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OUTSTANDING CONTRIBUTION

Y chromosome microdeletion in a father and his four infertile sons

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Microdeletions of Yq are associated with azoospermia and severe oligozoospermia. In general, men with deletions are infertile and therefore deletions are not transmitted to sons unless in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are performed. We report an unusual family characterized by multiple members with infertility and Yq microdeletion. Complete reproductive history, semen analyses and blood samples were elicited from relevant family members. DNA preparation and quantification were performed using commercial kits. A total of 27 pairs of sequence tagged sites based primer sets specific for the Y microdeletion region loci were used for screening. Southern blots using deleted in azoospermia (*DAZ*) and ribosomal binding motif (*RBM*) cDNAs were then analysed for confirmation. The proband, his three brothers and father were all found to be deleted for *DAZ* but not *RBM*. At the time of analysis, the proband's father was azoospermic whereas his four sons were either severely oligozoospermic or azoospermic. Unlike their father, the four sons are infertile and have no offspring, except for one of them who achieved a daughter only after IVF/ICSI treatment for infertility. Microdeletions of Yq involving the *DAZ* gene are associated with a variable phenotypic expression that can include evidently normal fertility.

Key words: azoospermia/gene deletion/genetics/infertility/Y chromosome

Introduction

Infertility occurs in ~14% of couples (Mosher, 1985) and abnormalities in the male partner are estimated to be present in up to half of the cases (Swerdloff *et al.*, 1985). Efforts to evaluate the causes of azoospermia have shown that after exclusion of traditionally recognizable causes (i.e. abnormal karyotype, obstruction, varicocoele, hormonal defect, etc.), most cases (50–75%) are unexplained and are termed idiopathic (Pryor *et al.*, 1997). Recently, it has been reported that up to 30% of men with 'idiopathic' azoospermia have microdeletions of the Y chromosome (Henegariu *et al.*, 1993; Ma *et al.*, 1993; Nagafuchi *et al.*, 1993; Kobayashi *et al.*, 1994; Najmabadi *et al.*, 1996; Reijo *et al.*, 1996; Vogt *et al.*, 1996; Pryor *et al.*, 1997). Exactly how and whether or not these microdeletions cause azoo/oligozoospermia is the subject of both intense investigation and debate.

Essential to the argument that Y microdeletions cause infertility is the observation that fertile men rarely manifest Y microdeletions. Microdeletions in four out of 200 fertile men studied have been reported (Pryor *et al.*, 1997). However, the deletions in these men were very small and most likely represented insignificant polymorphism. Relatively large deletions of the kind associated with male infertility have not been reported in men with normal fertility. Although it is generally assumed that these deletions arise *de novo* and that father to son transmission of Y microdeletion would not be expected, a few rare instances of father to one son transmission of Y chromosome microdeletion have been reported (Kobayashi *et al.*, 1994; Stuppia *et al.*, 1996; Vogt *et al.*, 1996; Pryor *et al.*, 1997). However, vertical transmission of a microdeletion involving the deleted in azoospermia (*DAZ*) locus from father to one son has been reported in only three cases (Kobayashi *et al.*, 1994; Vogt *et al.*, 1996; Pryor *et al.*, 1997). We now describe a four-generation family in which an azoospermic father and his four infertile sons all share an apparently identical microdeletion that includes the *DAZ* locus. This family represents the first and only report of spontaneous vertical transmission of *DAZ* deletion to multiple offspring. It provides evidence that a single Yq microdeletion can result in varying phenotypic expression in different individuals. It is clinically significant, in that the presence of a microdeletion is not an absolute marker for infertility and can be associated with apparently normal fertility.

Materials and methods

Screening for Yq microdeletion was performed on a routine basis for male infertility using a protocol reviewed and approved by the Institutional Review Board of College of Physicians & Surgeons, Columbia University. Samples were taken from patients after informed consent.

Semen analysis

Results were analysed using WHO criteria with a Nikon phase contrast microscope.

Serum hormone concentrations

Follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone were measured by solid-phase, two site chemiluminescent

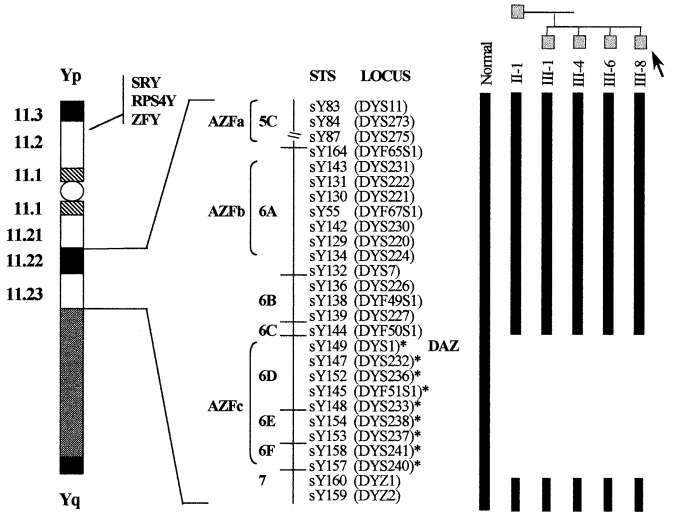

Figure 1. Y chromosome map and microdeletions in subinterval 6D–6F of the Y chromosome long arm in the proband (III-8), his father (II-1) and three brothers (III-1, III-4, III-6). The presence of a sequence tagged site (STS) is indicated by the solid portion of the column. The STS not amplified are marked with asterisks. The approximate boundaries of AZFa, AZFb, and AZFc regions (as per Vogt *et al.*, 1996) are shown.

enzyme immunometric assay (Immulite; Diagnostic Products Corporation, Los Angeles, CA, USA). Normal ranges for men are FSH <10 mIU/ml; LH <10 mIU/ml; and testosterone 270–1070 ng/dl.

Genomic DNA

Extraction of genomic DNA from whole blood was performed by lysis of red blood cells, followed by lysis of white blood cells and their nuclei. Cellular proteins were removed by salt precipitation, and genomic DNA was precipitated with isopropanol using Puregene DNA extraction kit (Gentra Systems, Inc. Minneapolis, MN, USA; catalogue no. D-5004).

Polymerase chain reaction (PCR)

Primers were produced as dried oligonucleotides on an automated DNA synthesizer (Perkin-Elmer Applied Biosystems Inc., Foster City, CA, USA). A total of 27 Y chromosome specific sequence tagged sites (STS) (Figure 1) were selected from an STS map (Vollrath *et al.*, 1992). They include the three proposed spermatogenesis loci AZFa, AZFb, and AZFc (as per Vogt *et al.*, 1996) spanning Yq intervals 5, 6 and 7. As a rapid screening protocol, a PCR multiplex system composed of two to six different primer pairs was used in a total of six multiplexed reactions (Table I). With each PCR run, a female control and a normal male control were included. All PCR reactions were run in polycarbonate (Techne®) plates in an MJ Research® machine. The PCR conditions were essentially as previously described (Henegariu *et al.*, 1993). Briefly, in a 14 µl total volume reaction, 50 ng of genomic DNA was used as template, 1 µl of primer standard solution (mix I or II or III or IV or V or VI consisting of 10 pmol per primer), 12 µl of 'PCR cold mix' (1.5 mmol/l $MgCl_2$, 0.2 mmol/l of each dNTP, 5% DMSO, 1× Taq polymerase reaction buffer without Mg^{2+}), 1.25 IU Taq DNA polymerase (Promega) and 1 drop of oil. The complete mixes were placed directly in a thermocycler preheated to 94°C. Cycling conditions for 27 cycles were: 94°C, 30 s (melting); 55°C, 45 s (annealing); and 72°C, 60 s (extension). The final extension time was 5 min. The PCR reaction products were then separated on 3% agarose gels (Bio-Rad, ultra-pure grade) by electrophoresis in TBE buffer.

Table I. Multiplex polymerase chain reaction (PCR) scheme used for the 27 STS primer pairs. The primers are ordered by decreasing expected lengths

Multiplex mix	Sequence tagged site (STS)	Expected PCR product length (bp)	Corresponding locus
I	157	285	DYS240
	154	245	DYS238
	142	196	DYS230
	145	160	DYF51S1
	131	143	DYS222
	139	120	DYS227
II	134	301	DYS224
	136	235	DYS226
	129	194	DYS220
	132	159	DYS7
	152	125	DYS236
III	143	311	DYS231
	55	256	DYF67S1
	130	173	DYS221
	149	132	DYS1 (*DAZ*)
	147	100	DYS232
IV	83	275	DYS11
	158	231	DYS241
	148	202	DYS233
	138	170	DYF49S1
	153	139	DYS237
V	164	690	DYF65S1
	84	326	DYS273
	87	252	DYS275
	144	143	DYF50S1
VI	159	550	DYZ2
	160	236	DYZ1

PCR products were stained with ethidium bromide and visualized by exposure to ultraviolet light. STS showing no amplification in multiplex reactions were confirmed by single reaction PCR with appropriate positive and negative controls. An STS was considered to be absent after three amplification failures.

Southern hybridization

Southern blotting was performed according to established protocol (Sambrook *et al.*, 1989). Briefly, 5 µg genomic DNA was digested with *HindIII* or *TaqI*, run on a 0.7% agarose gel in standard TBE buffer, transferred to a nylon membrane, and hybridized with ^{32}P-labelled probes. The *DAZ* probe was the purified insert of a plasmid (pDP1577) containing the full length cDNA (Reijo *et al.*, 1995). Similarly, the *RBM* probe was the plasmid insert of an *RBM* cDNA clone (MK5) (Ma *et al.*, 1993).

Paternity determination

Paternity of all four sons was confirmed by showing the expected segregation of four highly polymorphic autosomal markers (Weber and May, 1989). These were D21S156, D21S270, D13S132, and D13S159 with heterozygosities of 0.83, 0.86, 0.84 and 0.90 respectively.

Fluorescent in-situ hybridization (FISH)

FISH for *DAZ* was performed with Cosmid 63C9 (Saxena *et al.*, 1996), using established methods (Yu *et al.*, 1996).

Results

The proband (individual III 8 in Figure 2) and his spouse (III-9) presented to the Reproductive-Endocrinology-Infertility Clinic at Columbia-Presbyterian Medical Center with complaint of primary infertility for 3 years. Testing revealed normal

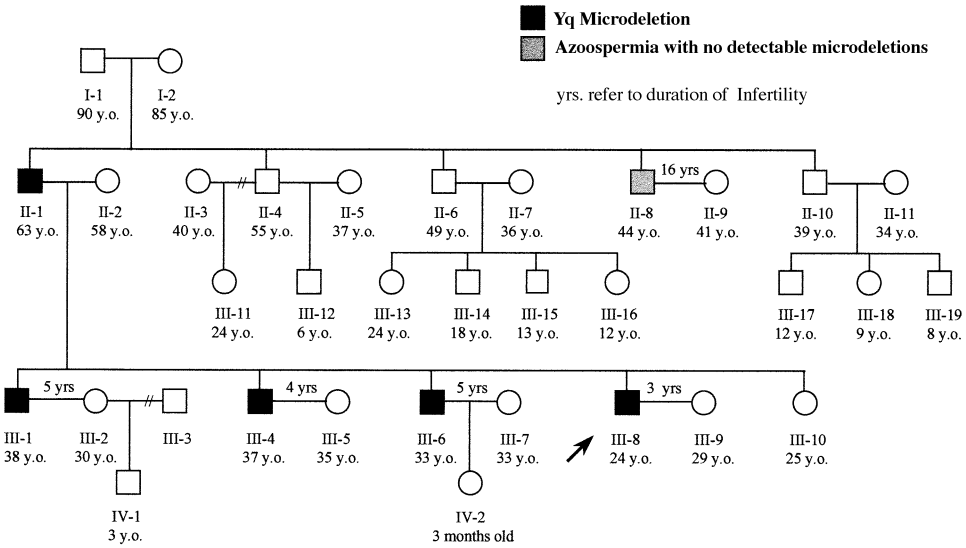

Figure 2. Pedigree of the four-generation family with results of Yq microdeletion testing. The proband (III-8) is indicated by an arrow. The proband (III-8) is severely oligozoospermic and microdeleted for subinterval 6D-6F of Yq. His father (II-1) was found to be azoospermic and the two brothers (III-4, III-6) were severely oligozoospermic. The third brother (III-1) declined semen testing. The proband, his father and three brothers were all found to have an apparently identical microdeletion including *DAZ*. The proband's uncle (II-8) was found to have azoospermia but no Yq microdeletion was detected.

Table II. Semen analyses and hormone profiles of relevant family members

ID	Relationship to proband	Age (years)	Sperm count ($\times 10^6$/ml)	FSH (mIU/ml)	LH (mIU/ml)	Testosterone (ng/dl)
III-8	Proband	24	0–0.5	3.5	4.5	485
III-6	Brother	33	3 spermatozoa	5.1	2.5	279
III-4	Brother	37	0.1	5.5	1.7	499
III-1	Brother	38	NA	6.3	1.6	414
II-1	Father	63	0	21.2	3.3	392
II-8	Uncle	44	0	40.7	8.7	37
		Normal values	>20	<10.0	<10.0	270–1070

FSH = follicle stimulating hormone; LH = luteinizing hormone; NA = not analysed.

karyotype and normal serum hormone levels while semen analyses showed severe oligozoospermia (Table II). Microdeletion screening by STS based PCR revealed the presence of a microdeletion in subinterval 6D–6F of the Y chromosome long arm (Figure 1). During discussion, the proband reported that his two older brothers (III-4 and III-6) were known to be azoospermic and infertile. Subsequent work-up revealed all three brothers had an apparently identical microdeletion. Testicular biopsy performed on one of them (III-6) demonstrated 'Sertoli cell only' syndrome.

The finding of microdeletion in three infertile brothers suggested their father was likely to carry the same deletion. A detailed study was undertaken on the remainder of the family who all resided in a small town in the Dominican Republic. A complete reproductive history was elicited from the adult family members. The familial relationships depicted in the pedigree (Figure 2) were confirmed by showing the expected segregation of several autosomal polymorphic markers (data not shown) for the relevant family members (I-1, I-2, II-1, II-8 and II-1, II-2, III-1, III-4, III-6, III-8, III-10). There was no evidence of non-paternity. Thorough cytogenetic studies on relevant family members (I-1, II-1, II-6, II-8, II-10, III-1, III-4, III-6, III-8, III-14, III-15, III-17, III-19, and IV-1) revealed normal karyotypes. Semen analysis was performed in seven of the 16 males and blood samples were obtained from most of the family members.

Table II summarizes the results of semen analysis and endocrine work-up on relevant family members. The proband (III-8), his father (II-1) and two of his three brothers (III-4, III-6) were found to be either azoospermic or severely oligozoospermic. The proband's oldest brother (III-1) declined semen analysis. In addition, the proband's uncle (II-8) was found to have azoospermia and elevated FSH with low testosterone. As shown in Figure 1, the proband, his father and three brothers were all found to have microdeletion of Yq by STS PCR analysis. Southern blotting with the *DAZ* (Figure 3) and *RBM* (data not shown) probes confirmed that the deletion included the *DAZ* locus but not the RNA binding motif (*RBM*) locus. FISH analysis with the *DAZ* Cosmid 63C9 (Saxena *et al.*, 1996) of the proband's father's (II-1) leukocytes showed uniform absence of the *DAZ* locus (Figure 4).

The finding that individual II-8 in the pedigree was azoospermic but did not have a microdeletion was a surprise. The *DAZ* locus in this individual was further tested by Southern

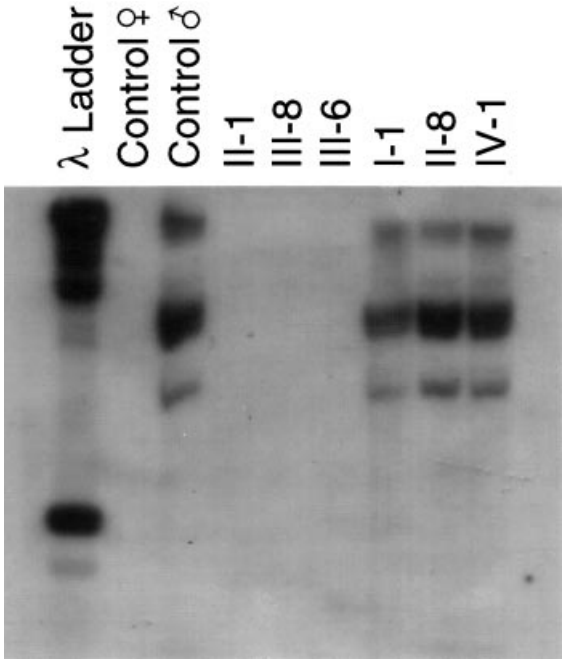

Figure 3. Southern blot with *DAZ* probe. The entire *DAZ* locus in individuals II-1, III-8, and III-6 is absent, whereas it appears to be present and normal in the control male and individuals I-1, II-8 and IV-1.

analysis using the *DAZ* cDNA and a different restriction enzyme (TaqI). It failed to show any abnormality of the *DAZ* locus. In addition, FISH with the *DAZ* Cosmid 63C9 showed normal intensity (data not shown).

The proband (III-8) and his older brother (III-6) were seeking infertility treatment. After extensive counselling, they opted for in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). The proband (III-8) and his wife (III-9) underwent two IVF-ICSI cycles that failed to produce a pregnancy secondary to poor ovarian response. The proband's brother (III-6) and his wife (III-7) underwent one cycle of controlled ovarian hyperstimulation and nine oocytes were retrieved. Eleven mature spermatozoa were found in three ejaculates on the day of retrieval and used for ICSI. Three oocytes fertilized which subsequently cleaved and were transferred. She delivered a healthy female baby (IV-2).

Discussion

We report an exceptional family in which an azoospermic father and his four infertile sons share an apparently identical Yq microdeletion involving the *DAZ* locus. The de-novo mutation that led to the microdeletion of *DAZ* appears to have originated in the proband's father (II-1). This deletion is expected to cause azoo/oligozoospermia and male infertility, and yet he spontaneously conceived five children and was unaware of any fertility problem. Interestingly, however, all four sons are infertile and are either azoospermic or severely oligozoospermic.

This family raises several issues with regards to the association between Yq microdeletion and infertility. First, it confirms that vertical transmission of Yq microdeletion is possible and

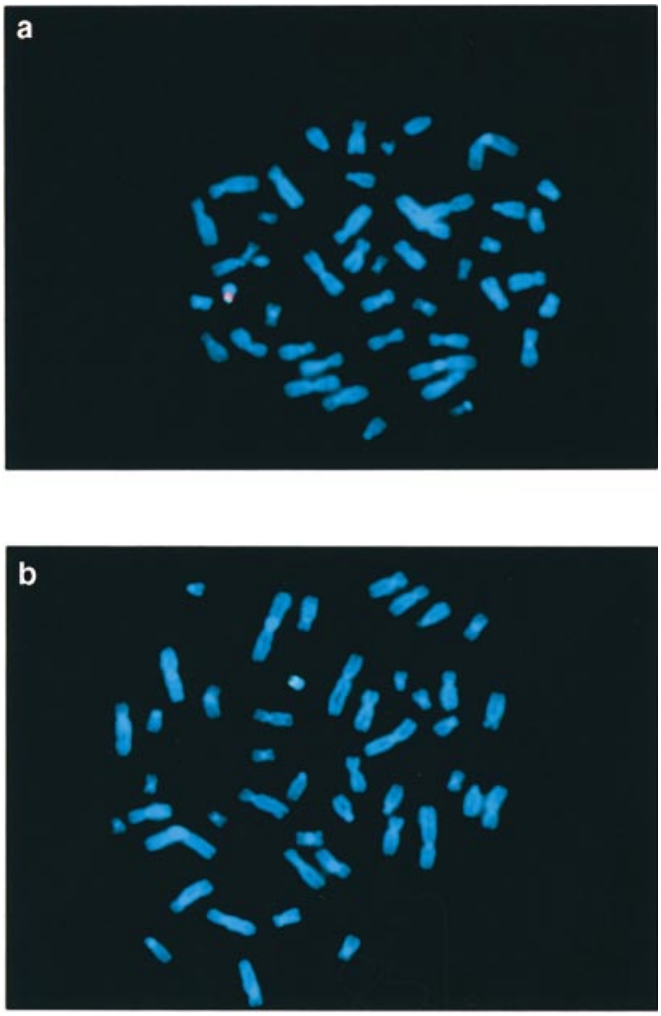

Figure 4. (**a**) Metaphase from individual I-1 (control) after FISH using probe DYZ3 (ONCOR) to identify the Y centromeric DNA (green) and probe Cosmid 63C9 to identify the *DAZ*-containing chromosome region (red). Both signals are seen on the Y chromosome. (**b**) Metaphase from individual II-1 using the same probes. Only the green signal is seen, identifying the Y chromosome, but no signal for *DAZ* region is present.

can lead to subsequent infertility in the male offspring. Second, it is obvious that the same deletion can result in different phenotypes in different individuals. Although the father (II-1) of the four boys in this family was azoospermic at the time of analysis, he fathered his first child at the age of 25 and his last one at the age of 38 years. Thus, he possessed some degree of fertility over a large span of years. Likewise, microdeletion of the Y chromosome, specifically *DAZ*, does not necessarily imply a lifelong history of azoospermia nor does it preclude the formation of a large family. His four sons, on the other hand, are infertile and either azoospermic or severely oligozoospermic.

The *DAZ* gene has been proposed as the azoospermia factor on the Y chromosome. This family shows clearly that while *DAZ* may have a critical role in spermatogenesis, it is not essential for fertility. Furthermore, total loss of the *DAZ* gene cluster can be associated with a histological picture of 'Sertoli cell only' as well as sperm maturation arrest (Foresta *et al.*,

1997; Pryor *et al.*, 1997). Several authors have found a poor correlation between the location of Y microdeletions (including *DAZ* deletions) with the clinical and histological phenotype of the patients (Reijo *et al.*, 1995, 1996; Vogt *et al.*; 1996; Silber *et al.*, 1998). The findings in this family agree that such a correlation may turn out to be quite problematic. Testicular biopsy of the proband's brother (III-6) showed a picture of 'Sertoli cell only' whereas the proband (III-8 with sperm count 0.5×10^6/ml) clearly would be expected to have some degree of sperm maturation on biopsy. Furthermore, testicular biopsy may not be representative of the entire testicle because there may be geographic heterogeneity for spermatogenesis as in individual III-6 whose ejaculates contained mature spermatozoa.

We can only speculate about the basis for phenotypic differences between family members with the same deletion. It is well known that identical deletions within autosomes may result in different phenotypes (Schinzel, 1994). One can postulate that such differences are consequences of each individual's exposure to his environment or expression of various modifying genes. A fertile father has been described with a microdeletion that widened when transmitted to his infertile son (Stuppia *et al.*, 1996). Although variable extensions at the borders of the deletion may exist between our different family members, these molecular extensions cannot be distinguished by interval mapping. By PCR analysis, the same STSs failed to amplify in our five individuals and Southern hybridization with the *DAZ* probe confirmed a complete deletion of this gene cluster. Although it is possible that the deletions observed are, in fact, not identical and adjacent areas may contain important genes that modulate the degree of phenotypic expression, these results still indicate a large overlap of deleted Y DNA (including the loss of *DAZ* gene cluster) in each individual of this unique family.

We were fascinated by the fact that the proband's uncle (II-8) has infertility and azoospermia but no apparent microdeletion by STS testing. Since southern blot analysis using both the *RBM* and *DAZ* probes as well as FISH analyses using a *DAZ* cosmid were all entirely normal, we are forced to conclude he has a different aetiology underlying his infertility. Admittedly, it is possible that he may have a smaller or point mutation/perturbation or proximal/distal rearrangement that is not detectable by current methods. He gave no history of exposure to gonadotoxins or other definable factors that were likely to affect spermatogenesis.

Until recently, Y microdeletion has had little clinical significance, since a man with a deletion will not, in general, reproduce. However, utilizing ICSI and testicular sperm aspiration (TESA), combined with IVF, it is now possible for oligo/azoospermic men with Y microdeletion to achieve pregnancies (Mulhall *et al.*, 1997; Silber *et al.*, 1998, and individual III-6). This has fostered concerns that such pregnancies may produce male offspring with similar microdeletions and subsequent infertility (Reijo *et al.*, 1996; Girardi *et al.*, 1997; Kremer *et al.*, 1997). Indeed, Yq microdeletion can be transmitted to male offspring via ICSI (Kent-First *et al.*, 1996). The family we report suggests that men with Yq microdeletions (such as individual II-1) who achieve pregnancies will transmit the same microdeletion and the risk of infertility to their sons (individuals III-1, III-4, III-6, III-8). Therefore, patients should be offered Y microdeletion screening prior to ICSI and they should be counselled on the certainty of transmitting the Yq microdeletion and possibly infertility to their sons. As more research is focused on genetic aetiologies of male infertility, identification of genes involved in spermatogenesis should provide insight into the pathophysiology of male infertility and a more rational basis for initiating therapy.

Acknowledgements

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