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Double-Blind Y Chromosome Microdeletion Analysis in Men with Known Sperm Parameters and Reproductive Hormone Profiles: Microdeletions Are Specific for Spermatogenic Failure*

CSILLA KRAUSZ, EWA RAJPERT-DE MEYTS, LONE FRYDELUND-LARSEN, LLUIS QUINTANA-MURCI, KEN McELREAVEY, AND NIELS E. SKAKKEBAEK

Immunogénétique Humaine (C.K., L.Q.-M., K.M.), Institut Pasteur, 75724 Paris Cedex 15 France; Unità di Andrologia (C.K.), Dipartimento di Fisiopatologia Clinica, Viale Pieraccini, 6, 50139 Firenze, Italy; and University Department of Growth and Reproduction (E.R.-D.M., L.F.-L., N.E.S.), Rigshospitalet, 9, Blegdamsvej, DK-2100 Copenhagen, Denmark

ABSTRACT

Y chromosome microdeletions have been reported as a possible genetic factor of male infertility. Despite a large number of studies in this subject, there is still considerable debate and confusion surrounding the role of Y chromosome microdeletions in male infertility. This has been further compounded by observations of Y microdeletions in fertile males. The aim of the present study was to evaluate: 1) the incidence of Y microdeletions in control male population and infertile males, where complete semen and hormonal analysis was available to define whether Y microdeletions are specific for spermatogenic failure or if they can be found also in normospermic men; and 2) whether the suboptimal semen quality reported in Denmark is associated with a higher incidence of Y microdeletions in respect to other populations. Double-blind molecular study of deletions was performed in 138 consecutive patients seeking intracytoplasmic sperm injection treatment, 100 men of known fertility, and 107 young military conscripts from the general Danish population. Microdeletions or gene-specific deletions were not detected in normospermic subjects or in subfertile men with a sperm count of more than 1×10^6/mL. Deletions of the Azoospermia factor (AZF)c region were detected in 17% of individuals with idiopathic azoo/cryptozoospermia and in 7% of individuals with nonidiopathic azoo/cryptozoospermia. The data indicate that: 1) the composition of the study population is the major factor in determining deletion frequency; 2) Y chromosome microdeletions are specifically associated with severe spermatogenic failure; therefore, the protocol described here is reliable for the routine clinical workup of severe male factor infertility; and 3) the frequency of Yq microdeletions in the Danish population is similar to that from other countries and argues against the involvement of microdeletions in the relatively low sperm count of the Danish population. (*J Clin Endocrinol Metab* **86:** 2638–2642, 2001)

RECENTLY, CONSIDERABLE attention has focused on the role of genetic factors in spermatogenic failure (1), with an emphasis on the Y chromosome. Deletions of three nonoverlapping regions of the Y chromosome long arm have been described, associated with male infertility. Deletions of the AZFa region are mainly associated with Sertoli cell only syndrome (SCOS), deletions of the AZFb region are mainly associated with spermatogenic arrest, and a range of phenotypes from azoospermia to oligozoospermia are associated with the absence of the AZFc region (2).

Individuals defined as idiopathic severe oligo- or azoospermic have the highest frequency of Y microdeletions, particularly involving the AZFc region. However, there remains considerable debate concerning the frequency, the position, and the phenotypes associated with Y chromosome microdeletions. For example, deletion frequency has been reported between 1–55% in different studies (3). It is not clear whether these wide variations, particularly in deletion frequency, are attributable to differences in the study design, the geographic or ethnic origins of the study population, or to experimental error. In addition, the contribution of Y microdeletions to male subfertility in the general population has not been evaluated; and the incidence of Y microdeletions in control males, where complete semen and hormonal analysis is available, has not been investigated. A complete investigation of the latter group is necessary because Y microdeletions have been repeatedly reported in fertile men (2, 4–6), which suggests that microdeletions may not be confined to infertile subjects. Until these questions have been definitively resolved, the utility of Y chromosome microdeletion screens in a clinical context remains controversial.

Here, for the first time, we describe a Yq microdeletion screen of infertile, subfertile, and normospermic men from the Danish population, where complete semen analysis and hormonal profile was available. The DNA samples were analyzed in a double-blind manner using 14 anonymous and 6 gene-specific Y chromosome markers. The choice of Danish subjects for this study was not completely coincidental. A recent study demonstrated that a relatively large fraction of the otherwise healthy Danish male population seems to have suboptimal semen quality (7). Thus, an additional aim of this

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Address all correspondence and requests for reprints to: Ken McElreavey, Immunogénétique Humaine, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France. E-mail: kenmce@pasteur.fr.

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study was to investigate a possible contribution of Y microdeletions to this worrying phenomenon. We show that Y chromosome microdeletions are specific for spermatogenic failure, that the variation in deletion frequency reported in other studies is probable attributable to study design, and that Y microdeletions do not seem to contribute to the relatively low sperm count of the general Danish population.

Materials and Methods

The study population has been recruited and studied through a comprehensive andrological examination, including semen analysis and hormonal analysis by the Department of Growth and Reproduction (Rigshospitalet, Copenhagen, Denmark). Samples were analyzed in a double-blind manner. DNA samples were sent from the Department of Growth and Reproduction in a coded way to the laboratory of Immunogénétique Humain (Institut Pasteur, Paris, France), where Y chromosome screening was performed. No exchange of information concerning clinical or molecular data took place until the completion of the study.

Subjects

A total of 345 subjects were studied. These consisted of 138 consecutive patients seeking intracytoplasmic sperm injection (ICSI) treatment, who were referred to the Rigshospitalet for a full andrological workup; 100 men of known fertility recruited from a Danish obstetric clinic who recently made their partners pregnant; and 107 young military conscripts from the general Danish population. All subjects gave an informed consent for molecular analysis of their blood samples, and the study was approved by a local ethical committee. A subdivision of the whole study population, on the basis of the sperm count, is shown in Table 1. Among patients seeking ICSI treatment, 62 were affected by idiopathic infertility; whereas, in 61 cases, abnormal andrological findings (including cryptorchidism and >2 degree varicocele) were reported; 12 patients were normozoospermic; and for 3 patients, no clinical information, other than infertility possibly due to a male factor, was available (Table 2).

Semen and hormonal analysis

Semen analysis was performed accordingly to the WHO 1992 guidelines (8). Serum concentrations of FSH, LH, and sex hormone binding globulin were measured using time-resolved immunofluorometric assays from DELFIA, Wallac, Inc., Turku, FIN. Testosterone and estradiol were measured by RIA (Coat-a-Count, Diagnostic Products, Los Angeles, CA) and Immuno, Diagostic Systems Laboratories, Inc. (Boldon, UK), respectively. Serum inhibin B was measured in duplicate in a double-antibody enzyme immunometric assay using a monoclonal antibody raised against the inhibin βB-subunit in combination with a labeled antibody raised against the inhibin α-subunit, as previously described.

Molecular analysis

PCR analysis of genomic DNA, extracted from lymphocytes, was performed. A total of 14 anonymous STS markers and 6 Y-specific genes, spanning the 3 AZF regions, were screened [in AZFa: sY82, sY86, sY87, and the gene DBY; in AZFb: sY114, sY116, sY125, sY133, and the genes *eIF-1AY*, *XKRY*, and *CDY*; in AZFc: sY145, sY147, sY152, sY158, and the genes *BPY2* and *DAZ* (sY254)]. sY98 and sY100 (between AZFa and AZFb) and sY160 (distal to AZFc in the heterochromatin) were also included (Fig. 1). The PCR primers and conditions were described previously, with modifications (9, 10). Duplex amplifications were performed using the following combinations: Mix A sY254, sY125; Mix 2 sY98 and sY100; Mix 3 sY160 and sY145; mix 4 sY87 and sY152. For each duplex reaction, the PCR conditions were 95 C for 5 min, followed by 35 cycles of 95 C for 1 min, 60 C for 1 min, and 72 C for 1 min.

The presence of deletions was confirmed by Southern blotting using a *DAZ* gene probe, as described elsewhere (10).

Results

Microdeletions were detected in nine infertile subjects affected by azoospermia and cryptozoospermia ($<1 \times 10^6$/mL; six and three patients, respectively). The deletions removed the entire AZFc region (sY145, sY147, sY152, and sY158, including the genes *DAZ* and *BPY2*) in each case. No difference in the extent of the deletion was detected. The clinical details of each patient with an AZFc deletion are described in Table 3. Microdeletions were not detected in the group of oligozoospermic ($>1 \times 10^6$/mL) or normospermic subjects.

The frequency of Y microdeletions in the subgroups with different sperm count shows the highest value among azoo/cryptozoospermic men (nine cases, 11.5%). This percentage has progressively decreased if severe oligozoospermic (8.9%)

TABLE 2. Subdivision of the ICSI candidate's group on the basis of their sperm count and aetiology deletion frequencies are calculated for the subgroups and for the overall study population

Category	Idiopathic	Nonidiopathic
Azoospermic	12	14
-With Yq deletion	4	2
$<1 \times 10^6$/mL	23	27
-With Yq deletion	2	1
Subtotal (deletion frequency)	35 (6/35; 17%)	41 (3/41; 7.3%)
1–5 $\times 10^6$/mL	15	7
5–20 $\times 10^6$/mL	12	13
Total (deletion frequency)	62 (6/62; 9.6%)	61 (3/61; 4.9%)

TABLE 1. Subdivision of the whole study population on the basis of sperm count

Category	Sperm count				
	Azoospermia	$<1 \times 10^6$/mL	1–5 $\times 10^6$/mL	5–20 $\times 10^6$/mL	$>20 \times 10^6$/mL
ICSI candidates	27	51	23	25	12
-with Yq deletion	6	3			
Military conscripts		1	5	16	83[a]
Fertile men			2	8	89
Total number	27	52	30	49	184
Deletion frequency		11.5%			
		8.9%			
		7.1%			

Deletion frequencies are indicated as a percentage of those individuals with different degrees of spermatogenic failure. Deletion frequency of 11.5%, 8.9%, and 7.1% in the subgroups of individuals with sperm count $<1 \times 10^6$/mL, $<5 \times 10^6$/mL, $<20 \times 10^6$/mL, respectively.

[a] Thirty-two military conscripts presented a sperm count of $<40 \times 10^6$/mL. A total of 53/105 (51%) of military conscripts have a sperm count $<40 \times 10^6$/mL.

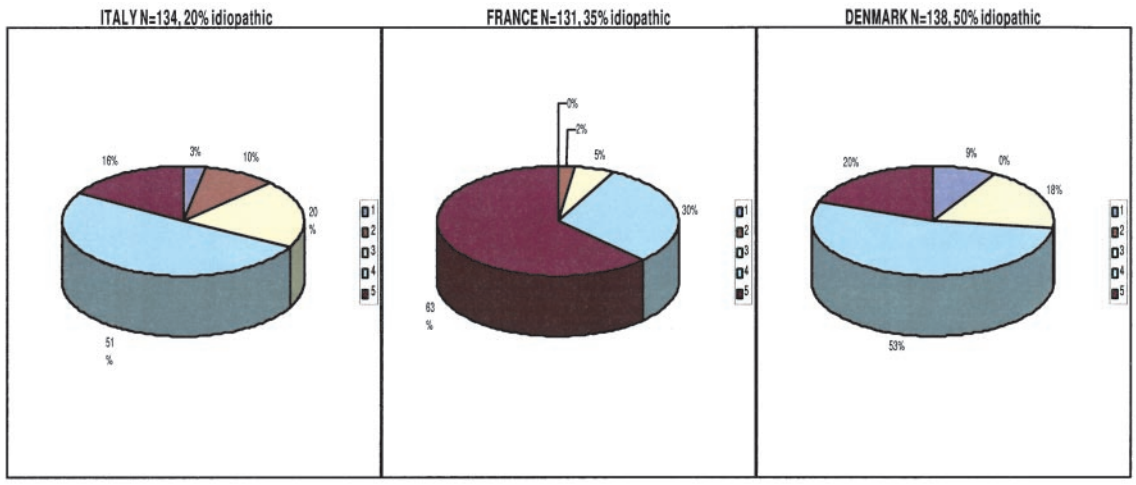
TABLE 3. Clinical details for the nine deleted patients

Code	Age	FSH U/L	LH U/L	T nmol/L	Inhibin B pg/mL	Sperm count $\times 10^6$/mL	Semen volume mL	Testis size L/R mL	Abnormal andrological findings	Histology	Type of deletion
P212	41	29.3	12.50	16.6	27	0.00	3.4	12/6	None	n.a.	AZFc
P283	39	23.4	4.16	8.6	29	0.10	7.25	10/12	None	n.a.	AZFc
P322	27	15.4	6.52	15.7	<20	0.00	3.7	15/15	None	n.a.	AZFc
P323	33	17.5	9.57	11.5	<20	0.00	3.65	12/12	None	Bil. SCO	AZFc
P325	31	13.4	4.68	8.51	23	0.01	2.40	10/10	Unilateral cryptorchidism (spont. desc. at 6 yr)	n.a.	AZFc
P327	33	14.0	4.36	3.51	<20	0.00	n.a.	6/6	Hypoandrogenism sexual dysfunction obesity	n.a.	AZFc
P340	40	6.35	4.06	10.1	61	0.00	2.2	8/12	Unilateral cryptorchidism (desc. horm. treatment at 12 yr)	L: spermatocytic arrest; R: SCOS	AZFc
P352	35	15.4	4.21	16.1	43	0.00	2.6	15/15	None	Bil. mixture of SCO (60–70%) and spermatocytic arrest; R: SCOS	AZFc
P354	28	5.72	3.26	17.4	75	0.115	6.2	12/12	Left varicocele 2°	Bil. spermatocytic arrest; few tubules SCO (3–5%)	AZFc

P, Sample code; T, testosterone; n.a., not available; spont., spontaneous; desc., descent; horm., hormonal; L, left; Bil, bilateral; R, right.

FIG. 1. Schematic representation of the long arm of the Y chromosome, divided into seven deletion intervals. The position of genes identified on Yq are shown, as are the relative positions of the AZF regions and the markers and genes used in this study.

and oligozoospermic subjects (7.1%) are included (Table 1). Microdeletions were only observed in the subgroup of men seeking ICSI treatment. Within this subgroup, the incidence of microdeletions in idiopathic azoo/cryptozoospermic men was 17% (Table 2). Microdeletions were also found in three cases of nonidiopathic infertility (7%). These subjects presented with unilateral cryptorchidism, varicocele grade 2, and hypoandrogenism with sexual dysfunction. In the first two of these three cases, gonadal histology was available,



DELETION FREQUENCY

TOTAL STUDY GROUP	1.5%	10.6%	6.5%
IDIOPATHIC SEVERE OLIGO-, AZOOSPERMIA SUBGROUP	10%	18%	12%

FIG. 2. Schematic representation of the results of the current study and those reported for the French (10) and Italian populations (9), using a similar set of markers and identical clinical definitions. The deletion frequency of the total study population and the deletion frequency of the subgroup comprising idiopathic azoospermia and severe oligozoospermia are indicated. 1, Azoospermia; 2, normospermia; 3, asthenozoospermia; 4, oligozoospermia (5–20 million/mL); 5, severe oligozoospermia (<5 million/mL).

which revealed both spermatogenic arrest and SCOS. These observations are consistent with the pattern of histology associated with Y chromosome microdeletions. Isolated gene-specific deletions were not detected. Although all the microdeletions seemed to have the same molecular extent in each case, a genotype/phenotype correlation could not be established on the basis of the semen analysis and testicular histology. Sperm concentration ranged from $0–0.135 \times 10^6$/mL. Testicular histology varied from spermatocytic arrest combined with Sertoli cell only (SCO) pattern to bilateral SCO pattern combined with diffused Leydig cell hyperplasia. The hormonal parameters showed a more homogeneous picture: serum inhibin B levels were below the normal range in each patient, whereas FSH levels were above the mean (3.94 U/L) in all cases, with abnormally high values in seven of nine patients.

Discussion

The data presented here clearly show that the composition of the study population is a major factor influencing deletion frequency. Patients affected by idiopathic infertility and severe oligo- or azoospermia have a greater probability of harboring microdeletions, compared with the nonidiopathic group or moderate oligospermic men, respectively. We found that the highest deletion frequency in the group defined as idiopathic azoospermic/cryptozoospermic was 17% (Table 2). A subdivision of the study population, on the basis of sperm number, indicates a deletion frequency of 11.5% in the group of azoospermic/cryptozoospermic patients (this includes both idiopathic and nonidiopathic patients). The incidence progressively decreases with the inclusion of less severe phenotypes, to 8.9% (oligospermic with $<5 \times 10^6$ sperm per mL) and 7.1% (oligospermic with $<20 \times 10^6$ sperm per mL) (Table 1). These figures are consistent with two other studies of the French and Italian populations, where uniformly defined clinical groups were studied using a similar study protocol (Refs. 9 and 10; Fig. 2). This suggests that variation in deletion frequency is mainly attributable to the clinical composition of the study group; and it also indicates that, at least in Europe, there is no evidence to support a variation in deletion frequency between different populations/ethnic groups or different geographic regions.

The data indicate a strict correlation between spermatogenic failure and the presence of Y microdeletions. Deletions were not detected in normospermic men, using the markers developed in the current study. In the group of fertile males, 10 subjects presented with reduced sperm counts. Yq microdeletions have been previously reported in some studies of fertile males (2, 4, 6). Because semen data for these men was not available, these microdeletions may have been associated with subnormal sperm count, or the markers used in the screening protocol may have been polymorphic.

All deletions detected in the current study involved the AZFc region, and the associated phenotype ranged from azoospermia to cryptozoospermia. This supports previous observations that AZFc deletions are the most frequent, and they are associated with a variable phenotype (3, 11). Because serum inhibin B concentrations were uniformly very low in the deleted patients, it is recommended that abnormally low levels of inhibin B should be included among the indications for a Y-chromosome-microdeletion screen. Gene-specific deletions were not detected, indicating that such events, which

have been recently reported (12), are either rare or associated with a well-defined clinical subgroup of patients. The use of a PCR-based deletion detection method, such as that used in this study, does not formally exclude other rearrangements of the Y chromosome, such as reduction in gene copy numbers or other structural rearrangements of the Y chromosome (inversions, duplications, and others) that may lead to male subfertility.

In this study, three men defined as nonidiopathic infertile (presenting cryptorchidism, varicocele, and hypoandrogenism) were found to harbor microdeletions of the AZFc region. The extent of these three deletions was identical to that observed in the idiopathic infertile men. Though it is possible that other factors could have contributed to reduced sperm production, the gonad histology in two of these cases was consistent with previous histological findings in cases where Y microdeletions have been detected. Microdeletions have occasionally been reported together with cryptorchidism, varicocele, obstructive azoospermia, and hypogonadotrophic hypogonadism (10, 11, 13) and are considered to be chance association. This finding indicates that Y microdeletion screening has to be extended to all patients affected by severe oligozoospermia, regardless of the presence of abnormal andrological findings.

Several reports indicated that male sperm counts have declined during the last 40–50 yr (14–16). This decline is associated with an increase in the frequency of anomalies of the male reproductive system, including ambiguous genitalia and testicular cancer. These data and the association between male subfertility and subsequent high risk of testicular cancer are consistent with the hypothesis that male subfertility and testicular cancer share important aethiological factors (17). Recently, a high frequency of young men with suboptimal semen quality has been reported in Denmark (18). More than 40% of young adult Danish men have sperm counts below 40×10^6/mL, which, according to a recent study (7), is associated with decreased fertility. Although a number of factors (such as pesticides, exogenous estrogens, and heavy metals) may have a negative impact on spermatogenesis, none of these factors have been formally demonstrated to be responsible for the reported decline in sperm quality in some countries, including Denmark.

Although Y microdeletions of specific regions on the long arm are a cause of male infertility, the data in this report argues against the involvement of microdeletions in the recently observed high incidence of reproductive abnormalities, including relatively low sperm count of the Danish population. The role of nongenetic factors, such as environmental pollutants, needs to be further investigated. The data also shows that the marker set used in the study is specific for reduced sperm production and can therefore be used in the routine clinical workup of both idiopathic and nonidiopathic severe oligozoospermic and azoospermic men. The demonstration of a Y chromosome microdeletion in an infertile man is not only important in defining the etiology of the condition but is also of clinical prognostic value (19).

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