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Original Article

Y-chromosome genes associated with sertoli cell-only syndrome identified by array comparative genome hybridization

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ABSTRACT

Background: The precise contribution of each chromosome gene or gene family in achieving male fertility is still the subject of debate. Most studies have examined male populations with heterogeneous causes of infertility, and have therefore reached controversial or uncertain conclusions. This study uses Y-chromosome array-based comparative genomic hybridization (aCGH) to examine a population of males with a uniform sertoli cell-only syndrome (SCOS) infertility phenotype.

Methods: Initial analysis of gene copy number variations in 8 SCOS patients, with determination of the log-ratio of probe signal intensity against a DNA reference, was performed using the Y-chromosome NimbleGen aCGH. To confirm the role of candidate genes, real-time quantitative RT-PCR was used to compare 19 patients who had SCOS non-obstructive azoospermia with 15 patients who had obstructive azoospermia but normal spermatogenesis.

Results: Our initial aCGH experiments identified *CDY1a* and *CDY1b* double deletions in all 8 patients who had total germ cell depletion. However, 5 patients had *DAZ1/2* and *DAZ3/4* deletions, 1 patient had a *DAZ2* and *DAZ3/4* deletion, and 2 patients had no *DAZ1/2* or *DAZ3/4* deletions. Examination of testicular mRNA expression in another 19 patients with SCOS indicated all patients had no detectable levels of *CDY1*.

Conclusions: Our findings indicate that CDY1 deletion in SCOS patients, and analysis of the expression of DAZ and CDY1 genes using aCGH and quantitative RT-PCR, may be useful to predict the presence of mature spermatozoa.

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At a glance of commentary

Scientific background on the subject

The precise contribution of each chromosome gene or gene family in achieving male fertility is still the subject of debate. Most studies have examined male populations with heterogeneous causes of infertility, and have therefore reached controversial or uncertain conclusions.

What this study adds to the field

Our findings indicate that CDY1 deletion in SCOS patients, and analysis of the expression of DAZ and CDY1 genes using aCGH and quantitative RT-PCR, may be useful to predict the presence of mature spermatozoa.

Male infertility is a complex disease with multiple causes, many of which have underlying genetic alterations. Previous studies of mouse models, mutation screening studies, and genetic association studies reported that genetic defects in spermatogenesis account for 10–15% of severe male infertility. These defects include chromosomal aberrations and single gene mutations [1–4]. However, many infertile males receive diagnoses of idiopathic infertility because of the currently incomplete understanding of the mechanisms that regulate spermatogenesis and sperm function. Identification of the causes of male idiopathic infertility requires the use of large-scale genetic association studies and testicular or spermatozoal expression studies in individuals who have well-defined alterations in spermatogenesis. Thus, identification of individuals with the same phenotype is the first step in elucidation of an underlying pathological mechanism. Unfortunately, most studies have examined male populations with heterogeneous causes of infertility, and have therefore reached controversial or uncertain conclusions. Moreover, variations in the outcomes of previous studies may be attributed to the use of different inclusion/exclusion criteria, different methodologies, inadequate sample sizes, and enrollment of men from different geographic regions and ethnicities. The array comparative genomic hybridization (aCGH) technique has many advantages relative to conventional methods used in male infertility analysis, especially for high-resolution studies. In particular, aCGH is an effective tool for identification of copy number variation (CNV) in clinical settings. It can also provide identification of other unrecognized structural chromosome defects associated with infertility, especially as an assisted reproductive therapy (ART) used for pre-implantation genetic diagnosis testing and other fetal pregnancy testing procedures. Since the introduction of aCGH, researchers have made significant advances in the identification and characterization of novel or previously known genes that are possibly associated with male infertility in mice and humans [5,6]. Although few previous studies of male infertility have used aCGH, the aCGH approach is a reliable alternative to multiplex PCR for discovery of pathogenic chromosome-Y microdeletions in male infertility [6,7] The purpose of this study was to identify candidate genes related to uniform Sertoli cell-only syndrome (SCOS) phenotype in infertile males by use of Y-chromosome aCGH to screen gene CNVs in the leukocyte genome, and to confirm these results by quantitative RT-PCR (qRT-PCR) and immunohistochemistry in another separate population using testicular specimens.

Material and methods

Study design

Our study design flow diagram is illustrated in Fig. 1. We initially screened deletions of the Y chromosome confined to patients with SCOS with high-resolution Y chromosome aCGH. Furthermore, we validated candidate genes being specific to men with impaired spermatogenesis in testicular biopsy samples in our second step analysis. Then males with obstructive azoospermia and males with non-obstructive azoospermia (NOA) were recruited from Kaohsiung Chang Gung Memorial Hospital (CGMH) during office visits for testicular sperm extraction (TESE) and ART. The urological services provide counseling for patients undergoing TESE using a previously described protocol [8]. Two distinct patterns were identified by histology of the seminiferous tubules; one group had tubules with germ cell aplasia and only sertoli cells (indicative of SCOS), and the other group had tubules containing mature germ cells (indicative of normal spermatogenesis). The diagnosis of SCOS was established if tubules with Sertoli cells alone were detected in at least two sites per testis based on testicular biopsies [9,10].

aGCH assay and data analysis

Patient genomic DNA and matched control DNA from a normal healthy male were isolated from peripheral blood nucleated cells collected in an EDTA tube. Then, 1 μg of each sample were verified by agarose gel electrophoresis. The aCGH analysis was performed using a Chromosome Y Tiling 385K Array (Roche NimbleGen Systems Inc., Madison, WI), with average probe spacing of ~20 Kb and probe length of 50–75 mer. Genomic DNA was sonicated to fragments of 600–2000 bp, and then re-purified with DNA labeling and hybridization. The fragmented genomic DNA from patients and matched controls were differentially labeled with the fluorescent cyanine-5 (Cy5) and cyanine-3 (Cy3). Specimen labeling, array hybridization, washing, and scanning were performed at NimbleGen Service Laboratory (Iceland) and array image analysis and data normalization were performed using NimbleScan software (Roche Diagnostics). The normalized data

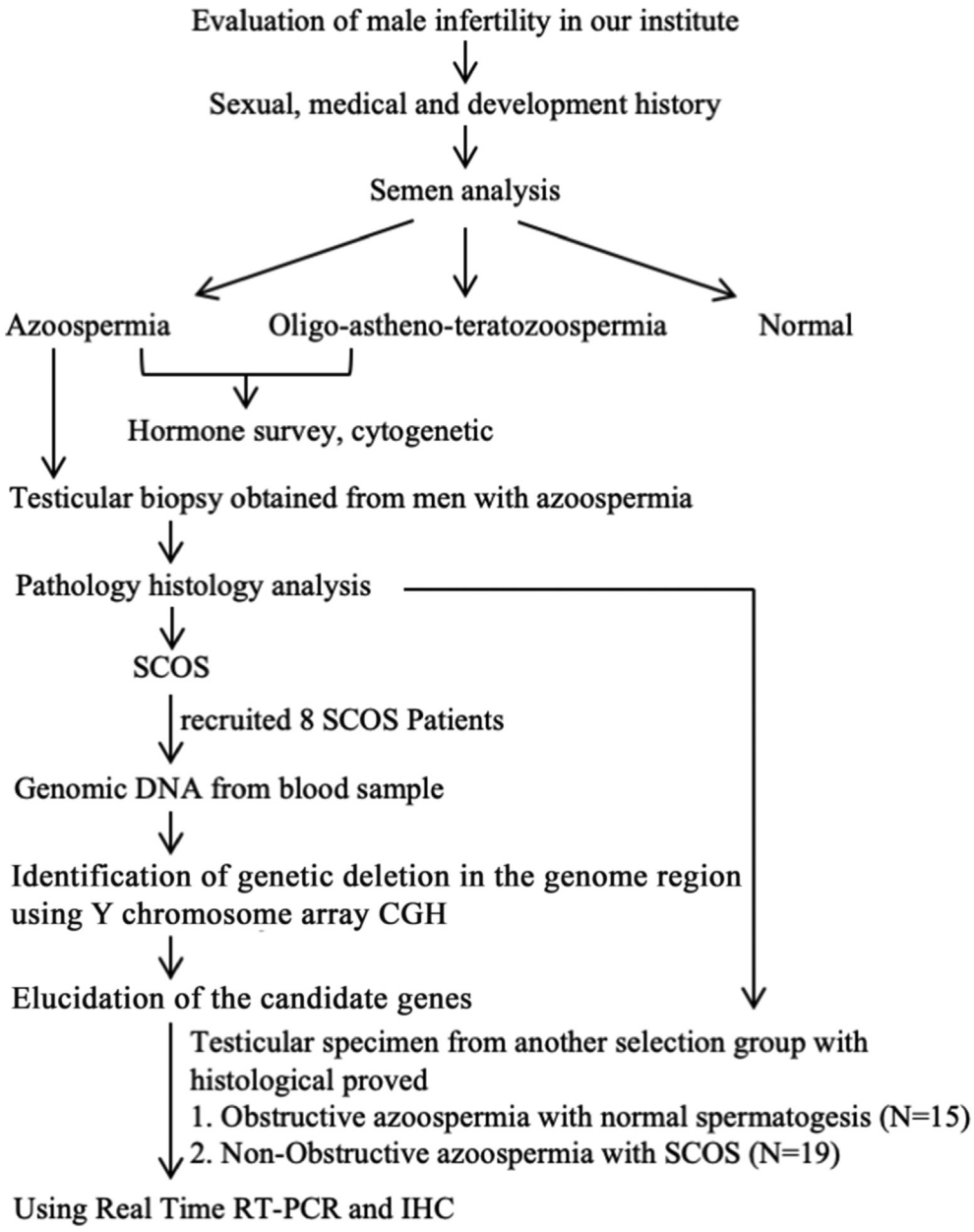

Fig. 1 Flow diagram for study design. Identification and validation of candidate genes.

were then processed using Nexus Expression software (Bio-Discovery, Inc., El Segundo, CA, USA).

DNA purification

DNA extraction was performed using the Gentra Puregene Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The genomic DNA was eluted into a final volume of 50 μL in sterile water, and DNA concentration was measured using NanoDrop ND-1000 (Thermo Scientific, Wilmington, MA). All DNA samples were stored at −20 °C until use.

Testicular tissue RNA extraction and qRT-PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen Inc., Carlsbad, CA, USA) and reverse-transcribed. Real-time reverse transcription-PCR (RT-PCR) was performed by using a SyBR green PCR master mix kit (PE Applied Biosystems, Foster City, USA). Sequence analysis was performed using an ABI Prism 7700 sequence detection system (PE Applied Biosystems). The gene-specific primer pairs were: *CDY1*-sense (5′-TCAGCCCAAGGGATAGAATCC-3′) and *CDY1*-antisense (5′-CAAGGGCACCATCTCTGATCA-3′); *DAZ*-sense (5′-GGCTCCTTTGACCACTCGAA-3′) and *DAZ*-antisense (5′-GACAGGCTCAAGGAGGAACAGA-3′); BPY2-sense (5′-CAGATTTTCACAGGTGCTGCTT-3′) and BPY2-antisense (5′-AGCAAGGTATTTCTCACATTCCC-3′); CSPG4P1Y-sense.

(5′-GAAGAGGCGAGTCCTGTTCA-3′) and CSPG4P1Y-antisense (5′-GCCACATAGAGGTAAGGCTCC-3′); GOLGA2LY1-sense (5′-GCAGCTAAAGCCCAGTTGAG-3′) and GOLGA2LY1-antisense (5′- CCCTGGAATGTTCAGCATGG-3′). Evaluation of the six potential reference genes (*GAPDH, 18S rRNA, HPRT1,*

TBP, β-actin and B2M) was initially performed for real-time PCR in testes. In agreement with a previous study that used the geNorm bioinformatic tool [11], *GAPDH* and 18S had the most stable expression among these housekeeping genes, so their average expression was used as the internal control. REST 2009 Software [12], a mathematic model that considers the variable PCR efficiencies for different genes of interest and reference genes, was used for this analysis.

Immunohistochemical staining

Testicular tissues from TESE were initially paraffin-embedded for immunohistochemical study. The sections were deparaffinized, rehydrated, retrieved with 10 mM citrate buffer, and then incubated with the primary antibodies. The anti-chromodomain Y (CDY) 1 (AV48645, Sigma, MO, USA) and anti-deleted-in-azoospermia (DAZ) 1 (WH0001617M1, Sigma) antibodies were diluted 1:50. After addition of the appropriate secondary IgG antibody, sections were incubated with DAB (Dako, Glostrup, Denmark) and counterstained with Gill's hematoxylin (Merck, Whitehouse, NJ, USA). The images were observed using a Nikon microscope (Nikon E800, Melville, NY) and analyzed using Nikon's NIS-Elements software (LIM, Prague).

Statistical analysis

SPSS version 10.0 (SPSS, Inc., Chicago, IL, USA) was used for data analysis. Proportions were compared using the chi-square or Fisher's exact test, and false discovery rate (FDR) correction by Benjamini and Hochberg method for multiple comparisons as appropriate, and a *P* value below 0.05 was considered statistically significant.

Results

We initially analyzed DNA samples from 8 unrelated infertile men (median age:38.3 (29–48)) with otherwise normal karyotypes who had SCOS and reference fertile man without known Y-chromosome microdeletions (YCM) using NimbleGen aCGH. The incidence of potentially susceptible loci mapping to known genes was high in our patient cohort, and the highly overlapping recurrent deletions on Yq11.223 and Yq11.23 seemed pathogenic (Fig. 2 and Supplemental Fig. 1). Analysis of the 8 SCOS patients with total germ cell depletion all had CDY1a and CDY1b double deletions, 5 patients had DAZ1/2 and DAZ3/4 deletions, 1 patient had DAZ2 and DAZ3/4 deletions, and 2 patients had no DAZ1/2 or DAZ3/4 deletions (Fig. 2 and Supplemental figures 1, Table 1). Analysis of the BPY gene cluster indicated only 2 patients had total deletion (6 patients had *BPY2* deletion, 3 patients had *BPY2B* and *BPY2C* deletion), 4 patients had *CSPG4LYP1* deletion, and 3 patients had GOLGA2LY1 deletion (Table 1).

We further validated candidate CDY1 and DAZ genes in another separate population using testicular specimens. We used real-time RT-PCR to quantitatively analyze the expression of *CDY1*, *DAZ*, *BPY*, Golgi autoantigen, golgin subfamily a2-like, Y-linked 1 (GOLGA2LY1) and chondroitin sulfate proteoglycan 4-like, Y-linked pseudogene 1 (CSPG4LYP1) in testes specimens from 15 IVF/ICSI patients with obstructive azoospermia (but normal spermatogenesis), and 19 patients with SCOS. The serum levels of LH (11.0 ± 4.8 versus 4.0 ± 1.8, mIU/mL) and FSH (24.5 ± 8.8 versus 5.0 ± 2.7, mIU/mL) were higher in SCOS patients compared to the obstructive azoospermia patients; however, the age and serum testosterone were similar. None of the 19 SCOS patients had detectable CDY1 expression and 18 of them had no detectable *DAZ* expression. In contrast, samples from patients with normal spermatogenesis had *CDY1* and *DAZ* expression (Table 2). Analysis of specimens from men with obstructive azoospermia and SCOS indicated the presence of DAZ1 in spermatogonia. Interestingly, CDY1 immunostaining was present in late-stage germ cells of specimens from men with obstructive azoospermia (Fig. 3).

Discussion

Structural abnormalities of the Y chromosome usually are not life-threatening, but they often adversely affect spermatogenesis. Microdeletions in the azoospermia factor (AZF) region of the Y chromosome are among the most common genetic causes of male infertility [13]. However, the roles of specific AZF genes in spermatogenesis are not fully understood. Many studies have examined the AZFc subregion, because of its important role in male infertility. In particular, studies of the AZFc subregion indicated that the frequency of AZFc deletion is high among men with azoospermia and oligozoospermia. Recent studies examined partial deletions in the AZFc subregion at the molecular level, and identified 4 significant partial deletions (b2/b4, gr/gr, b2/b3, and b1/b3) [14,15]. The AZFc locus encodes 21 candidate genes and 11 families of transcription units whose expression is specific to the testes. Seven of these families are in the AZFc deletion interval, including GOLGA2LY1 and CSPG4LYP1. However, specific deletions of these genes are unknown, so their possible roles as AZFc candidate genes remain uncertain.

The candidate genes in the AZFc deletion interval are DAZ, BPY2, and CDY1. The *BPY2* gene (Spermatogenic failure Y-linked 2) is in the non-recombinant portion of the Y chromosome, and is expressed exclusively in the testes. The encoded protein interacts with ubiquitin protein ligase E3A, and may function in the development of male germ cells and male infertility. The Y chromosome has 3 nearly identical copies of this gene, and 2 copies are in a palindromic region. However, our analysis of the BPY gene cluster indicated only 2 SCOS patients had total deletion (6 patients had *BPY2* deletion, 3 patients had *BPY2B* and *BPY2C* deletion). By contrast, our aCGH experiments identified *CDY1* and *DAZ* are the highly overlapping recurrent deletions in SCOS patients. However, we found the presence of DAZ1 expression in spermatogonia, early spermatocytes (Fig. 3 C). Deletions of the *DAZ* gene are the most common cause of azoospermia, but do not correlate with total spermatogenic failure. This may be because of compensatory expression of the different DAZ proteins, which may have different functions [16].

Many studies of the DAZ genes have documented their expression in germ cells and showed that all 4 copies in this gene family (*DAZ1*, *DAZ2*, *DAZ3* and *DAZ4*) are in AZFc [17–19].

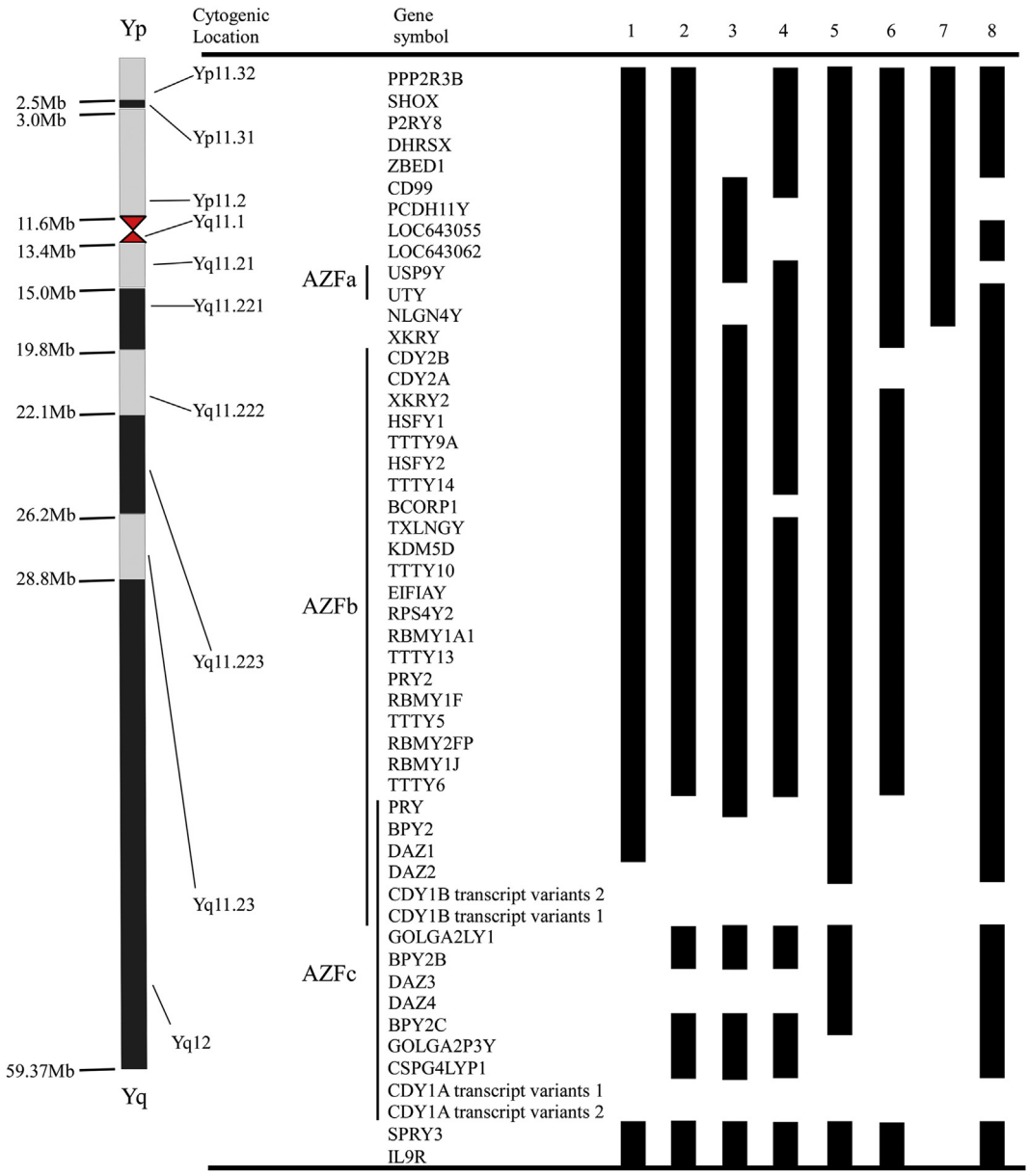

Fig. 2 **Schematic representation of the deletions in the eight patients with sertoli cell only syndrome through array CGH analysis.** The human Y chromosome shows the AZF loci between Y chromosome Yq11.223 to Yq11.23, associated genes. The aCGH analysis was performed using a Chromosome Y Tiling 385K Array, with average probe spacing of ~20 Kb and probe length of 50–75 mer. Common deletion in all eight cases are *CDY1a* gene and *CDY1b* gene.

Table 1 The distribution of AZFc deletion in the eight patients.

	DAZ1	DAZ2	DAZ3	DAZ4	CDY1	CDY1B	BPY2	BPY2B	BPY2C	CSPG4LYP1	GOLGA2LY1
1	+	−	−	−	−	−	+	−	−	−	−
2	−	−	−	−	−	−	−	+	+	+	+
3	−	−	−	−	−	−	−	+	+	+	+
4	−	−	−	−	−	−	−	+	+	+	+
5	+	+	+	+	−	−	+	+	+	−	+
6	−	−	−	−	−	−	−	−	−	−	−
7	−	−	−	−	−	−	−	−	−	−	−
8	+	+	+	+	−	−	+	+	+	+	+

+: Present, −: Deleted

Table 2 Comparative analysis of CDY1, DAZ, *BPY2*, *CSPG4P1Y* and *GOLGA2LY1* expression in adult human testis.

	Normal spermatogenesis (n = 15)	SCOS (n = 19)	*p* value
CDY1 expression rate	100% (15/15)	0% (0/19)	<0.001
DAZ expression rate	100% (15/15)	5.2% (1/19)	<0.001
BPY2 expression rate	100% (15/15)	78.9% (15/19)	0.141
CSPG4P1Y expression rate	100% (15/15)	84.2% (16/19)	0.238
GOLGA2LY1 expression rate	100% (15/15)	57.8% (11/19)	0.008

Expression rate (positive expression number/totally number).
n = number.
Chi-square or Fisher's exact test, and false discovery rate (FDR) correction by Benjamini and Hochberg method for multiple comparisons.

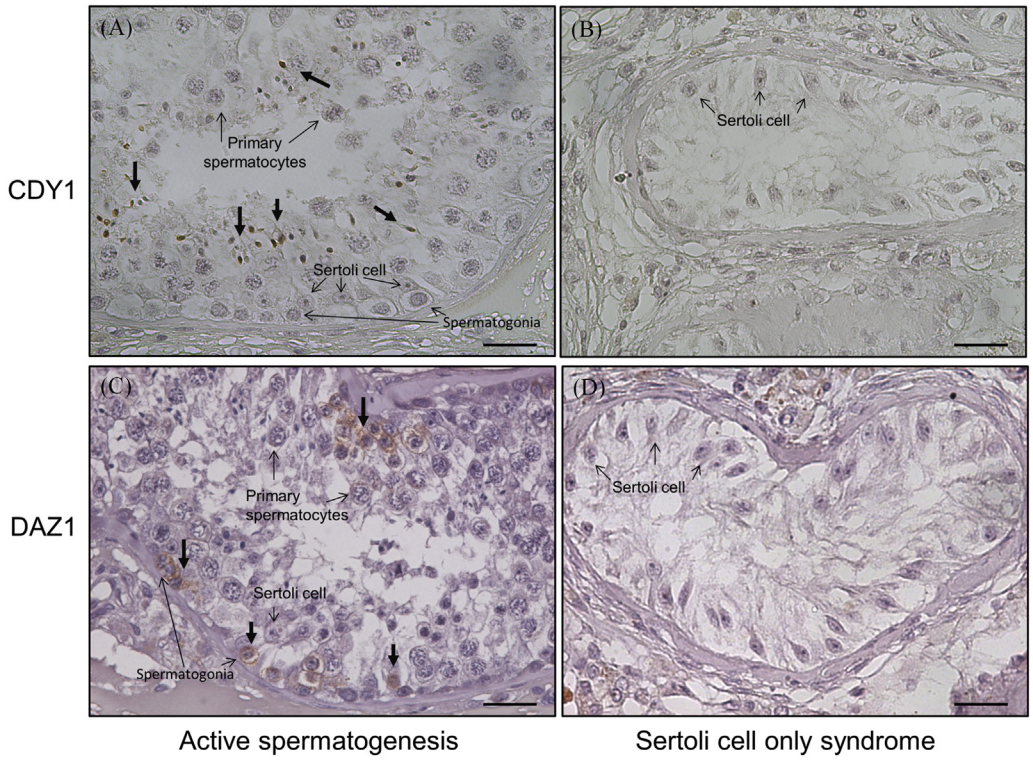

Fig. 3 **The localization and expression of CDY1 and DAZ1 proteins in normal (A, C) and SCOS (B, D) testes were analyzed by immunohistochemical analysis.** The sections were incubated with the anti-CDY1 (A, B) and -DAZ1 (C, D) primary antibodies, respectively. After addition of the appropriate secondary IgG antibody, sections were incubated with DAB and counterstained with Gill's hematoxylin. Note that CDY1 expression was predominantly observed in the late-stage germ cells (A) and DAZ1 immunostaining was present in spermatogonia and early-stage spermatocytes(C). No CDY1 or DAZ1 expression were detected in SCOS testes (B, D). Magnification × 400. bar = 20 μm.

DAZ gene copy deletions, in combination with alterations in other proteins, impact spermatogenesis [20,21], and this is a common cause of infertility in human males. DAZ is expressed in spermatogonia, in early- and late-stage spermatocytes, and in postmeiotic germ cells up to mature sperm cells. *DAZ* genes, encoding for RNA binding proteins, contain a highly conserved RNA recognition motif and at least one *DAZ* repeat encoding for a 24 amino acids able to bind other mRNA binding proteins and function as adaptors for target mRNA transport and activators of their translation [21]. Both distinctive processes of gametogenesis, the determination/specification of germ cells and meiosis, appear controlled by the *DAZ* family proteins through the post-translation regulation of germline-specific proteins at various levels, including the transport, degradation/stabilization, and transduction of the coding mRNAs [21]. DAZ is involved in RNA translation during sexual differentiation of XY germ cells, acting as an antagonist of NANOS, another essential protein required for meiosis initiation [22]. Besides, DAZ also acts as a master regulator of a post-transcriptional mRNA program essential for germ cell survival [23,24].

Interestingly, CDY1 immunostaining was present in late-stage germ cells of specimens from men with obstructive azoospermia (but normal spermatogenesis) (Fig. 3A). CDY1 is one of the candidate genes for male dyszoospermia related to YCM [25]. The human *CDY* gene encodes for the chromodomain Y-linked protein, whose expression is testes-specific. There are two identical copies of this gene within the AZFc region (*CDY1A*

Table 3 Summary of studies focusing on Y chromosome gene for the investigation of NOA azoospermia.

	Phenotype (number of patients)	Method of analysis	Identified gene deletions in the Y-chromosome,
Our study	SCOS (27)	Y chromosome aCGH RT-PCR, immunohistochemistry	*DAZ1/2 and DAZ3/4, CDY1, BPY2, CSPG4LYP1, GOLGA2LY1*
Heydarian et al., 2016	NOA (29)	(qRT-PCR) and ELISA assay	*CDY1*
Vijesh et al., 2015	NOA (120)	Multiplex PCR	*DAZ and CDY*
Kumari et al., 2015	Azoospermia (34)	STS mapping TaqMan Assays.	*SRY, DAZ and BPY2*
Malcher et al., 2013	Various types of NOA (18)	Microarrays quantitative polymerase chain reaction Western blot and immunohistochemistry	NO identified gene in the Y chromosome
Tuttelmann et al., 2011	SCOS(29)	244A/400K array −CGH (median probe spacing 8.9Kb-5.3 Kb	Sex-chromosomal, mostly private CNVs were significantly overrepresented in patients with SCOS. NO identified gene in the Y chromosome
Kleiman et al., 2011	SCOS(21)	histological RT-PCR	*CDY1 and BOULE*
Ravel et al., 2009	Azoospermia (5)	PCR	*DAZ1/2 and DAZ3/4, CDY1*
Kleiman et al., 2003	SCOS (26)	Multiplex PCR	*CDY1*
Kleiman et al., 2001	SCOS (21)	histological RT-PCR	*CDY1 DAZ, RNA-binding motif (RBM)*
Ferlin et al., 2001	SCOS(6)	PCR	*CDY1 and DAZ*

and *CDY1B*) and a pair of closely related genes in the P5 palindrome (*CDY2A* and *CDY2B*) [26]. The mouse CDY-like (Cdyl) gene is the homolog of human *CDY*. The protein products of either human *CDY/CDYL* or mouse *Cdyl* genes share high similarity. The protein contains a chromatin organization modifier (chromodomain) domain and a histone acetyltransferase domain [26]. Chromodomains are structural components of heterochromatin-like complexes that can act as gene repressors. In mammals, dramatic chromatin remodeling occurs during spermiogenesis, whereby histones are displaced from chromatin, first by transition proteins and later by protamines [30]. A major reduction in total CDY protein might interfere with the histone-to-protamine transition during spermiogenesis, thus contributing to overall spermatogenic failure [26]. Liu et al. demonstrate that the chromodomain Y-like protein CDYL acts as a crotonyl-CoA hydratase to regulate histone crotonylation negatively. This activity is implemented to react to sex chromosome-linked genes and histone replacement during spermatogenesis [31].

Germline deletion of Cdyl causes teratozoospermia and progressive infertility in male mice [27]. The mouse CDYL protein is abundantly expressed in the nuclei of step 9−12 spermatids [26] and the human CDY1 protein is present in the nucleus of late spermatids (mature spermatids/spermatozoa), where histone hyperacetylation takes place [28]. This implies that CDY1 plays an important role in late spermatogenesis [29]. Thus, *CDY1* deletion is a plausible mechanism for spermatogenic failure. Kleiman et al. report *CDY1* transcripts have the best correlation with complete spermatogenesis [32]. The presence of *CDY1* expression as a predictive tool for sperm retrieval has been described not only by the Kleiman et al. but also by Heydarian et al. [29]. They found the expression level of CDY1 was a potential marker for prediction of sperm recovery in NOA. Ravel et al. determine the relationship between various forms of partial AZFc deletions and spermatogenic failure [33].

We found that *CDY1a* or *CDY1b* deletion alone is common in fertile or infertile men with mixed ethnic origin and heterogeneous phenotype. Furthermore, CDY1a and CDY1b double deletions may be associated with SCOS and defective spermatogenesis. Azoospermia is uncommon but not rare, occurring in about 1% of men in the general population and 10% of men receiving care in infertility centers [8]. However, the prevalence of SCOS of NOA in the overall population is estimated to be very low [34]. This is the first study that uses Y chromosome array-based comparative genomic hybridization (aCGH) to examine a population of males with a uniform SCOS infertility phenotype [28,29,32,33,35−40] (Table 3). Deletion screening by array-CGH has many potential advantages over the multiplex PCR systems. So far as is known, only limited study has been published reporting on the use of microarray technology for the detection of Y chromosome deletions [6,7,25,41]. While the high cost of microarrays may initially make their use prohibitive in a routine clinical laboratory, it is expected that the cost will decrease as the technology becomes further developed and more widely applied.

Conclusions

In conclusion, testicular histopathological results provide the strongest evidence for successful TESE, but AZF deletion analysis also has potential prognostic value. Our findings indicate that *CDY1* deletion in SCOS patients, and analysis of

the expression of *DAZ* and *CDY1* genes using aCGH and real-time quantitative RT-PCR, may be useful to predict the presence of mature spermatozoa.

Ethics approval and consent to participate

Written informed consent was obtained from all patients, and this study was approved by the Ethics Committee of CGMH and the local institutional review board (CGMH 96–1363B and CGMH 97–2399).

Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Conflicts of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bj.2022.03.009.

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