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ORIGINAL RESEARCH

DAX-1 and DAX-1A expression in human testicular tissues with primary spermatogenic failure

M.C. Lardone[1], A. Parada-Bustamante[1], M. Ebensperger[2], R. Valdevenito[3], E. Kakarieka[4], D. Martínez[1], R. Pommer[1], A. Piottante[5], and A. Castro[1,*]

[1]Institute of Maternal and Child Research, School of Medicine, University of Chile, Santiago P.C. 8360160, Chile [2]Department of Urology, San Borja Arriaran Clinical Hospital, Santiago P.C. 8360160, Chile [3]Department of Urology, José Joaquín Aguirre Clinical Hospital, School of Medicine, University of Chile, Santiago C.P. 8380456, Chile [4]Department of Pathology, San Borja Arriaran Clinical Hospital, Santiago P.C. 8360160, Chile [5]School of Medicine, Andres Bello University, Santiago P.C. 8370186, Chile

*Correspondence address. Tel: +56-2-9770850; Fax: +56-2-4247240; E-mail: acastro@med.uchile.cl

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ABSTRACT: DAX-1 [dosage-sensitive sex reversal-adrenal hypoplasia congenital (AHC) critical region on the X chromosome gene 1; *NR0B1*] is an orphan nuclear receptor that acts as a transcriptional repressor in adrenal/gonadal development, steroidogenesis and probably spermatogenesis. An alternatively spliced form called DAX-1A (*NR0B1A*) has been described in several tissues including the testis, and *in vitro* studies have shown an inhibitory effect on DAX-1 transcriptional function. We aimed to study the mRNA and protein expression of DAX-1 in testicular tissues of 65 men with primary spermatogenic failure [complete Sertoli cell only syndrome (SCOS), focal SCOS, maturation arrest and mixed atrophy] compared with 33 controls with normal spermatogenesis. As a novel finding, we observed intense immunostaining, not only in the nucleus of Sertoli cells, but also in pachytene spermatocytes and round spermatids. The quantitative mRNA expression of DAX-1 and DAX-1A was similar between cases and controls and was not associated with the levels of gonadotrophins and steroids. Moreover, DAX-1 transcript expression level was ~750-fold higher than DAX-1A, and there was a strong positive correlation between them ($r = 0.52$; $P < 0.001$). We conclude that, in addition to Sertoli cells, DAX-1/DAX-1A is expressed in germ cells from spermatogonia to round spermatids. Besides, the similar mRNA expression of DAX-1 and DAX-1A in testicular tissues from cases and controls does not support the involvement of DAX-1 in the etiology of primary spermatogenic failure. Finally, the low level of expression of the alternative transcriptional variant DAX-1A would not support its putative inhibitory function *in vivo*.

Key words: DAX-1 / DAX-1A / NR0B1 / NR0B1A / spermatogenic failure

Introduction

DAX-1 [dosage-sensitive sex reversal-adrenal hypoplasia congenital (AHC)-critical region on the X chromosome gene 1; *DAX1*] is an orphan nuclear receptor that plays an important role in adrenal development, gonadal function and steroidogenesis (Clipsham and McCabe, 2003; Lalli and Sassone-Corsi, 2003; Niakan and McCabe, 2005; McCabe, 2007). DAX-1 is expressed in several endocrine tissues of mouse, rat and human, including adrenal cortex, pituitary, hypothalamus and gonads (Yu *et al.*, 1998a). DAX-1 is also known to act as a transcriptional repressor of several genes encoding enzymes for the synthesis of steroid hormones in adrenal and gonadal tissues (Wang *et al.*, 2001; Lalli and Sassone-Corsi, 2003; Jo and Stocco, 2004). In addition, DAX-1 is a nucleocytoplasmic shuttling protein associated with ribonucleoprotein structures in the nucleus and polyribosomes in the cytoplasm (Lalli *et al.*, 2000). These findings suggest that DAX-1 plays an additional regulatory function in post-transcriptional processes.

Point mutations or deletions in *DAX1* cause an X-linked syndrome characterized by neonatal or childhood onset adrenal insufficiency, AHC and hypogonadotropic hypogonadism (HHG), which is diagnosed later in life by the absence of pubertal development. HHG is thought to be caused by disorders at both the hypothalamic and pituitary levels (Tabarin *et al.*, 2000); affected males, however, may have an intrinsic defect in spermatogenesis that is not responsive to gonadotrophin therapy (Seminara *et al.*, 1999). In mice, disruption of Dax-1 causes progressive degeneration of the testicular germinal epithelium until complete loss of germ cells after 14 weeks, while maintaining levels of gonadotrophins and testosterone production comparable with those of wild-type mice, suggesting

primary testicular failure rather than a dysfunction at the pituitary level (Yu et al., 1998b).

In the human testis, it has been shown that DAX-1 expression is regulated during development, and in the adult testis it is mainly restricted to Sertoli cells and a weaker expression may be observed in Leydig cells (Kojima et al., 2006). Moreover, diminished DAX-1 mRNA testicular expression has been found in azoospermic patients with maturation arrest and SCOS compared with normal tissues, suggesting a critical role in spermatogenesis (Kojima et al., 2006). In sexually mature rats, it has been shown a stage-specific pattern with intense immunostaining in Sertoli cells during spermatogenesis stages VII–XII, coinciding with the stages of major events in spermatogenesis (Tamai et al., 1996; Kojima et al., 2006).

An expanded transcription regulatory network under DAX-1 has been evidenced by the finding of an alternatively spliced variant called *DAX1A* (Ho et al., 2004; Hossain et al., 2004), and the formation of DAX-1 homodimers and heterodimers with a number of transcription factor partners, including DAX-1A and SHP (Iyer et al., 2006; McCabe, 2007). DAX-1A is encoded by exons 1 and 2A of *DAX1*, with exon 2A located within the *DAX1* intron 1. DAX-1A transcript expression has been observed in several steroidogenic tissues, including adrenal gland, ovary and testis (Ho et al., 2004; Hossain et al., 2004; Nakamura et al., 2009). Comparison between DAX-1 and DAX-1A transcript expression has shown contradictory results, but the evidence points to DAX-1 as the major isoform present in steroidogenic tissues (Ho et al., 2004; Hossain et al., 2004; Nakamura et al., 2009).

The aim of the present study was to elucidate the testicular transcripts and protein expression of *DAX1* in testicular tissue of men with primary impairment of spermatogenesis. We studied the cellular localization of DAX-1/DAX-1A by immunohistochemistry and quantified the transcriptional expression of both transcripts by quantitative real-time RT–PCR (qPCR) in testicular tissue of patients with SCOS, maturation arrest and mixed atrophy compared with controls.

Materials and Methods

Testicular samples and human steroidogenic tissues

Testicular tissues were obtained from azoospermic or severe oligozoospermic infertile patients (<5 million sperm/ml) who underwent testicular biopsy for sperm retrieval at the Institute of Maternal and Child Research, and at the José Joaquín Aguirre Hospital of the University of Chile. We also obtained testicular tissues from normozoospermic patients who underwent testicular biopsy during genital surgery procedures for non-neoplastic disorders such as hydrocele, varicocele or epididymal cysts. All subjects gave their informed consent, and the study was approved by the Institutional Review Boards of the University of Chile, School of Medicine, San Borja Arriarán and José Joaquín Aguirre Hospitals.

All participants underwent a complete physical examination, semen analysis (according to WHO criteria) (WHO, 2001), karyotype and study of Y chromosome microdeletions. Testis volume was measured by ultrasonography and/or Prader orquidometer. Patients with abnormal karyotype, Y chromosome microdeletion, HHG and chronic diseases, in addition to those undergoing hormonal treatments or who had been exposed to alcohol or drugs were excluded from the study.

A piece of testicular tissue was fixed in Bouin's solution for 6 h at room temperature and 6-μm-thick paraffin sections were stained with hematoxylin-eosin, periodic acid-Schiff, Masson (trichomic) and orcein staining for histopathology. The evaluation included a qualitative and quantitative analysis of germinal epithelium in 25 seminiferous tubules, the calculation of the modified Johnsen Score (JS) (Johnsen, 1970; Jezek et al., 1998), the counting of Leydig cells per cluster and Sertoli cells per tubule.

Among cases with spermatogenic failure, the most common andrological abnormalities were mild or moderate varicocele (16%) operated at least 2 years before the study, cryptorchidism corrected during childhood (16%) and 14% of cases showed no other testicular alteration than decreased testicular volume.

We also collected adrenal tissue from an 82-year-old woman and from a 2 day-old new born who died of unrelated causes from the Pathology Department of San Borja Arriaran Clinical Hospital. In addition, two corpora lutea from mid-luteal phase were generously provided by the laboratory of Reproductive Endocrinology at the Institute of Maternal and Child Research for total RNA and protein extraction.

Hormonal analysis

Serum LH, FSH and sex hormone-binding globulin (SHBG) were measured by immunoradiometric assays (Diagnostic Product Corporation, CA, USA). Total testosterone and estradiol (E_2) were measured by radioimmunoassays (Testosterone RIA DSL-4100 and Estradiol RIA DSL-4400, Diagnostic System Laboratories, Webster, TX, USA) in blood samples obtained between 8 and 10 a.m. Intratesticular testosterone and E_2 were measured by radioimmunoassay as described previously (Lardone et al., 2010).

Immunohistochemistry

Immunohistochemistry was performed on 6-μm-thick paraffin sections mounted on silanized slides of the same testicular pieces of tissues fixed for histology assessment. After deparaffination, the sections were immersed in 10 mM citrate buffer (pH 6.0) and heated at 121°C for 7 min. Non-specific staining was avoided by incubating with H_2O_2 30% (diluted 1:10 in methanol) for 10 min, followed by successive incubation with bovine serum albumin (2% in phosphate-buffered saline) for 30 min, then with Biotin Blocking System (Dako, Glostrup, Denmark) and finally with blocking solution (Histostain® SP Broad Spectrum, Zymed® Laboratories, Invitrogen, San Francisco, CA, USA). Subsequently, sections were incubated at 37°C for 1 h with anti-DAX-1 rabbit polyclonal immunoglobulin G (IgG) antibody sc-841 (Santa Cruz Biotechnology, Santa Cruz, CA, USA, diluted at 2 μg/ml) or anti-DAX-1 ab60144 (Abcam, Cambridge, UK, diluted at 20 μg/ml). Both anti-DAX-1 antibodies are directed against the N-terminus of human DAX-1, which is common to both DAX-1 and DAX-1A proteins.

To assess the specificity of the immunoreaction, we used different negative controls: omitting of primary antibody, the primary antibody preabsorbed with an excess of purified antigen (sc-841P, Santa Cruz Biotechnology) at a ratio of 1:10, or normal rabbit IgG (sc-2027, Santa Cruz Biotechnology) at the same concentration of DAX-1 antibody. Subsequently, broad spectrum biotinylated secondary antibody and streptavidin-horseradish peroxidase was used following the manufacturer's specifications (Histostain® SP Broad Spectrum, Zymed® Laboratories, Invitrogen).

The H-Score approach was used to accurately describe the extent of immunohistochemical staining of the different types of somatic and germ cells of cases and controls. One representative slide of every case was selected and the cells of 10 to 15 seminiferous tubules and five to eight clusters of Leydig cells were assessed using an intensity grade scale ranging from 0 for no staining, 1 for weak, 2 for moderate and 3 for intense staining.

Total RNA isolation and cDNA synthesis

At the time of testicular biopsy, two additional pieces of testicular tissue (16–36 mm^3) contiguous to that used for histology assessment were stored in liquid nitrogen until total RNA extraction was carried out using standard methods (TRIzol Reagent, Invitrogen Life Technologies, Carlsbad, CA, USA). Subsequently, total RNA was treated with recombinant Deoxyribonuclease I (DNA-free kit, Ambion, Austin, TX, USA) for DNA removal. RNA was quantified by absorbance at 260 nm (ND-1000 Spectrophotometer, NanoDrop, Wilmington, DE, USA) and assessed by observing the preservation of 28S and 18S ribosomal RNA species. The synthesis of complementary DNA (cDNA) was performed following the manufacturer's specifications (RevertAid^{TM} H Minus M-MulV, MBI Fermentas, Burlington, ON, Canada) using aliquots of 1 μg of RNA. cDNA samples were stored at −20°C until use.

Quantitative real-time PCR analysis

The mRNA expression level of *DAX1* and *DAX1A* and the internal control gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), were determined using a standard curve method for absolute quantification by real-time PCR and SYBR Green. Primer sequence for DAX-1 (Hossain *et al.*, 2004) and DAX-1A (Ho *et al.*, 2004) transcripts were previously reported. For the amplification of GAPDH transcript, specific primers were designed using Primer 3 Software (Rozen and Skaletsky, 2000) (Table I).

PCR was performed using Platinun® SYBR® Green qPCR Super Mix-uracil-DNA glycosylase (UDG) reactive (Invitrogen Life Technologies) in a Chromo4 detector (MJ Research Inc., St Bruno, QC, Canada). All PCR reactions were carried out in triplicate, in a 20 μl final volume containing: 10 μl Platinun® SYBR® Green qPCR Super Mix-UDG with 3.0 mmol/l $MgCl_2$; 1 μl 500 nmol/l (DAX-1) or 400 nmol/l (DAX-1A) forward plus reverse primers; 1 μl or 3 μl cDNA for DAX-1 or DAX-1A transcripts amplification respectively; and diethylpyrocarbonate-treated water up to 20 μl. cDNA samples for GAPDH transcript amplification were diluted 1:5. PCR profile was identical for the three pair of primers: after an initial heating at 50°C for 2 min (UDG incubation) and denaturation at 95°C for 5 min, temperature cycling was initiated. Each cycle consisted of denaturation at 95°C for 15 s, hybridization at 57°C (DAX-1 and GAPDH primers) or 59°C (DAX-1A primers) for 30 s and elongation at 72°C for 15 s. The fluorescence signal was acquired at the end of the elongation step. A total of 40 cycles were performed.

Standard curves were generated from serial dilutions (10^1 to 1×10^7 copies/μl) of a synthesized plasmid that contains an identical fragment to that amplified in the samples. The amount of mRNA present in the original RNA extract was determined by interpolation on the standard curve. To compensate for variation in RNA amount and reverse transcription efficiency, the copy number of DAX-1 and DAX-1A transcripts was normalized to that of GAPDH. Intra-assay coefficients of variation (CV) were <1.5% according to the C_t value accepted, and the inter-assay CV were 1.7%, 3.0 and 2.0% for GAPDH and DAX-1 and DAX-1A transcripts expression, respectively.

Table I Primers for quantitative real-time RT–PCR.

Primer	Sequence	Fragment length (bp)
DAX-1A forward	CTACCTCAAGGGGACCGTCCT	237
DAX-1A reverse	ATGCTGACTGTGCCGATGATG	
DAX-1 forward	TCCAAATGCTGGAGTCTGAAC	222
DAX-1 reverse	AGGAGGCTGCTTTTGAAGG	
GAPDH forward	AGCCGCATCTTCTTTTGC	163
GAPDH reverse	AATGAAGGGGTCATTGATGG	

Statistical analysis

Differences among groups were tested by Kruskal–Wallis test, multiple pairwise comparisons were performed using Mann–Whitney *U*-test, and correlations between continuous variables were tested by Spearman's test using the SPSS version 11.5. *P*-values <0.05 were considered statistically significant.

Results

Patients

Among 91 azo/oligozoospermic infertile patients, 65 were diagnosed as non-obstructive azoospermia, of which 26 were diagnosed as complete Sertoli cell only syndrome (SCOS), 12 focal SCOS (>60% tubules with only Sertoli cells and some with complete spermatogenesis), 16 spermatogenic arrest at different stages of maturation (1 at spermatogonia, 10 at spermatocyte and 5 at round spermatid) and 11 mixed atrophy (mixture of the earlier-mentioned types of tubular histology with hyalinization of some tubules). Obstructive azoospermia was diagnosed in 26 infertile patients because their histological finding was complete spermatogenesis (all the tubules evaluated had complete spermatogenesis or elongated spermatids at least, JS ≥ 8). In addition, seven normozoospermic subjects were recruited and all had normal testicular histology.

Additional histological evaluation is summarized in Table II.

Hormonal profile

No differences were found in the hormonal parameters between obstructive azoospermic controls ($n = 26$) and normozoospermic controls ($n = 7$); so they were analyzed as a single group. The serum and intratesticular hormonal profile of cases and controls is shown in Tables III and IV, respectively.

Correlations between serum LH or FSH, and DAX-1 mRNA expression showed a negative relationship among cases ($r = -0.245$; $P = 0.053$ and $r = -0.306$; $P = 0.015$, respectively) (Fig. 1). In contrast, no correlation was observed between DAX-1 transcript expression and serum or intratesticular testosterone and E_2. In the case of DAX-1A transcript, no correlation with gonadotrophins or steroids was observed.

Protein expression of DAX-1/DAX-1A in testicular tissue

We examined the localization of DAX-1/DAX-1A immunoexpression in testicular tissues of patients with impairment of spermatogenesis and in controls. A positive immunoreaction was observed in almost all nuclei of Sertoli cells, spermatogonia, round spermatids and Leydig cells, and in the cytoplasm of pachytene spermatocytes in both cases and controls. On the other hand, no staining or weak staining was observed in the cytoplasm of Sertoli and Leydig cells (Fig. 2). The summary of immunostaining assessment for DAX-1/DAX-1A by H-Score is shown in Table V.

In order to corroborate the detection of immunoexpression in germ cells, a different anti-DAX-1 antibody was assayed (ab60144, Abcam, Cambridge, UK), which also detected intense

Table II Histological evaluation in 25 seminiferous tubules.

	No. of biopsies	Johnsen Score	Leydig cells/cluster	Sertoli cells/tubule	% of atrophic tubules
cSCOS	26	2.0 ± 0.0	14 ± 5	23 ± 9	0.8 ± 2.5
fSCOS	12	2.5 ± 0.5	11 ± 6	23 ± 6	1.0 ± 1.8
MA	16	4.8 ± 1.4	7.0 ± 4	13 ± 4	1.5 ± 6
MxA	11	2.7 ± 0.8	14 ± 3	10 ± 5	40 ± 19
NS	33	8.7 ± 0.4	5.0 ± 3	13 ± 3	0

Values are expressed as mean ± SD. Atrophic tubule: no cells in the tubular section. cSCOS, complete Sertoli cell only syndrome; fSCOS, focal SCOS; MA, maturation arrest; MxA, mixed atrophy; NS, normal spermatogenesis.

Table III Serum hormonal profile in cases and controls.

	Normal spermatogenesis	Spermatogenic failure			
		cSCOS	fSCOS	MA	MxA
n	33	26	12	16	11
Age (years)	34 (18–46)	34.0 (19–42)	33 (23–40)	31 (26–35)	36 (27–48)
FSH (mIU/ml)	2.6 (1.5–7.6)	17.5 (8–28)*	11.7 (3.5–25.1)*	8.2 (1.9–23.6)*	22.9 (7.2–9)*
LH (mIU/ml)	2.5 (1–5)	5.1 (2–9.4)*	4.0 (1.8–10.3)*	3.0 (15–6.2)	6.1 (2.5–24)*
Testosterone (ng/ml)	3.5 (2–5.1)	3.5 (1.8–5.1)	2.6 (1.7–4.5)*	3.3 (1.9–6)	2.7 (0.8–4.9)
Estradiol (pg/ml)	38.4 (22.9–70)	33.0 (19–54)	43.8 (25–69)	40.5 (24–78)	34.5 (23–47)
Testosterone/LH ratio	4.7 (2.3–9.5)	2.7 (0.9–6.7)*	2.4 (0.8–6.4)*	4.1 (1.4–10)	2.1 (0.2–3.7)*

Values are expressed as median (2.5–97.5 percentiles).
cSCOS, complete Sertoli cell only syndrome; fSCOS, focal SCOS; MA, maturation arrest. MxA, mixed atrophy.
*$P < 0.05$ compared with normal spermatogenesis. Reference values: FSH 1.0–7.0 mIU/ml; LH 1.0–8.0 mIU/ml; testosterone 2.0–8.0 ng/ml; estradiol up to 60 pg/ml; SHBG 10–80 nmol/l.

Table IV Intratesticular testosterone and E_2 concentrations in cases and controls.

	Normal spermatogenesis obstructive controls	Spermatogenic failure			
		cSCOS	fSCOS	MA	MxA
n	8	14	8	3	8
Testosterone (ng/mg protein)	14 (2.5–60)	26 (0.8–183)	41 (3–71)	40 (38–44)	81 (22–279)
Estradiol (pg/mg protein)	49 (5–116)	95 (17–2083)	95 (4–349)	143 (105–198)*	329 (108–1681)*
Estradiol/testosterone (pmol/nmol $\times 10^{-3}$)	2.6 (1.12–6)	8 (1.5–49)*	2 (0.7–9)	4 (1.3–5)	5 (2.5–12)

Values are expressed as median (2.5–97.5 percentiles).
*$P < 0.05$ compared with normal spermatogenesis.

immunoreaction in the nucleus of Sertoli cells and round spermatids, and in the cytoplasm and nucleus of pachytene spermatocyes (Fig. 2C and D).

Transcriptional levels of *DAX1* and *DAX1A*

We performed real-time RT-PCR with standard curves to assess the absolute quantification of DAX-1 and DAX-1A mRNA expression. The primers used for the detection of these two transcripts did not cross-react, were specific for mRNA and produced a single peak in the melting curves. Both PCR products were sequenced, and using the BLAST alignment tools, we corroborated that DAX-1A amplicons matched to the sequence proposed by Ho *et al.* (2004) for the boundaries of exons 1 and 2A. No amplification was observed in breast, placental and lever human tissues (data not shown).

The copy number of GAPDH transcript and the normalized copy number of DAX-1 and DAX-1A transcripts were similar between obstructive and normozoospermic controls, ($P = 0.078$, $P = 0.301$, $P = 0.792$, respectively), so that they were analyzed as a single group. The copy number of GAPDH mRNA/μg total RNA was similar among the different histological groups ($P = 0.351$, Kruskal–Wallis test).

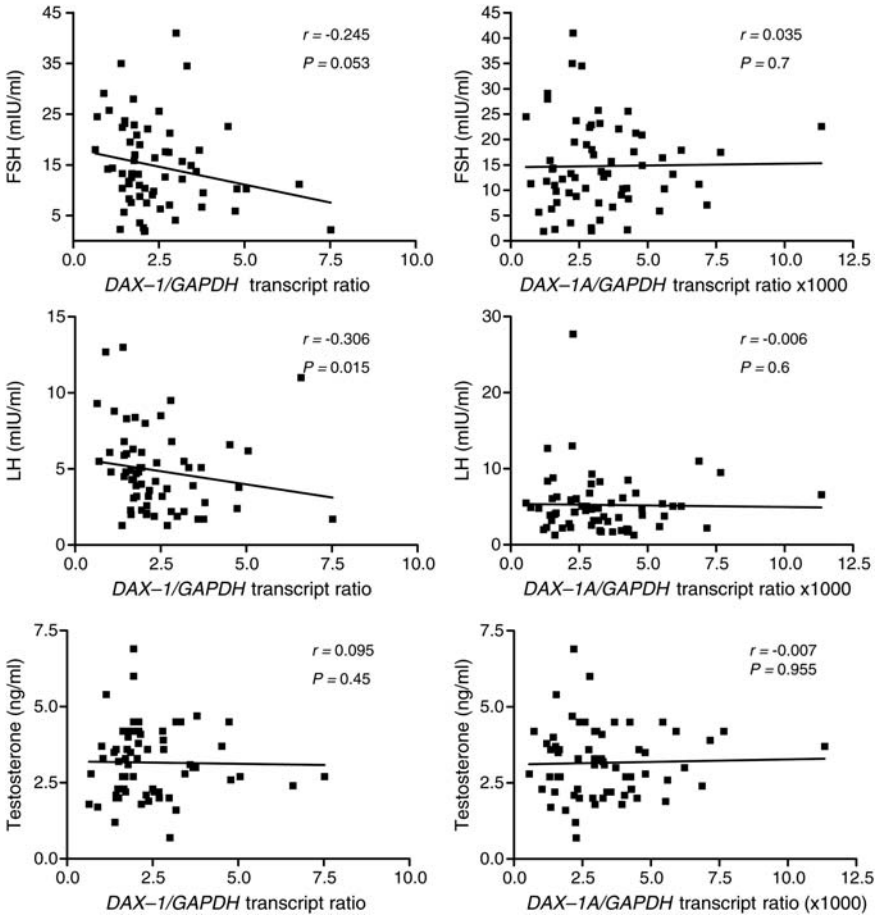

Figure 1 Correlation between *DAX1* or *DAX1A* mRNA expression levels and serum FSH, LH and testosterone concentrations in patients with primary spermatogenic impairment.

The median mRNA expression level of DAX-1 normalized by GAPDH was significantly lower in the mixed atrophy group compared with controls ($P = 0.017$), whereas the other groups did not show any difference. On the other hand, the normalized mRNA expression level of DAX-1A was higher in the complete SCOS group compared with controls ($P = 0.024$), whereas mixed atrophy patients had decreased DAX-1A mRNA expression ($P = 0.002$) (Fig. 3).

In general, DAX-1A mRNA levels were significantly lower (~750-fold) than those of DAX-1; however, there was a strong positive correlation in the expression level of these two transcripts (Fig. 4). In other steroidogenic tissues, we also observed that the transcriptional expression of DAX-1A is ~1000-fold less than the expression of DAX-1 (423 copies/μg RNA versus 444 900 copies/μg RNA in corpus luteum; 94 copies/μg RNA versus 43 760 copies/μg RNA in the adult adrenal gland and 323 copies/μg RNA versus 599 400 copies/μg RNA in the fetal adrenal gland).

In addition, we observed that the mRNA expression level of DAX-1 and DAX-1A correlated positively with the number of Sertoli cells/tubule among cases with spermatogenic impairment ($r = 0.298$, $P = 0.017$ and $r = 0.497$, $P = 0.001$, respectively), whereas this correlation was not observed in tissues with normal spermatogenesis.

Discussion

The present study examined the pattern and level of expression of the transcription factor DAX-1, and the more recently described spliced variant DAX-1A, in human testicular tissues with spermatogenic impairment.

The intense expression of DAX-1 protein observed in the nucleus of Sertoli cells, independently of testicular histological pattern, is congruent with its functional role as a nuclear receptor in the establishment and maintenance of testicular function (Lalli and Sassone-Corsi, 2003), as well as a modulator of steroidogenesis, by inhibiting the transcriptional activity of steroidogenic factor-1 (SF-1) (Ito *et al.*, 1997; Lalli *et al.*, 1998; Gurates *et al.*, 2003), but our results do not suggest an association with spermatogenic defects.

Likewise, we also found a strong expression in germ cells; however, its function in these cells remains unclear. In the testis, gene transcription is maximum during the early stages of spermatogenesis, especially during the stages of spermatogonia and pachytene spermatocytes, whereas during the late phases, transcription declines and stored mRNAs are activated to synthesize proteins (Eddy and O'Brien, 1998; Schlecht *et al.*, 2004; Bettegowda and Wilkinson, 2010). *In vitro* studies have suggested that DAX-1 may have a role in RNA

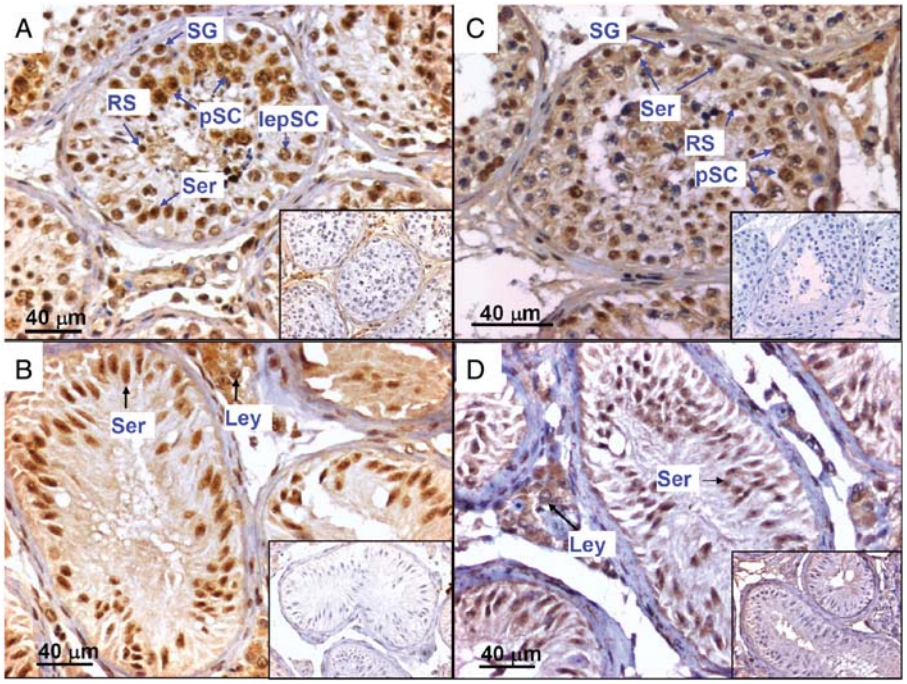

Figure 2 Immunohistochemistry of DAX-1 in human testicular tissue. Normal spermatogenesis (**A**) and SCOS (**B**) tissues immunostained with IgG anti DAX-1 (sc-841, Santa Cruz). DAX-1 is intensely expressed in the nucleus of Sertoli cells (Ser), round spermatids (RS) and in the nucleus and cytoplasm of pachytene spermatocytes (pSC); positive expression is observed in the nucleus of Leydig cells (Ley) and spermatogonia (SG). Immunostaining of testicular tissues with normal spermatogenesis (**C**) and SCOS (**D**) using a different anti-DAX-1 antibody (ab60144, Abcam) show positive immunoreaction in SG, RS, pSC, Ley and Ser. Arrows show representative positive cells. Negative isotype controls are shown in the insets.

Table V H-Score of DAX-1/DAX-1A immunoexpression in the nuclei of testicular somatic and germ cells.

	#	Spermatogonia	Pachytene spermatocyte	Round spermatid	Sertoli cell	Leydig cell
SCOS	11	1.3[a]	2.6[a]	1.95[a]	3.2 ± 0.4	2.7 ± 0.5
MA	6	2.3 ± 0.6	3.2 ± 0.5	2.7[b]	3.1 ± 0.4	2.4 ± 0.6
MxA	6	2.7 ± 0.0	3.3 ± 0.6	2.7 ± 0.0	3.3 ± 0.3	2.7 ± 0.5
NS	14	2.0 ± 0.5	3.2 ± 0.4	2.5 ± 0.8	2.8 ± 0.3	2.8 ± 0.5

Number of biopsies analyzed. Values represent the mean ± SD of H-Score in each group.
SCOS, Sertoli cell only syndrome; MA, maturation arrest; NS, normal spermatogenesis.
[a]Analyzed in two patients with focal SCOS.
[b]Analyzed in one patient with incomplete spermatocyte maturation arrest.

metabolism acting in the transport of messenger ribonucleoproteins to the cytoplasm and in translational control (Lalli *et al.*, 2000). In this context, we speculate that the intense localization of DAX-1 in pachytene spermatocytes and in round spermatids is congruent with a possible function of DAX-1 in post-transcriptional control. In addition, an *in vitro* study has shown that most of the severe testicular pathologies associated with *Dax-1* knock-out model remain unchanged when the expression of this protein is selectively restored in Sertoli or Leydig cells, suggesting that Dax-1 is required by other cell types in the male gonad (Yu *et al.*, 1998a, b; Jeffs *et al.*, 2001; Meeks *et al.*, 2003).

Although the pattern of expression of DAX-1 in the testis suggests an important function in spermatogenesis, our transcript expression studies do not suggest that DAX-1 or DAX-1A accounts for the failure of spermatogenesis. The finding that DAX-1 mRNA is decreased in tissues with mixed atrophy may be related to the fact that these tissues have ~40% of atrophic tubules (denuded of germ and Sertoli cells). In support of this view, a positive correlation was observed between mRNAs expression levels and the number of Sertoli cell/tubule in our cases, indicating that most DAX-1 mRNA expression emanates predominantly from Sertoli cells. On the contrary, in tissues with normal spermatogenesis, the contribution of germ cells eliminates this correlation.

Except for the report by Kojima *et al.* (2006), DAX-1 has not been studied in human testicular tissues with spermatogenic failure.

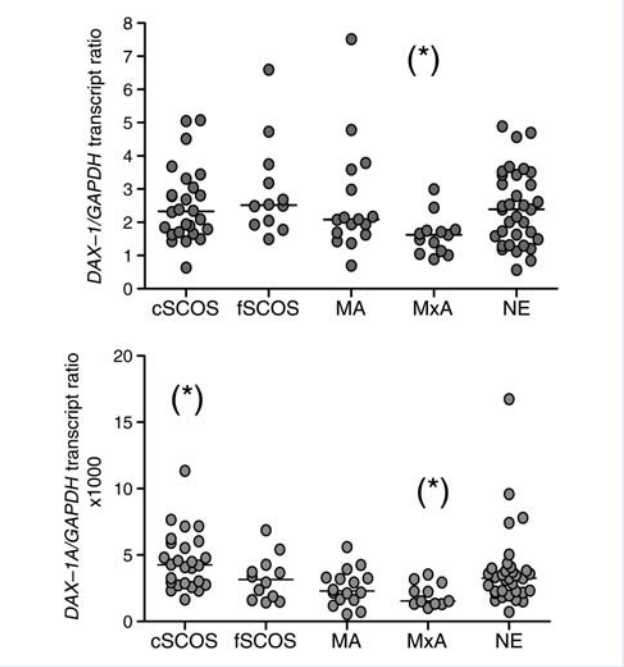

Figure 3 Absolute quantification of DAX-1 and DAX-1A transcript levels in different testicular histological groups by quantitative real-time PCR. *$P < 0.05$ compared with NS. cSOSC, complete Sertoli cell only syndrome; fSCOS, focal Sertoli cell only syndrome; MA, maturation arrest; MxA, mixed atrophy; NS, normal spermatogenesis.

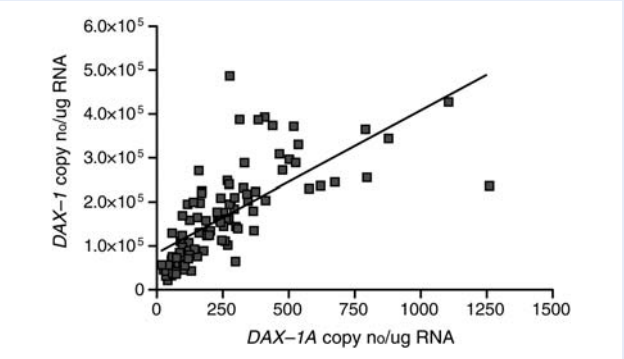

Figure 4 Correlation between DAX-1 and DAX-1A mRNA expression level in testicular tissue. A positive strong correlation is shown between the copy number of DAX-1 and DAX-1A mRNAs ($r = 0.834$; $P = 0.001$) in testicular tissues of cases and controls.

Interestingly, these authors found lower DAX-1 transcript expression in two unrelated pathologies, maturation arrest and SCOS, using a relative quantification method in a reduced number of patients. On the contrary, we used quantitative RT-PCR with standard curves in a large population of histological well-characterized patients, in an attempt to counteract the inherent dispersion of human samples.

The novel transcriptional variant DAX-1A has shown an inhibitory effect on DAX-1 *in vitro*, suggesting a role for DAX-1A in the regulation of adrenal and gonadal differentiation and function (Hossain *et al.*, 2004). In the same study, DAX-1A was unable to repress the SF-1-mediated induction of a reporter gene; instead DAX-1A increased StAR promoter-luciferase gene expression when SF-1 was present in low amounts, and relieved DAX-1 mediated repressor activity when it was cotransfected with SF-1 and DAX-1, although the amounts of DAX-1A construct were up to 10 times higher than those of DAX-1. In our study, the lower levels of the DAX-1A transcript compared with DAX-1 in testicular and in other steroidogenic tissues would not be congruent with an inhibitory effect of DAX-1A over DAX-1. However, we cannot exclude a functional effect of DAX-1A over some of the multiples inhibitory targets of DAX-1, i.e. in tissues with complete germ cell aplasia, where we observed a more uniform ratio of these two transcripts, depending upon the cellular compartment and promoter context. In this regard, opposite functions on SF-1-mediated transcription have been observed for Dax-1 depending on the doses of Dax-1 transfected in adrenal and Leydig cells, suggesting that gene dose is critical for regulatory influences on gene expression, and that the protein ratio may define the overall transcriptional output (Xu *et al.*, 2009).

Our secretory azoospermic patients showed serum testosterone levels within or near the lower normal range; however, their testosterone/LH ratio was significantly lower than controls in the most severe testicular phenotypes, suggesting Leydig cell dysfunction (Lardone *et al.*, 2010). To better understand the hormonal environment, we measured the intratesticular levels of testosterone and E_2. Tissues with impairment of spermatogenesis seem to have higher levels of both steroids, but this may be explained by a high concentration of intratesticular steroids per area of tissue, due to Leydig cell hyperplasia and reduced testicular volume. However, patients with complete SCOS showed an increased E_2/testosterone ratio, suggesting an overexpression of aromatase (Lardone *et al.*, 2010). Nevertheless, our analysis did not reveal a correlation between DAX-1 or DAX-1A mRNA expression and intratesticular hormones that would explain the increase in E_2 concentrations by a deregulation of aromatase expression. In fact, studying the same patients, we observed that CYP19 transcriptional expression does not correlate with DAX-1 or DAX-1A transcript expression levels (Lardone *et al.*, unpublished data).

Additionally, we observed a negative correlation between DAX-1 mRNA expression and serum LH and FSH in our cases; most cases with higher levels of gonadotrophins had a mixed atrophy histological pattern and lower number of Sertoli cells; therefore, we cannot conclude that there is a direct relationship between gonadotrophin levels and DAX-1 testicular transcript expression.

In conclusion, this is the first report regarding the expression of the two isoforms of DAX-1 in testicular tissues of men with spermatogenic impairment. Our results show that DAX-1/DAX-1A is not only expressed in Sertoli and Leydig cells, but also in the nucleus and/or cytoplasm germ cells, suggesting an additional function for this transcriptional factor. In addition, the similar gene expression of DAX-1 in testicular tissues from cases and controls, and the lack of significant correlation with hormonal markers of Leydig cell dysfunction, does not support the involvement of DAX-1 in the etiology of primary spermatogenic failure. Finally, the fact that the alternative transcriptional variant DAX-1A is expressed in a very low amount does not support its putative function *in vivo*.

Authors' roles

M.C.L. wrote the paper, performed PCR assays and contributed substantially to the design, statistical analyses and interpretation of the results. A.P.-B. and D.M. contributed substantially with immunoreaction techniques. M.E. and R.V. performed clinical evaluation and testicular biopsies of azo/oligozoospermic patients. R.P. collaborated in the recruitment and clinical evaluation of patients. E.K. contributed with non-testicular tissues. A.P. analyzed testicular biopsies. A.C. contributed with the conception of the study, direction of experimental protocols and revision of the manuscript.

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