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Men with infertility caused by *AZFc* deletion can produce sons by intracytoplasmic sperm injection, but are likely to transmit the deletion and infertility

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Deletion of the *AZFc* region of the Y chromosome is the most frequent molecularly defined cause of spermatogenic failure. We report three unrelated men in whom azoospermia or severe oligozoospermia was caused by de-novo *AZFc* deletions, and who produced sons by intracytoplasmic sperm injection (ICSI). We employed polymerase chain reaction (PCR) assays to examine the Y chromosomes of their four infant sons. All four sons were found to have inherited the Y chromosome deletions. Such sons are likely to be infertile as adults. This likelihood should be taken into account when counselling couples considering ICSI to circumvent infertility due to severe oligozoospermia or non-obstructive azoospermia.

Key words: AZFc/chromosome deletions/ICSI/PCR

Introduction

Intracytoplasmic sperm injection (ICSI) has become the preferred method for circumventing severe male-factor infertility (Palermo *et al*., 1992; Tarlatzis and Bili, 1998). Such infertility is common: ~2% of men exhibit severe oligozoospermia or azoospermia (de Kretser, 1997). Deletion of the *AZFc* region of the Y chromosome is the most frequent molecularly-defined cause of spermatogenic failure. *AZFc* deletions have been reported by various investigators to account for 2–21% of cases of non-obstructive azoospermia and severe oligozoospermia (Reijo *et al*., 1995, 1996; Najmabadi *et al*., 1996; Nakahori *et al*., 1996; Qureshi *et al*., 1996; Vogt *et al*., 1996; Foresta *et al*., 1997; Girardi *et al*., 1997; Kremer *et al*., 1997; Pryor *et al*., 1997; Simoni *et al*., 1997; van der Ven *et al*., 1997; Vereb *et al*., 1997). *AZFc*-deleted men and their partners are candidates for ICSI, since in most cases spermatozoa or mature spermatids suitable for the procedure can be recovered from semen or from testis biopsies (Mulhall *et al*., 1997; Silber *et al*., 1998). In fact, *AZFc*-deleted men have fathered daughters via ICSI; in these cases the men transmitted their intact X chromosomes rather than their deleted Y chromosomes (Mulhall *et al*., 1997). Concerns have been voiced that *AZFc* deletions, and the resultant infertility, might be transmitted readily from fathers to sons via ICSI (Bhasin *et al*., 1994; Silber *et al*., 1995; Engel *et al*., 1996; Morris and Gleicher, 1996). However, such transmission has not been documented, and its likelihood is unknown.

Kent-First and colleagues conducted the first study of Y-chromosomal DNA sequences in boys conceived by ICSI (Kent-First *et al*., 1996). In that study, none of the 32 ICSI fathers tested were found to have a deletion of the *AZFc* region of the Y chromosome. In one family, the ICSI-conceived son was found to have a de-novo *AZFc*/*AZFb* deletion, which was not present in his father. In another family, transmission via ICSI was found of a small Y deletion that mapped outside the regions that have been clearly implicated in spermatogenic failure (*AZFa*, *AZFb*, *AZFc*) (Vogt *et al*., 1996). This small deletion might be an 'insignificant polymorphism' of no clinical importance (Kent-First *et al*., 1996). In no case was transmission observed of an *AZFc* deletion from father to son (Kent-First *et al*., 1996).

The pressing clinical question is whether infertile, *AZFc*-deleted men are likely to father sons via ICSI, and if so, whether those sons will carry the same deletions and thus be infertile. In theory, if simple Mendelian principles apply, Y chromosome deletions should be transmitted to all sons. The possibility that *AZFc* deletions would be transmitted via ICSI has been underscored by the finding of *AZFc*-deleted, Y-bearing spermatozoa in the semen of an oligozoospermic, *AZFc*-deleted man (Reijo *et al*., 1996), and by reports that, on rare occasions, *AZFc* deletions have been transmitted naturally (Vogt *et al*., 1996). Could this event, which is uncommon under natural circumstances (where *AZFc*-deleted men are rarely fertile), become routine when ICSI (which circumvents male infertility) is employed? These concerns have been so great that the possible effects of ICSI on the frequency of *AZFc* deletions in the general population have been modelled mathematically (Kremer *et al*., 1998). However, in no case has ICSI-mediated father-to-son transmission of an *AZFc* deletion been documented. Given the dearth of direct evidence, some investigators have prudently cautioned against premature conclusions as to the risks of genetic transmission via ICSI (Morris and Gleicher, 1996; Kupker *et al*., 1997).

Rather than studying the Y chromosomes of a set of unselected ICSI boys, (see Kent-First *et al*., 1996), we chose to focus on the ICSI sons of men with demonstrated *AZFc* deletions. From a large, ongoing study of Y-chromosomal DNA sequences in men with spermatogenic failure (Reijo *et al*., 1995; Mulhall *et al*., 1997; Silber *et al*., 1998), we identified three infertile couples who had had at least one son via ICSI, and in which the man carried an *AZFc* deletion. Using DNA probes of the Y chromosome, we investigated

whether the sons had inherited the *AZFc* deletions present in their fathers' Y chromosomes.

Materials and methods

Three unrelated couples were referred to the Infertility Center at St Luke's Hospital after 2–3 years primary infertility attributed to non-obstructive azoospermia. All six adults were otherwise healthy, had normal karyotypes as judged by analysis of peripheral blood leukocytes, and were aged 30–33 years. In three of the men, standard semen analyses had failed to reveal spermatozoa, and vasography had demonstrated no obstruction. As described below, we identified a few spermatozoa in ejaculates from two of the men, but only after semen centrifugation and extensive searching. In each case, testicular biopsy had revealed maturation arrest, with normal appearing spermatogonia and spermatocytes, but virtually no spermatids, mature or immature, except in rare tubules. The hormone concentrations for men from families 1, 2, and 3 were: follicle stimulating hormone (FSH) 1.6, 5.5, and 15 IU/l; luteinizing hormone (LH) 6.0, 4.3, and 6.0 IU/l; and testosterone 413, 320, and 393 ng/100 ml respectively.

Family 1

The couple underwent a single cycle of in-vitro fertilization (IVF) with ICSI. The woman was treated with s.c. leuprolide acetate, a gonadotrophin-releasing hormone (GnRH) agonist, to inhibit gonadotrophin secretion and then with FSH and human menopausal gonadotrophin (HMG), given i.m. to stimulate ovarian follicle development. Human chorionic gonadotrophin (HCG) was administered i.m., and 22 oocytes were retrieved 36 h later. Spermatozoa ($n = 15$) were recovered by centrifuging the man's ejaculated semen and searching through 25 concentrated microdroplets. A single spermatozoon was injected into each of 15 oocytes, nine of which underwent normal fertilization, as indicated by the presence of two pronuclei and two polar bodies. After 2 days, six embryos were transferred to the woman's Fallopian tube. She received daily i.m. injections of progesterone in oil (50 mg) until fetal cardiac activity was demonstrated by ultrasonography. By 2 weeks after embryo transfer, serum concentrations of β-HCG had increased to 478 IU/l. Ultrasonography was carried out 4 weeks later and demonstrated two intrauterine sacs, both with fetal heartbeats. At 28 weeks gestation, twin A appeared normal as judged by ultrasonography, but twin B exhibited pulmonary atresia and a small right ventricle. Two boys were delivered by Caesarian section at 37 weeks gestation; twin A weighed 2640 g and twin B 1900 g. Twin A was healthy at birth and remains so at age 11 months. Twin B required intubation shortly after birth. Echocardiography of twin B confirmed the pulmonary atresia and right ventricular hypoplasia detected prenatally. Balloon atrial septostomy was attempted, but was unsuccessful due to redundant atrial septal tissue. Poor myocardial contractility led to Twin B's death shortly after an attempted atrial septectomy. We know of no other case in which congenital heart disease has been associated with deletion of the *AZFc* region. No increase in the incidence of such cardiac defects has been observed among babies conceived by ICSI (Bonduelle *et al*., 1998). We suspect that congenital heart disease, observed in ~1% of all newborn children (Hoffman, 1995), was not caused by Y deletion in this case. Instead, it is probably an unfortunate coincidence of independent aetiology. Chromosome 22, whose anomalies have been implicated in some congenital heart defects (Goldmuntz and Emanuel, 1997), was scrutinized by cytogenetic methods in both parents; no abnormalities were detected (M.Watson, personal communication).

Family 2

The couple underwent a single cycle of IVF with ICSI, as described for Family 1. A total of 13 oocytes were retrieved. Eight spermatozoa were recovered by centrifuging the man's ejaculated semen and searching through 25 concentrated microdroplets. A single spermatozoon was injected into each of eight oocytes, five of which underwent normal fertilization. After 2 days, four embryos were transferred to the woman's Fallopian tube. Serum concentrations of β-HCG increased to 640 IU/l by 2 weeks after embryo transfer, and to 1684 IU/l 2 days later. Three weeks later, ultrasonography revealed two intrauterine sacs, both with fetal heartbeats. Both twins appeared normal by ultrasonography at 28 weeks gestation, and healthy twins were born by vaginal delivery at 35 weeks: a boy (1560 g) and a girl (1790 g). Both twins are healthy at age 4 months.

Family 3

The couple underwent four cycles of IVF with testicular sperm extraction (TESE) and ICSI. No spermatozoa were observed after centrifuging ejaculated semen. In the first cycle of TESE/ICSI, 10 oocytes were injected with spermatozoa retrieved from the testis, three oocytes underwent normal fertilization, and three embryos were transferred. In the second cycle, eight oocytes were injected, two oocytes were fertilized, and two embryos were transferred. In the third cycle, eight oocytes were injected, six oocytes were fertilized, and five embryos were transferred. No pregnancy was achieved in the first three cycles. In the fourth cycle, 10 oocytes were injected, four oocytes were fertilized, and four embryos were transferred. Serum concentrations of β-HCG increased to 268 IU/l by 2 weeks after embryo transfer, and to 969 IU/l 3 days later. Four weeks later, ultrasonography revealed a single intrauterine sac, with a fetal heartbeat. A healthy boy, weighing 3008 g, was delivered by Caesarian section at 38 weeks gestation. The boy required monitoring at home for several months because of gastric reflux, but is healthy at 3 months.

Y DNA analysis

All tests were performed on DNAs purified from blood leukocytes, obtained by venipuncture or from umbilical cords. All Y-DNA markers employed had been placed previously on a physical map of the chromosome (Foote *et al*., 1992; Vollrath *et al*., 1992). The markers represented all known genes and gene families in the non-recombining region of the Y chromosome (Lahn and Page, 1997; Vogt *et al*., 1997).

Results

In each of the three families studied, we employed polymerase chain reaction (PCR) to test the father, the ICSI son or sons, and the paternal grandfather for the presence or absence of 38 DNA landmarks distributed across the entirety of the Y chromosome. The DNA landmarks tested included all three regions of the Y chromosome that have been clearly implicated, when deleted, in spermatogenic failure. The results of our Y-DNA analysis are summarized in Table I. Data for selected Y-DNA markers are shown in Figure 1. The three infertile men, although unrelated, have similar deletions on their Y chromosomes. Each of these infertile men (WHT3305, WHT3362, and WHT3111) carries most of the Y chromosome, including the sex-determining gene *SRY* (Sinclair *et al*., 1990), the *AZFa* and *AZFb* regions (implicated in some cases of spermatogenic failure) (Vogt *et al*., 1996), and the heterochromatic region comprising the distal long arm. However, in each

Table I. Y-DNA markers (sequence-tagged sites, or STS) and their presence (+) or absence (−) in control and experimental subjects

Y DNA marker (STS)	GenBank STS accession no. or reference	Gene or locus	Deletion interval	Control male	Control female	Family 1				Family 2			Family 3			
						WHT3513 grandfather	WHT3305 father	WHT3470 twin son A	WHT3469 twin son B	WHT3449 grandfather	WHT3362 father	WHT3613 son	WHT3257 grandfather	WHT3111 father	WHT3676 son	
sY14*	G38356	*SRY*	1A1A	+	−	+	+	+	+	+	+	+	+	+	+	
sY274	G38351	*RPS4Y*	1A1B	+	−	+	+	+	+	+	+	+	+	+	+	
sY238	G38352	*ZFY*	1A2	+	−	+	+	+	+	+	+	+	+	+	+	
sY19	G12010	*DYS252*	1A2−1B	+	−	+	+	+	+	+	+	+	+	+	+	
sY211	G38342		2A	+	−	+	+	+	+	+	+	+	+	+	+	
sY57	G38358	*DYS257*	3C	+	−	+	+	+	+	+	+	+	+	+	+	
sY634	G38353	*RBM*		+	−	+	+	+	+	+	+	+	+	+	+	
sY594	G34978	*TTY1*	3C, 4A	+	−	+	+	+	+	+	+	+	+	+	+	
Ex9A	Schiebel *et al.*, 1997	*PRKY*	3G−4A	+	−	+	+	+	+	+	+	+	+	+	+	
sY276	G38362	*AMELY*	4A	+	−	+	+	+	+	+	+	+	+	+	+	
sY601	G34984	*PRY*	4A, 6C, 6E	+	−	+	+	+	+	+	+	+	+	+	+	
sY200	G38360	*TSPY*	3C, 4B	+	−	+	+	+	+	+	+	+	+	+	+	
sY78*	G38359	*DYZ3*	centromere	+	−	+	+	+	+	+	+	+	+	+	+	
sY600	G34980	*TTY2*	4A	+	−	+	+	+	+	+	+	+	+	+	+	
sY620*	G38348	*DFFRY*	5C	+	−	+	+	+	+	+	+	+	+	+	+	
sY610	G38346	*DBY*	5C	+	−	+	+	+	+	+	+	+	+	+	+	*AZFa*
sY592	G34977	*UTY*	5C	+	−	+	+	+	+	+	+	+	+	+	+	
sY593	G34981	*TB4Y*	5D	+	−	+	+	+	+	+	+	+	+	+	+	
sY90	G38357	*KALY*	5E	+	−	+	+	+	+	+	+	+	+	+	+	
sY595	G34985	*BPY1*	5G	+	−	+	+	+	+	+	+	+	+	+	+	
sY210	G38361	*STSP*	5I	+	−	+	+	+	+	+	+	+	+	+	+	
sY638	G38355	*CDY*	5L, 6F	+	−	+	+	+	+	+	+	+	+	+	+	
sY591	G34987	*XKRY*	5L	+	−	+	+	+	+	+	+	+	+	+	+	
sY119*	G11997	*DYS211*	5M	+	−	+	+	+	+	+	+	+	+	+	+	
sY121	G38341	*DYS212*	5N	+	−	+	+	+	+	+	+	+	+	+	+	
SH34Y/ SH35Y	Agulnik *et al.*, 1994	*SMCY*	5O	+	−	+	+	+	+	+	+	+	+	+	+	
sY603	G34991	*EIF1AY*	5Q	+	−	+	+	+	+	+	+	+	+	+	+	*AZFb*
F20/ E355	Ma *et al.* 1993	*RBM*		+	−	+	+	+	+	+	+	+	+	+	+	
sY142	G38345	*DYS230*	6C	+	−	+	+	+	+	+	+	+	+	+	+	
sY143	G38347	*DYS231*	6C	+	−	+	+	+	+	+	+	+	+	+	+	
sY205*	G38344	*DAZ*	6D, 6E	+	−	+	−	−	−	+	−	−	+	−	−	
sY254	G38349	*DAZ*	6D, 6E	+	−	+	−	−	−	+	−	−	+	−	−	
sY624*	G38350	*DAZ*	6D, 6E	+	−	+	−	−	−	+	−	−	+	−	−	
sY602	G34986	*BPY2*	6E	+	−	+	−	−	−	+	−	−	+	−	−	*AZFc*
sY202	G38340		6E	+	−	+	−	−	−	+	−	−	+	−	−	
sY158	G12006	*DYS241*	6F	+	−	+	−	−	−	+	−	−	+	−	−	
sY159*	G38354	*DYZ1*	7	+	−	+	+	+	+	+	+	+	+	+	+	
sY160	G38343	*DYZ2*	7	+	−	+	+	+	+	+	+	+	+	+	+	

*Primary data for these seven Y-DNA markers are shown in Figure 1.

GenBank STS accession numbers provide electronic access to PCR primer sequences and reaction conditions. Deletion intervals on the Y chromosome are numbered according to Vollrath *et al.*, 1992. Much of the Y-DNA data for two of the fathers (WHT3305 and WHT3111) was reported previously (Silber *et al.*, 1998); the third father (WHT3362) was not studied previously.

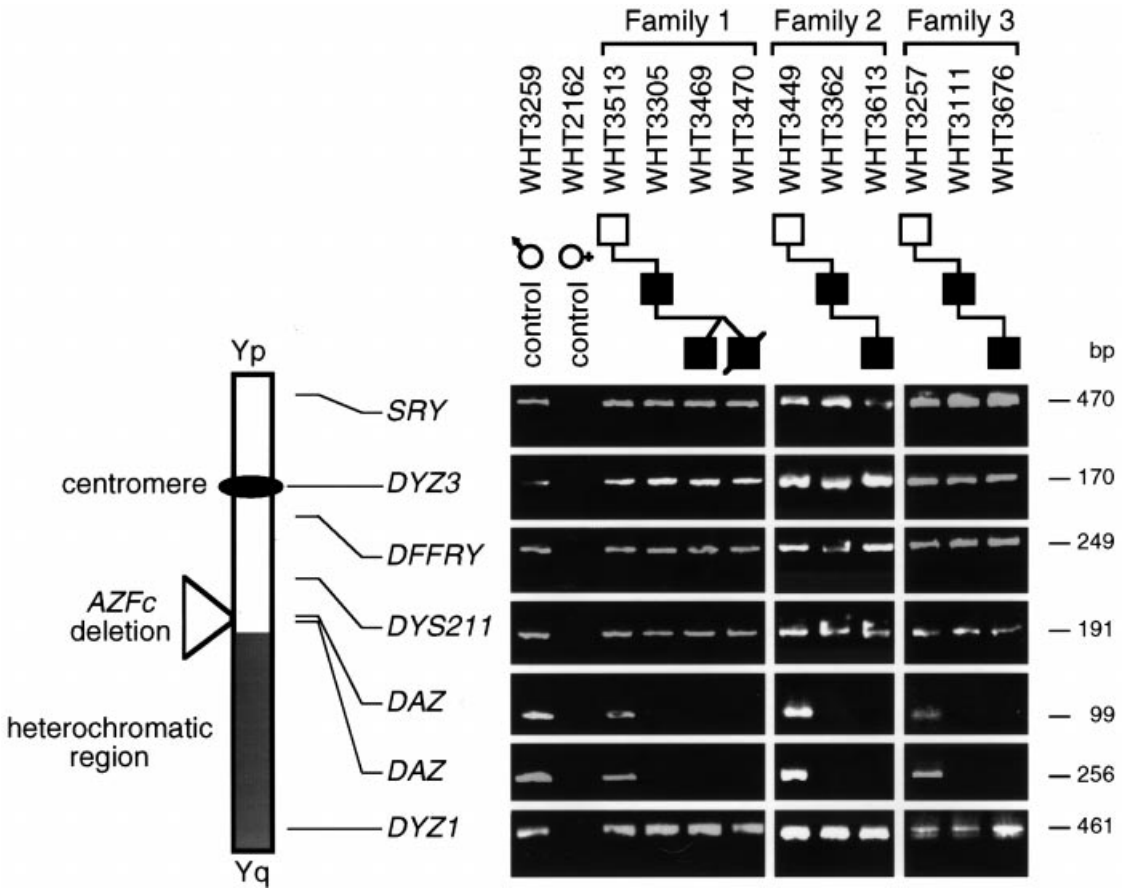

Figure 1. Polymerase chain reaction (PCR) analysis of Y chromosome DNA markers in three infertile men (WHT3305, WHT3362, and WHT3111), their fathers, and their sons. Results are shown for seven Y-DNA markers, identified with asterisks in Table I; the *DAZ* markers shown are sY205 and sY624. In family trees, filled square denotes deletion of *AZFc* region. Sizes (bp) of PCR products are listed at right.

of these three men, the Y chromosome lacks the *DAZ* gene cluster as well as other markers of the *AZFc* region. As judged by these assays, the extent of these Y deletions is typical of those that have been observed in many azoospermic or severely oligozoospermic men (Reijo *et al*., 1995, 1996; Najmabadi *et al*., 1996; Nakahori *et al*., 1996; Qureshi *et al*., 1996; Vogt *et al*., 1996; Foresta *et al*., 1997; Girardi *et al*., 1997; Kremer *et al*., 1997; Pryor *et al*., 1997; Simoni *et al*., 1997; van der Ven *et al*., 1997; Vereb *et al*., 1997). The Y deletions found in these ICSI fathers were also found in each of their sons (Table I, Figure 1).

If the Y deletions are the cause of the ICSI fathers' infertility, then the deletions should be *de novo*, i.e., they should not exist in the paternal grandfathers of the ICSI children. Indeed, the Y chromosomes of the paternal grandfathers (WHT3513, WHT3449, and WHT3257) appear to be intact (Table I, Figure 1). We conclude that, in each of these families, the infertile man's deletion arose *de novo*. This is in keeping with previous evidence that nearly all cases of *AZFc* deletions examined represent de-novo mutations (Reijo *et al*., 1995, 1996; Vogt *et al*., 1996; Foresta *et al*., 1997; Girardi *et al*., 1997; Kremer *et al*., 1997; Pryor *et al*., 1997). In each of the families described here, we can be confident that the de-novo deletion of the *AZFc* region is the cause of the infertile man's spermatogenic failure. These de-novo *AZFc* deletions could have arisen either in germ cells of the paternal grandfathers, or zygotically or post-zygotically in the ICSI fathers (Edwards and Bishop, 1997).

Discussion

The three families that we describe demonstrate that *AZFc*-deleted men can father sons via ICSI. In keeping with simple Mendelian expectations, all four boys inherited their fathers' *AZFc*-deleted Y chromosomes. When used in ICSI, *AZFc*-deleted spermatozoa are capable of fertilizing oocytes and eliciting full developmental potential. Nearly all *AZFc* deletions identified in previous studies represented new mutations (Reijo *et al*., 1995, 1996; Vogt *et al*., 1996; Foresta *et al*., 1997; Girardi *et al*., 1997; Kremer *et al*., 1997; Pryor *et al*., 1997). This observation implies that the great majority of *AZFc*-deleted men are infertile, and leads to the prediction that the three surviving boys in these three families will themselves be azoospermic or oligozoospermic as adults, barring the discovery of an effective therapeutic intervention. At present, we anticipate no further evaluation of these boys' fertility potential until puberty, at which point it may be advisable to begin periodic evaluation of testicular volume, semen quality, and reproductive hormone concentrations.

Our observations provide a concrete foundation for alerting couples to the likelihood of transmitting infertility-causing Y deletions by ICSI. Since *AZFc* deletions are the most common

molecularly defined cause of spermatogenic failure (Reijo *et al*., 1995, 1996; Najmabadi *et al*., 1996; Nakahori *et al*., 1996; Qureshi *et al*., 1996; Vogt *et al*., 1996; Foresta *et al*., 1997; Girardi *et al*., 1997; Kremer *et al*., 1997; Pryor *et al*., 1997; Simoni *et al*., 1997; van der Ven *et al*., 1997; Vereb *et al*., 1997), we expect that significant numbers of *AZFc*-deleted boys will be fathered through ICSI.

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