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EXTENSIVE CLINICAL EXPERIENCE

Molecular and Clinical Characterization of Y Chromosome Microdeletions in Infertile Men: A 10-Year Experience in Italy

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Context: An explosive growth in Y chromosome long arm (Yq) microdeletion testing demand for male infertility occurred in the past few years. However, despite the progresses in the biology of this chromosome, a number of molecular and clinical concerns are not supported by definitive data.

Objective: The objective was to provide information on the type and prevalence of microdeletions in infertile males, indication for testing, genotype-phenotype correlation, sperm aneuploidies, and genetic counseling.

Design and Setting: We performed a prospective study from January 1996 to December 2005 in an academic clinic.

Patients: We studied 3073 consecutive infertile men, of which 625 were affected by nonobstructive azoospermia and 1372 were affected by severe oligozoospermia. Ninety-nine patients with microdeletions are described here.

Main Outcome Measures: Yq microdeletions, seminal analysis, reproductive hormones, testicular cytology/histology, and sperm sex chromosomes aneuploidies were used as outcome measures.

Results: The prevalence of microdeletions was 3.2% in unselected infertile men, 8.3% in men with nonobstructive azoospermia, and 5.5% in men with severe oligozoospermia. Only 2 of 99 deletions were found in men with more than 2 million sperm/ml. No clinical data are useful to identify *a priori* patients with higher risk of Yq microdeletions. Most deletions are of the AZFc-b2/b4 subtype and are associated with variable spermatogenic phenotype, with sperm present in 72% of the cases. Complete AZFa and AZFb (P5/Proximal P1) deletions are associated with Sertoli cell-only syndrome and alterations in spermatocyte maturation, respectively, whereas partial deletions in these regions are associated with milder phenotype and frequent presence of sperm. Men with AZFc-b2/b4 deletions produce a higher percentage of sperm with nullisomy for the sex chromosomes and XY-disomy.

Conclusions: This extensive clinical research expands the knowledge on genotype-phenotype relationships and confirms that the identification of Yq microdeletions has significant diagnostic and prognostic value, adding useful information for genetic counseling in these patients. **(*J Clin Endocrinol Metab* 92: 762–770, 2007)**

MICRODELETIONS OF THE Y chromosome long arm (Yq) represent the most frequent molecular genetic cause of severe male infertility (for review, see Refs. 1 and 2), represented by severe oligozoospermia and nonobstructive azoospermia. The intense effort of many laboratories in past years contributed to a significant understanding of the clinical and molecular significance of this genetic alteration, and now Y chromosome microdeletions are routinely screened worldwide.

Almost 20 yr after the initial cytogenetic observation in 1976 suggesting the presence of an azoospermia factor (AZF) on Yq (3), progress in molecular biology allowed the first analysis of Yq microdeletions in infertile men (4–7). The first genes identified in AZF and thought to be involved in spermatogenic failure were the RBMY (RNA binding motif on the Y) gene (8) and the DAZ (deleted in azoospermia) gene (5). Subsequently, in 1996, the long arm was shown to contain in fact three AZF regions, designated AZFa, AZFb, and AZFc, from proximal to distal Yq (7).

However, only the completion of the sequencing of the Y chromosome revealed the real structure and organization of this chromosome, and previous data were reinterpreted on the basis of this new molecular information. In particular, it was shown that most of the AZF microdeletions are generated by intrachromosomal homologous recombination between repeated sequence blocks organized into palindromic structures showing nearly identical sequences (9–11). The original classification in the three AZF regions was therefore modified according to the mechanism of deletion in: AZFa, P5/Proximal-P1 (previous AZFb), P5/Distal-P1, P4/distal P1, and b2/b4-AZFc. Together with the refinement of Yq

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Abbreviations: AZF, Azoospermia factor; FISH, fluorescence *in situ* hybridization; FNA, fine-needle aspiration; HS, hypospermatogenesis; ICSI, intracytoplasmic sperm injection; MA, maturation arrest; SCOS, Sertoli cell-only syndrome; STS, sequence tagged site; TESE, testicular sperm extraction.

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mapping, the methods for Yq microdeletion screening improved. In fact, initial analyses were based on anonymous and heterogeneous markers [sequence tagged sites (STSs)] that frequently were noninformative and gave false results. The precise mapping of Yq allowed the development of more specific STSs of high quality, and guidelines for molecular diagnosis of Yq microdeletions were published (12, 13). As a consequence, Yq testing has become more homogeneous and reliable in different laboratories.

Despite this progress during the past 10 yr, new molecular and clinical aspects of Yq microdeletions have arisen, for which definitive data have not been produced, mainly based on the limited number of cases. This is the case, for example, of prevalence of nonclassical (partial) AZF deletions, possible decline in testicular function over time, and production of sperm with aneuploidies.

In this study, we present a summary of all the cases, well characterized at the molecular and clinical level, that we analyzed in our center during a 10-yr period (1996–2005).

Subjects and Methods

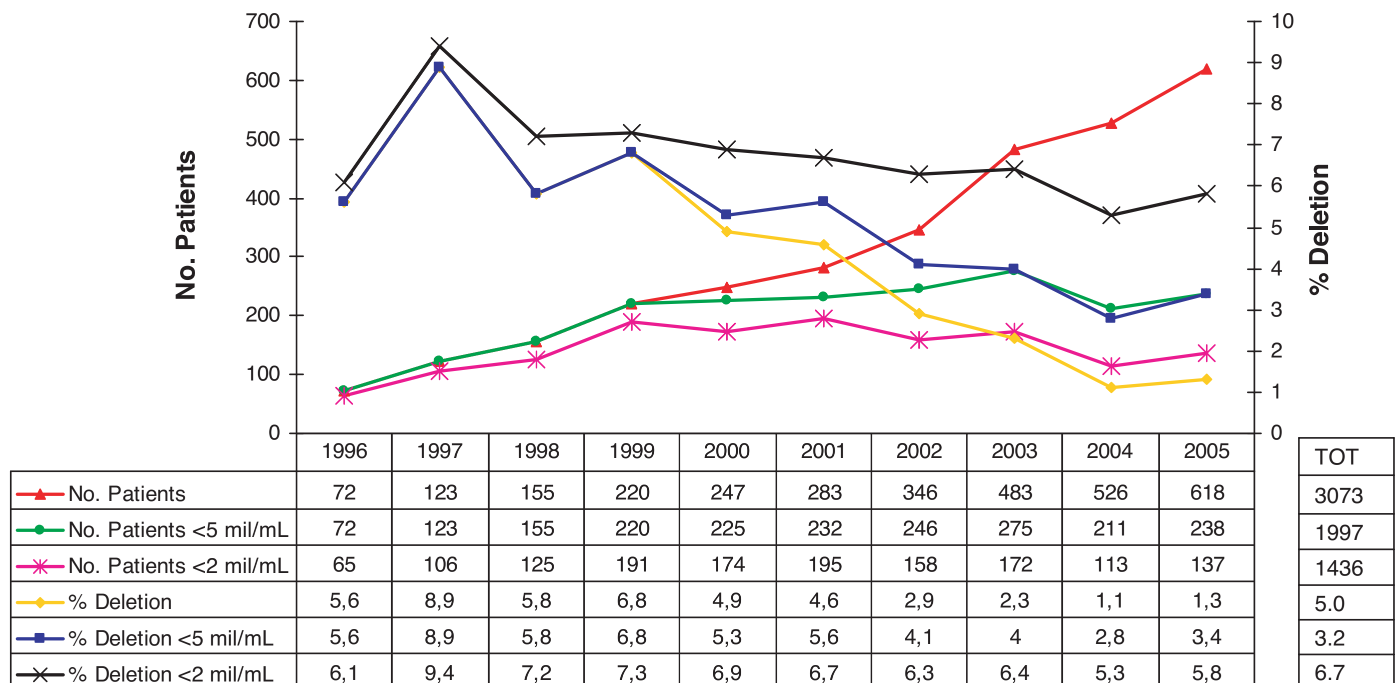
Subjects

The study was approved by the local Ethics Committee of the University of Padova and was in accordance with the Helsinki II Declaration. All participants were asked for and provided their informed consent. A total of 3073 consecutive infertile men attended our university center from January 1996 to December 2005 and were screened for Yq microdeletions. Patient selection was different during these 10 yr. In 1996–1999, selection criteria were stringent, and we screened for microdeletions only in men affected by azoospermia and severe oligozoospermia (sperm concentration < 5 million/ml) with a testicular phenotype of Sertoli cell-only syndrome (SCOS) or severe hypospermatogenesis (HS). Most of these subjects were classified as idiopathic or were affected by cryptorchidism (14–18) and were primarily recruited in our center. From year 2000, our center offered molecular diagnosis for external patients, and selection criteria were less restrictive; thus, men with nonidiopathic infertility or men with sperm concentration more than 5 million/ml were also studied. Therefore, as shown in Fig. 1, from 2000–2005, the proportion of men who actually had indication for Y microdeletion screening (azoospermia, severe oligozoospermia) was declining. Patients were excluded if they had: 1) congenital absence of vas deferens and obstructive azoospermia; 2) treatment with chemotherapeutic agents or radiotherapy, or testicular tumors; 3) karyotype abnormalities including Y chromosome alterations; or 4) androgen receptor gene mutations (19). Of the 3073 men screened for Y microdeletions, 625 presented azoospermia, 1372 had severe oligozoospermia, 833 had a sperm concentration more than 5 million/ml but no greater than 10 million/ml, and 243 had a sperm concentration more than 10 million/ml.

As described, microdeletions of the Y chromosome were found only in the cohort of subjects affected by azoospermia/severe oligozoospermia, and only two of them had more than 2 million sperm/ml. Therefore, to compare clinical data of men with and without Yq microdeletions, we considered only men with a sperm concentration less than 2 million/ml (97 and 1339, respectively). All these men were carefully and clinically analyzed (see below). As controls, we recruited 310 subjects with proven fertility and normal sperm concentration (>20 million/ml); these men had wives in the first trimester of pregnancy. All patients and controls were of Caucasian origin and came from different Italian regions.

Molecular analysis

Patients and controls have been analyzed by routine diagnostic Y chromosome homemade multiplex PCR screening using three STSs for the AZFa region (sY86, DBY1 for the DBY gene, DF3.1 for the USP9Y gene), three for the AZFb region (sY117, sY125, sY127), two for the AZFc region (sY254 and sY255 for the DAZ gene), and one between AZFa and AZFb (sY95). The screening assay was organized into three multiplex PCRs, each including a positive control marker (sY14 for the SRY gene). PCR was performed on genomic DNA extracted from peripheral blood cells as previously described (20). Analysis was always performed with a male control sample, a female sample, and a blank sample. Negative results (no amplification) were considered only after three amplification



	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	TOT
No. Patients	72	123	155	220	247	283	346	483	526	618	3073
No. Patients <5 mil/mL	72	123	155	220	225	232	246	275	211	238	1997
No. Patients <2 mil/mL	65	106	125	191	174	195	158	172	113	137	1436
% Deletion	5,6	8,9	5,8	6,8	4,9	4,6	2,9	2,3	1,1	1,3	5.0
% Deletion <5 mil/mL	5,6	8,9	5,8	6,8	5,3	5,6	4,1	4	2,8	3,4	3.2
% Deletion <2 mil/mL	6,1	9,4	7,2	7,3	6,9	6,7	6,3	6,4	5,3	5,8	6.7

FIG. 1. Results of 10-yr experience on Yq microdeletion testing. The total number of men, the number of men with indication for the testing (sperm count < 5 million/ml), and the total number of men with sperm count less than 2 million/ml are indicated with the respective prevalence of deletions.

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failures, repeating the experiments on new DNA extracted from a second blood collection. Furthermore, before assuming a deletion, each STS was analyzed separately in a single PCR. Patients with microdeletions were analyzed with a second step analysis for additional STSs to obtain confirmation of the deletion and to define the deletion breakpoints, as follows: sY82 and sY83 for proximal AZFa breakpoint; sY88 for distal AZFa breakpoint; sY1264, sY1227, and sY1228 for breakpoint in P5; sY1197, sY1192, and sY1191 for breakpoint in P3/b2; sY1291, sY1125, sY1190, and sY1206 for breakpoint in P1; and sY1201, sY159, and sY160 for distal P1 and heterochromatic region. Analysis of DAZ gene copies when indicated was performed by single nucleotide variant analysis as previously described (20).

Clinical analyses

Detailed medical history and physical examination were obtained by the same three physicians. Testicular volume was measured by orchidometer. Semen analysis was performed according to the World Health Organization guidelines (21). The diagnosis of azoospermia was established by pellet analysis after semen centrifugation. LH and FSH were measured by immunoradiometric assay and testosterone by RIA with commercial kits (Adaltis Italia, Bologna, Italy).

Testicular cytology and testicular sperm extraction (TESE)

Bilateral testicular fine-needle aspiration (FNA) was performed in Yq microdeleted patients, as previously described (22). This method allows the identification of all germ cells in their different maturation steps (spermatogonia, primary and secondary spermatocytes, round and elongated spermatids, mature spermatozoa) and of Sertoli cells and permits clarification of the different kinds of tubular damage. Testicular phenotype was classified as follows: 1) SCOS, characterized by complete absence of all germ cells in both testes; 2) HS, characterized by a quantitative reduction in the number of germs cells; and 3) maturation arrest (MA), characterized by normal presence of germ cells until a definite step of spermatogenesis and total lack of germ cells in the later stages (23). We did not distinguish between HS and partial MA because FNA cytological analysis is not the optimal technique to differentiate with sufficient adequacy between these two conditions. Azoospermic men without mature sperm identified at FNA (45 subjects) underwent bilateral TESE to assess clearly the presence or absence of mature sperm and to retrieve sperm for assisted reproductive techniques. In these cases, the classification of testicular phenotype considered FNA and TESE results: SCOS and MA at FNA were classified as SCOS or HS and complete MA and HS when sperm were absent or present, respectively, at TESE (23).

Sperm aneuploidies

Eleven men with the b2/b4 deletion (sperm concentration, 1.4 ± 1.1 million/ml; range, 0.5–4.2 million/ml) and 387 severely oligozoospermic men without microdeletions (sperm concentration, 1.8 ± 0.8 million/ml; range, 0.1–4.0 million/ml) were analyzed for numerical alterations of sperm sex chromosomes by means of fluorescence *in situ* hybridization (FISH), as previously reported (24, 25). As controls for this analysis, 103 normozoospermic fertile men were used. At least 5000 sperm were analyzed for each subject, and percentages of sperm with the Y chromosome, the X chromosome, nullisomies (absence of sex chromosomes), and XX, XY, and YY disomies (abnormal presence of the two sex chromosomes) were calculated.

Statistical analysis

In general, nonparametric statistics were used because most data did not have a Gaussian distribution. Comparisons of proportions (prevalence of anomalies in patients and controls) were performed by means of the Fisher's exact test. Comparisons of the data among groups were analyzed by Wilcoxon rank sum test. Statistical analysis was performed with the open-source statistical software R (http://cran.r-project.org). *P* values (two-sided) of less than 0.05 were considered to indicate statistical significance. Data are expressed as mean ± SD of the mean.

Results

Prevalence of Yq microdeletions

The trend in mutation detection during 1996–2005 is represented in Fig. 1. The overall frequency of Yq microdeletions was 3.2% (99 of 3073). However, if we consider only men who were truly candidates for Y microdeletion screening (azoospermic and severely oligozoospermic), this figure is 5.0% (99 of 1997). Starting from year 2000, there is a progressive decline in mutation frequency due to the less stringent patient selection criteria. In contrast, the prevalence of deletions in the cohort of subjects with sperm count less than 5 million/ml is relatively constant during the 10 yr, although in the past years this frequency tends to be lower mainly due to inclusion also of nonidiopathic infertile men from year 2000. In the subgroup of men with sperm concentration less than 2 million/ml, the deletion frequency is constant during the 10 yr (Fig. 1). Overall, the frequency of deletions is 8.3% (52 of 625) in men with azoospermia and 3.4% (47 of 1372) in men with severe oligozoospermia (<5 million/ml) (Table 1). If we consider only men with sperm concentration less than 2 million/ml, this figure is 6.7% (97 of 1436). Only two men out of 99 with microdeletions had a sperm concentration more than 2 million/ml, only six had a sperm concentration more than 1 million/ml, and all of them had AZFc (b2/b4) deletion. The great majority (83 of 99 or 83.8%) of patients with microdeletions had a sperm count of 0.1 million/ml or less.

We never found Yq microdeletions in men with more than 5 million sperm/ml (1076 men) and in controls (310 men). Two severely oligozoospermic men with AZFc-b2/b4 deletion were brothers and have inherited an identical deletion from their father. Unfortunately, sperm analysis of the father was not available. DNA from male relatives of another 53 men with Yq microdeletions was obtained, and we did not find any deletions; therefore, these cases were considered as *de novo* events.

TABLE 1. Frequency of the different Yq microdeletions in 1997 men grouped on the basis of sperm concentration

	Azoospermia	>0 ≤ 2 million/ml	>2 ≤ 5 million/ml	Total
AZFa	6/625 (1.0)	5/811 (0.6)	0/561 (0)	11/1997 (0.6)
AZFb	7/625 (1.1)	1/811 (0.1)	0/561 (0)	8/1997 (0.4)
AZFb-c	12/625 (1.9)	1/811 (0.1)	0/561 (0)	13/1997 (0.6)
AZFc-b2/b4	25/625 (4.0)	38/811 (4.7)	2/561 (0.4)	65/1997 (3.2)
AZFa-b-c	2/625 (0.3)	0/811 (0)	0/561 (0)	2/1997 (0.1)
Total	52/625 (8.3)	45/811 (5.5)	2/561 (0.4)	99/1997 (5.0)

Data are presented as number of patients/total number (percent).

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Type of microdeletions

The type of the 99 Yq microdeletions is represented in Fig. 2. The most frequent deletion was in the AZFc region (b2/b4) (65 of 99, 65.7%), followed by AZFb-c region (13 of 99, 13.1%), AZFa region (11 of 99, 11.1%), AZFb (8 of 99, 8.1%), and complete AZFa-b-c region (2 of 99, 2.0%). Of the 11 AZFa deletions, three were complete deletions, one was a deletion of the USP9Y gene, and seven were of the DBY gene. In AZFb region, we observed three patients carrying the P5/proximal-P1 deletion as originally described (10) (confirmed also by DAZ single nucleotide variant analysis that confirmed the absence of DAZ1 and DAZ2), four with a partial AZFb deletion probably originating from b5/b1 recombination as described previously (26), and one that we define "unique," indicating a particular P5/proximal-P1 deletion. With the standard set of STS primers, this patient was classified as AZFb deleted (absence of sY117, sY125, sY127, and presence of sY254 and sY255). With the second set of STSs, we confirmed the proximal breakpoint in P5 (presence of sY1264 and absence sY1227 and sY1228), and we had no amplification of sY1197 and sY1291 (as for the other P5/proximal P1 deletions), but we obtained normal amplification of sY1192 and sY1191. Then we confirmed the deletion of the cluster DAZ1/DAZ2. To explain this particular case, we hypothesized a complex mechanism of rearrangements between palindromes: a b2/b4 inversion followed by a g1/g2 and a subsequent P5/distal-P1 deletion.

All deletions in the AZFc region were classical b2/b4 deletion. Deletions in the AZFb-c region were mainly of the P4/distal-P1 subtype (11 cases), whereas only two men had a terminal deletion with proximal breakpoint in P5 (Fig. 2). The two complete AZFa-b-c deletions had proximal breakpoint in proximal AZFa and distal breakpoint in b4.

Seminal and testicular phenotype of Yq microdeletions

Men with deletions in the AZFa region had invariably a severe quantitative reduction of spermatogenesis, represented by severe HS or SCOS (Table 2). In particular, men with the complete AZFa deletion invariably presented azoospermia associated with SCOS, whereas men with partial AZFa deletions (USP9Y gene only, DBY gene only) frequently presented sperm in the ejaculate, although with a sperm concentration of 0.1 million/ml or less (cryptozoospermia) associated with severe HS. Therefore, combining seminological and testicular data, complete AZFa deletions were always associated with absence of sperm both in the ejaculate and testicular tissue, whereas deletions of single AZFa genes were associated with the presence of sperm in six of eight cases. In only one of six cases of AZFa deletions presenting with azoospermia was sperm recovered at TESE.

Men with deletions in the AZFb and AZFb-c regions frequently had azoospermia (19 of 21, 90.5%), and cryptozoospermia was observed only in one case of partial AZFb deletion and one case of P5/terminal deletion. Men with deletions in the AZFb region showed severe HS or a specific MA at spermatocyte level. Maturation disturbances at the spermatocyte level (higher number of spermatogonia and spermatocytes with respect to spermatids and spermatozoa)

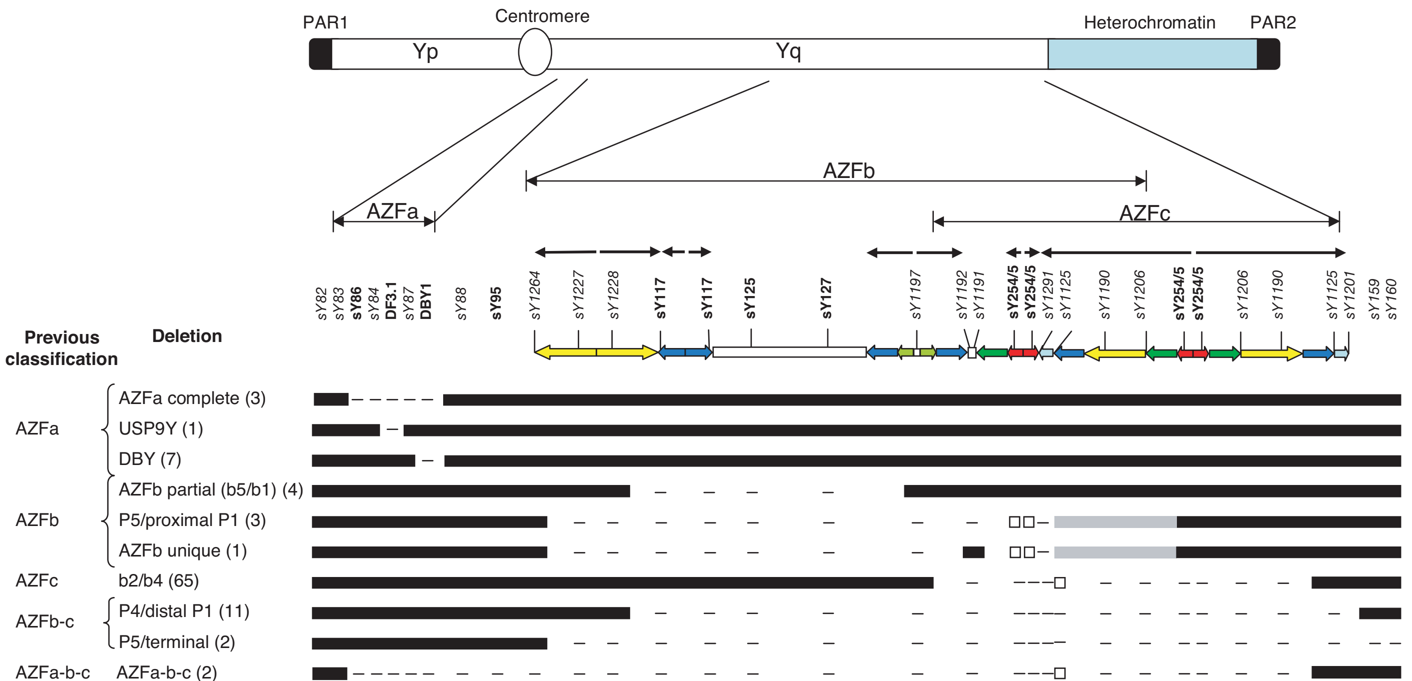

FIG. 2. Representation of Yq microdeletions detected. *Top*, The Y chromosome and the three AZF regions. *Middle*, STSs used to detect deletions (the STSs used for the initial first step screening are in *bold*, and the STSs used for the second step extension analysis are in *italics*) and the ampliconic structure of AZFb-c region forming five palindromes (P1–P5) with homologous amplicons marked with the color code of Kuroda-Kawaguchi *et al.* (9). *Bottom*, Results of STS analysis and classification of Yq microdeletions with the number of patients in *parentheses*. *Solid black bars*, STS present; *lines*, STS absent; *white boxes*, repetitive STSs that normally amplify by PCR but are assumed to be absent in the context of the deletion pattern; *solid gray bars*, repetitive STSs that normally amplify by PCR, but their presence or absence cannot be determined.

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TABLE 2. Combined seminological and testicular cytology/TESE of the 99 men with Yq microdeletions

	n	Azoospermia			≤0.1 million/ml; HS	>0.1 ≤ 2.0 million/ml; HS	>2.0 ≤ 5.0 million/ml; HS
		SCOS	HS	MA			
AZFa							
Complete	3	3					
USP9Y	1				1		
DBY	7	2	1		4		
AZFb							
P5/ProxP1	3		2	1Spc			
Partial (b5/b1)	4		2	1Spc	1		
Unique	1		1				
AZFb-c							
P4/DistP1	11	9		2Spc			
P5/term	2	1			1		
b2/b4	65	16	7	2Spc	24	14	2
AZFa-b-c	2	2					
Total	99	33	13	6Spc	31	14	2

Spc, At the spermatocyte level.

were frequently observed in men with HS. Combining seminological and testicular data, men with the classical P5/Proximal P1 AZFb deletion, although azoospermic, may have few mature sperm in the testes because HS was seen in two of three cases. Patients with partial deletions of the AZFb region frequently have sperm in the ejaculate or in the testes: in only one of four cases was a complete MA observed, and no mature sperm were present.

Men with deletions including the AZFb-c regions have a more severe spermatogenic impairment. In only one of 13 cases were few sperm observed in the ejaculate and in the testes, and this was a nonclassical AZFb-c deletion. In the other cases, azoospermia was associated with complete absence of germ cells (SCOS, 10 cases) or complete spermatocyte arrest (two cases).

Men with the b2/b4 deletion (65 cases) have variable seminal and testicular phenotypes. Sperm concentration varied from azoospermia (25 of 65, 38.5%), to cryptozoospermia (24 of 65, 36.9%), to severe oligozoospermia (16 of 65, 24.6%). Overall, 61.5% (40 of 65) of men with b2/b4 deletion had sperm in the ejaculate, but only six had more than 1 million/ml. Testicular phenotype was represented frequently by a quantitative reduction of spermatogenesis (SCOS, 16 cases; HS, 47 cases) and more rarely by MA at the spermatocyte (two cases). Therefore, combining seminological and testicular data, b2/b4 deletions were associated with the presence of sperm in the ejaculate and/or in the testes in 47 of 65 (72.3%) cases, and the chance of finding sperm in the testes in men with azoospermia is 28.0% (7 of 25, of which three had sperm identification at FNA and four had sperm recovery at TESE). Men with complete AZFa-b-c deletions invariably showed azoospermia with SCOS.

Clinical manifestation of Yq microdeletions

Comparisons between men with and without Yq microdeletions were performed for andrological data and hormonal concentrations. Because only two men with deletions had more than 2 million sperm/ml, only men with a sperm concentration less than 2 million/ml (97 with and 1339 without deletions) were considered for this analysis. Infertility-related symptoms were less frequent in men with Yq microdeletions (38.1%) with respect to men without microdeletions (54.7%) ($P < 0.05$). Importantly, the prevalence of the most frequent andrological diagnosis (varicocele and history of cryptorchidism) was similar (23.7 and 9.3%, respectively, in men with Yq microdeletions; and in 31.3 and 10.1% in men without Yq microdeletions). Taken together, the prevalence of Yq microdeletions was not statistically different between men with idiopathic severe infertility (azoospermia <2 million sperm/ml) and men with nonidiopathic infertility (varicocele, cryptorchidism, infections, orchitis, testicular trauma or torsion) (60 of 714, 8.4%; 37 of 625, 5.9%). Of 23 men with deletions presenting varicocele, eight underwent varicocelectomy elsewhere before they were screened for Yq microdeletions in our center.

Hormonal data (Table 3) showed no differences in LH and testosterone plasma concentrations between men with and without Yq microdeletions. FSH levels in men with Yq microdeletions were higher than controls but lower than levels in men without deletions (13.8 ± 7.2 *vs.* 15.3 ± 5.7 IU/liter; $P < 0.05$). In particular, significantly lower concentrations of FSH were observed in men with deletions in AZFb regions and men with the b2/b4 deletions.

Sperm sex chromosome aneuploidies

Sperm aneuploidy rate was assessed by FISH in 11 men with the b2/b4 deletions and compared with 387 nondeleted severely oligozoospermic men and 103 normozoospermic control men (Table 4). Patients with the b2/b4 deletion, but not nondeleted men, had a significant reduction in the percentage of normal Y-bearing spermatozoa with respect to controls (34.7 ± 2.1 *vs.* 49.0 ± 1.7; $P < 0.01$) and a concomitant increase in nullisomic sperm (10.4 ± 1.5 *vs.* 1.1 ± 0.2; $P < 0.01$). We also found a significant increase in XY-disomic sperm (4.1 ± 1.2 *vs.* 0.2 ± 0.5; $P < 0.01$). This percentage is also statistically higher than that observed in oligozoospermic men without Y chromosome microdeletions (1.4 ± 1.8; $P < 0.01$).

Correlation with age

Although we did not find a correlation between age at diagnosis and sperm count, analysis of the 65 men with the b2/b4 deletion showed that the age of men with azoospermia

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TABLE 3. Reproductive hormones in azoospermic–severely oligozoospermic men (sperm count < 2 million/ml) with Yq microdeletions (n = 97) and without deletions (n = 1339) and in normozoospermic controls (n = 310)

	n	FSH (IU/liter)	LH (IU/liter)	Testosterone (nmol/liter)	Mean testicular volume (ml)
AZFa	11	16.3 ± 4.1	5.7 ± 1.9	18.4 ± 6.4	9.6 ± 3.0
AZFb	8	9.5 ± 3.7[b]	5.0 ± 0.7	18.6 ± 5.7	12.0 ± 3.9
AZFb-c	13	15.5 ± 5.3	5.6 ± 2.1	16.5 ± 5.7	10.3 ± 2.6
b2/b4	63	13.7 ± 7.9[a]	4.9 ± 2.5	17.0 ± 5.1	10.7 ± 2.4
AZFa-b-c	2	26.1 ± 14.0	6.8 ± 7.4	12.0 ± 9.8	8.5 ± 0.7
Total	97	13.8 ± 7.2[a]	5.1 ± 2.3	17.1 ± 5.4	10.6 ± 2.6
Without Yq microdel	1339	15.3 ± 5.7	5.5 ± 3.0	16.6 ± 4.8	10.9 ± 3.1
Controls	310	2.9 ± 1.2	3.1 ± 0.9	19.1 ± 4.4	16.4 ± 3.3

For all data but testosterone in AZFa, AZFb, and AZFb-c, $P < 0.01$ *vs.* controls.
[a] $P < 0.05$ *vs.* without deletions.
[b] $P < 0.01$ *vs.* without deletions.

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(25 cases, 36.4 ± 4.5 yr) was significantly higher with respect to that of men with sperm in the ejaculate (40 cases, 34.2 ± 5.2 yr; $P < 0.05$). A temporal trend for hormonal and seminological data was available in five cases of b2/b4 deletions and two cases of P4/Distal P1 deletions. In all these cases, there was an increase in FSH plasma levels over 3–10 yr, associated in two cases (one b2/b4 and one P4/Distal P1 deletion) with reduction in sperm concentration from cryptozoospermia to azoospermia and SCOS.

Discussion

The progress in molecular biology of the Y chromosome in the past 10 yr and the intense effort of many laboratories connected to andrology have definitively clarified that Yq microdeletions represent the most frequent genetic cause, together with certain chromosomal abnormalities, of severe spermatogenic impairment (1, 24). Many clinical and molecular questions were answered, and Yq screening has become a routine test worldwide. A recent survey in Italy of the Italian Society of Human Genetics indicates that Yq screening is one of the most frequent postnatal molecular genetic tests, with almost 5000 tests performed by 70 laboratories in 2004 (27). This impressive demand for Yq genetic testing requires a rationalization, with the goals of providing tests of the highest quality and indications for their appropriate use, which should follow the good clinical practice. In this regard, some important issues can be answered only by careful clinical assessment of a large group of men with well-characterized microdeletions. The present study, conducted over 10 yr in our center where both molecular and clinical analyses are performed, represents the largest series of infertile men with Yq microdeletions published to date.

Prevalence, clinical aspects, and indications for Yq microdeletion testing

Yq microdeletions are specific for severe spermatogenic impairment. In 10 yr, we never found deletions in men with more than 5 million sperm/ml, in accordance with the majority of published studies within and outside Italy (for review, see Refs. 1, 2, 13, 28, and 29). However, we found an AZFc-b2/b4 deletion naturally transmitted from a father to two infertile men. Some other rare cases of deletions in fertile men and natural transmission of AZF deletions have been described (7, 30–33), exemplifying the concept that fertility is not synonymous with normal sperm count.

The majority of our cases presented a sperm concentration less than 2 million/ml, and 84% of them had azoospermia or cryptozoospermia with 0.1 million sperm/ml or less. The prevalence of Yq microdeletions in the present study is in accordance with the data of the literature (1, 2, 28), with 8.3% in men with nonobstructive azoospermia, 3.4% in men with severe oligozoospermia (<5 million sperm/ml), and 5.5% in the subgroup of men with less than 2 million sperm/ml. Although initial screening for Yq microdeletions was mainly conducted in men with idiopathic infertility, it is evident that the prevalence of deletions is similar in men with idiopathic and nonidiopathic testicular damage (8.4 and 5.9%, respectively), even if preliminary data on a limited number of cases form Italy suggested a higher prevalence of deletions in men with idiopathic infertility (19 *vs.* 7%) (34). Yq screening in severely infertile men with other apparent causes of testicular damage is particularly important to avoid unnecessary treatments. For example, in our series eight of 23 men with deletions underwent avoidable varicocelectomy (35) before Yq microdeletion testing was performed. No clinical data

TABLE 4. Sperm sex chromosome aneuploidies in 11 men with the b2/b4 deletion, compared to severely oligozoospermic men without microdeletions and normozoospermic controls

Patients	Normal spermatozoa		Disomy			Nullisomy	Other
	X-bearing	Y-bearing	XX	YY	XY		
b2/b4 (n = 11)	49.8 ± 1.9	34.7 ± 2.1[a]	0.4 ± 0.3	0.5 ± 0.7	4.1 ± 1.2[a]	10.4 ± 1.5[a]	0.1 ± 0.2
Non-Yq deletion severe oligospermia (n = 387)	48.3 ± 1.5	48.0 ± 1.6	0.6 ± 0.8[b]	0.6 ± 0.8[b]	1.4 ± 1.8[b]	1.0 ± 0.2	0.1 ± 0.3
Controls (n = 103)	49.4 ± 1.6	49.0 ± 1.7	0.1 ± 0.5	0.1 ± 0.9	0.2 ± 0.5	1.1 ± 0.2	0.1 ± 0.4

[a] $P < 0.01$ *vs.* controls and non-Yq deletions.
[b] $P < 0.01$ *vs.* controls.

from medical history or physical examination are able to identify *a priori* patients with higher risk of Yq microdeletions. Hormonal data are also not useful to this aim. We found significant lower plasma FSH concentrations in men with Yq microdeletions with respect to men without deletions, according to a previous preliminary study (36). We have interpreted these findings as suggestive for a less severe damage of the Sertoli cells in this group of patients with respect to men with other causes of testicular damage. However, these data have not been confirmed by other authors (37), and the range of FSH concentrations is highly overlapping between men with and without microdeletions. Therefore, individual data cannot be used as indicators of Yq abnormalities.

Type of microdeletions and screening procedure

Diagnostic testing of Yq microdeletions is generally performed by PCR amplification of selected regions of the Y chromosome designated STSs. The precise sequencing and mapping of the male-specific region of the Y chromosome (9–11) clarified that many STSs used in initial screening were polymorphic, ambiguous, heterogeneous, and unreliable, and frequently gave technical problems and false results (12, 13). This fact, together with the use of heterogeneous STSs in the diverse laboratories and the different patient selection criteria, were important factors determining the great variability in deletion frequency reported in initial studies (28). The methods for Yq testing have now been standardized for an "optimal" detection rate (13), and some commercial kits for Yq screening are available. We adopted a homemade multiplex PCR following the general policy of EAA/EMQN guidelines (13), but with a different set of primers, both for the initial screening and for the second-step extension analysis. The most evident differences are the use of gene-specific primers in the AZFa region (DBY and USP9Y genes) and the use of primers located in specific amplicons for the second-step analysis able to give information on the mechanism and, therefore, the extension of the deletions.

Confirming previous data (1, 2, 13, 30), our findings showed that classical AZFc deletions caused by b2/b4 recombination represent the most frequent finding (66% of deletions in our cases). An important finding of our survey is the definition of less frequent deletions in AZFa and AZFb regions. The relative frequency of deletions in AZFa in our survey (11.1%) is somewhat higher with respect to many published reports (3–5%) (13, 28), and this is mainly due to a high detection rate of partial deletions in this region, such as isolated deletions of DBY gene. In fact, the relative deletion frequency of the complete AZFa region in our survey is 3.0% (3 of 99). Comparison of our data with the two most extensive analyses on AZFa deletions shows similar deletion frequency in infertile men: 11 of 3073 (0.3%) in our survey, 1 of 576 (0.2%) in the survey of Sun *et al.* (38), and 2 of 995 (0.2%) in the survey of Krausz *et al.* (39). However, descriptions of high frequency of partial AZFa deletions have been published in highly selected cases of severe testiculopathies, especially SCOS, when analyzed in details for AZFa markers (18, 40–42). These studies also highlighted the improved value of inclusion of DBY and USP9Y gene markers in the minimal set of markers for the analysis of AZFa deletions (18, 40). These markers are not included in the EAA–EMQN scheme (13). Other studies clarified that the use of different STS markers allowed some deletions previously classified as complete AZFa deletions to be diagnosed correctly as partial AZFa (40). Other than patient selection and type and number of STSs used, differences in the incidence, type of microdeletions, and genotype-phenotype relation may theoretically also be influenced by ethnic factors, genetic background, or Y chromosome haplogroups. However, we have no direct data to clarify these aspects, and published reports do not seem to support this view (43). Although the molecular mechanisms inducing unusual AZFa and AZFb deletions are not understood and they do not involve known intrachromosomal recombination events, it is essential to distinguish between complete and partial deletions in these regions that are associated with different phenotype. Therefore, although partial AZFa and partial AZFb deletions are rare events, we suggest including primers for DBY and USP9Y genes in the first-step screening and to perform a second-step analysis to define proximal and distal breakpoints when the absence of AZFb markers are observed.

Genotype-phenotype correlation

The definition of the precise testicular phenotype by means of bilateral FNA and TESE in all subjects with Yq microdeletions allowed us to draw important conclusions on the genotype-phenotype correlation and to expand our knowledge on the role of this chromosome in spermatogenesis. We now have further evidence that deletions removing the entire AZFa or AZFb (P5/Proximal P1) regions are associated with distinct phenotypes, represented by SCOS and spermatocyte arrest, respectively (7, 18, 40, 44, 45). In these cases, patients are invariably azoospermic, and the chance of finding sperm in the testes is virtually zero (complete AZFa) or very poor (P5/Proximal P1). However, deletions of isolated genes of the AZFa region, involving only the DBY gene or the USP9Y genes, or partial deletions in the AZFb region (b5/b1 as defined here), frequently are associated with some sperm in the ejaculate or in the testes and are rarely associated with spermatogenic arrest (18, 26, 40, 46, 47). These data suggest that genes in the proximal or distal end of AZFb region are involved in the regulation of meiosis, whereas AZFa genes are probably involved in the differentiation process of premeiotic germ cells and mitotic phases of spermatogenesis.

Men with deletions involving AZFb-c or AZFa-b-c regions have the most severe spermatogenic damage, resulting frequently in SCOS or rarely in complete spermatocyte arrest, confirming initial data (28).

A strict genotype-phenotype correlation is not evident for AZFc-b2/b4 deletions that are associated with variable phenotypes, ranging from oligozoospermia to azoospermia with HS to SCOS, and more rarely to complete spermatogenic arrest, as previously reported (5–7, 23, 48, 49). Among men with Yq microdeletions, those with AZFc-b2/b4 have the most favorable chance to have sperm in the ejaculate or in the testes (72% in our series and in accordance with the literature). The variable phenotype observed in men with AZFc-

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b2/b4 deletion might be related to the progressive decline of the spermatogenic potential over time (50). We had some evidence for such phenomenon observing a higher age at diagnosis of men with azoospermia with respect to men with sperm in the ejaculate and a progressive increase in FSH levels associated with decline in spermatogenic activity in 5 b2/b4 cases. Although the data set is limited for drawing conclusions about the evolution of the Yq deleted phenotype, these findings are extremely useful in the clinical practice. Oligozoospermic subjects should be encouraged to cryopreserve sperm to avoid the risk of becoming completely azoospermic or to avoid more invasive techniques such as TESE/ intracytoplasmic sperm injection (ICSI) in the future (51).

Sperm aneuploidies and genetic counseling

Most men with Yq microdeletions require ICSI (with ejaculated or testicular spermatozoa) to overcome their infertility. Because all spermatozoa from Y-deleted men harbor the same microdeletion (6), ICSI male offspring of men with Yq microdeletions will also carry the deletion and will have spermatogenic impairment in adulthood. Although the severity of spermatogenic failure cannot be predicted, preventive cryopreservation of sperm in the sons at a relatively young age should be recommended to the parents.

However, preliminary reports (52) suggested that men with Yq microdeletions may also produce a higher percentage of sperm nullisomic for sex chromosomes. Our findings of FISH analysis on sperm of men with AZFc-b2/b4 deletion confirm these data and suggest that, concomitant with a reduction in the percentage of normal Y-bearing spermatozoa and an increase in nullisomic sperm, there is an increase in XY-disomic sperm. The occurrence of nullisomic sperm in these men suggests a general instability of the Y chromosome, with loss of complete Y chromosomes in sperm. In this view, Yq microdeletions could be considered as "premutations" for a subsequent complete loss of the Y chromosome in the AZF-deleted patients' sperm, increasing the risk of embryonic X0 cells (Turner syndrome). The higher percentage of XY disomic sperm suggests that the presence of a Yq microdeletion can interfere with the meiotic process, leading more frequently to a nondisjunction of the bivalent XY. The theoretical risk after ICSI is the birth of 47,XXY boys (Klinefelter syndrome). It has to be noted, however, that these risks are more theoretical because in the 31 children already born from men with AZF deletion (23, 37, 53–56), no consequences other than transmission of the Yq deletion have been reported. Nevertheless, clear information regarding implantation rate and incidence of spontaneous abortion for the partners of men with Yq microdeletions is not available.

Conclusions

Y chromosome microdeletion screening has become a routine practice, and an explosive growth in Yq testing demand was observed in the last few years. We now have clearer information regarding who should be tested for Yq microdeletions and how they should be tested, and we have identified that specific AZF deletions have significant diagnostic and prognostic value. There is a precise etiological diagnosis of male infertility, and unnecessary medical and surgical treatment can be avoided. We also have information about the chance of finding sperm in the testes of azoospermic men and about sperm cryopreservation in oligozoospermic men. Genetic counseling in men with Yq microdeletions candidates for ICSI should consider the obligate transmission of the deletion to male offspring, the possibility of sperm cryopreservation in these boys as soon as they reach adulthood, and the theoretical higher risk of generating 45,X0 and 47,XXY embryos.

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