucidated the difficulty in precisely identifying borders of the AZF regions (Kuroda-Kawaguchi *et al*., 2001; Repping *et al*., 2002). Localization of breakpoints for the AZFb and c regions has demonstrated that these two regions overlap by 1.5 Mb, and the combined AZFb+c deletion has yet another distinct set of breakpoints in the same region (Repping *et al*., 2002).

Vogt *et al*. (1996) first demonstrated that deletion of the three different AZF regions appears to have different effects on spermatogenesis. The correlation between detection of sperm within testicular tissue and specific AZF deletion has been made in only a few studies with small numbers of subjects. Whereas sperm have been detected within the testes of men with AZFc deletion by microdissection testicular sperm extraction (TESE) (Schlegel, 1999), no spermatids were detected in men with deletion of the AZFb region for use in ICSI, suggesting that complete AZFb deletions are a significantly adverse prognostic finding for TESE (Girardi *et al*., 1997; Brandell *et al*., 1998; Silber *et al*., 1998; Krausz *et al*., 2000). A recent, thorough characterization of 42 men with AZFc deletion determined that 81% of men with this deletion produce sperm overall, 38% with sperm in the ejaculate and 67% of those that are azoospermic on diagnostic biopsy or with TESE (Oates *et al*., 2002).

We have identified and characterized a large number of men with deletion of AZFa, AZFb and/or AZFc to determine the prevalence of complete spermatogenesis for each type of microdeletion by assessing the presence of sperm on semen analysis and the presence of intratesticular sperm identified either on diagnostic testis biopsy or with TESE. These findings provide prognostic information on the chance of successful sperm retrieval for these patients based upon the specific region of the Y chromosome deleted.

## Materials and methods

### *Patient selection and clinical evaluation*

Men who presented for evaluation of infertility and were found to have Y chromosome microdeletion were included in this study. Informed consent was obtained from all patients who underwent Y chromosome microdeletion testing. Charts were reviewed for semen analysis, diagnostic testis biopsy results, findings on microdissection TESE, patient age, serum testosterone, serum FSH and LH, testicular volume and karyotype. For patients with multiple semen analyses available for analysis, the mean of individual sperm concentration results was used and reported as an average sperm concentration. The presence of elongating spermatids on diagnostic biopsy or the finding of sperm on TESE was correlated with the specific AZF region deleted. For patients referred to our laboratory only for diagnostic micro-Y testing, results of semen analysis, diagnostic biopsy and TESE were obtained directly from the referring physician; those patients for whom this information was not available were excluded from analysis.

### *Y chromosome analysis*

Genomic DNA was extracted from peripheral blood using two methods, the Stratagene DNA Extraction Kit (Stratagene, USA) and the Genomic DNA Purification Kit (Promega, USA). Thirty STS within the long arm of the Y chromosome were selected emphasizing the AZFa, b and c regions. Previously published primer sequences were used for each STS (Henegariu *et al*., 1994; Reijo *et al*., 1995; 1996). Testing for the presence of the short arm of the Y chromosome (Yp) was performed with the STS sY14, located within the SRY (sex-determining region on the Y chromosome) gene. The most distal STS on Yq, sY160DYZ1, is located within the heterochromatic region, whereas all others are located within the euchromatic region. Multiplex PCR was performed for analysis of microdeletions. PCR products were run by electrophoresis on a 4% agarose gel impregnated with ethidium bromide for visualization under UV light. Failure of amplification for a given STS was confirmed twice with single primer PCR. DNA from a fertile male served as positive control, whereas water and DNA from a female served as negative control for multiplex and single primer PCR respectively.

### *Microdissection TESE technique*

A semen specimen was obtained immediately prior to planned TESE to confirm azoospermia. If no sperm were identified within the pellet produced by centrifugation of the sample at 3000 *g* for 15 min, microdissection TESE was performed on either the same day of planned oocyte retrieval or 1 day prior to oocyte retrieval with incubation of the sperm overnight. Microdissection TESE was performed as previously described (Schlegel, 1999) utilizing the operating microscope and one transverse incision in the tunica albuginea through which spermatogenic tubules were selected for extraction.

### *Statistical analysis*

Outcome variables (age, serum testosterone, serum FSH and LH, testicular volume) for azoospermic men with AZFc deletion with successful surgical sperm retrieval were compared to those with unsuccessful sperm retrieval using the Mann–Whitney *U*-test. Men with AZFc deletion and azoospermia were also compared to the group of AZFc-deleted men with oligozoospermia using the same variables and the Mann–Whitney *U*-test.

## Results

A total of 78 men with AZF deletion was evaluated. The different STS deletion patterns and the number of patients found to have each pattern are shown in Figure 1. The most common region deleted was AZFc. Further distribution of these patients by specific deletion, the number of patients in each group with azoospermia and the number of patients within each group to undergo TESE, diagnostic testis biopsy or neither surgical procedure are shown in Table I. All men with AZFa, AZFb, AZFb+c and Yq deletions were azoospermic. While 62% (26/42) of men with isolated AZFc deletion were azoospermic, 38% (16/42) sustained a level of spermatogenesis sufficient to produce sperm within the ejaculate (Table II). The highest average sperm concentration reported for the one

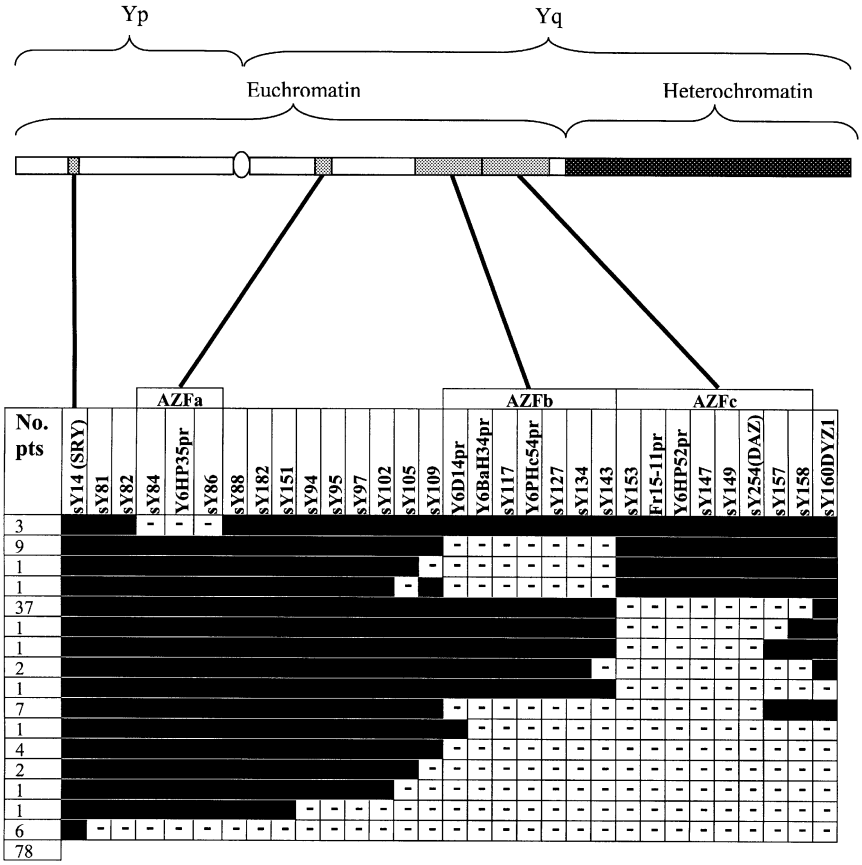


**Figure 1.** Schematic diagram of Y chromosome illustrating sequence-tagged sites (STS), AZF regions and the different deletion patterns of all patients in this study. '–' denotes deletion of specific STS. The column on the left shows the number of patients in this study with the deletion pattern indicated.

**Table I.** Total number of patients in each deletion group, number of patients in each group with azoospermia and surgical procedure evaluated

| Deletion | Total no. of patients | No. with azoospermia (%) | No. of patients to undergo: | | |
|---|---|---|---|---|---|
| | | | TESE | Diagnostic biopsy | Neither procedure |
| AZFa | 3 | 3 (100) | 1 | 2 | 0 |
| AZFb | 11 | 11 (100) | 6 | 3 | 2 |
| AZFc | 42 | 26 (62) | 12 | 20 | 10 |
| AZFb+c | 16 | 16 (100) | 6 | 4 | 6 |
| Yq (AZFa+b+c) | 6 | 6 (100) | 3 | 1 | 2 |

TESE = testicular sperm extraction.

**Table II.** Average sperm concentration for 42 men with AZFc deletion

| Classification | Average sperm concentration ($\times 10^6$/ml) | No. of patients (%) |
|---|---|---|
| Azoospermia | 0 | 26 (62) |
| Virtual azoospermia | <1 | 13 (31) |
| Severe oligozoospermia | 1–5 | 2 (5) |
| Moderate oligozoospermia | >5–10 | 1 (2) |
| Mild oligozoospermia | >10–20 | 0 |

patient (age 38 years) with moderate oligozoospermia was $5.3\times10^6$/ml (from three semen analyses showing 3, 5 and $8\times10^6$/ml).

Table III shows the surgical sperm retrieval rate for each deletion group. All men with AZFa, AZFb, AZFb+c and Yq deletion had complete absence of sperm on both TESE and diagnostic biopsy. A total of seven patients with the entire AZFa region deleted (three men with AZFa deletion + four men with Yq deletion) and 23 patients with deletions that involved at least the entire AZFb region (nine men with AZFb deletion + 10 men with AZFb+c deletion + four men with Yq deletion) did not have sperm located within testicular tissue by TESE or on diagnostic testis biopsy. The only group of patients with evidence of sperm production were men with isolated AZFc deletions. Surgical techniques demonstrated sperm in 75% (9/12) of men with isolated AZFc deletion who underwent

TESE and in 45% (9/20) who underwent diagnostic biopsy alone allowing documentation of sperm production for 56% (18/32). All men with AZFc deletion who underwent TESE were azoospermic on the day of the TESE/ICSI cycle, although three of these men had demonstrated 100–300 sperm on semen analysis at one time prior to the TESE, stressing variable intra-individual spermatogenic capacity; TESE was successful for all three men. Five men with AZFc deletion who underwent biopsy had sperm documented on semen analysis prior to biopsy, and of those, four had spermatids detected on the biopsy, while one did not. Two of the 10 men with AZFc deletion that did not undergo TESE or biopsy were consistently azoospermic. Thus, documentation of spermatogenesis throughout the patient's medical histories, whether within surgically retrieved testicular tissue or within the ejaculate, for AZFc-deleted men occurred in 9/12 (TESE group) + 10/20 (biopsy group) + 8/10 (neither surgical procedure group) for a total of 27/42 or 64%.

Karyotype analysis was available for 47 men in this study. Of one patient with AZFa deletion and four with AZFb deletion, all had 46,XY karyotype. A total of 25 men with AZFc deletion underwent karyotype analysis and all with the exception of three were normal; one patient was 47,XXY/46,XY, one was 46,XY/45,X/46,XidicY(q12) and the third 46,XY with an inversion on chromosome 9. A total of 12 men with AZFb+c deletion underwent karyotype analysis, and with the exception of three men with 45,X/46,XY mosaicism and one with 46XinvY(q11.2q12) karyotype, all were normal. Five men with Yq deletion were evaluable for karyotype which showed 46,XX(SRY$^+$) for four and 46X,i(Y)(p10) in one patient.

Age, serum testosterone, serum FSH and LH levels and testicular volume were correlated with surgical detection of sperm by TESE or diagnostic biopsy in azoospermic men with AZFc deletion for whom information on all parameters was available. No statistically significant differences were noted between median values of the variables between the two groups (Table IV). Comparison of men with AZFc deletion who were azoospermic to the group of AZFc-deleted men that were oligozoospermic also demonstrated no statistically significant differences in median values for age, testosterone, FSH, LH and testicular volume (Table V).

**Table III.** Surgical sperm retrieval rate (%) in AZF deletion

| Deletion | TESE | Diagnostic biopsy | Overall |
|---|---|---|---|
| AZFa | 0 (0/1) | 0 (0/2) | 0 (0/3) |
| AZFb | 0 (0/6) | 0 (0/3) | 0 (0/9) |
| AZFc | 75 (9/12) | 45 (9/20) | 56 (18/32) |
| AZFb+c | 0 (0/6) | 0 (0/4) | 0 (0/10) |
| Yq (AZFa+b+c) | 0 (0/3) | 0 (0/1) | 0 (0/4) |

## Discussion

The association between Y chromosome microdeletion and defective spermatogenesis has been well established (Reijo *et al*., 1995; 1996; Vogt *et al*., 1996; Girardi *et al*., 1997; Mulhall *et al*., 1997a; Brandell *et al*., 1998; Silber *et al*., 1998; Martinez *et al*., 2000; Krausz *et al*., 2000; Oates *et al*., 2002). Although it has been shown that a proportion of patients with AZFc deletion may have sperm in the ejaculate and also may have sperm within the testis on diagnostic biopsy or with testicular sperm extraction (Silber *et al*., 1998; Oates *et al*., 2002), a large series of patients with deletions involving the AZFa and AZFb regions has not been characterized in published literature. During evaluation of infertility, testis biopsy may be done to determine the extent of spermatogenesis present within the testicle in equivocal cases to distinguish obstructive from non-obstructive azoospermia. A diagnosis of

**Table IV.** Correlation of age, serum testosterone, FSH, LH and testicular volume with sperm retrieval in azoospermic men with AZFc deletion

| | Age (years) | Testosterone (270–1730 ng/dl)[a] | FSH (1–8 IU/l)[a] | LH (2–12 IU/l)[a] | Left testicular volume (ml) | Right testicular volume (ml) |
|---|---|---|---|---|---|---|
| Sperm + ($n$ = 9) | 33 ± 5.4 | 315 ± 115 | 14.9 ± 5.3 | 5.7 ± 2.4 | 12 ± 2.7 | 11 ± 3.3 |
| Sperm – ($n$ = 11) | 32 ± 5.0 | 405 ± 209 | 13.9 ± 8.2 | 5.1 ± 2.4 | 14 ± 3.5 | 13 ± 3.5 |
| $P$-value | NS | NS | NS | NS | NS | NS |

Values are mean ± SD.
[a]Normal range.
NS = not significant.

**Table V.** Correlation of age, serum testosterone, FSH, LH and testicular volume with presence of sperm in the ejaculate of men with AZFc deletion

| | Age (years) | Testosterone (nl 270–1730 ng/dl) | FSH (nl 1–8 IU/l) | LH (nl 2–12 IU/l) | Left testicular volume (cc) | Right testicular volume (cc) |
|---|---|---|---|---|---|---|
| Azoospermic ($n$ = 21) | 32 ± 5.3 | 377 ± 180 | 14.7 ± 6.9 | 5.5 ± 2.3 | 13 ± 3.3 | 12 ± 3.7 |
| Oligozoospermic ($n$ = 8) | 34 ± 5.3 | 381 ± 147 | 13.1 ± 4.3 | 5.4 ± 2.1 | 12 ± 2.8 | 12 ± 4 |
| $P$-value | NS | NS | NS | NS | NS | NS |

Values are mean ± SD.
nl = normal range; NS = not significant.

non-obstructive azoospermia should prompt microdeletion testing (Jarow *et al*., 2002). Microdissection TESE has been shown to be a successful technique for sperm retrieval in men with non-obstructive azoospermia of various aetiologies and appears to be the procedure of choice for sperm retrieval for use with ICSI in these patients (Schlegel, 1999). We characterized 78 men with AZFa, AZFb and/or AZFc deletion to determine the incidence of sperm appearance in the ejaculate, on histological evaluation of testis tissue from diagnostic biopsy and with TESE to evaluate prognostic information for sperm retrieval in this population based on the specific region deleted.

The most common microdeletion found in our patient population was AZFc. The association between AZFc deletion and impaired, but variable, spermatogenesis has been demonstrated previously (Mulhall *et al*., 1997a; Silber *et al*., 1998; Oates *et al*., 2002). Here, we report that 38% of men with isolated AZFc deletion had sperm present within the ejaculate and a surgical sperm retrieval rate of 56% overall (75% with TESE and 45% with diagnostic biopsy). These results are consistent with the characterization of a large cohort of men with AZFc deletion by Oates *et al*. (2002), which showed that 38% of men with AZFc deletion had sperm in their ejaculate and 67% had some degree of complete spermatogenesis demonstrated either with TESE or diagnostic biopsy. Clearly men with AZFc deletion have variable capacity to produce sperm, some producing none within the seminiferous tubules, while some produce a quantity of sperm sufficient to survive epididymal transit and appear in the ejaculate (Silber *et al*., 1997). Although rare cases of transmission of the AZFc deletion have been described from fertile father to infertile sons by natural conception (Chang *et al*., 1999; Saut *et al*., 2000), the majority of men with AZFc deletion have significantly compromised spermatogenesis, such that ICSI is necessary for sperm from these patients to produce biological offspring. The highest sperm concentration detected in an AZFc patient in this study was $8\times10^6$/ml. It is therefore unlikely that Y chromosome microdeletion testing in men with sperm concentration greater than this will yield a positive result.

Table III illustrates the success rates with which sperm were surgically retrieved from the testes by two techniques, standard diagnostic testis biopsy and microdissection TESE. Routine diagnostic biopsy is usually done by removal of a small piece of tissue from one superficial area of the testis, whereas microdissection TESE entails thorough microscopic assessment of a large area of seminiferous tubules accessed with one generous transverse incision within the tunica albuginea to identify spermatogenic tubules. Our results of sperm retrieval in men with AZFc deletion show that the microdissection TESE technique is nearly twice as effective when compared with routine biopsy technique for sperm retrieval, consistent with previous observations in men with nonobstructive azoospermia (Schlegel, 1999).

Age, serum testosterone, FSH, LH and testicular volume did not correlate with sperm retrieval in AZFc-deleted patients (Table IV). Success of surgical sperm retrieval in azoospermic men with AZFc deletion cannot be predicted using these variables. As previously noted by multiple investigators, FSH levels and testicular volume do not predict sperm retrieval as these factors reflect overall testicular function (Mulhall *et al*., 1997b; Schlegel *et al*., 1997; Oates *et al*., 2002). The single 'best' region of spermatogenesis determines the chance of sperm retrieval and does not affect FSH or testicular size. Likewise, age, serum testosterone, FSH, LH and testicular volume did not correlate with the presence of sperm in the ejaculate in men with AZFc deletion (Table V). Mean age of the oligozoospermic group was not less than that of the azoospermic group. The capacity to maintain spermatogenesis at a level sufficient for spill into the ejaculate appears to vary between individuals in a manner independent of age, testosterone, FSH, LH and testicular volume.

Isolated microdeletion of the AZFa region is relatively rare, whereas deletion of the AZFb region occurs more commonly, either in isolation or in conjunction with deletion of the AZFc region (Figure 1). We have identified nine men with deletion of the seven STS spanning the AZFb region only, whereas an additional seven patients had the entire AZFb region deleted plus the proximal six (out of a total of eight) STS of the AZFc region. Although the STS used in our study are different from those used by Repping *et al*. (2002), the deletion pattern of these seven patients may correlate with the P5/distal-P1 type of deletion pattern in which the distal portion of AZFc is spared. Several deletion patterns involving the AZFb and c regions are evident in Figure 1, and although spermatogenesis is variable with deletion of the AZFc region alone, none of the patients in this study with deletion of the AZFb region, either isolated, in continuity with the AZFc region, or as part of Yq deletion demonstrated evidence of mature spermatid production.

Incomplete spermatogenesis has been demonstrated for men with deletions involving the AZFa and AZFb regions on testis histology (Vogt *et al*., 1996; Pryor *et al*., 1997; Martinez *et al*., 2000). Kamp *et al*. (2001) described in detail a series of nine patients with complete AZFa deletion; all patients had a Sertoli cell-only pattern on histology with no sperm identified. Consistent with reported testis biopsy findings, attempted TESE in men with complete deletions of the AZFa or AZFb regions has been unsuccessful. Brandell *et al*. (1998) described seven azoospermic patients with deletions including the entire AZFb region; all underwent TESE with no evidence of elongating spermatids or sperm. Silber *et al*. (1998) did not detect sperm by TESE in five azoospermic patients with deletions encompassing the AZFa and/or AZFb regions. To our knowledge, completion of spermatogenesis in men with deletions of the full AZFa or AZFb regions has not been demonstrated. Partial deletions within an AZF region are not prognostically revealing.

A total of seven men with deletion patterns that include deletion of the AZFa region (three with deletion of AZFa alone + four with Yq deletion) and 23 men with deletion including the AZFb region (nine with AZFb deletion, 10 with AZFb+c deleted and four with Yq deletion) underwent either diagnostic testis biopsy or TESE and all had evidence of abnormal spermatogenesis with complete absence of elongating or mature spermatids. The majority of men with isolated AZFc deletion, however, did have adequate sperm production so that sperm from the ejaculate or surgically retrieved from the testis

were available for ICSI. Vertical transmission of the deletion has been demonstrated, and all sons inherit the same deletion, likely to cause defective spermatogenesis, though to an uncertain degree (Chang *et al*., 1999; Saut *et al*., 2000; Oates *et al*., 2002). Boys with inherited AZFc deletion are otherwise healthy, and girls are unaffected. Couples in which the male partner has AZFc deletion should be informed of this inheritance pattern prior to the decision to proceed with ICSI.

Karyotype analysis demonstrated that large chromosome abnormalities can coexist with Y chromosome microdeletions, most commonly sex chromosome mosaicism. The five men with Yq deletion by microdeletion testing were found to lack the entire Yq on karyotype analysis. If the first genetic test done is that of the karyotype, and this demonstrates absence of Yq (i.e. 46,XX male), then microdeletion testing is likely unnecessary. We did not observe translocation of Yq regions to other chromosomes in this study.

In conclusion, deletions of the AZFa, b and c regions are associated with abnormal spermatogenesis, and certain deletions are of prognostic value. Microdeletion of either the entire AZFa or AZFb regions of the Y chromosome heralds an exceptionally poor prognosis for sperm retrieval, whereas the majority of men with AZFc deletion have sperm retrieved successfully for use in IVF with ICSI. The prognostic significance of complete deletions involving either the AZFa or AZFb regions should be discussed with patients prior to attempted TESE.

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