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Quantification of *DDX3Y*, *RBMY1*, *DAZ* and *TSPY* mRNAs in testes of patients with severe impairment of spermatogenesis

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Y chromosome microdeletion is the most important genetic cause of impairment of spermatogenesis. Nevertheless, a significant proportion of patients with spermatogenic failure do not have this condition. This study investigated the expression level of AZF genes, *DDX3Y* (*DBY*), *RBMY1*, *DAZ* and *TSPY* in testicular tissues of 42 subjects with impaired spermatogenesis compared with 33 with normal spermatogenesis. Histopathological evaluation was performed in all subjects and tissues were classified according to Johnsen Score. Transcript amounts were determined by quantitative-competitive RT–PCR. Patients with complete Sertoli cell-only syndrome (SCOS) did not exhibit *RBMY1*, *DAZ* and *TSPY* gene expression, however, we detected very low expression of *DDX3Y* transcript. Tissue samples with focal SCOS showed significantly decreased expression of all genes ($P < 0.001$). Maturation arrest and hypospermatogenesis tissues expressed significantly low levels of *DDX3Y* testicular transcript ($P < 0.001$), while the mRNA levels of the other genes were similar to that in tissues from the normal spermatogenesis group. Negative or diminished gene expression of *DDX3Y*, *RBMY1*, *DAZ* and *TSPY* in tissues samples with SCOS or focal SCOS reflects the absence or the lower number of germ cells, respectively. The finding that the testicular transcript of *DDX3Y* is significantly decreased in patients with severe spermatogenenic failure, especially in those presenting maturation arrest, suggests an important role of *DDX3Y* during spermatogenesis.

Keywords: spermatogenesis impairment; AZF genes; DDX3Y; testicular transcripts; DBY

Introduction

Spermatogenesis is a complex process that requires highly regulated expression of several genes that operate in a precise temporal sequence to produce normal mature spermatozoa (Matzuk and Lamb, 2002). Recently, the discovery of Y chromosome azoospermia factors (AZFs) and its deletion in infertile men, added to the knowledge of techniques that disrupt AZF homologous genes in animal models, has permitted the identification of many genes related to spermatogenesis. Deletions of Y chromosome genes represent an important genetic cause of idiopathic male infertility. These deletions occur as three AZFs on the euchromatic region of Yq (Yq11), named AZFa, AZFb and AZFc (Vogt *et al.*, 1996). The AZF regions include genes that encode proteins implicated in male spermatogenesis. Among these genes, *DDX3Y* (DEAD-box RNA helicase Y, formerly DBY) in AZFa, *RBMY1* (RNA-binding motif on the Y) in AZFb and *DAZ* (deleted in azoospermia) in AZFc are considered strong AZF candidates because they are frequently deleted in infertile men, they are exclusively expressed in human testes, and their homologues in other species have a role in spermatogenesis (Foresta, 2001). Among other Y chromosome genes, likely implicated in spermatogenesis but not related to microdeletions, *TSPY* (testis-specific protein, Y-linked) is a candidate oncogene that, due to its limited expression pattern in germ cells, is thought to function as a proliferation factor during spermatogenesis (Schnieders *et al.*, 1996).

However, the exact function of these genes in human spermatogenesis has not been elucidated.

DDX3Y is the major AZFa gene candidate since it is frequently deleted in infertile patients and its absence is correlated with severe spermatogenic damage with a significant reduction or even complete absence of germ cells. Expression analysis of the *DDX3Y* gene revealed the presence of two transcripts, one which is ubiquitously expressed (alternative transcript 2), and a shorter transcript (alternative transcript 1) which is exclusively expressed in the testis, strongly suggesting a specific role for *DDX3Y* in the spermatogenic process (Foresta *et al.*, 2000). This hypothesis is further supported by the syntenic homology between the human AZFa region and the mouse ΔSxr^b interval (Mazeyrat *et al.*, 1998), whose deletion causes a severe block in spermatogenesis affecting the proliferation of spermatogonia. The DDX3Y protein is a member of the DEAD-box protein family of RNA helicases exclusively expressed in testis tissue, predominantly

in spermatogonia. Moreover, the observation that other members of this family may play a crucial role in human and mouse spermatogenesis (Tsai-Morris *et al.*, 2004) reinforces the hypothesis that DDX3Y may be responsible for impairment of spermatogenesis.

RBMY1 is considered the most important AZFb gene candidate. From the complete sequence of the human Y chromosome, it is now known that there are six functional RMBY copies mapping within this region (Skaletsky *et al.*, 2003). RBMY1 encodes a RNA-binding protein, which probably functions in pre-RNA splicing, operating as a germ-cell-specific co-factor for more ubiquitously expressed splicing factors, providing tissue specificity in the complex mechanism of splicing (Elliott, 2004). A human RBMY1 protein has been detected in all the transcriptionally active stages of germ cells from spermatogonia to round spermatids (Elliott *et al.*, 1997,1998). Its function may be important for normal germ cell development and may be compromised in men with altered *RMBY* expression.

AZFc deletion is the most frequent Y chromosome microdeletion and accounts for ~60% of Yq microdeletions in infertile men (Foresta, 2001). The *DAZ* gene family represents the stronger candidate for AZFc phenotype and is composed of four gene copies with high sequence homology between them (Kuroda-Kawaguchi *et al.*, 2001). They encode a germ-cell-specific RNA-binding protein, which may bind several not yet identified transcripts, regulating their translation during gametogenesis (Yen, 2004). Despite the high frequency of *DAZ* deletions in infertile men, it is still not clear if *DAZ* genes are essential for human spermatogenesis. Different phenotypes ranging from complete absence of testicular spermatozoa to mild oligozoospermia and fertility are found in subjects with AZFc deletions. Moreover, partial deletions of AZFc, such as the 'gr/gr' subtype, have been documented in patients with severe impairment of spermatogenesis, as well in subjects with normal spermatogenesis (Hucklenbroich *et al.*, 2004; Carvalho *et al.*, 2006; Ravel *et al.*, 2006; Yang *et al.*, 2006; Lardone *et al.*, 2007).

TSPY is part of a heterogenous repetitive gene family previously described in the proximal part of human Yp, with an estimated 20–40 copies of related sequences. Additional copies were mapped to the long arm of Y chromosome ($Yq_{11.23}$) (Ratti *et al.*, 2000). The main prototypic transcript, termed TSPY-S, previously $TSPY^{major}$, is expressed exclusively in testis and the protein has been located predominantly in spermatogonia, and to a lesser degree in primary spermatocytes (Schnieders *et al.*, 1996). TSPY protein is a member of a superfamily, which includes SET and NAP, which are activating factors of the replication process, as binding-partners of cyclin B (Schnieders *et al.*, 1996).

Considering that an significant proportion of patients with spermatogenic failure do not have Y chromosome microdeletions that would explain their spermatogenic defect, hence the present study was designed to investigate the possible alteration in transcriptional expression of AZF genes in these subjects. With this aim, the quantities of transcripts for *DDX3Y*, *RBMY1*, *DAZ* and *TSPY* were determined by quantitative competitive-reverse transcription-polymerase chain reaction (QC-RT–PCR). The levels of transcripts were compared between subjects with normal and impaired spermatogenesis and were related to different histological patterns.

Materials and Methods

Patients

This study was approved by the Institutional Review Board of the School of Medicine at the University of Chile, San Borja Arriarán Clinical Hospital and José Joaquín Aguirre Hospital, and all subjects gave their informed consent. Patients were Chilean and were referred to the Institute of Maternal and Child Research, to the Urology Service of the San Borja Arriarán Hospital or to the José Joaquín Aguirre Hospital. The study included 65 patients with severe oligozoospermia or azoospermia, and 10 normozoospermic subjects. The patients agreed to provide a piece of testicular tissue for QC-RT–PCR and for histopathological diagnosis, during their testicular biopsy for sperm retrieval or during their testicular surgery for non-neoplastic reasons such as hidrocele ($n = 2$), varicocele ($n = 6$) or epididymal cysts ($n = 2$). All participants underwent an evaluation that included complete physical examination, hormonal tests and karyotype. Testis volume was measured by ultrasonography and/or Prader orquidometer. Serum total testosterone and estradiol were determined using radioinmunoassay. LH and FSH were measured by immunoradiometric assay. Patients with hypogonadotrophic hypogonadism, hypoandrogenism, chronic diseases or Y chromosome microdeletions were excluded. The Y chromosome microdeletion standard analysis was performed as previously described (Castro *et al.*, 2004). The analysis included specific markers for *DDX3Y* (Lahn and Page, 1997); *RBM1* [MK5, F19/E55 (Kostiner *et al.*, 1998)]; *DAZ* [sY255 (Reijo *et al.*, 1995), *sY283* (Reijo *et al.*, 1996), *sY254* (Reijo *et al.*, 1995)] and *TSPY* (forward 5′-CCAGATGTCAGCCCTGATCA-3′ and reverse 5′-CATAATCCGGATACC-3′).

Semen analysis

Semen analysis was performed according to normal standard parameters using the World Health Organization (WHO criteria, 1999). The diagnosis of azoospermia was based on the absence of sperm in at least two separate semen analyses after centrifugation of semen samples (1000*g*, 5 min). The selected infertile men were classified as azoospermic or severe oligozoospermic patients (sperm count $\leq 5\ 10^6$/ml).

Testicular biopsy

A small piece of testicular tissue was fixed in Bouiń s solution for histopathological evaluation. Testicular histology assessment included a qualitative and quantitative analysis of germinal epithelium in 25 tubules, and the modified Johnsen score was calculated (Johnsen, 1970; Jezek *et al.*, 1998). According to this score, the tissues were classified as: Sertoli cell-only syndrome (SCOS), complete or focal (i.e. some foci of spermatogenesis); maturation arrest (germ cells until spermatogonia, spermatocyte or spermatid, which may be complete or incomplete, i.e. some tubules show germ cells more developed than the maturation detention stage) or hypospermatogenesis (proportional and quantitative reduction of the different types of germ cells).

Total RNA isolation and cDNA synthesis

The pieces of testicular tissues (30–40 mm^3) were collected in RNA stabilization solution (RNA later™, Ambion, Inc, TX, EE.UU), and incubated at 4°C overnight. They were subsequently removed from RNA later™ and stored at −80°C until RNA extraction. Total cellular RNA was extracted using standard methods (TRIZOL Reagent, Invitrogen Life Technologies), quantified by absorbance at 260 nm, aliquoted and stored at −20°C. RNA integrity was assesed in a 1% gel agarose electrophoresis by observing the preservation of 28S and 18S ribosomal RNA species. Prior to the synthesis of complementary DNA (cDNA), RNA was treated with deoxyribonuclease I, Amplification Grade (Invitrogen Life Technologies: Gran Island, NY, USA). Reverse transcription (RT) was performed following the manufacturer specifications (SuperScript™ II Rnase H Reverse Transcriptase, Invitrogen Life Technologies) using aliquots of 1 μg of DNA-free RNA, and cDNA was stored at −20°C until use.

Preparation of native and competitor RNA

The synthesis of native and competitor RNA templates was performed according to the method described previously (Celi *et al.*, 1993; Raga *et al.*, 1998). A 1 μg aliquot of human testicle total RNA (Ambion) was subjected to RT as described above. PCR was performed using the forward, reverse and reverse competitor primers for each gene. For quantitative RT–PCR, the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. Sequences for *DDX3Y*, *RBMY1*, *DAZ*, *TSPY* and *GAPDH* primers and amplicon sizes are shown in Table 1. The specificity of primers was tested using the Basic Local Alignment Search Tool (BLAST) using Nucleotide Sequence Databases from GenBank and performing single PCR reactions with genomic DNA as template to determine mRNA specificity.

Table 1: Primers used for polymerase chain amplification

Gene	Primer	Sequences	Amplicon size (bp)
DAZ	Forward	5′-GGC GGT TCT ACC TCC GAG-3′	186
	Reverse	5′-TGG TGG TGG CAG GCA GCA-3′	
	Reverse (competitior)	5′-TGG TGG TGG CAG GCA GCA TTT CAC CCA CCA CAA C-3	151
DDX3Y	Forward	5′-ATG GAC AAA GTA GTG GTT CC-3′	450
	Reverse	5′-TTT TTT TTT TGG GGT GGC AC-3′	
	Reverse (competitior)	5′-TTT TTT TTT TGG GGT GGC ACG TGA CTT TGA GCT CCC TTG A-3′	351
RBMY1	Forward	5′-ATG CAC TTC AGA GAT ACG G-3′	505
	Reverse	5′-CTT TGA AAA CAA TTC CTT TTC -3′	
	Reverse (competitior)	5′-CTT TGA AAA CAA TTC CTT TTC CAC ATT TCA ATT TTT GAT TTG-3′	403
TSPY	Forward	5′-CCA GAT GTC AGC CCT GAT CA-3′	224
	Reverse	5′-CAT AAT CCG GAT ACC ACT CAA-3′	
	Reverse (competitior)	5′-CAT AAT CCG GAT ACC ACT CAA ATA TTC CAA TTT TTG ATT TGC-3′	181
GAPDH	Forward	5′-TGG CGT CTT CAC CAC CAT-3′	679
	Reverse	5′-CAC CAC CCT GTT GCT GAT-3′	
	Reverse (competitior)	5-CAC CAC CAC CCT GTT GCT GTA GTA GCC CAG GAT-3′	532

DDX3Y primers amplify a sequence of alternative transcript 1 (GenBank accession no. AF000985), but not of alternative transcript 2 (GenBank accession no. AF000984). *DAZ* primers amplify one sequence common to the mRNA of the four *DAZ* genes (GenBank accession no. NM_020363, AF414183, NM_020364, AF248482). *RBMY1* primers amplify the *RBMY1* mRNA (GenBank accession no. NM_005058) and *TSPY* primers amplify the human *TSPY* mRNA (GenBank accession no. NM_003308).

PCR amplifications were performed in 15 μl reactions containing 0.2 μM of each primer, 1.5 mM of $MgCl_2$, 100 μM of each dNTP (Invitrogen Life Technologies), 1 μl of cDNA, 1× PCR buffer (MBI Fermentas: Burlington, ON, Canada) and 1 U of *Taq* DNA polymerase (MBI Fermentas). The temperature profile was an initial cycle of 3 min at 94°C followed by 30 cycles of amplification (94°C for 45 s, 60°C for 1 min for *DDX3Y*, *DAZ*, *TSPY* and *GAPDH* or 58°C for *RBMY1* primers, and 72°C for 1 min), and a final extension at 72°C for 10 min. The PCR products were separated on a 3% agarose gel. The bands were purified using BIOCLEAN for DNA band purification system (Biotools, B&M Labs, S.A. Spain) and subcloned into a pGEM®-T Easy Vector (Promega, USA). *Escherichia coli* competent cells JM109 (Promega) were transformed. The inserts were confirmed by sequencing using standard automatic sequencing.

Quantification of DDX3Y, RBMY1, DAZ, TSPY and GAPDH mRNA transcripts by QC-RT–PCR

Quantification by QC-RT–PCR was performed as described previously (Tsai and Wiltbank, 1996; Lin *et al.*, 2001; Kuo *et al.*, 2004). Competitor and native fragments were purified from the correspondig plasmid and RNA synthesized by *in vitro* transcription (RiboProbe *In Vitro* Transcription System; Promega). The RNA product was quantified by total absorbance at 260 nm (NanoDrop® ND-1000 Spectrophotometer).

The QC-RT–PCR assay involved the reverse transcription and subsequent amplification of a constant amount of competitor RNA -[2 or 4 attomoles (amol)], of serial dilutions of native RNA (from 0.015 to 4 amol for *DAZ* and *RBMY1*, from 0.25 to 24 amol for *TSPY*, from 0.125 to 16 amol for *DDX3Y* and from 2 to 64 amol for *GAPDH*) or of RNA from unknown samples. The RNA was added to a RT master mix (50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$, pH 8.3, 10 mM DTT, 5.25 ng of random primers, 0.5 mM of each dNTP, 40 U of ribonuclease inhibitor (RNaseOUT™, Invitrogen Life Technologies) and 50 U of reverse transcriptase (SuperScript™ II Rnase H Reverse Transcriptase, Invitrogen Life Technologies). RT was performed at 42°C for 50 min followed by heating to 70°C for 15 min and quick chilling on ice.

PCR was performed with 1 μl of native or unknown sample and 1 μl of competitor RT products, diluted according to protocols, added to 13 μl of PCR mix [final concentration: 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP each, 1 U *Taq* DNA polymerase (MBI Fermentas) and 0.35 μM of primers]. This was subjected to an initial cycle of 3 min at 94°C followed by 30 cycles of amplification (94°C for 45 s, 60°C for 1 min for *DDX3Y*, *DAZ*, *TSPY* and *GAPDH* or 58°C for *RBMY1* primers and 72°C for 1 min), and a final extension at 72°C for 10 min.

The PCR products were separated on a 3% agarose gel, stained with ethidium bromide and scanned with the Typhon 9200 instrument (Amersham Biosciences: Buckinghamshire, UK). The digital images were analysed using the Image Quant 5.0 software and the intensity of each band was obtained. To construct the standard curve, the logarithmic ratio of native to competitor was plotted against the logarithmic initial amounts of native. Concentrations of mRNA transcripts in the samples were calculated by interpolation on the standard curve. The amount of transcript for *DDX3Y*, *RBMY1*, *DAZ* and *TSPY* in each testicular sample was normalized by the amount of *GAPDH* in the same testicular sample (transcript ratio, gene/*GAPDH* transcript).

Statistical analysis

Differences in transcript concentrations and ratios among the four histological groups were analysed by the Kruskal–Wallis test. Multiple pairwise comparisons were performed using Mann–Whitney test. *P*-values <0.05 were considered statistically significant.

Results

Patients

Among 65 azo/oligozoospermic infertile patients, 42 were diagnosed with non-obstructive azoospermia, and their histopathological findings were maturation arrest ($n = 13$), complete SCOS ($n = 19$), focal SCOS ($n = 7$) and hypospermatogenesis ($n = 3$) (Table 2). Among the patients exhibiting spermatogenic maturation arrest, seven showed spermatogenic arrest at the spermatocyte stage, three were at the level of spermatogonium and three were at the level of early spermatid. Obstructive azoospermia was diagnosed in 23 infertile patients who had normal spermatogenesis in their histological evaluation (Johnsen score ≥ 8) and were classified as obstructive controls. In most cases, the cause of obstruction was unknown, although the cause in some patients was vasectomy. Patients with bilateral absence of vas deferens and orchitis were excluded from this study. The 10 normozoospermic patients had normal testicular histology. All subjects had a normal karyotype and did not exhibit Y chromosome microdeletions by PCR standard analysis. LH and testosterone serum levels were within normal limits in all patients. FSH serum levels were observed to be significantly higher in patients with complete and focal SCOS ($P < 0.01$, Mann–Whitney test). Serum estradiol level was similar among the histological groups, although four patients exceded the normal range (1 SCOS, 1 focal SCOS, 1 maturation arrest and 1 normozoospermic control) (Table 2).

Table 2: Hormonal profile and sperm count in cases and controls

	n	Age (years)	FSH (mUI/ml)	LH (mUI/ml)	Testosterone (ng/ml)	Estradiol (pg/ml)	Sperm count (5×10^6/ml)
Cases							
SCOS	19	33 ± 7	12.8 ± 4.1[a]	4.1 ± 1.5	3.8 ± 1.6	46 ± 27.8	0.00 ± 0
Focal SCOS	7	34 ± 4	11.5 ± 6.6[a]	4.0 ± 2.1	4.5 ± 2.1	42.3 ± 28.4	0.014 ± 0.038
Maturation arrest	13	31 ± 3	7 ± 4.9	4 ± 3.3	4.2 ± 1.3	41 ± 16	0.031 ± 0.067
Hypospermatogenesis	3	36 ± 2	5.7 ± 3	3.7 ± 1.3	4.9 ± 2.1	29 ± 7	0.167 ± 0289
Total	42	33 ± 5	10.3 ± 5.4	4 ± 2.2	4.1 ± 1.6	42.6 ± 23	0.023 ± 0.087
Controls (normal spermatogenesis)							
Obstructive azo/oligozoospermic	23	36 ± 8	3.1 ± 62	3.0 ± 1.8	4 ± 1.7	37.26 ± 12	0.078 ± 0.33
Normozoospermic	10	32 ± 11.5	2.8 ± 1.6	3.1 ± 1.6	5 ± 2.7	42 ± 13	63.7 ± 44.8
Total	33	34 ± 9	3.5 ± 2.3	3.1 ± 1.7	4.0 ± 1.3	39 ± 12.5	

The values are expressed as median ± SD. Reference values: FSH, 1.0–7.0 mIU/ml; Testosterone, 2.0–8.0 mIU/ml; Estradiol up to 60 pg/ml. [a]significant difference compared with controls ($P = 0001$).

Quantification of DDX3Y, RBMY1, DAZ and TSPY mRNA transcripts

The standard curve methodology was highly reproducible. The inter-assay coeficients of variation calculated as the mean of the inter-assay CV of each subject, were 18.6, 12.2, 18.1, 22.1, 27.1% for *GAPDH*, *TSPY*, *DAZ*, *DDX3Y* and *RBMY1*, respectively, and the R^2 values ranged between 0.97 and 0.99. Because of the relatively low inter-assay CV (7.5 ± 5%), the samples were measured one time in every assay, but between two and five independent assays were performed for each transcript studied.

The mean ± SD of *GAPDH* transcript level (copies/ng total RNA) for obstructive controls, normozoospermic controls, hypospermatogenesis, maturation arrest, complete SCOS and focal SCOS groups was 1978123.6 ± 1026222.2; 1727919.37 ± 981142.32; 1716223 ± 345671.8; 1307884 ± 729612; 1763882.55 ± 1216438.20; 1588583.82 ± 1301676.80, respectively (Fig. 1). No significant differences were observed among the histological groups.

The copy number of *DDX3Y*, *RBMY1*, *DAZ* and *TSPY* is shown in Table 3. Patients with complete SCOS did not exhibit *RBMY1*, *DAZ* and *TSPY* gene expression, however, we detected very low expression of *DDX3Y* transcript. Tissue samples with focal SCOS showed significantly decreased expression of all genes. Additionally, in the hypospermatogenesis and maturation arrest groups, *DDX3Y* and *DAZ* mRNA expression was decreased, whereas the mRNA amounts of the other genes were similar to the normal spermatogenesis groups.

The *GAPDH*, *RBMY1*, *DAZ* and *TSPY* transcriptional levels were similar between the two groups with normal spermatogenesis, however, *DDX3Y* gene expression was slightly lower in the normozoospermic control groups ($P = 0.02$).

The results of normalizing gene mRNA expression (*DDX3Y*, *RBMY1*, *DAZ* and *TSPY*) by the internal reference *GAPDH* in the different histological groups are shown in Fig. 2. The transcript ratio of *DDX3Y*, *RBMY1*, *DAZ* and *TSPY* was significantly lower in patients with focal SCOS compared with the control groups ($P < 0.001$). When comparing normalized *DDX3Y* expression level in patients with maturation arrest, we also found that it was significantly lower ($P < 0.001$) with respect to the normal spermatogenesis groups. Further analysis among the different stages of maturation arrest did not show any significant differences. *DAZ*, *RBMY1* and *TSPY* transcript ratios, in the maturation arrest group, did not exhibit any difference with the control groups.

Although the sample size was small, the patients with hypospermatogenesis showed normalized *DDX3Y* expression levels significantly lower compared with the normal spermatogenesis group ($P = 0.007$). In addition, the normalized expression of *DAZ* gene was significantly diminished compared with the control groups. However, the transcript ratios of *RBMY1* and *TSPY* were not different from controls.

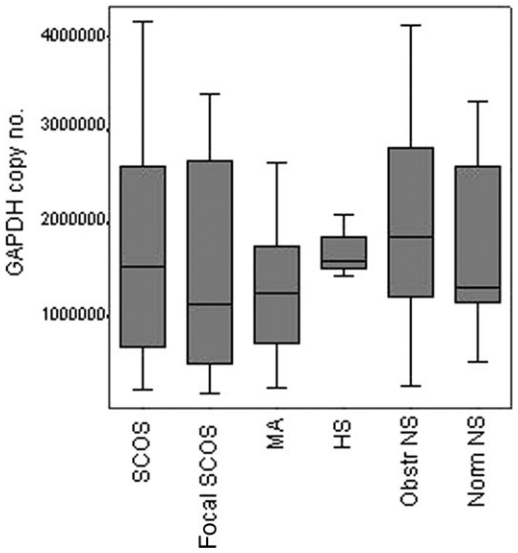

Figure 1: *GAPDH* mRNA copy number of different testicular histological groups
Bars represent the 75% of the values. Horizontal lines indicates median values and errors lines indicates the 99% of the values. No significant differences were noted among the six groups ($P = 0.58$, Kruskal–Wallis test). MA, maturation arrest; HS, hypospermatogenesis; Obstr NS, obstructive controls with normal spermatogenesis; Norm NS, normozoospermic controls with normal spermatogenesis

Discussion

The most important genetic cause of spermatogenesis impairment is Y chromosome microdeletions. In the present study, we studied the hypothesis that an expression defect in spermatogenesis-linked genes may produce spermatogenesis failure in patients that do not have Y chromosome microdeletions and who do not manifest other clinical, cytogenetic or endocrinologic abnormalities. Gene expression was assessed by quantification of the mRNAs levels of four candidate genes in patients with different types of spermatogenic failure compared with subjects with normal spermatogenesis. Transcript amounts were measured using QC-RT–PCR with standard curve methodology, a reliable method that allows the evaluation of

Table 3: Transcript copy number per ng RNA in each histological group

Transcript	Obstructive control	Normozoospermic control	Hypospermatogenesis	Maturation arrest	Focal SCOS	SCOS
DDX3Y	81 401 ± 37 938[a]	48 459 ± 39 136[a]	9011 ± 5634[b]	7366 ± 8059[b]	3260 ± 1719[b]	1553 ± 844[b]
RBMY1	1220 ± 1173	650 ± 422	658 ± 776	613 ± 637	58 ± 65[b]	0.0[b]
DAZ	518 ± 435	421 ± 380	116 ± 15[b]	269 ± 226[c]	149 ± 26[b]	0.0[b]
TSPY	102835 ± 97 524	85 719 ± 90 359	86 233 ± 73 369	83 767 ± 50 838	2196 ± 1803[b]	0.0[b]

The values are expressed as median ± SD. [a]significant difference between obstructive and normozoospermic controls ($P = 0.015$); [b]significant difference compared with controls ($P < 0001$); [c]significant difference compared with controls ($P < 0001$).

limited amounts of experimental material with high sensitivity and reproducibility.

No gene expression of *RBMY1*, *DAZ* and *TSPY* was found in patients diagnosed with complete SCOS, although very low expression of *DDX3Y* transcript was detected in these tissues. In patients with focal SCOS, transcript expression of these genes was present, although significantly reduced. In both cases, the results were not surprising because these genes are expressed predominantly in germ cells (Schnieders *et al.*, 1996; Foresta *et al.*, 2000; Ditton *et al.*, 2004; Elliott, 2004; Szczerba *et al.*, 2004), so it reflects the absence or the lower number of germ cells, respectively. A previous study (Kuo *et al.*, 2004) found that *RBMY1* and *DAZ* were expressed in patients with SCOS, although at significantly lower levels than in patients with normal spermatogenesis. The authors explained their findings by suggesting that these genes may be expressed in other cells different from germ cells. However, it is known that *DAZ* and

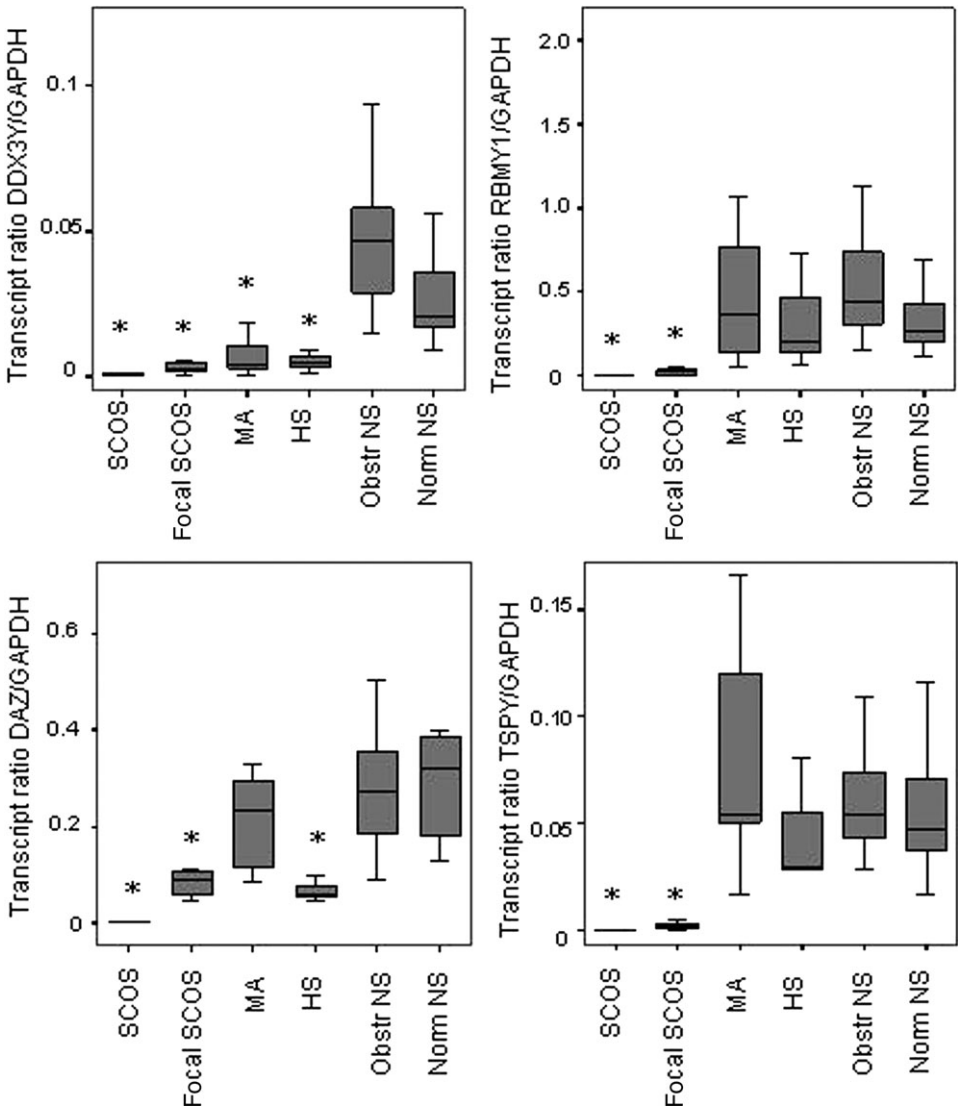

Figure 2: Transcript ratios (gene/*GAPDH*) for *DDX3Y*, *RBMY1*, *DAZ* and *TSPY* in different testicular histologic groups
Bars represent the 75% of the values. Horizontal lines indicate median values and errors lines indicate the 99% of the values. *indicates significant difference between the histologic group and normal spermatogenesis groups (*P* 0.001). SCOS patients group has gene transcript ratio zero because of no gene expression (*DAZ*, *RBMY1* and *TSPY*).

RBMY1 genes are expressed only in germ cells. We corroborated this observation by showing that we did not find mRNA expression in tissues diagnosed as complete SCOS, whereas tissues with some foci of spermatogenesis, and therefore presence of germ cells, showed expression of germ-cell-specific genes such as *DAZ*. Consequently, we separated the results of patients with complete SCOS (Johnsen score = 2) from those with focal SCOS. In addition, the fragment of testicular tissue used for histopathological evaluation was contiguous to that used for RNA extraction and therefore the mRNA quantification results would correspond to the respective histological phenotype.

Interestingly, tissues exhibiting different degrees of spermatogenic maturation arrest or hypospermatogenesis showed significantly decreased expression of *DDX3Y*, compared with normal tissues. This finding could not be explained, at least in the case of maturation arrest, by a reduced number of germ cells, since the number of spermatogonias and/or spermatocytes, was similar to that in normal tissues (Fig. 3). Furthermore, the amount of transcripts of the other genes studied was not different compared with normal spermatogenesis, reinforcing the idea that it is not a matter of germ cell number.

The analysis of two patients with testicular hystology of maturation arrest and who also exhibited a Y chromosome microdeletion in AZFc, did not present a reduced transcriptional level of *DDX3Y* (data not shown). This data support our hypothesis that a reduced expression level of *DDX3Y* may play an important role in the aetiology of spermatogenic impairment in subjects without Y chromosome microdeletion.

DDX3Y is an AZFa single copy gene that produces two transcripts, a shorter transcript which is only expressed in the testis (alternative transcript 1) (Foresta *et al.*, 2000), and a ubiquitously expressed transcript (alternative transcript 2) (Lahn and Page, 1997). Despite this, the protein is detected only in the male germ line, predominantly in the cytoplasm of spermatogonia and occasionally in the cytoplasm of some pre-leptotene/leptotene spermatocytes (Ditton *et al.*, 2004). These findings suggest that the testicular transcript (alternative transcript 1) would be the one translated and that DDX3Y protein has a cytoplasmic function in these germ cells.

In contrast to our results, a previous study (Kuo *et al.*, 2004) reported no significant difference in *DDX3Y* transcription levels in testicular tissues of patients with impairment of spermatogenesis including hypospermatogenesis, maturation arrest and SCOS compared with normal spermatogenesis. The authors explained this finding by suggesting that *DDX3Y* is expressed ubiquitously. The expression of *DDX3Y* (alternative transcript 1) in our complete SCOS tissues was almost undetectable by competitive RT–PCR, which may represent a minimal constitutive expression of this transcript.

DDX3Y belongs to the highly conserved DEAD-box family of RNA helicases which has multiple members in the human genome. These proteins are involved in various aspects of RNA metabolism, including nuclear transcription, pre-mRNA splicing, ribosome biogenesis, nucleocytoplasmic transport, translation, RNA decay and organellar gene expression (de la Cruz *et al.*, 1999).

The exact role of *DDX3Y* in RNA metabolism is unknown. It has been suggested that its X chromosome homologue, *DBX* (*DDX3*) has a role in RNA splicing since it has been localized in nuclear speckles, which are known spliceosome components, and is known to have an RS-like domain which is present in several splicing factors (Abdelhaleem, 2005). The autosomal homologue for *DDX3Y* in the mouse, D1Pas1/PL10, also appears to play a role in spermatogenesis since its exclusive testicular expression and nuclear localization in meiotic and post-meiotic germ cells would suggest a role for this protein in the regulation of mRNA processing (Session *et al.*, 2001). The low levels of *DDX3Y* mRNA in patients with germ cell differentiation defects, showed in this study, suggest that this gene acts during the complex cascade of genes that controls germ cell differentiation. The cytoplasmic location of DDX3Y protein could indicates a role in the translation process for this DEAD-box RNA helicase.

Meiosis and spermiogenesis are the main checkpoints for the correct operation of numerous genes that regulate germ cell maturation. It has been reported that the wrong formation of the synaptonemal complex during prophase I of meiosis results in a complete early maturation arrest of spermatogenesis (Miyamoto *et al.*, 2003). A similar phenotype was found when the protein expression of *BOULE* and its putative target gene *CDC25A*, a phosphatase required to activate the maturation promoting factor, was absent in a group of azoospermic men (Luetjens *et al.*, 2004). On the other hand, male mice with a null mutation of GRTH/Ddx25, a testis-specific RNA helicase

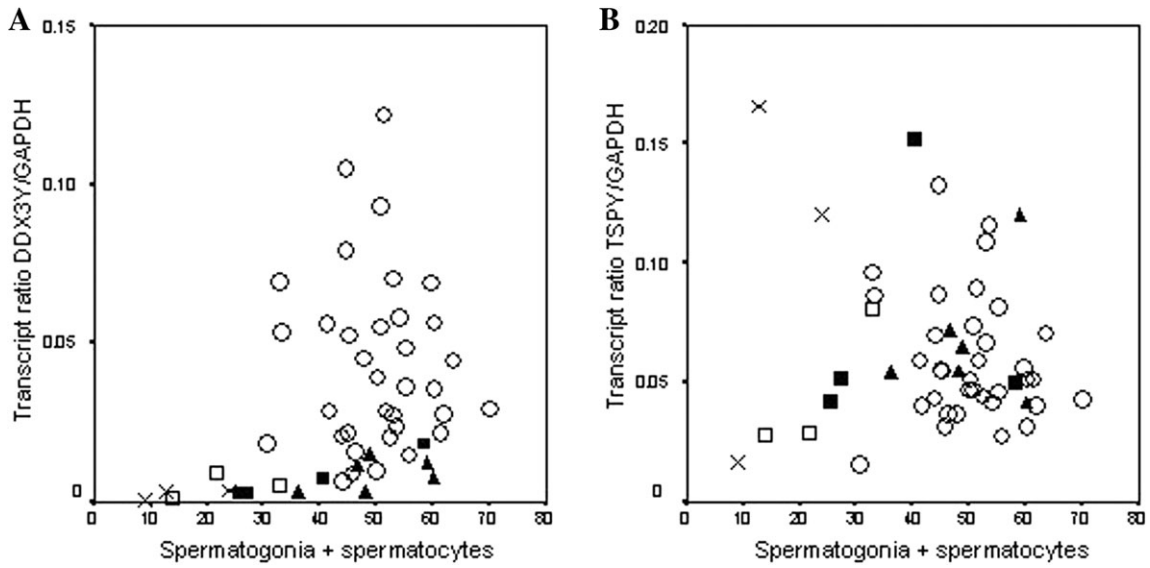

Figure 3: Relationship of the number of spermatogonia plus spermatocytes per seminiferous tubule with (**A**) the DDX3Y transcript copies number and (**B**) the transcript ratio of TSPY (not down regulated gene) in maturation arrest (MA) subgroups, hypospermatogenesis and control group.
Open circle, control; filled square, spermatid MA; filled triangle, spermatocyte MA; cross, spermatogonia MA; open square, hypospermatogenesis.

member of the DEAD-box family, were sterile with azoospermia caused by complete arrest of spermiogenesis (Tsai-Morris *et al.*, 2004).

Although preliminary, our results show that *DDX3Y* gene expression is reduced in all stages of maturation arrest with some patients presenting only spermatocytes in their tubules, which indicates an arrest possibly in the prophase of the first meiotic division, whereas other patients have some tubules where cell maturation progresses mainly to round spermatids. It is tempting to speculate that the spermatogenic failure in our patients arises from factor(s) upstream of *DDX3Y*, which would be involved in regulating its transcription and possibly that of many others genes not yet investigated. The distinct testicular phenotypes may be the consequence of different environmental and genetic backgrounds present in our patients.

The histological pattern of tissues with hypospermatogenesis was associated with some level of maturation arrest in their tubules, which may explain the similarity in the *DDX3Y* expression profile of hypospermatogenesis with that of the maturation arrest group.

Nevertheless, a larger number of patients with hypospermatogenesis would be useful to corroborate any assumption.

In summary, the mRNA level of *RBMY1* and *TSPY* are not altered in patients with impairment of spermatogenesis, such as maturation arrest and hypospermatogenesis. We describe for the first time that the mRNA level of *DDX3Y*, the candidate gene for AZFa deletions, is significantly decreased in testicular tissues of patients with severe spermatogenenic failure, especially in those with maturation arrest. This study suggests an important role for *DDX3Y* during spermatogenesis. An expression profile of DDX3Y protein in these patients and the possible targets of this DEAD-box RNA helicase may be useful to elucidate the role of *DDX3Y* in spermatogenesis.

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References

Abdelhaleem M. RNA helicases: regulators of differentiation. *Clin Biochem* 2005;**38**:499–503.

Carvalho CM, Zuccherato LW, Bastos-Rodrigues L, Santos FR, Pena SD. No association found between gr/gr deletions and infertility in Brazilian males. *Mol Hum Reprod* 2006;**12**:269–273.

Castro A, Zambrano N, Kaune H, Madariaga M, Lopez P, Mericq V. YqTER deletion causes arrest of spermatogenesis in early puberty. *J Pediatr Endocrinol Metab* 2004;**17**:1675–1678.

Celi FS, Zenilman ME, Shuldiner AR. A rapid and versatile method to synthesize internal standards for competitive PCR. *Nucleic Acids Res* 1993;**21**:1047.

de la Cruz J, Kressler D, Linder P. Unwinding RNA in Saccharomyces cerevisiae: DEAD-box proteins and related families. *Trends Biochem Sci* 1999;**24**:192–198.

Ditton HJ, Zimmer J, Kamp C, Rajpert-De Meyts E, Vogt PH. The AZFa gene DBY (DDX3Y) is widely transcribed but the protein is limited to the male germ cells by translation control. *Hum Mol Genet* 2004;**13**:2333–2341.

Elliott DJ. The role of potential splicing factors including RBMY1, RBMX, hnRNPG-T and STAR proteins in spermatogenesis. *Int J Androl* 2004;**27**:328–334.

Elliott DJ, Millar MR, Oghene K, Ross A, Kiesewetter F, Pryor J, McIntyre M, Hargreave TB, Saunders PT, Vogt PH *et al.* Expression of RBM in the nuclei of human germ cells is dependent on a critical region of the Y chromosome long arm. *Proc Natl Acad Sci USA* 1997;**94**:3848–3853.

Elliott DJ, Oghene K, Makarov G, Makarova O, Hargreave TB, Chandley AC, Eperon IC, Cooke HJ. Dynamic changes in the subnuclear organisation of pre-mRNA splicing proteins and RBM during human germ cell development. *J Cell Sci* 1998;**111**:1255–1265.

Foresta C. Y chromosome microdeletions and alterations of spermatogenesis. *Endocr Rev* 2001;**22**:226–239.

Foresta C, Ferlin A, Moro E. Deletion and expression analysis of AZFa genes on the human Y chromosome revealed a major role for DBY in male infertility. *Hum Mol Genet* 2000;**9**:1161–1169.

Hucklenbroich K, Gromoll J, Heinrich M, Hohoff C, Nieschlag E, Simoni M. Partial deletions in the AZFc region of the Y chromosome occur in men with impaired as well as normal spermatogenesis. *Hum Reprod* 2004;**20**:191–197.

Jezek D, Knuth UA, Schulze W. Successful testicular sperm extraction (TESE) in spite of high serum follicle stimulating hormone and azoospermia: correlation between testicular morphology, TESE results, semen analysis and serum hormone values in 103 infertile men. *Hum Reprod* 1998;**13**:1230–1234.

Johnsen SG. Testicular biopsy score count–a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males. *Hormones* 1970;**1**:2–25.

Kostiner DR, Turek PJ, Reijo RA. Male infertility: analysis of the markers and genes on the human Y chromosome. *Hum Reprod* 1998;**13**:3032–3038.

Kuo PL, Lin YH, Teng YN, Hsu CC, Lin JS, Lin YM. Transcriptional levels of four Y chromosome-linked AZF genes in azoospermic men and their association with successful sperm retrieval. *Urology* 2004;**63**:131–136, discussion 136.

Kuo PL, Wang ST, Lin YM, Lin YH, Teng YN, Hsu CC. Expression profiles of the DAZ gene family in human testis with and without spermatogenic failure. *Fertil Steril* 2004;**81**:1034–1040.

Kuroda-Kawaguchi T, Skaletsky H, Brown LG, Minx PJ, Cordum HS, Waterston RH, Wilson RK, Silber S, Oates R, Rozen S *et al.* The AZFc region of the Y chromosome features massive palindromes and uniform recurrent deletions in infertile men. *Nat Genet* 2001;**29**:279–286.

Lahn BT, Page DC. Functional coherence of the human Y chromosome. *Science* 1997;**278**:675–680.

Lardone MC, Parodi DA, Ebensperger M, Peñaloza P, Cornejo V, Valdevenito R, Pommer R, Castro A. AZFc partial deletions in Chilean men with severe spermatogenic failure. *Fertil Steril* 2007 Apr 6; [Epub ahead of print].

Lin YM, Chen CW, Sun HS, Tsai SJ, Hsu CC, Teng YN, Lin JS, Kuo PL. Expression patterns and transcript concentrations of the autosomal DAZL gene in testes of azoospermic men. *Mol Hum Reprod* 2001;**7**:1015–1022.

Luetjens CM, Xu EY, Rejo Pera RA, Kamischke A, Nieschlag E, Gromoll J. Association of meiotic arrest with lack of BOULE protein expression in infertile men. *J Clin Endocrinol Metab* 2004;**89**:1926–1933.

Matzuk MM, Lamb DJ. Genetic dissection of mammalian fertility pathways. *Nat Cell Biol* 2002;**4**(Suppl):s41–s49.

Mazeyrat S, Saut N, Sargent CA, Grimmond S, Longepied G, Ehrmann IE, Ellis PS, Greenfield A, Affara NA, Mitchell MJ. The mouse Y chromosome interval necessary for spermatogonial proliferation is gene dense with syntenic homology to the human AZFa region. *Hum Mol Genet* 1998;**7**:1713–1724.

Miyamoto T, Hasuike S, Yogev L, Maduro MR, Ishikawa M, Westphal H, Lamb DJ. Azoospermia in patients heterozygous for a mutation in SYCP3. *Lancet* 2003;**362**:1714–1719.

Raga F, Casan EM, Kruessel JS, Wen Y, Huang HY, Nezhat C, Polan ML. Quantitative gonadotropin-releasing hormone gene expression and immunohistochemical localization in human endometrium throughout the menstrual cycle. *Biol Reprod* 1998;**59**:661–669.

Ratti A, Stuppia L, Gatta V, Fogh I, Calabrese G, Pizzuti A, Palka G. Characterization of a new TSPY gene family member in Yq (TSPYq1). *Cytogenet Cell Genet* 2000;**88**:159–162.

Ravel C, Chantot-Bastaraud S, El Houate B, Mandelbaum J, Siffroi JP, McElreavey K. GR/GR deletions within the azoospermia factor c region on the Y chromosome might not be associated with spermatogenic failure. *Fertil Steril* 2006;**85**:229–231.

Reijo R, Alagappan RK, Patrizio P, Page DC. Severe oligozoospermia resulting from deletions of azoospermia factor gene on Y chromosome. *Lancet* 1996;**347**:1290–1293.

Reijo R, Lee TY, Salo P, Alagappan R, Brown LG, Rosenberg M, Rozen S, Jaffe T, Straus D, Oea Hovatta. Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. *Nat Genet* 1995;**10**:383–393.

Schnieders F, Dork T, Arnemann J, Vogel T, Werner M, Schmidtke J. Testis-specific protein, Y-encoded (TSPY) expression in testicular tissues. *Hum Mol Genet* 1996;**5**:1801–1807.

Session DR, Lee GS, Wolgemuth DJ. Characterization of D1Pas1, a mouse autosomal homologue of the human AZFa region DBY, as a nuclear protein in spermatogenic cells. *Fertil Steril* 2001;**76**:804–811.

Skaletsky H, Kuroda-Kawaguchi T, Minx PJ, Cordum HS, Hillier L, Brown LG, Repping S, Pyntikova T, Ali J, Bieri T *et al.* The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. *Nature* 2003;**423**:825–837.

Szczerba A, Jankowska A, Andrusiewicz M, Karczewski M, Turkiewicz W, Warchol JB. Distribution of the DAZ gene transcripts in human testis. *Folia Histochem Cytobiol* 2004;**42**:119–121.

Tsai SJ, Wiltbank MC. Quantification of mRNA using competitive RT-PCR with standard-curve methodology. *Biotechniques* 1996;**21**:862–866.

Tsai-Morris CH, Sheng Y, Lee E, Lei KJ, Dufau ML. Gonadotropin-regulated testicular RNA helicase (GRTH/Ddx25) is essential for spermatid development and completion of spermatogenesis. *Proc Natl Acad Sci USA* 2004;**101**:6373–6378.

Vogt PH, Edelmann A, Kirsch S, Henegariu O, Hirschmann P, Kiesewetter F, Kohn FM, Schill WB, Farah S, Ramos C *et al.* Human Y chromosome azoospermia factors (AZF) mapped to different subregions in Yq11. *Hum Mol Genet* 1996;**5**:933–943.

WHO. Laboratory manual for the examination of human semen and sperm-cervical mucus interaction. Cambridge University Press, 1999.

Yang Y, Xiao CY, A ZC, Zhang SZ, Li X, Zhang SX. DAZ1/DAZ2 cluster deletion mediated by gr/gr recombination per se may not be sufficient for spermatogenesis impairment: a study of Chinese normozoospermic men. *Asian J Androl* 2006;**8**:183–187.

Yen PH. Putative biological functions of the DAZ family. *Int J Androl* 2004;**27**:125–129.

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