AZFa deletions in Sertoli cell-only syndrome: a retrospective study

O. Blagosklonova1, F. Fellmann, M.-C. Clavequin, C. Roux and J.-L. Bresson

Service de Cytogénétique-Immunocytologie-Biologie du Développement et de la Reproduction, CHU et CNRS UPRESA 6025, Faculté de Médecine et de Pharmacie, Place Saint-Jacques, 25030 Besançon cedex, France

1To whom correspondence should be addressed at: Service de Cytogénétique-Immunocytologie-Biologie du Développement et de la Reproduction, CHU et CNRS UPRESA 6025, Faculté de Médecine et de Pharmacie, Place Saint-Jacques, 25030 Besançon cedex, France. E-mail: oxana_blagoski@hotmail.com

Lack of data on the genotype–phenotype relationship in cases of AZF microdeletions is due to the limited number of histological investigations in human male infertility cases. We investigated the possibility of retrospective detection of Yq11 microdeletions by using DNA extracted from diagnostic testicular biopsies. We used histological criteria to select two series of material: 22 biopsies with Sertoli cell-only syndrome and 14 biopsies with maturation arrest at the spermatocyte I stage. Two markers, DFFRY and DAZ, were tested by nested polymerase chain reaction (PCR) in the two series. In the Sertoli cell-only syndrome series, we found four deletions affecting the DFFRY gene (18.2%). In the second series, no deletions were detected. Two conclusions may be considered, although the number of specimens analysed is limited: (i) the frequency of deletions observed in Sertoli cell-only syndrome allows us to suggest that deletion in the AZFa region may be involved in this pathology; and (ii) retrospective studies may yield some additional elements in our search for eventual genotype–phenotype relationships.

Key words: AZF/DFFRY/male infertility/Sertoli cell-only syndrome/Y chromosome

Introduction

The azoospermia factor (AZF) region of the Y chromosome was so named because of cytogenetic deletions only observed in infertile men (Tiepolo and Zuffardi, 1976). Over the last decade many studies have been carried out to investigate the role of this region in spermatogenesis. Molecular studies in infertile men have suggested the localization of genes controlling spermatogenesis in the Yq11 region (Ma et al., 1993; Reijo et al., 1995; Lahn and Page, 1997). For many of these genes, the function is unknown as is their degree of their involvement in spermatogenesis. According to current data, genes located in Yq11 may be divided into two groups: Y-specific multi-copy genes (CDY, DAZ, PRY, RBM, TSPY, TTY1, TTY2) and X–Y homologous genes (DBY, DFFRY, EIF1AY, SMCY, UTY) (Lahn and Page, 1997).

It has been postulated that Yq11 molecular deletions affecting different loci (AZFa, AZFb, AZFc) could be responsible for the disruption of spermatogenesis at different stages and that there might be a correlation between the site of deletion and the histological gonad phenotype (Vogt et al., 1996).

Until now, we have considered proximal Yq11 microdeletions (locus AZFa) to be associated with Sertoli cell-only syndrome (Vogt et al., 1992, 1996; Qureshi et al., 1996; Pryor et al., 1997; Brown et al., 1998; Grimaldi et al., 1998; Ferlin et al., 1999), despite some contradictory publications (Qureshi et al., 1996; Ferlin et al., 1999). The DFFRY gene is currently considered as a candidate gene for the AZFa phenotype. The DFFRY gene might be involved in maintaining male germ cell lineage, like its X-homologue (DFFRX), which is involved in oocyte development (Brown et al., 1998).

Deletions involving the AZFb locus appear to be associated with spermatogenic arrest at the spermatocyte in the majority of cases (Najmabadi et al., 1996; Stuppia et al., 1996, 1998; Vogt et al., 1996; Girardi et al., 1997; Brandell et al., 1998; Lee et al., 1998; Krausz et al., 1999; Kleiman et al., 1999) with several expections (Najmabadi et al., 1996; Lee et al., 1998; Ferlin et al., 1999). The RBM1 and RBM2 genes have been mapped inside the AZFb region (Ma et al., 1993; Vogt et al., 1996). The RBM1 gene copy which is missing in the Y chromosome of AZFb-deleted patients can be considered as a candidate gene for expression of AZFb phenotype (Chandley and Cooke, 1994; Vogt et al., 1996; Elliott et al., 1997).

The AZFc locus containing the DAZ gene cluster is the most frequently deleted region in men with non-obstructive infertility (Kostiner et al., 1998; Simoni et al., 1998). Histologically, these deletions are associated with various spermatogenic alterations, e.g. Sertoli cell-only syndrome, maturation arrest and hypospermatogenesis (Reijo et al., 1995; Najmabadi et al., 1996; Qureshi et al., 1996; Vogt et al., 1996; Foresta et al., 1997; Girardi et al., 1997; Pryor et al., 1997; Simoni et al., 1997; Brandell et al., 1998; Grimaldi et al., 1998; Lee et al., 1998; Stuppia et al., 1998). The question of genotype–phenotype relationships remains open, because of insufficient data on the histological state in testis of patients with AZF microdeletions. However, 20 years ago infertile men underwent histological examination almost systematically. Here, we present a study on the use of diagnostic
testicular biopsies for retrospective detection of microdeletions in the AZF genes, DAZ and DFFRY.

Materials and methods

Materials selection

Our study was approved by the Hospital Ethical Committee. Diagnostic testicular biopsies were collected over the last 35 years. All biopsy samples were fixed in a Bouin mixture, embedded in paraffin, and 7 µm thick tissue sections were stocked on glass slides. Two histologically homogeneous series were selected: 14 specimens with and 22 specimens with Sertoli cell-only syndrome.

DNA extraction and polymerase chain reaction (PCR)

Tissue sections on slides were deparaffinized in three xylene baths, and rehydrated by washing in serial dilutions of ethanol (100, 95 and 70%). Then the slides were soaked in a 1% Lugol iodine solution for 5 min, washed in double-distilled water, bleached for ~3 s in a 2.5% sodium thiosulphate aqueous solution, and then thoroughly washed in double-distilled water (Tbakhi et al., 1998). The sections were completely scraped off the slides with care to avoid cross-contamination, and transferred to a 1.5 ml microcentrifuge tube. 100 µl of TE buffer (EDTA pH 8.5 mmol/l, Tris–HCl pH 8.10 mmol/l) was then added to the tube, and mixed by vortexing for 10 s. 500 µl of LSN solution [lithium acetate 0.3 mmol/l, EDTA pH 8.1 mmol/l, Tris–HCl pH 8.10 mmol/l, sodium dodecyl sulphate (SDS) 2%] was added to the tube. The incubation was carried out at 37°C for 2 h with agitation. Then DNA was purified by the phenol–chloroform method followed by ethanol precipitation and air drying. DNA was taken up in 80 µl of ultrapure water. For one PCR reaction, 15–20 µl of such purified DNA was used. At the first stage, we carried out the amplification of GAPDH to control for DNA extraction.

The first duplex PCR reaction was carried out in a final volume of 100 µl, a reaction mixture consisting of 1× PCR buffer, MgCl₂ 3 mmol/l, DyNAzyme II DNA polymerase 9 IU (Finnzymes Oy, Finland), dNTP 250 mmol/l, external primer pairs 250 µmol/l each, and 20 µl of target DNA. Thermocycling (TouchDown thermal cycling system; Hybaid, UK) consisted of 5 min at 94°C, 25 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min and finally the reaction was heated at 72°C for 5 min. The external primers for DAZ and DFFRY genes are presented in Table I.

A 1 µl aliquot of the first PCR reaction was transferred into a microcentrifuge tube containing 20 µl of a mix consisting of 1× PCR buffer, MgCl₂ 2 mmol/l, DyNAzyme II DNA polymerase 1.5 IU (Finnzymes Oy), dNTP 250 mmol/l, and internal primer pairs 125 µmol/l each. The internal primers for DAZ and DFFRY genes are shown in Table I. Thermocycling consisted of 5 min at 94°C for one cycle, 1 min at 94°C, 1.5 min at 60°C, and 1 min at 72°C for 25 cycles, with a final 5-min extension at 72°C. Reactions were analysed on a 3% agarose gel. Each PCR experiment contained two negative controls (female DNA, and DNA from a patient with a cytogenetically detectable Yq deletion), plus a positive control and a water control. In the absence of amplification for one or both markers, we repeated the first and second amplification. If the result remained negative, then the DNA extraction was repeated.

Southern blot analysis

After 35 cycles of first amplification, 20 µl of mix were separated by electrophoresis on 1% agarose gel. The samples in the gel were depurinated by treatment with HCl 0.25 mol/l, denatured with NaCl 1.5 mol/l, NaOH 0.5 mol/l and then neutralized with Tris–HCl 1 mol/l, NaCl 1.5 mol/l. The samples were transferred to Hybond-N+ nylon

Figure 1. Duplex-nested polymerase chain reaction (PCR) analysis of testicular DNA samples. Internal DAZ marker (180 bp) and internal DFFRY marker (110 bp). Lane 1 = water control, lane 2 = female DNA, lane 3 = DNA of patient with cytotogenetically detectable Yq (AZFc) deletion, lane 4 = positive control, lanes 5 and 6 = DNA of Sertoli cell-only testicular samples (nos. 302 and 257) with DFFRY deletion, lanes 7 and 8 = DNA of Sertoli cell-only testicular samples with positive DAZ and DFFRY markers, lanes 9 and 10 = DNA of testicular samples with maturation arrest at the spermatocyte I stage.

Figure 2. Southern blotting analysis. Products of first amplification were hybridized with reverse internal primer for the DFFRY gene labelled with α-[³²P]. Lane 1 = positive control, lanes 2, 3 and 4 = Sertoli cell-only testicular samples (nos. 302, 257, and 221) with DFFRY deletion, lane 5 = Sertoli cell-only testicular sample with positive DFFRY marker.

Table I. Primer set used for duplex nested polymerase chain reaction (PCR)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Marker</th>
<th>Sequence</th>
<th>STS size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAZ</td>
<td>sY277 forward</td>
<td>GGGTTTTGCTGCAAATCGTAAATTA</td>
<td>494</td>
<td>Reijo et al. (1996)</td>
</tr>
<tr>
<td></td>
<td>sY254 reverse</td>
<td>GAAACGTGTAAAAACAGGCAAG</td>
<td>180</td>
<td>Reijo et al. (1995)</td>
</tr>
<tr>
<td></td>
<td>sY254 forward</td>
<td>GGGTTGAGTTACAAATTCCGAAA</td>
<td>268</td>
<td>Unpublished data</td>
</tr>
<tr>
<td>DFFRY</td>
<td>External forward</td>
<td>CCTGTTGCTACTAGTGGCAGAT</td>
<td>111</td>
<td>Lahn and Page (1997)</td>
</tr>
<tr>
<td></td>
<td>External reverse</td>
<td>AATAGCTTAAATGGTTAAGGCGCC</td>
<td></td>
<td>Unpublished data</td>
</tr>
<tr>
<td></td>
<td>Internal forward</td>
<td>GAGCGGAGCAGTTGCTAGTCGAC</td>
<td></td>
<td>Lahn and Page (1997)</td>
</tr>
<tr>
<td></td>
<td>Internal reverse</td>
<td>CTGCAATTTTCCCACATCAACC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
membrane (Amersham, Orsay, France) in 20× sodium chloride/sodium citrate (SSC) and then fixed 20 min in NaOH 0.4 mol/l. The probes used in our analysis were reverse internal primers for DAZ and DFFRY genes labelled with α-[32P]-dCTP, by the Tailing DNA labelling kit (Amersham). Prehybridization was performed at 65°C for 30 min in a QuikHyb hybridization solution (Stratagene, Montigny le Bretonneux, France), followed by hybridization in the same sperm solution for 3 h at 60°C in the presence of 100 µg/ml of salmon sperm DNA and a radiolabelled probe. The membrane was first washed twice with 2× SSC, 0.1% SDS for 15 min at room temperature and then with 0.2× SSC, 0.1% SDS for 15 min at 60°C. The membrane was exposed for autoradiography.

Results

We performed DNA extraction for 36 samples of testicular biopsies which were 1–30 years old. For 35 testicular biopsies, DNA extraction was successful; for only one biopsy with

Table II. Histological characteristics of biopsies with DFFRY (AZFa) microdeletion

<table>
<thead>
<tr>
<th>Biopsy no.</th>
<th>Tubular diameter</th>
<th>Thickness of tunica propria</th>
<th>Intertubular space</th>
<th>State of Leydig cells</th>
<th>Sampling data</th>
</tr>
</thead>
<tbody>
<tr>
<td>302</td>
<td>Very reduced</td>
<td>Slightly thick</td>
<td>Increased</td>
<td>Hyperplasia</td>
<td>1978</td>
</tr>
<tr>
<td>257</td>
<td>Reduced</td>
<td>Slightly thick</td>
<td>Increased</td>
<td>Considerable hyperplasia</td>
<td>1977</td>
</tr>
<tr>
<td>236</td>
<td>Reduced</td>
<td>Normal</td>
<td>Increased</td>
<td>Hyperplasia</td>
<td>1976</td>
</tr>
<tr>
<td>221</td>
<td>Very reduced</td>
<td>Slightly thick with hyalinosis</td>
<td>Normal</td>
<td>Slight hyperplasia</td>
<td>1976</td>
</tr>
</tbody>
</table>

Figure 3. (A) Maturation arrest at spermatocyte I stage; scale bar = 50 µm. (B) Biopsy no. 257, Sertoli cell-only syndrome with Leydig cell hyperplasia; scale bar = 100 µm. (C) Biopsy no. 257, Sertoli cell-only syndrome; scale bar = 50 µm.

Table III. Bibliographic data concerning actual relationships between site of Yq microdeletion detected in peripheral blood DNA and gonadal histology.

<table>
<thead>
<tr>
<th>Deletion in</th>
<th>Histology</th>
<th>n</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZFa</td>
<td>Sertoli cell-only</td>
<td>6</td>
<td>Qureshi et al., 1996; Vogt et al., 1996; Pryor et al., 1997; Grimaldi et al., 1998</td>
</tr>
<tr>
<td></td>
<td>Hypospermatogenesis</td>
<td>3</td>
<td>Qureshi et al., 1996; Ferlin et al., 1999</td>
</tr>
<tr>
<td>AZFb</td>
<td>Sertoli cell-only</td>
<td>5</td>
<td>Najmabadi et al., 1996; Lee et al., 1998; Ferlin et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Maturation arrestc</td>
<td>15</td>
<td>Najmabadi et al., 1996; Stuppia et al., 1996; Vogt et al., 1996; Elliott et al., 1997; Girardi et al., 1997; Brandell et al., 1998; Lee et al., 1998; Stuppia et al., 1998; Krausz et al., 1999; Kleiman et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Hypospermatogenesis</td>
<td>3</td>
<td>Ferlin et al., 1999</td>
</tr>
<tr>
<td>AZFc</td>
<td>Sertoli cell-only</td>
<td>12</td>
<td>Reijo et al., 1995; Simon et al., 1997; Grimaldi et al., 1998; Ferlin et al., 1999; Chang et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Maturation arrestc</td>
<td>26</td>
<td>Reijo et al., 1995; Najmabadi et al., 1996; Qureshi et al., 1996; Stuppia et al., 1996; Vogt et al., 1996; Girardi et al., 1997; Grimaldi et al., 1998; Lee et al., 1998; Krausz et al., 1999; Kleiman et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Hypospermatogenesis or near normal spermatogenesis</td>
<td>28</td>
<td>Stuppia et al., 1996; Foresta et al., 1997; Girardi et al., 1997; Pryor et al., 1997; Brandell et al., 1998; Stuppia et al., 1998; Ferlin et al., 1999; Krausz et al., 1999; Kleiman et al., 1999</td>
</tr>
<tr>
<td>AZFa/b</td>
<td>Hypospermatogenesis</td>
<td>2</td>
<td>Ferlin et al., 1999</td>
</tr>
<tr>
<td></td>
<td>Sertoli cell-only</td>
<td>11</td>
<td>Najmabadi et al., 1996; DeRosa et al., 1997; Elliott et al., 1997; Foresta et al., 1997; Girardi et al., 1997; Pryor et al., 1997</td>
</tr>
<tr>
<td>AZFb/c</td>
<td>Maturation arrestc</td>
<td>6</td>
<td>Stuppia et al., 1996; Girardi et al., 1997; Brandell et al., 1998; Lee et al., 1998; Stuppia et al., 1998</td>
</tr>
<tr>
<td></td>
<td>Hypospermatogenesis</td>
<td>5</td>
<td>Foresta et al., 1997; Brandell et al., 1998; Ferlin et al., 1999</td>
</tr>
<tr>
<td>AZFa/b/c</td>
<td>Sertoli cell-only</td>
<td>18</td>
<td>Reijo et al., 1995; Elliott et al., 1997; Foresta et al., 1997; Girardi et al., 1997; Brandell et al., 1998; Ferlin et al., 1999; Kleiman et al., 1999</td>
</tr>
</tbody>
</table>

aIn these cases the testicular structure was analysed by means of bilateral fine needle aspiration.
bSome tubules had limited spermatogonial proliferation.
cIn some cases maturation arrest occurred at different stage of spermatogenesis.
dIncludes multiple deletions involving several loci simultaneously.

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a histological picture of Sertoli cell-only syndrome, DNA amplification failed with DAZ/DFFRY markers as well as with the GAPDH marker. In the group of 14 biopsies with maturation arrest at the spermatocyte I stage, 13 biopsies were found to be positive for DAZ/DFFRY markers. In one case, DAZ amplification was negative, while DFFRY was positive, but Southern blotting with a DAZ oligoprobe showed the presence of a DAZ locus. In the Sertoli cell-only syndrome series (21 biopsies), DNA amplification for the DAZ gene marker was positive for all samples; however, for four samples (18.2%), the DFFRY gene marker (AZFa) was negative (Figure 1). These negative results were confirmed by the Southern blot technique (Figure 2). The histological characteristics of biopsies with DFFRY gene marker deletion are summarized in Table II; examples are shown in Figure 3.

Discussion

Testicular biopsy was widely used for diagnosis 20 years ago; however, over the last two decades, this technique has been replaced by other, less aggressive, techniques. The successful use of spermatozoa or spermatids recovered from the testis for IVF has generated renewed interest in testicular biopsies. Studies concerning potential links between Yq microdeletions and non-obstructive infertility are relatively recent: therefore, at the present time, the number of reported cases with historical data remains limited.

Our work may provide some helpful data concerning possible relationships between Yq molecular deficit and the histological conditions of gonads. Bouin fixation has not been favoured for successful DNA extraction. However, the use of old diagnostic material (Bouin-fixed and paraffin-embedded) for molecular biology techniques has been demonstrated in some recent studies (Longy et al., 1997; Tbakhi et al., 1998). We confirm that the use of an adapted protocol for nucleic acid isolation and nested amplification technique is feasible for detecting microdeletions in archived material. In 35 out of 36 cases, we were able to extract DNA from testicular biopsies that allowed PCR amplification. The choice of markers is particularly important in our approach, because of the lower quality of the extracted DNA. Nevertheless, our approach may be useful because like all retrospective studies, it enables us to define histological criteria and select a histologically homogeneous series.

We selected Sertoli cell-only and maturation arrest cases for two reasons: these two pathologies have clear histological characteristics and a high frequency of Yq microdeletions in Sertoli cell-only syndrome has been reported in prospective studies (Foresta et al., 1998; Ferlin et al., 1999).

In the two histological series, two genes (DFFRY and DAZ) located in AZFa and AZFc loci respectively, were tested. Until now, rare deletions in AZFa loci have been most frequently associated with Sertoli cell-only syndrome (Qureshi et al., 1996; Vogt et al., 1996; Pryor et al., 1997; Grimaldi et al., 1998; Ferlin et al., 1999). On the other hand, a prevalence of deletions affecting the DAZ gene cluster (AZFc) have been observed in patients with non-obstructive infertility (Simoni et al., 1998) and associated with variable histological pheno-types (Table III). A co-existence of deletions affecting AZFa and AZFc loci has also been shown in patients with idiopathic Sertoli cell-only syndrome (Ferlin et al., 1999).

All four samples with DFFRY microdeletions were in the Sertoli cell-only syndrome group. The high frequency (18.2%) of AZFa deletions observed in Sertoli cell-only syndrome allows us to suggest that the AZFa region may be involved in this pathology, although, at the present time, we cannot define the size of the four deletions detected in the Sertoli cell-only series. The absence of amplification of the DFFRY marker in association with the positive DAZ marker seems to favour the presence of interstitial microdeletions in the AZFa or AZFa/b loci, rather than major terminal deletions involving the entire AZF region or double interstitial microdeletions in the AZFa/c loci.

Beyond all selection considerations, the fact that deletions were not found in the series of 14 biopsies with maturation arrest in meiosis I, may suggest that we must look for other causes of these situations: deletion of RBM copies in AZFb region (Vogt et al., 1996) or Y chromosome gene mutations (Sun et al., 1999). Some other genetic origin may be considered, as in the case of mice where maturation arrest is conditioned by several autosomal genes (A-myb, Atm, Hsp70.2, Mih1) (Grootegoed et al., 1998; Kim et al., 1998).

In conclusion, our results show that Yq microdeletion detection is possible in fixed, paraffin-embedded, archived testicular material. This approach may be used to answer some current questions concerning the consequence of Y chromosome molecular pathology at the gonadal level. Moreover, the results of this study suggest the involvement of DFFRY (AZFa) deletions in Sertoli cell-only pathogenesis. In some cases of Sertoli cell-only syndrome, ultrastructural analyses have allowed information to be obtained on possible aetiological factors (Nistal et al., 1990; Terada and Hatakeyama 1991). Ultrastructural analysis was not applicable to our study material. However the application of ultrastructural, histochemical and immunohistochemical methods may be useful in understanding the repercussions of AZF microdeletions on the morphological aspects of testes.

References

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