SUBMICROSCOPIC DELETIONS OF THE Y CHROMOSOME ARE NOT LIMITED TO AZOOSPERMIC MEN, BUT ARE ALSO DETECTED IN INFERTILE MEN WITH IDIOPATHIC OLIGOZOOSPERMIA


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ABSTRACT

It is now agreed that 10-25% of infertile men with azoospermia have submicroscopic deletions of the Y chromosome long arm (Yq), consistent with the proposed location of the azoospermia locus (AZF) in Yq 11.23. However, it is not known whether Yq microdeletions are unique to men with azoospermia or whether they are also observed in infertile men with less severe defects of spermatogenesis (oligozoospermia). The objective of this study was to determine the prevalence of Yq deletions in infertile men with idiopathic oligozoospermia.

DNA was extracted from blood lymphocytes of 45 oligozoospermic men (sperm densities <20 million/mL) in whom known causes of infertility had been excluded. All subjects were typed for the 27 Y-specific STSs that have been mapped to deletion interval 6. An STS was considered negative if no PCR product was observed in 3 reactions, in which a fertile male gave a specific PCR product and a normal female DNA did not. Whenever sufficient DNA was available, deletions detected by PCR were verified by Southern hybridization.

Of the 45 oligozoospermic men, 4 (9%) had deletions of one or more STSs. These deletions were verified by Southern hybridization. All 4 deletions were located in distal interval 6 (6C and 6D) and included the DAZ (Deleted in Azoospermia) gene, a Y-specific gene that has been proposed as a candidate for male infertility. All four patients had sperm densities of less than 1 million/mL and three out of these 4 patients had mean testis volume of less than 15 mL. Two of the infertile men with Y deletions had a testicular biopsy; testicular histology in both of these patients was consistent with germ cell arrest, spermatocyte stage.

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also in infertile men with oligozoospermia. Taken together with previously reported studies on azoospermic men, these results indicate that Yq deletions are predominantly observed in, but are not limited to, infertile men with relatively severe defects of spermatogenesis.

**MATERIAL AND METHODS**

**Subjects**

Blood samples were collected from infertile men following protocols approved by the Monash Medical Center Human Ethics Committee, and the Institutional Review Boards of the Harbor-UCLA Medical Center, Charles R. Drew University of Medicine and Science, and Karolinska Institute. Peripheral blood or DNA samples from 45 infertile men, 24-41 years of age, who were oligozoospermic, were available for this study. Each individual had undergone evaluation for infertility, and known causes of infertility or hypogonadism had been excluded. Patients with clinical phenotype consistent with Klinefelter’s syndrome (very small testes, eunuchoid proportions, gynecomastia and hypergonadotropic hypogonadism) were also excluded. Isolation of DNA from blood samples was performed by the Mini-Prep (Quiagen Inc., Chatsworth, CA) procedure or a modified phenol chloroform method. Serum FSH, LH, and testosterone were measured at the Monash University by specific radioimmunoassays. In Los Angeles and Stockholm, serum testosterone levels were measured by an iodinated immunoassay, and serum and FSH levels were measured by an iodinated immunoassay, and by specific and sensitive fluoroimmunometric assays us-
STS MAP OF OLIGOZOOSPERMIC INDIVIDUALS

Fig. 1. A list of sequence-tagged sites corresponding to interval 6 of the Y-chromosome. A. Drawing of the Y-chromosome. B. A list of the STSs used for typing patients is shown across the top; a list of 4 individuals bearing deletions in different locations of interval 6 is shown below. STSs that are underlined have been confirmed by Southern analysis. Solid bars represent the presence of STS.

ing reagents obtained from Delphia-Wallace Corporation (Turko, Finland).37 Based on the normal ranges established for these hormones in the Los Angeles37 and Monash Laboratories, 57% of the subjects had elevated serum FSH levels, 3% had elevated LH levels and 30% had low testosterone levels. Eleven patients had chromosomal studies and normal 46,XY karyotypes. Testicular biopsy information was available in 13 subjects. Six patients had germ cell arrest, one had a Sertoli Cell Only (SCO) phenotype, 4 had hypospermatogenesis, one had SCO and hypospermatogenesis, and one biopsy was reported as “normal”. No biopsy was done solely for the purpose of this research.

To ascertain if microdeletions were associated only with idiopathic oligozoospermia, we also typed DNA samples from 8 infertile men with oligozoospermia associated with maldescent of the testes.

STSs

Twenty five STSs, used to map the Yq interval 6, were selected from the STS map of Vollrath et al.38 based on the following criteria: first, these STSs were Y-specific and did not have homologues on the X chromosome or autosomes; second, these STSs had been localized to Yq deletion interval 6.29,38 Two additional STSs, sY254 and sY255, corresponding to DAZ sequences, were also examined. The STSs used in this study are listed in Figure 1. The PCR primer pairs for these 27 STSs have been previously published.38

We have previously demonstrated that Y deletions occur extremely infrequently, if at all, in normal fertile men.

PCR conditions

Each patient sample was types for all 27 STSs using polymerase chain reaction (PCR). For each PCR, one µL (100 ng to 200 ng) of patient DNA (extracted from peripheral blood cells) was used. A mix containing 8.6 µL water, 2 µL (25 µM) MgCl₂, 2 µL 10X buffer, 1 µL (10 mM) of each dNTP, 1 µL (1 mM) each of the forward and reverse primers, and 2 units of Amp Taq DNA polymerize (Perkin-Elmer Cetus Corporation, Emeryville, CA) was added to the patient’s DNA. The PCR reaction was carried out at different annealing temperatures depending on the specificity of the STS primers. An annealing temperature of 55°C was used for all reactions except for STSs 164, 139, 148, 154, 155, 156, 158, and 157, which had variable annealing temperatures. Two µL of tracking dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) was added to each sample before running it on a 3% agarose/TAE gel. For STS products longer than 400 bp, 1% gel was used instead.

Criteria used for defining microdeletions

Each set of PCR reactions included at least two nor-
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Table I. Clinical phenotype of oligozoospermic men with Yq deletions.

<table>
<thead>
<tr>
<th>Number</th>
<th>Age</th>
<th>Sperm density million/mL</th>
<th>Testis volume Rt.</th>
<th>Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31</td>
<td>&lt;1</td>
<td>30</td>
<td>GCA</td>
</tr>
<tr>
<td>2</td>
<td>39</td>
<td>&lt;1</td>
<td>8</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>29</td>
<td>&lt;1</td>
<td>15</td>
<td>GCA</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>&lt;1</td>
<td>15</td>
<td>NA</td>
</tr>
</tbody>
</table>

T, serum testosterone levels nmoVL; LH, serum luteinizing hormone U/L; FSH, serum follicle-stimulating hormone, U/L.
NA, not available; GCA, germ cell arrest at the primary spermatocyte stage.
Sperm densities are in million/mL.
LH, normal range is 2-12 IU/L at Monash and 2-13 IU/L in Los Angeles Laboratories; FSH, normal range is 1-9 IU/L at Monash and 1-11 IU/L in Los Angeles Laboratories; T (testosterone), normal range is 10-23 nmoVL at Monash and 7.3-30 nmoVL in Los Angeles Laboratories.
Testicular volumes measured by Pradeer orchidometer, refer to the volumes of right (Rt.) and left (Lt.) testes, respectively.

mal male (positive control) and a normal female control (negative control). A PCR reaction was considered satisfactory if the normal male DNA produced the PCR product of the specific size and the normal female did not. A patient sample was considered "positive" for an STS if it produced the PCR product of the expected size under the same condition, and negative if a product of the expected size was not obtained after 3 PCR attempts.

Verification of microdeletions by Southern analysis
All Y deletions detected by PCR were verified by Southern analysis. For the Southern analysis, 10 μL of genomic DNA was digested with EcoRI, run on 0.7% agarose gel, transferred to nylon membrane and hybridized with 32p-labeled probe. The PCR products for each STS were labeled and used as probe.

RESULTS

The validation of the STS mapping strategy has been previously published.

STS typing of infertile men
We studied 45 infertile men with oligozoospermia using this STS-based mapping strategy. Four patients (9%) failed to amplify one or more STS. Figure 1 lists the STSs that were found to be deleted in these 4 patients. All 4 of these deletions are distal (i.e. they are located in intervals 6C and 6D). These deletions overlap with the DAZ (deleted in azoospermia) gene region. Indeed, sY254 repressing DAZ sequences was deleted in all 4 of these patients. All of the deletions included multiple STSs.

Southern hybridization data
In each of the 4 patients with Yq deletions, the deletions were verified by Southern hybridization, similar to our previous publication.

Phenotypes of men with oligozoospermia and Yq microdeletions
The characteristics of these men are shown in Table I. All the oligozoospermic men with microdeletions had sperm densities of less than 1 million/mL (Table I). In 2 of these patients, testicular histology was available; the biopsy appearance consisted of maturation arrest at the primary spermatocyte stage in both of these patients. Testicular volumes were less than 15 mL in 3 of these 4 patients. Karyotype was available only in one patient with microdeletion; this patient had a normal 46, XY karyotype. Serum FSH levels were elevated in 2 patients.

Family studies
DNA was available from the father and brother of one oligozoospermic man with Y deletion. Southern analysis of these patients’ genomic DNA with DAZcCNA probe revealed a Y deletion that included the DAZ gene. Examination of the father’s genomic DNA reveals the presence of all the DAZ bands (Figure 2). These data indicate that in this patient, the deletion occurred de novo and was not inherited from his father.

DISCUSSION

Our data suggest significant (9%) prevalence of Yq microdeletions in infertile men with idiopathic oligozoospermia. This prevalence is similar to the 10-20% prevalence of Yq deletions previously reported in azoospermic men by others and us. However, it is notable that all 4 infertile patients with Y deletions had a sperm density of less than 1 million/mL. Taken together with the previous data on pre-
Fig. 2. This figure represents an example of Southern analysis using the DAZ probe, that was kindly provided by David Puge, to screen patients for DAZ deletions. Patient P 260 is an oligozoospermic individual that has a deletion in DAZ gene, but his father (P 261) showed no detectable deletion in DAZ gene. Male and female controls are also shown.

Dominantly azoospermic men, these results suggest that Yq deletions are observed mostly in infertile men with more severe defects of spermatogenesis (azoospermia or severe oligozoospermia). It is also remarkable that 3 out of 4 men with deletions in this study, and 6 of 7 azoospermic men with Y deletions (on whom data were available) in our previous study29 had mean testis volumes of less than 15 mL.

Because all the deletions were in the distal region and only a small number of patients had microdeletions, we are unable to ascertain a correlation between the location or the size of the microdeletions and the clinical phenotype; this issue needs further examination in a larger sample of infertile men. Also, to-date only infertile men with azoospermia and oligozoospermia have been examined. Further studies are needed on larger samples of infertile men with other infertility phenotypes, such as morphology and motility defects and obstructive lesions to establish genotype-phenotype correlation.

The deletions in these subjects appear not to be contiguous. This observation has also been reported previously by others and us. Some of the intervening STSs represent repetitive sequences, and the PCR product may represent amplification from a different site. It is also possible that the order of these STSs may not be correct in the published map. This conclusion is supported by known discrepancies between the deletion map and the YAC contiguous map in some STS location.29 We did not have access to blood samples from the fathers or brothers of all the patients. However, DNA samples were available from the father and one brother of one individual with Yq deletion. In this patient, Southern analysis of the infertile son's DNA revealed a large-deletion resulting in deletion of the DAZ bands. On the other hand, the father and brother had all the expected bands (Figure 2). Thus the Y deletion in this patient must have occurred de novo, and was not inherited from his father, although vertical transmission from father to son has also been reported.40

It is now agreed that genes on the Y chromosome play an important role in germ cell development. Growing bodies of data from several laboratories have demonstrated the association of Y microdeletions with azoospermia. The study reported in this manuscript adds oligozoospermia to the infertility phenotypes that are associated with Y deletions. While this manuscript was in preparation, Reijo et al. reported the occurrence of Y deletion in a group of oligozoospermic men.41 These data only demonstrate associations; a cause and effect relationship between Y deletions and infertility has not been established. Two Y-specific gene families have been cloned and been proposed as candidates for putative azoospermia factor.22,27,30,33 The RBM (RNA Binding Motif containing) gene family is a multiple copy gene family. Most of these copies are located in the proximal part of deletion interval 6. At least two members of this gene family are expressed uniquely in the testis. The genomic structure and organization of the RBM gene family has been recently published by Najmabadi et al. and reveals a 15 kb gene with 12 exons.33 There is a high degree of homology between exons VI, VII, VII, IX and X and also between some of the introns within the same gene.42 The role of the RBM gene family as a candidate for the AZF has been challenged by several observations. First, large deletions of the RBM gene are infrequent in infertile men.33 More importantly, RBM sequences have been shown to be present even in infertile men with Y deletions. These data do not exclude the formal possibility that point mutations, small deletions or rearrangement of the RBM gene may be present in some of the patients. Reijo et al. reported the cloning of a novel, RNA-binding protein gene, DAZ (deleted in azoospermia).33 Although this gene was originally reported to be a single-copy gene, additional copies have been identified in the distal region of Yq. The presence of the RNA binding motif suggests a role in RNA processing. However, its exact role in spermatogenesis remains to be determined. Deletions of the Y-chromosome that include the DAZ gene appear to be the most frequent deletions in infertile men with azoospermia and oligozoospermia. Although all the deletions described in this manuscript are in the DAZ region, microdeletions that do not overlap with the DAZ region have
been reported by several groups. These findings raise the possibility that gene(s) in Yq deletion interval 6 might be associated with subsets of male infertility. Whether there are other Y-specific genes in interval 6 or whether point mutations in DAZ or RBM genes are responsible for infertility remains to be seen. A recent case control study of subfertile men suggested that subfertility might be inherited as an autosomal recessive disorder. Thus it is likely additional Y-specific and autosomal genes will be implicated in subsets of male infertility.

The occurrence of chromosomal deletions in infertile men with oligozoospermia has significant implications from the perspective of assisted reproductive technologies. Because intracytoplasmic sperm injection may allow partners of these oligozoospermic men with Yq deletions to achieve pregnancy, this genetic defect will be transmitted to the offspring. The expression of infertility in the offspring as a consequence of these therapeutic maneuvers will not become apparent for several decades. This raises issues of informed consent and ethical concerns. The substantial prevalence of microdeletions detected in this study suggests that infertile men with oligozoospermia who are undergoing intracytoplasmic sperm injection should be screened for Yq deletions. Considering the ease and accessibility of the PCR technology, the STS-based screening for microdeletions could become potentially available for screening in clinical cytogenetic laboratories. At the very minimum, infertile couples with azoospermia and/or oligozoospermia who are considering assisted reproductive technologies, particularly intracytoplasmic sperm injection, should be counseled about the potential for transmitting genetic disorders to the offspring.

REFERENCES


35. Higuchi R: Rapid, efficient DNA extraction for PCR from cells or blood. Amplifications 2: 1-3, 1989.


