Analysis of Yq microdeletions in infertile males by PCR and DNA hybridization techniques

Paola Grimaldi¹, Claudia Scarponi¹, Pellegrino Rossi¹, Massimiliano Rocchietti March², Andrea Fabbri², Aldo Isidori², Giovanni Spera², Csilla Krausz³ and Raffaele Geremia¹,4

¹Cattedra di Anatomia, Dipartimento di Sanità Pubblica e Biologia Cellulare, Università degli Studi di Roma ‘Tor Vergata’, 00133 Roma, ²Cattedra di Andrologia, Dipartimento di Fisiopatologia Medica, Università degli Studi di Roma ‘La Sapienza’, 00161 Roma, and ³Cattedra di Andrologia, Dipartimento di Fisiopatologia Clinica, Università di Firenze, 50139 Firenze, Italy

4To whom correspondence should be addressed

Defects in spermatogenesis have been found associated with deletions of different portions of Y chromosome long arm (Yq), suggesting the presence of the azoospermia factor in the control of spermatogenesis. We studied 67 men with idiopathic azoospermia and severe oligozoospermia, cytogenetically normal, for the presence of microdeletions on Yq chromosome. By using polymerase chain reaction (PCR) and Southern blotting techniques we analysed the AZFa, AZFb and AZFc loci on Yq, where deletions have been associated with defects in spermatogenesis. Deletions of a portion of the Y chromosome were detected in five patients. Four of these patients shared deletions in distal Yq11 interval 6, including the DAZ gene, while one patient lacked loci in the proximal Yq11. Testicular histology of two patients bearing distal Yq11 deletions showed two different spermatogenic defects including Sertoli cell-only (SCO) syndrome and maturation arrest, while the patient with microdeletions in the proximal Yq11 showed a SCO phenotype.

Key words: AZF/male infertility/microdeletions/Y chromosome

Introduction

Male infertility in subjects with azoospermia or severe oligozoospermia with unknown aetiology was first associated with a possible genetic cause by Tiepolo and Zuffardi (1976), in a report of six azoospermic patients carrying a deletion of the distal portion of Y chromosome long arm. On the basis of this finding they proposed the existence of a spermatogenesis gene, the ‘azoospermia factor’ (AZF) on Yq.

Confirmatory data for the actual presence of genes of spermatogenesis in Yq had to wait until molecular maps of Y chromosome and molecular tools for mapping microdeletions were made available. DNA probes specific for Y chromosome loci (Vergnaud et al., 1986) or polymerase chain reaction (PCR) amplification of sequence-tagged sites (STS) (Vollrath et al., 1992) have allowed the identification of microdeletions in Yq regions known not to pair or recombine with the X chromosome.

Up to now, three non-overlapping loci have been identified on Yq11; and their deletion is associated with sterility (azoospermia or severe oligospermia) (Vogt et al., 1996). Two have been localized to interval 6 of Yq11 and are called AZFb and AZFc, and one is more proximal to the centromere in Yq11 and is called AZFa. In interval 6 of Yq11, two genes have been identified that are deleted in a number of infertile men; one is the Y chromosome RNA-recognition motif (YRKM/RBM) identified by Ma et al. (1993), the other is the Deleted in AZoospermia gene (DAZ) identified by Reijo et al. (1995). The first gene RBM, has been tentatively linked with AZF (Ma et al., 1993) and is a member of an RNA-binding protein gene family, which is represented in multiple copies in different regions of the Y chromosome, and at least one copy is localized to the AZFb region (Elliott et al., 1997). The second gene, DAZ, is at present the strongest candidate for AZF, and its deletion has been found to be associated with a wide range of spermatogenic defects from bona fide Sertoli cell-only (SCO) syndrome to reduced spermatogenesis resulting in severe oligozoospermia (Reijo et al., 1995, 1996). In the proximal region of Yq11 the gene DFRY has been recently linked to AZFa. This gene is the Y-linked homologue of the DFFRX (Drosophila fat-facets related X gene), and it has been shown to be deleted in three azoospermic males (Brown et al., 1998). However it must be stressed that the rare deletions in this locus (from 1–5% in different surveys) generally associate with a SCO phenotype (Ma et al., 1992; Vogt et al., 1996), with the exception of one published case showing reduced spermatogenesis (Qureshi et al., 1996), thus suggesting that this locus is important for early steps of spermatogenesis, possibly in prenatal development. As a whole the data available would indicate that AZF is a multi-gene complex preferentially located in Yq11, whose members might act at specific differentiative steps of spermatogenesis.

In the present report we analysed 67 azoospermic and severe oligozoospermic patients for the presence of microdeletions in Yq11 using 18 STS and two DNA probes. We found four subjects with deletions in AZFc, including the DAZ gene, and one patient with deletions in AZFa.
Materials and methods

Clinical subjects

Patients affected by azoospermia \((n = 60)\) and oligozoospermia \((n = 7)\) (sperm concentration \(< 1 \times 10^6/\text{ml}\) were analysed in this study. A total of 40 patients were recruited for a prospective study from the andrology clinic clientele of the University of Roma 'La Sapienza' and Florence, while 27 were analysed retrospectively. Patients with varicocele and with a history of cryptorchidism were not excluded from our screening, while patients with obstruction of the seminal ducts, as evaluated with the analysis of the seminal markers of duct patency such as fructose, carnitine and citric acid, were excluded.

Semen analysis was performed according to the World Health Organization parameters. Serum follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone were measured by radioimmunoassay, according to methods of Fabbrì et al. (1988).

All patients had a normal 46,XY karyotype, thus excluding chromosomal aberrations associated with male sterility.

PCR amplification of STS

Genomic DNA of each patient was obtained from peripheral blood leukocytes using the Nucleon Kit II (Scotlab, Wiesloch, Germany). PCR amplification was performed with a set of 18 pairs of oligonucleotide primers for Y-specific STS (Vollrath et al., 1992; Reijo et al., 1995). The STS analysed were: sY83, sY84, sY86, sY87 (located in AZFa), sY117 (located in AZFb), and sY164, sY55, sY132, sY112, sY152, sY155, sY242, sY254, sY277, sY236, sY202, sY157 (located in AZFc). The STS selected in this study were normally amplified in control males and were absent in control females, thus suggesting Y chromosome specificity. One STS specific for the gene SRY, located in Yp, was also analysed. PCR reactions were carried out by 35 cycles with 94°C for 1 min, 60°C for 1 min and 72°C for 1.2 min. Each PCR reaction was generally performed using two pairs of primers. The PCR products were separated on 1.5% agarose gel.

Southern blot analysis

Samples (10 µg) of each genomic DNA were digested with EcoRI for probe p50f2 and with TaqI for probe p49f and separated by electrophoresis on 1% agarose gel in tris-borate-EDTA (TBE) buffer. The DNA samples in the gel were first denatured by treatment with 1.5 M NaCl, 0.5 M NaOH, and then neutralized with 1.5 M NaCl, 0.5 M Tris–HCl (pH 8). The DNA samples were transferred to Hybond-N nylon membrane (Amersham, UK) in 10× sodium chloride/sodium citrate (SSC) and then fixed by 1200 J UV irradiation. The probes used in our analysis were either purified PCR-amplified STS or Y-chromosome DNA fragments (p50f2 and p49f), generously provided by J.Weissembach. The probes were labelled with α-[32P]-dATP, by using the random-primed DNA labelling kit (Boehringer Mannheim, Mannheim, Germany). Prehybridization was performed at 68°C for 1 h in QuikHyb hybridization solution (Stratagene, La Jolla, CA) and was followed by hybridization in the same solution for 1 h at 68°C in the presence of 100 µg/ml of salmon sperm DNA and radiolabelled probe at 1×10⁶ c.p.m./ml. The membrane was washed first twice with 2× SSC, 0.1% sodium dodecyl sulphate (SDS) for 15 min at room temperature and then with 0.2× SSC, 0.2% SDS for 30 min at 60°C and then exposed for autoradiography.

Results

The 67 patients with azoospermia or severe oligozoospermia, selected as described in Methods, were screened by PCR amplification and Southern blot analysis for potential deletions of 20 loci on Yq11. STSs and Y chromosome DNA probes used in this study, and their localization on Yq11 are shown in Figure 1. Most of the STS map to interval 6 of Yq11, which includes the DAZ gene in AZFc locus (sY254, sY277, sY283). Four STS (sY83, sY84, sY86, sY87) map to AZFa, and another STS (sY117) maps to AZFb.

We detected deletions on Yq11 in five patients by PCR amplification. Figure 2 shows the Y-specific STS found deleted: patients SP8, FO3, FA16, and FA45 showed deletions in
The absence of the DAZ gene cluster (Saxena et al., 1996), agrees with PCR data. The available DNA from the other DAZ-deleted patient FA45 was not sufficient for Southern analysis.

Three more patients showed microdeletions of a single STS in AZFc by PCR amplification, being sY283 negative in two cases and sY157 in the third one. Although the PCR amplification was repeatedly negative in separate reactions, we demonstrated that they were false negatives, by a Southern blot study using as probe the same PCR product found deleted.

The FA14 patient showed microdeletions only in AZFa, including the STS sY84, sY86, sY87, and sY83 (Figure 2). All other STS analysed in patient FA14 were normally amplified. Southern blot analysis using these STS as probes did not give conclusive results due to cross-reactivity with autosomal DNA.

The clinical parameters of the five patients with microdeletions are summarized in Table I. Four patients were azoospermic and only the DAZ-deleted FO3 patient showed very rare and aberrant spermatozoa.

Testicular histology was available in three deleted patients. FA14, carrying microdeletions in AZFa, had Sertoli cell-only syndrome with complete lack of germ cells. Patients carrying deletion of the DAZ gene (SP8 and FA45) showed different spermatogenic defects including Sertoli cell-only syndrome and maturation arrest. Testicular histology of patient FA45 carrying a DAZ deletion was associated with a SCO phenotype and is shown in Figure 4.

**Discussion**

Spermatogenesis is a complex event leading to the formation of mature spermatozoa, occurring under the control of a wide variety of factors acting from prenatal to adult life (Vogt, 1997). An important role in the regulation of spermatogenesis is certainly played by the genetic programming of germ cell itself based both on autosomal and sex chromosomal genes. A group of idiopathic infertility has been, in fact, associated with genetic causes linked to the Y chromosome and many studies have recently shown deletions on Y chromosome long arm in idiopathic azoospermic males. The frequencies of deletions of Yq, reported in different studies, range between 3 and 18% of males with non-obstructive azoospermia or severe oligozoospermia (Reijo et al., 1995; Stuppia et al., 1996; Vogt et al., 1996; Girardi et al., 1997; Pryor et al., 1997; Qureshi et al., 1997; Simoni et al., 1997; Van der Ven et al., 1997).

In our screening of 67 infertile males we have found five patients carrying microdeletions, corresponding to a frequency of 7.5%, that perfectly agrees with the statistical value obtained averaging all the surveys reported to date (Simoni et al., 1998). The frequency of microdeletions reported here is the result of a correction of the PCR data, made on the basis of Southern blot analyses. In fact, we could exclude three more patients showing a single STS deletion by PCR amplification, not confirmed by Southern blotting, thus lowering the microdeletion rate from ~12% to 7.5%. Even though it is not possible from our result to evaluate statistically whether some STS are more prone to give false negatives, or whether this technical problem might be relevant in explaining the higher frequency of microdeletions found by others, in our opinion Southern
As for the mechanism of the appearance of microdeletions, it has been suggested the occurrence of an intrachromosomal recombination event between repeated sequences flanking the affected gene, causing a loop that is deleted (Edwards and Bishop, 1997). If this is the case, the possibility exists of more such events in the same chromosome causing discontinuous deletions, e.g. the one observed in patient SP8.

Many efforts have been made to identify the genes on Yq that are important for a normal spermatogenesis (Lahn and Page, 1997), but up to now the gene/s that are absolutely required for germ cell development have not been characterized. The first genes identified on Yq that could be involved in the control of spermatogenesis belong to the RBM gene family, and include up to 30 copies of genes and pseudogenes, spread over both arms of the Y chromosome (Ma et al., 1993). RBM genes encode RNA-binding proteins and are specifically expressed in spermatogonia and early spermatocytes (Chandley and Cooke, 1994). The RBM1 gene has been mapped to the AZFb region of the Yq (Vogt et al., 1996; Elliott et al., 1997), but the precise role of this gene in spermatogenesis is not clear, since it is present in many azoospermic males.

The other candidate gene for AZF is DAZ which, similar to the RBM1 gene, encodes a protein with an RNA-binding motif and is present in multiple copies (at least two or three copies) on Yq. The DAZ gene has been mapped to the AZFc region of the Yq (Vogt et al., 1996) and is specifically expressed in spermatogonia in the testis (Menke et al., 1997). Deletions of DAZ gene are found frequently in azoospermic or in severe oligozoospermic males and no point mutations have been identified up to now (Vereb et al., 1997). However, the observation that loss-of-function mutations of Boule, the DAZ homologue in Drosophila, results in azoospermia (Eberhart et al., 1996), strongly supports a critical role for the DAZ gene in spermatogenesis. The functional role of DAZ has not been characterized, and the range of testicular histology observed in DAZ-deleted patients varies from very severe defects in spermatogenesis, e.g. SCO, to severe reduction in sperm production with oligozoospermia (<1×10^6/ml) (Reijo et al., 1996). In our study, testicular histology of the patients carrying deletion of DAZ showed in patient FA45 a Sertoli cell-only syndrome and in patient SP8 a maturative arrest. Moreover, patient FO3, also carrying the DAZ deletion but whose biopsy was not available, showed cryptoazoospermia in the ejaculate. These observations suggest that different co-factors could modulate the strength of the mutation and that deletion of DAZ is compatible with completion of spermatogenesis at very low levels (Mulhall et al., 1997).

The correlation between DAZ mutations and testis phenotype is further complicated by two other considerations: the first derives from the observation that the structure of the DAZ gene is characterized by 16 exons and multiple copies of a repeat in tandem array (Saxena et al., 1996), and by the presence in the male population of a polymorphism (Mulhall et al., 1997).
number of the DAZ repeats resulting in the possible transcription of different functional DAZ genes (Yen et al., 1997). The second consideration is based on the presence of a DAZ homologue, called DAZLA (DAZ-Like Autosomal), which has been identified on chromosome 3 (Saxena et al., 1996). DAZLA is expressed specifically in the testis and could be involved in autosomal recessive male infertility. A possible involvement of the autosomal homologue in spermatogenesis is supported by the observation that in mouse the homologue of DAZ, dazl, is autosomal (Cooke et al., 1996) and its targeted disruption leads to a complete absence of gamete production in both testis and ovary demonstrating that dazl is essential for development and survival of germ cells (Ruggiu et al., 1997).

In the AZFa region of the Y chromosome, the gene DFFRY, the Y-linked homologue of the Drosophila DFFRX gene, has been identified recently (Brown et al., 1998). This gene encodes a protein related to ubiquitin action and, hence, is involved in protein turnover. We have identified one patient (FA14) with microdeletions in the AZFa region, whose testicular histology showed Sertoli-cell only syndrome in which all the tubules analysed lacked germ cells. This is in line with observations by Ma et al. (1992) and Vogt et al. (1996), which confirm that deletions proximal to the centromere in Yq are linked to a spermatogenic defect that probably occurs during the proliferation of spermatogonia, before puberty.

A final consideration should be made on the possible occurrence of mosaicism of Yq microdeletions arising postzygotically, which might decrease the probability of detection of genetic defects by PCR or Southern techniques, using DNA from blood cells as a template. It is important to verify the frequency of this situation, in order to evaluate the necessity to extend PCR analysis of DNA from peripheral blood to sperm samples or testis biopsies, to avoid mis-detection due to mosaicism. In our opinion, however, molecular diagnosis, optimized by Southern blotting to confirm PCR results, should be recommended to those patients with azoospermia or severe oligozoospermia (<1×10⁹/ml), who are seeking assisted fertilization by intracytoplasmic sperm injection for correct information on the possibility of the transmission of molecular defects to male offspring (Silber et al., 1995; Bonduelle et al., 1996).

Acknowledgements

Work supported by MURST 60% and by CNR grant no. 98.00509. We are indebted to Dr Weissembach for the kind gift of the plasmids p49f and p50f2.

References


Analysis of Yq microdeletions


Received on May 27, 1998; accepted on September 10, 1998