

Manifestation of Y-chromosomal deletions in the human testis: a morphometrical and immunohistochemical evaluation

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BACKGROUND: Deletions of the AZF (azoospermia factor) subregions on the Y chromosome are accompanied by a diverse spectrum of spermatogenic disturbances ranging from hypospermatogenesis to total depletion of germ cells causing infertility. The AZF region encodes gene products which are candidates for the genetic control of spermatogenesis. Although it is known which genes are involved, a general principle of cause and effect cannot yet be deciphered and the deletion type has non-uniform histological phenotypes. **METHODS AND RESULTS:** We analysed morphological parameters of testicular biopsies from 17 patients diagnosed for Y chromosome microdeletions. As control groups we analysed testes from patients with idiopathic Sertoli cell-only (SCO) syndrome ($n = 11$), mixed atrophy ($n = 10$) and complete spermatogenesis ($n = 11$). A detailed genetic analysis on the extension of the observed microdeletions revealed similar breakpoints in the distal and proximal region of the AZFc region, indicating a common mechanism of homologous recombination for such deletions, as has been suggested before. Morphometric parameters such as the diameter of the tubules, lumen, thickness of the lamina propria and height of the tubule epithelia were investigated. The diameter of the tubules from patients with microdeletions was found to be significantly smaller compared with patients with mixed atrophy. Considering also the size of the tubules, lumen and epithelia, a Y-chromosomal microdeletion represents an intermediate state between an idiopathic SCO and normal spermatogenesis. The immunohistochemical analysis of six different Sertoli cell markers, cytokeratin 18, vimentin, inhibin α subunit, 14-3-3 θ , FSH receptor and androgen receptor, revealed no impact of AZF deletion on the specific expression pattern of these genes. **CONCLUSIONS:** Our results suggest that, notwithstanding the deletion of a common region in the AZFc region, microdeletions of the Y chromosome lead to an intermediate status between idiopathic SCO and complete spermatogenesis, resulting in a heterogeneous histological profile regardless of the seminiferous activity. The Sertoli cell function seems not to be altered.

Key words: azoospermia/male infertility/microdeletions/testis morphology/Y deletions

Introduction

Infertility affects 10–15% of couples seeking children (Nieschlag, 2000; review). In about half of the cases the male has disturbed fertility. Approximately 22% of almost 14 000 patients seeking help at our institute are diagnosed with severe oligozoospermia or azoospermia possibly treatable only by ICSI. The low numbers or total lack of sperm may have many causes such as endocrine disorders, infections, cryptorchidism, systemic diseases or obstruction of the seminal pathway. In half the cases testicular biopsies identify the men as belonging to the category of idiopathic spermatogenic defects and show a total range of spermatogenic failures from complete absence of germ cells [Sertoli cell-only (SCO) syndrome] through maturation arrest up to hypospermatogenesis (Foresta *et al.*, 2001b). Between 1.5–20% of the severe cases are associated

with Y-chromosomal microdeletions in the azoospermia factor (AZF) region (Reijo *et al.*, 1995; Krausz *et al.*, 2001) which is divided into three subregions (a, b and c) (Vogt *et al.*, 1996). The distribution of these microdeletions seems to be a stochastic event independent of the Y chromosome haplotypes (Paracchini *et al.*, 2000; Quintana-Murci *et al.*, 2001). Most investigations concerning patients with these microdeletions deal with effects of the missing or non-functioning gene products. The origin of microdeletion is presently the object of intense investigation. Recent data have shown that both AZFa and AZFc complete deletions arise from homologous recombination. In the case of AZFa complete deletions, this is due to recombination between proviral sequences (Sun *et al.*, 2000), while complete AZFc deletion results from recombination between the b2–b4 elements of this palindromic region (Kuroda-Kawaguchi *et al.*, 2001). In any case, the mechanism by which these deletions

effect germ cell depletion is not immediately evident (Krausz *et al.* 2000; Liow *et al.*, 2001).

Apart from azoospermia and severe oligozoospermia, no typical clinical characteristics associated with the AZF deletions have been identified. In order to characterize these patients more intensively we have analysed testicular biopsies from patients with Y-chromosomal microdeletions. Up to now >2200 patients with azoospermia or severe oligozoospermia have been screened in our institute for Y chromosome deletions by simplex or multiplex PCR amplification of genomic DNA (Maurer *et al.*, 2001). Twenty-four of these patients had deletions in the AZFc region, three in AZFb and one in AZFa. Three others had a combination of either deletions in the AZFa and AZFb or in the AZFb and AZFc regions, and one patient showed a complete loss of the entire AZF region. Testis biopsies were obtained from 17 of these 32 patients.

Although many papers have compared morphometrically analysed human testis parameters with histology (Lanz and Neuhäuser, 1963; Bustos-Obregon and Holstein, 1973; Zukerman *et al.*, 1978; Matsuda *et al.*, 1996), to our knowledge, no reports have investigated in detail the morphometrical parameters in cases of Y-chromosomal microdeletions. The diameter of the lamina propria directly depends on the Sertoli cells and is associated with their maturation status (Paniagua *et al.*, 1990). In this study we have compared the morphometric parameters of testicular histology samples obtained from patients with and without deletions of the Y chromosome. In addition, Sertoli cells were analysed by immunocytochemical staining of Sertoli cell-specific proteins. Six different markers for the differentiation status of Sertoli cells were studied in order to elucidate how AZF deletions may contribute to spermatogenic dysfunction. These markers were: (i) two intermediate filaments—cytokeratin 18, a marker for immature Sertoli cells (Bergmann and Kliesch, 1994, Steger *et al.* 1996, 1999), and vimentin, expressed consistently in Sertoli cells; (ii) two hormone receptor proteins, the FSH receptor and the androgen receptor, which both induce spermatogenesis-stimulating factors locally in Sertoli cells (Shan *et al.*, 1997, Levalle *et al.*, 1998); and (iii) inhibin α , a subunit of inhibin B which is positively correlated with spermatogenesis, and 14-3-3 θ , a kinase regulator potentially important in the prevention of apoptosis (Chaudhary and Skinner 2000). Finally, a complete genetic characterization of the deletions was carried out in order to identify possible genotype–phenotype correlations.

Subjects and methods

Patients

Testicular biopsy specimens from patients (average age 31.3 ± 3.0 years) from our infertility clinic with complete SCO ($n = 5$) or focal SCO ($n = 12$) with Y-chromosomal deletions were analysed. The patients with focal SCO were subdivided into those with hypospermatogenesis ($n = 5$) and maturation arrest (spermatocytes; $n = 7$). From three patients, a biopsy of only one testis was taken. All men gave informed written consent to the procedures.

As a control group, fixed specimens of testes were taken from patients diagnosed with mixed atrophy with different degrees of

spermatogenic impairment characterized by a variable proportion of tubules displaying SCO ($n = 10$), but no Y-chromosomal deletions. Five of these patients had hypospermatogenesis and three spermatogenic arrest (spermatocytes or round spermatids). Two other control groups with no Y-chromosomal microdeletions were composed of complete idiopathic SCO ($n = 11$) and patients with testes diagnosed as having complete spermatogenesis in all tubules ($n = 11$). The average age of the patients with mixed atrophy was 34.3 ± 6.1 years, with idiopathic SCO 33.3 ± 2.0 years and with complete spermatogenesis 36.4 ± 5.1 years.

Hormones

Peripheral blood was obtained for analysis of hormone values. FSH and LH were analysed by immunofluorometric assays (Autodelphia; Wallac, Inc., Freiburg, Germany) and serum testosterone was measured by radioimmunoassay (Diagnostic Systems Laboratories, Inc., Sinsheim, Germany).

Genomic DNA isolation

Genomic DNA was obtained from the peripheral leukocytes of patients using the Nukleon Kit II (Scotlab, Wiesloch, Germany). PCR amplification of genomic DNA was performed according to guidelines published by the European Academy of Andrology using the first and second set of primers. For the patients diagnosed as bearing an AZFb/c or AZFc deletion a more precise breakpoint localization analysis was performed. For this we employed recently published sequence tagged sites (STSs), which allow the delineation of the breakpoint to a proximal region of 349 kb and a distal region of 229 kb (Kuroda-Kawaguchi *et al.*, 2001). In brief, the following primers were used. sY1196 (393 bp): 5'-GTTGGC-AACTTGCCTGCT-3' and 5'-CCTTCTCTCAAAGTCCCC-3'; sY1197 (453 bp): 5'-TCATTGTGTCTCTCTTGGGA-3' and 5'-CTAAGCCAGGAAGTCCAC-3'; sY1192 (255 bp): 5'-ACTA-CCATTTCTGGAAGCCG-3' and 5'-CTCCCTTGGTTCATGCC-ATT-3'; sY254 (401 bp): 5'-GGGTGTTACCAGAAGGCAAA-3' and 5'-GAACCGTATCTACCAAAGCAGC-3'; sY1054 (340 bp): 5'-ACCTAAGGGAACCCAGGAGA-3' and 5'-CGACACTTTTGGG-AAGTTTCA-3'; sY1125 (223 bp): 5'-GTGGGGGTTTCACATT-ATGG-3' and 5'-GGTCACAGACTCACATTTAAGCA-3'; sY1201 (677 bp): 5'-CCGACTTCCACAATGGCT-3' and 5'-GGGAGAAAA-GTTCTGCAACG-3'.

For the different STSs the following PCR cycling parameters were used. A single denaturation step at 94°C for 2 min was followed by 35 cycles consisting of 94°C for 30 s, 54°C for 45 s and 72°C for 45 s. The reaction was terminated by a final extension step at 72°C for 7 min. The amplicons were subjected to a 2% agarose gel electrophoresis and documented. This analysis was repeated at least twice for every patient. In addition BPY 2 (sY602) and Cdy1 (sY639) were analysed and were found to be deleted in all patients with an AZFb/c or c deletion (data not shown).

The AZFa patient and the molecular characterization of the deletion extension has been previously described (Sun *et al.*, 2000).

Morphometrical analysis

Bouin's fixed specimens, embedded in paraffin, were sectioned at 4 μ m. After deparaffinization and rehydration, the sections were stained with periodic acid Schiff and haematoxylin, dehydrated and mounted. The sections were randomized and surveyed under an upright microscope (Axioskop; Zeiss, Oberkochen, Germany) at two different magnifications (objectives 10 \times and 63 \times). Images of the sections were captured with a computer-assisted camera system (KS400 2.0; Zeiss) and five different parameters were measured. The total biopsy was used as a frame and the tubules were randomly

chosen. For each parameter, 20 individual tubules per testis biopsy were investigated. From each specimen the diameters of the testis tubules and lumen were determined at a magnification of 10 \times . The diameters of the lamina propria and tubule epithelia were determined at a magnification of 63 \times . To achieve comparable results, the measurements were always taken on the axis running the shortest distance through the centre of the tubule. Images were made with a charged coupled device (CCD) camera (AxioCam; Zeiss) controlled by image software (Axiovision; Zeiss).

Immunohistochemistry

Bouin's fixed specimens of testes from patients with AZF deletions, embedded in paraffin, were sectioned at 4 μ m. Control sections were made from patients whose biopsies were diagnosed with normal spermatogenesis, idiopathic SCO or mixed atrophy. All sections were treated equally. After deparaffinization and rehydration, primary antibodies against six different Sertoli cell-specific antigens were applied. Three different mouse monoclonal antibodies, cytokeratin 18 (IQ Products, Groningen, Netherlands), vimentin (Progen, Heidelberg, Germany) and inhibin A (α subunit) (RD Systems, Minneapolis, USA), and three different rabbit polyclonal antibodies, 14-3-3 θ (Santa Cruz Biotechnology, Heidelberg, Germany), androgen receptor (ABR Golden, CO, USA) and FSH receptor (donated by Dr James Dias, Mab 106-156 IgG2A unpurified culture media), were applied for 30 min at room temperature in blocking buffer.

After washing, Dako-EnVision (Dako Diagnostika, Germany) was added for 30 min, followed by a washing and incubation with Newfuchsin (Dako) for 30 min. The sections were counterstained with haematoxylin for 10 s and mounted under coverslips with Dako Faramount (Dako) prior to observation using an upright microscope (Axioskop) at different magnifications (objectives 10 \times and 25 \times). Digital images of equal exposure were acquired with a CCD camera (AxioCam) controlled by image software (Axiovision).

Statistics

The results of the bilateral testes evaluation are shown per patient. Data are given as means \pm SD. For statistical comparison, *t*-test or one-way analysis of variance followed by Tukey's test were employed. *P* < 0.05 was considered to be statistically significant.

Results

Of 17 patients, 11 with AZFc deletions, two with a deletion encompassing AZFa and AZFb or AZFb and AZFc, two with deletions in the AZFb region, and one with a deletion in the AZFa region were evaluated (see Table I). A detailed molecular analysis revealed that in the AZFc-deleted patients the extension of the deletion can be restricted in the proximal boundary to sY1197 and sY1192 and at the distal side to the boundary of sY1054 and sY1125 (Figure 1). Thus, all deletions display similar or identical breakpoints and cover ~3.3–3.8 MB. In the two AZFb/c patients the extension of each deletion is different. Whereas patient 1 (Figure 1, lane 1) lacks all proximal STSs like sY1196 and sY1197, the distal STSs sY1125 and sY1201 are present. This is in accordance with a breakpoint which is identical to a distal AZFc deletion (see above), but spans more into the proximal region of the Y chromosome, thereby removing AZFb. The second patient (Figure 1, lane 2) displays a more extended deletion, where not only the proximal STSs for AZFc are missing as expected, but the patient also lacks the distal STSs of AZFc. Thus, this deletion must encompass a bigger portion of this part of the Y chromosome. A karyotype analysis for one patient (1) was performed, but did not yield any Y-chromosomal abnormalities. For the second AZFb/c deletion this analysis was not performed because of the unavailability of the patient.

Both patients with a deletion that included the AZFa region showed SCO in both testes, whereas two of the patients with a deletion in the AZFb region had germ cells in both testes, but spermatogenesis was arrested at the primary spermatocyte stage with one patient having some late spermatids. Among the group of patients having only deletions in the AZFc region, four had a few early spermatids, but most of the tubules merely contained primary spermatocytes, spermatogonia or only Sertoli cells. The LH and testosterone levels of all patients were in the normal range, but the average FSH levels were elevated (15.9 ± 7.4 IU/l). Only four patients with at least

Table I. Comparison of hormone and testicular characteristics of the AZF patients investigated. Data are means \pm SD, *n* = 17

	Deletion				
	AZFa	AZFa,b	AZFb	AZFc	AZFbc
<i>n</i>	1	1	2	11	2
Age at date of biopsy (years)	30.3	32.3	34.3 \pm 1.0	30.3 \pm 4.0	33.3 \pm 1.0
Sperm concentration ($\times 10^6$ /ml)	Azoo	Azoo	Azoo (<i>n</i> = 2)	Azoo (<i>n</i> = 7) 0.1–0.5 (<i>n</i> = 3) 1 (<i>n</i> = 1)	Azoo (<i>n</i> = 2)
Testis volume (ml)					
Right	16	8	14.0 \pm 2.0	13.3 \pm 5.4	13.5 \pm 5.5
Left	16	12	13.5 \pm 6.5	12.6 \pm 5.6	17.3 \pm 1.6
Hormone levels					
LH (IU/l)	3.2	6.1	5.4 \pm 1.3	5.6 \pm 2.5	5.3 \pm 1.6
FSH (IU/l)	10	10.1	17.5 \pm 3.0	13.9 \pm 7.2	17.3 \pm 1.6
Testosterone (nmol/l)	25.6	18.1	33.3 \pm 19.3	31.3 \pm 43.5	13.0 \pm 3.0
Histology right testis	SCO	SCO	Arrest Hypo 3 Hypo	3 SCO 5 Arrest	SCO Arrest
Histology left testis	SCO	SCO	2 Arrest	5 SCO 6 Arrest	2 Arrest

SCO = Sertoli cell-only; arrest = spermatogenesis arrest; hypo = hypospermatogenesis; azoo = azoospermia.

some germ cells had normal FSH levels (4.0, 4.5, 6.1 and 6.4 IU/l), and the others had elevated FSH levels (range 9.6–31.0 IU/l). Among the control groups, the average FSH level of the SCO patients (17.9 ± 9.1 IU/l) was elevated and the patients with complete spermatogenesis had average FSH levels of 5.0 ± 5.6 IU/l.

Analysis of six different Sertoli cell-specific antigens revealed their expression in all men with AZF deletions. The cytoplasmic distributed microfilament cytokeratin 18 was almost never expressed in Sertoli cells with very few exceptions (Figure 2a). These were in two tubules out of 30

in one patient with an AZFc deletion. Although this patient had germ cells, the Sertoli cells of these two tubules were atypically flat showing an arrest at the stage of spermatogonia and spermatocytes (Figure 2a). AZF patients or the control patients with SCO syndrome had Sertoli cells with such a morphology, but in testis sections of three control patients with normal spermatogenesis several Sertoli cells were cytokeratin 18-positive, showing spermatogenic arrest.

An intense staining of another microfilament, vimentin, was found in all SCO patients independent of their AZF status (data not shown). Sertoli cells in tubules with germ cells present showed a less intense staining of vimentin, but compared with the control sections no qualitative expression differences could be observed. In all Sertoli cells FSH receptor, 14-3-3 θ and inhibin α subunit were strongly expressed independent of the presence of germ cells in the testes tubules. Among the AZF patients and the control patients no differences in the immunostaining could be detected. As an example, inhibin α subunit distribution is shown in Figure 2b. The nuclear staining of the androgen receptor in Sertoli cells showed a more differential picture. In tubules with germ cells present almost all nuclei were positively stained independent of the deletion status (Figure 2c). In contrast, five patients with AZFc deletions and SCO syndrome had many unstained Sertoli cell nuclei. In the control group with idiopathic SCO, many Sertoli cell nuclei were also unstained for the androgen receptor protein. The Sertoli cells with unstained nuclei showed no morphological differences compared with the positively stained cells of the same tubules.

The morphometrical evaluation was categorized according to the deletion type and is shown in Table II. The tubule parameters (diameter of the tubules, lumen, lamina propria and epithelium) of patients with microdeletions showed no significant differences in patients with focal or complete SCO. A comparison of the parameters from patients with microdeletion types AZFa, AZFb, and the combinations of AZFa+b or AZFb+c with patients with an AZFc microdeletion, also yielded no significant differences. However, by comparing the testis parameters of the Y-chromosomal deletion group with patients with mixed atrophy, idiopathic SCO and complete spermatogenesis, an intermediate state between total SCO and complete spermatogenesis emerged.

(i) The diameter of the average tubule of patients with an AZFc deletion and patients with idiopathic SCO was significantly smaller than that of patients with complete spermatogenesis (Figure 3). The tubular size of patients with mixed atrophy was similar to that of AZFc patients.

(ii) The lumen diameter was significantly smaller compared with the control group with complete spermatogenesis and larger than in patients with idiopathic SCO (Figure 3a). The tubules and lumen diameters of patients with mixed atrophy were similar to those of AZFc patients.

(iii) The diameter of the lamina propria of idiopathic SCO patients was increased compared with the AZFc patients and with the control group with complete spermatogenesis (Figure 3b).

Patients with idiopathic SCO had the thickest lamina propria (12.1 ± 2.4 μ m) compared with all other groups (Figure 3b).

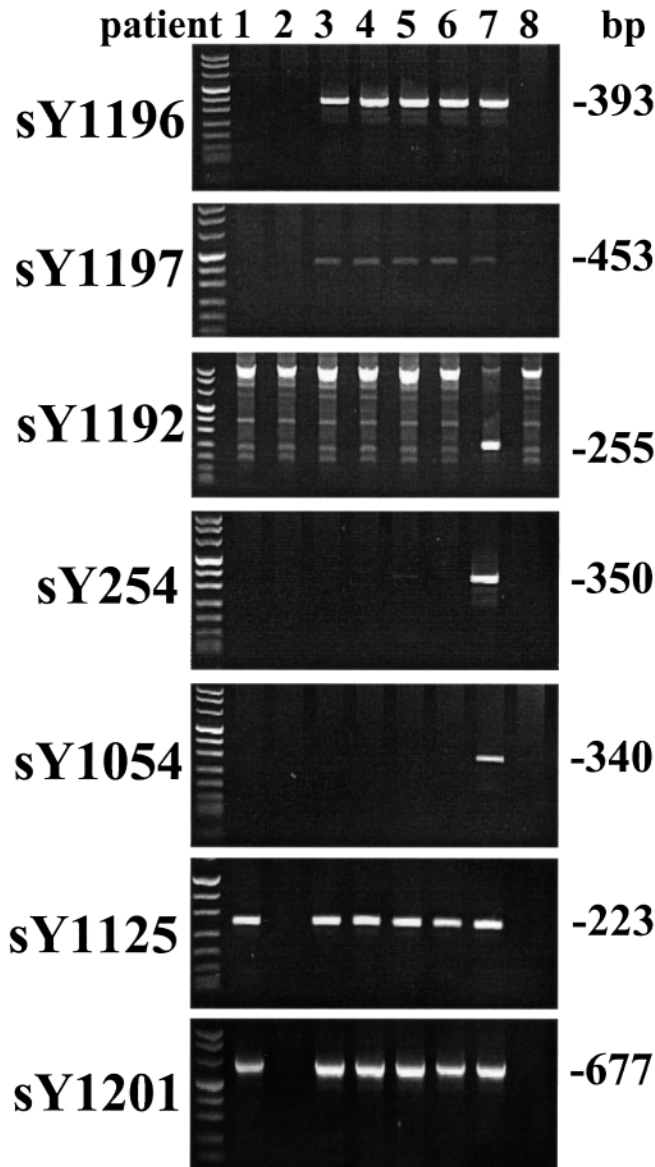


Figure 1. PCR assay in six patients displaying AZFb/c (1,2) and AZFc (3–6) deletions using sequence tagged sites (STSs) which delineate the breakpoints of AZFc deletions. On the left the different STSs are given and on the right the corresponding amplicon sizes. As a control the DNA of a fertile man (lane 7) and a woman (lane 8) were used. The STSs could either be not amplified, e.g. sY1197, or gave rise to unspecific amplification, as was the case for sY1192. The PCR assay for the AZFc deletion boundaries gave identical results for the remaining patients with an AZFc deletion (data not shown).

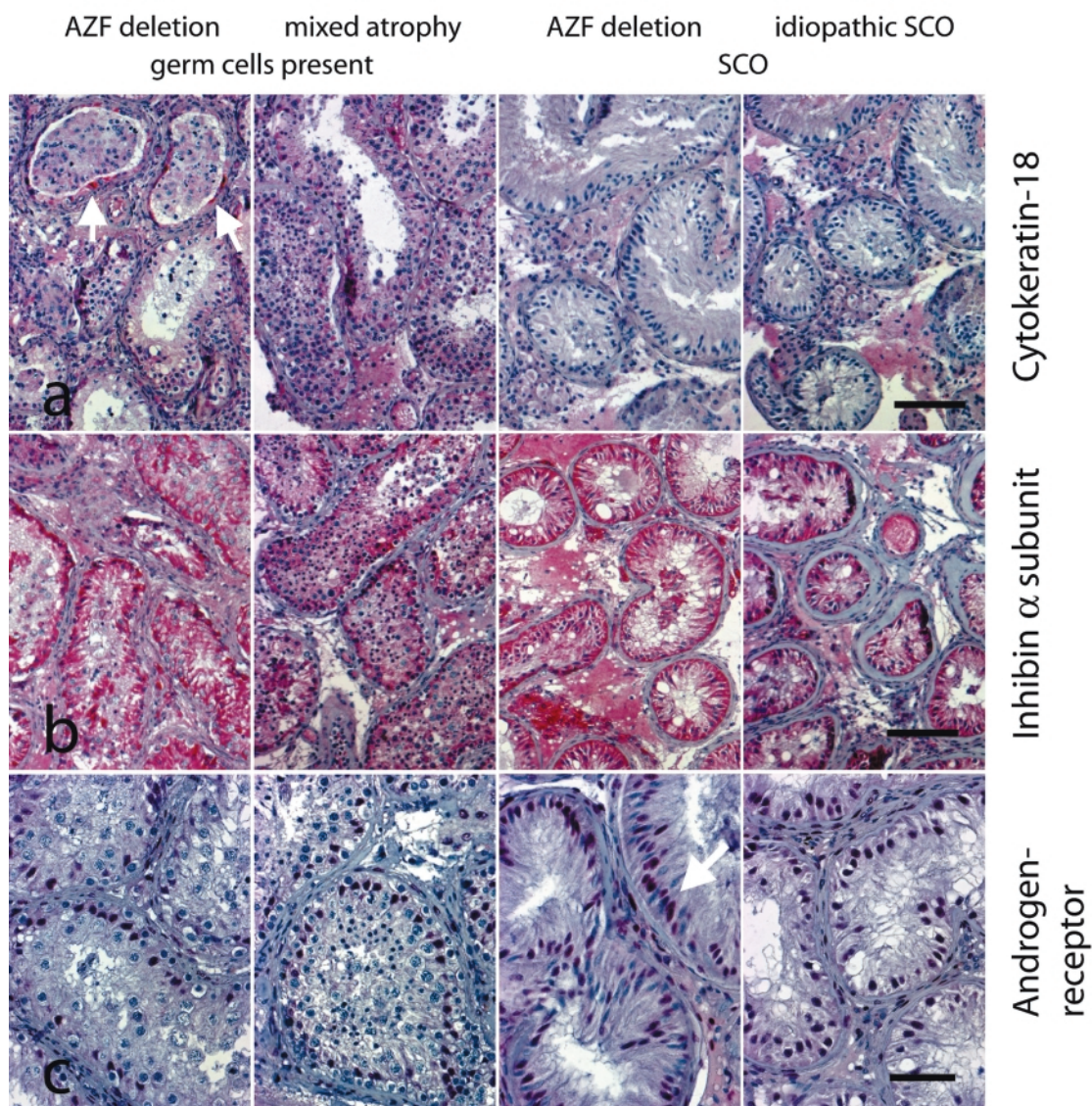


Figure 2. Immunohistochemical staining of cyto­keratin 18 (a), inhibin α subunit (b), and androgen-receptor (c). The first two columns represent sections of biopsies with germ cells present and the third and fourth columns represent biopsies of patients with Sertoli cell only (SCO) syndrome. The first and third columns are representative images of patients with Y chromosome deletions and the other two are images from control patients with idiopathic dysfunctions. (a) Cytokeratin 18 is only expressed in Sertoli cells of very few tubules with germ cells present (arrows) independently of the Y-chromosomal deletion status (image of AZFc-deleted man). In biopsies of SCO patients (image of AZFa-deleted man), no expression was observed. Scale bar = 100 μ m. (b) Inhibin α subunit was always detected in the Sertoli cells and the expression level seemed to be equally intense in all patients (images of an AZFb/c-deleted man with germ cells and an AZFc-deleted man with SCO). Scale bar = 100 μ m. (c) Nuclear staining of the androgen receptor. Almost all Sertoli nuclei were positively stained for the androgen receptor (arrow) and less Sertoli nuclei of AZFc patients with SCO syndrome were negative. (Both images are from patients with AZFc deletion.) Scale bar = 50 μ m

The lamina propria diameter from AZFc patients tended to be increased ($9.2 \pm 3.2 \mu\text{m}$) and was significantly different from the group with complete spermatogenesis ($6.0 \pm 1.1 \mu\text{m}$) and was also significantly lower ($P = 0.028$) than that of patients with idiopathic SCO. The average diameter of the tubule epithelium was also significantly increased ($P = 0.048$). Interestingly, both average diameters were significantly decreased compared with the control groups with mixed atrophy and complete spermatogenesis ($P < 0.01$). The tubular epithelium of the control group with mixed atrophy was also not significantly higher than that of patients with complete spermatogenesis. Among the patients with AZFc deletions and

germ cells present, the epithelium diameter (range 31.2–51.7 μm) could be lower than that in patients with idiopathic SCO (range 25.2–38.1 μm).

Discussion

After the description of the three AZF regions (Vogt *et al.*, 1996), analysis of Yq chromosome microdeletions has become a major tool in the diagnostics of male infertility. It is routinely performed by a PCR method on DNA extracted from leukocytes (Simoni *et al.*, 1999). Our analysis of the Y chromosome deletion position and size demonstrates that in

Table II. Individual testicular phenotype of the AZF patients. Data are average values from 28 testes from 17 different patients. For each parameter 20 individual tubules per testis biopsy were investigated

AZF deletion type	Patient no.																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Diameter (µm)	a + b	a	b	b	c + b	c + b	c	c	c	c	c	c	c	c	c	c	c
Tubulus	121.1	129.9	124.4	146.7	151.4	146.1	130.3	159.6	150.1	129.9	118.1	153.2	106.1	145.4	155.9	145.9	142.7
Lumina	44.9	52.9	25.8	58.0	54.2	47.5	45.6	56.1	61.7	49.9	36.2	53.0	42.8	48.9	66.5	51.1	49.1
Lamina propria	10.6	7.2	15.7	9.5	8.6	9.5	5.8	7.5	10.1	5.9	10.3	9.1	17.4	8.3	11.1	7.9	8.2
Sertoli cell nuclei	7.8	9	8.7	8.3	8.9	8.8	9.2	10.9	8.2	8.4	8	10.5	8.5	9.2	8.8	7.8	7.4
Epithelium	31.6	31	29	42.6	42.8	44.4	38.7	39	32.2	36.5	31.2	41.7	30.7	42.1	33.2	40.9	40.8
Lamina propria + Epithelium	38.1	38.5	49.3	44.4	48.6	49.3	42.4	51.7	44.2	40	41	50.1	31.6	48.3	44.7	47.4	46.8
Germ cells present	SCO	SCO	Arrest	Hypo	Arrest	Arrest	SCO	Hypo	Arrest	Arrest	Hypo	Arrest	SCO	Arrest	Hypo	Hypo	SCO
SCO tubules (%)	100	100	52	15	3	0	100	95	98	91	54	61	100	91	15	0	100

SCO = Sertoli cell-only.

Morphometrical aspects of Y-chromosomal microdeletions

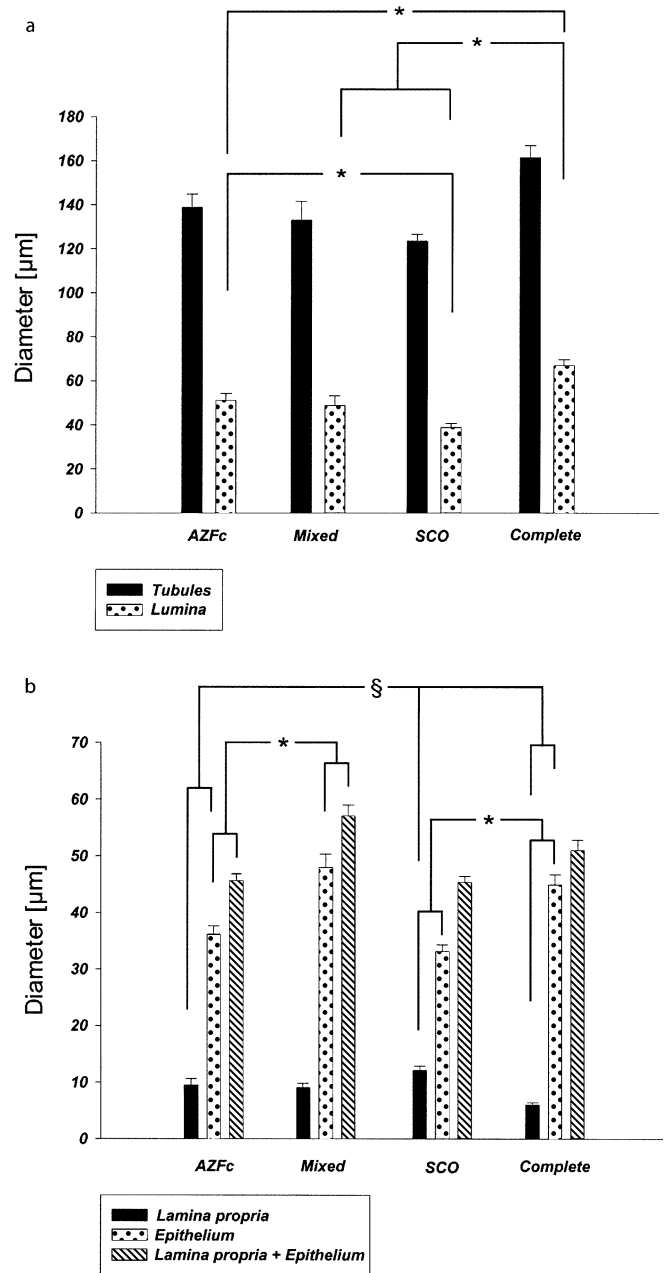


Figure 3. (a) Comparison of the testicular tubules and lumen diameters measured (10×). Biopsies of patients with microdeletions, AZFc ($n = 11$), were compared with three control groups: patients with mixed atrophy (Mixed; $n = 10$), idiopathic Sertoli cell-only patients (SCO; $n = 11$), and patients with complete spermatogenesis (Complete; $n = 11$). (b) Diameters of laminae propriae, epithelia and the combination of the diameters of both (63×). All four patient groups are specified by the size of their epithelia. The size of the lamina propria is inversely related to the tubular epithelium diameter. Data are means \pm SD. * $P < 0.01$, § $P < 0.05$.

all our patients with deletions in the AZFc region, the deletions encompass the entire region between the palindromic complex (Kuroda-Kawaguchi *et al.*, 2001). Even the two patients with a larger deletion which included the AZFc region also had the entire AZFc part excised. This result verifies the observation of Kuroda-Kawaguchi *et al.* that deletions in the AZFc region almost always encompass the palindromic structures, resulting

in uniform recurrent deletions via homologous recombination (Kuroda-Kawaguchi *et al.*, 2001). The two different deletion sizes in the two patients who also had an excised part in the AZFb region suggests that the palindromic structure could also be repeated several times in the AZFb region. The uniform excision demonstrates that the palindromic complex strongly favours a total deletion of the AZFc region. The size difference of the AZFc flanking region of direct repeats that are the corresponding targets for recombination is >20-fold longer than those of the AZFa region (Kamp *et al.*, 2000). This seems to be responsible for the much higher rate of Y deletions in AZFc compared with the other two regions, namely AZFa and AZFb.

Sertoli cells in patients with Y chromosome deletions do not seem to be altered. The tested Sertoli cell markers were not significantly different in patients with a deletion and in control patients with the same histological picture. Cytokeratin 18, a marker for the maturity of Sertoli cells, is absent in almost all patients and is found only occasionally in single Sertoli cells. Vimentin, another intermediate microfilament, is strongly expressed in all Sertoli cells independent of the histological status. By testing the expression of these cytoskeleton proteins, e.g. cytokeratin 18 and vimentin, it has been shown that patients with idiopathic SCO have a mosaic pattern of differentiated and undifferentiated Sertoli cells (Steger *et al.*, 1996; Bar-Shira Maymon *et al.*, 2000). This cannot be verified for our patients so far. All tested patients with idiopathic SCO were negative for cytokeratin 18 and positive for vimentin in their Sertoli cells. Nevertheless, such patients have a much smaller tubule diameter compared with patients with complete spermatogenesis (Paniagua *et al.*, 1990). Surprisingly, the tested Sertoli cell-specific proteins were not significantly altered from Sertoli cells which have been shown to be unaffected by Y chromosome microdeletions (Bar-Shira Maymon *et al.*, 2000; Kleiman *et al.*, 2001). This suggests that the dysfunction of the germ cells is a direct result of the Y chromosome deletion and not indirectly mediated by the dysfunction of Sertoli cells in accordance with earlier observations (Foresta *et al.*, 2001b).

In this study, we morphometrically analysed biopsies from 17 patients with Y chromosome microdeletions. Most of the genomic deletions were limited to the AZFc subregion and were therefore grouped together for statistical evaluation (11 cases). The genomic analysis showed that all patients in this group had an identical deletion size and position. The main testicular dysfunction cause in all these patients was very similar. Therefore a histological comparison with patient groups that have testicular dysfunctions without Y-chromosomal deletions is very useful. In spite of the same genomic pattern, the testicular histology among the patients with deletions varied from complete SCO to hypospermatogenesis. The patients with AZFc deletions compared with patients with mixed testicular atrophy had a similar average tubule diameter that was only slightly larger than that of patients with idiopathic SCO. Although in the AZFc group some tubules showed signs of spermatogenesis, the average lumen diameter was significantly larger than that of the idiopathic SCO group ($P = 0.002$). The tubules with spermatogenic cells among the

SCO tubules may explain these greater values because no significant difference was found between AZFc and mixed atrophy. Both average diameters were clearly smaller than the lumen of the control group with complete spermatogenesis ($P < 0.05$). This may seem obvious because when germ cells are missing only the Sertoli cells can contribute to the inner tubule size.

The results of the morphometrical evaluation demonstrate that the testis morphology of men with Y-chromosomal deletions has an intermediate status between that of normal spermatogenesis and SCO. Paniagua *et al.* and Matsuda *et al.* showed that the lamina propria increases its diameter according to the severity of seminiferous damage (Paniagua *et al.*, 1990; Matsuda *et al.*, 1996). For the AZFc patients, the thickness of the lamina propria takes an intermediate state between total SCO and complete spermatogenesis, similar to the mixed atrophy morphology. Significant size differences between the AZFc group compared with the patients with mixed atrophy and with patients with idiopathic SCO were detected in the epithelium diameter. The height of the seminiferous epithelium of patients with complete spermatogenesis was comparable with that of other patients of the same average age (Lanz and Neuhäuser, 1963; Pajarinen, 1997), whereas the epithelium of patients with mixed atrophy was even slightly larger than the latter group. In contrast, the AZFc group represents an intermediate state between complete spermatogenesis and idiopathic SCO. Testicular tubules lacking germ cells do not need a large lumen to channel sperm out of the testis and therefore may not be widened *per se*. Matsuda *et al.* showed that patients with testicular obstructions in childhood have fewer germ cells and smaller tubule diameters compared with an age-matched control group (Matsuda *et al.*, 1996). Unfortunately, it is unknown at which age idiopathic SCO patients lose their germ cells. On the other hand, AZFc patients, being a group with a mild form of spermatogenic damage, have a reduced number of germ cells. A depletion of germ cells with time has already been demonstrated in two patients (Österlund *et al.*, 2000; Calogero *et al.*, 2001), which correlates with previous findings that sperm counts decrease in patients sequentially followed-up (Girardi *et al.*, 1997; Simoni *et al.*, 1997). This could mean that patients with AZFc deletions may have normal or at least only subnormal germ cell numbers after puberty and that the quality of spermatogenesis decreases over time, leading to total SCO.

An analysis of the type of microdeletion compared with its phenotype from published data of open biopsies and fine needle aspirations (FNAs) demonstrates that spermatogenic damage is partly correlated with the Y-chromosomal genotype. Deletion of the entire AZF region is accompanied by the absence of germ cells. Deletion of genes in the AZFa region has the most severe effects (Foresta *et al.*, 2000). A truncation of the gene product of USPY9 (DFFRY, *Drosophila* fat-facets related Y), located in the AZFa region, has been demonstrated to lead to non-obstructive azoospermia (Sun *et al.*, 1999). Conversely, deletions in the AZFc or AZFb region show less severe damage of spermatogenesis and only 24% ($n = 28$) of the cases evaluated histologically ($n = 118$) had complete SCO syndrome. Most of the affected genes in these regions

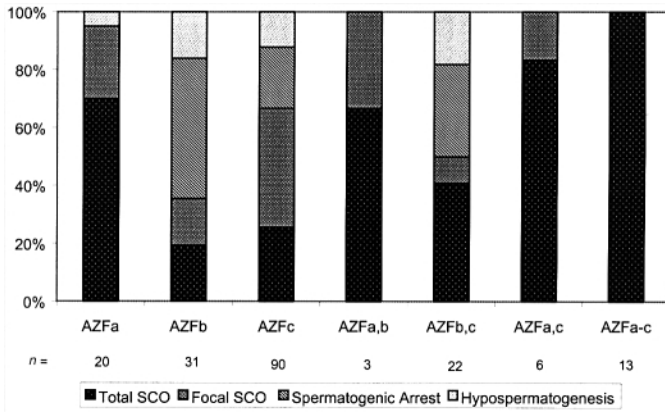


Figure 4. Testicular histologies from 185 patients. Published results from 111 biopsies and 57 fine needle aspirations from 25 publications (Najmabadi *et al.*, 1996; Qureshi *et al.*, 1996; Stuppia *et al.*, 1996; Vogt *et al.*, 1996; De Rosa *et al.*, 1997; Elliott *et al.*, 1997; Girardi *et al.*, 1997; Pryor *et al.*, 1997; Simoni *et al.*, 1997; Vereb *et al.*, 1997; Brandell *et al.*, 1998; Foresta *et al.*, 1998; Grimaldi *et al.*, 1998; Lee *et al.*, 1998; Ferlin *et al.*, 1999; Kim *et al.*, 1999; Kleiman *et al.*, 1999; Krausz *et al.*, 1999; Seifer *et al.*, 1999; Bar-Shira Maymon *et al.*, 2000; Blagosklonova *et al.*, 2000; Foresta *et al.*, 2000; Lin *et al.*, 2000; Österlund *et al.*, 2000; Krausz *et al.*, 2001) combined with 17 histologies from the presented patients.

(e.g. DAZ, CDY, TSPY or RBMY) are multicopy genes and a deletion does not seem to prevent spermatogenesis completely, but rather works in a dose-related fashion (Foresta *et al.*, 2001a). Analysis of the literature (Figure 4) reveals that deletion in the AZFc region leads to SCO in many tubules and >41% of the patients show a focal SCO syndrome, whereas a complete deletion of the AZFb region leads to spermatogenic arrest (Krausz *et al.*, 2000). An AZFc deletion seems to deplete testes of germ cells up to total SCO, rather than prevent spermatogenic progression. A clear correlation between microdeletion and testicular phenotype cannot be drawn solely from the location of the deletion, but a combination of location (AZFa, AZFb or AZFc) and size is clearly relevant. It can be concluded that an increase in microdeletion size correlates with a depletion of germ cells, and spermatogenesis of the remaining seminiferous tubules is retarded.

It is concluded that Y-chromosomal microdeletions lead to alterations in testis morphology different from those in patients with mixed atrophy, although the germ cell status of both is identical. The Sertoli cell function seems not to be altered. Regardless of the seminiferous activity, biopsies of patients with Y-chromosomal microdeletions show a morphometric intermediate status between idiopathic SCO and complete spermatogenesis in all the parameters measured. Together with the data in the literature, our results give evidence that microdeletions of the Y chromosome may lead to alterations in spermatogenesis which can become progressively worse with time, resulting in the heterogeneous histological profile observed among patients.

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