

## CASE REPORT

## Detection of aromatase and estrogen receptors (ER $\alpha$ , ER $\beta_1$ , ER $\beta_2$ ) in human Leydig cell tumor

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### Abstract

A Leydig cell tumor is a rare neoplasm, deriving from the interstitial cells, whose pathogenesis has not been still defined. Leydig cells of normal adult testis are known as physiological targets for estrogens. However, some studies on transgenic rodents suggest a role of estrogens in the development of Leydig cell hyperplasia and Leydig cell tumor. Therefore, with the aim to evaluate a possible link between estrogens and testicular tumorigenesis, this study investigated the expression of aromatase and estrogen receptors (ER $\alpha$ , ER $\beta_1$ , ER $\beta_2$ ) in testes from two patients with Leydig cell tumor. A strong immunoreactivity for aromatase, ER $\beta_1$ , and ER $\beta_2$ , together with a detectable ER $\alpha$  immunostaining, was revealed in tumoral tissues. These findings were confirmed by western blot analysis of tumor extracts detecting a 55 kDa P450arom, a 67 kDa ER $\alpha$  band, a 59 kDa ER $\beta_1$  band, and a 53 kDa ER $\beta_2$  band. The pattern of ER expression in neoplastic cells appears different from that of control Leydig cells exhibiting only ER $\beta_1$  and ER $\beta_2$  isoforms. The authors hypothesize how the high estrogen production could play a role in the neoplastic transformation of Leydig cells, while the exclusive presence of ER $\alpha$  in tumoral cells could amplify estradiol-17 $\beta$  signaling contributing to the tumor cell growth and progression.

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### Introduction

A Leydig cell tumor is a rare neoplasm of the male gonadal interstitium, accounting for 2–3% of all testicular tumors (1). It is usually observed in adult men (30–60 years) but it may also occur in prepubertal boys. This tumor is generally associated with endocrine disturbance, as adulthood gynecomastia and precocious puberty in children (2, 3). A Leydig cell tumor is commonly benign, but ~10% malignant variants have been reported in adults. A malignant neoplasm is characterized by large size, marked nuclear pleomorphism, high mitotic rate, extensive necrosis, and vascular invasion (4, 5).

Estrogen receptor (ER) expression has been reported by different authors in normal Leydig cells of adult testis (6–8) indicating these cells as physiological targets for estrogens. Furthermore, cytochrome P450 aromatase, the enzyme catalyzing androgen aromatization into estrogens, has been detected in Leydig cells of normal human testis (9), suggesting a paracrine action of estrogens, locally produced, in the steroidogenesis control (10).

However, estrogens are able to regulate cell growth and apoptosis (11, 12); therefore, these hormones could also be involved in testicular neoplastic proliferation. A possible involvement of estrogens in tumorigenesis in the human male gonad has been suggested by the increased

incidence of testicular germ cell tumors after prenatal or occupational estrogen exposure (13–17). Conversely, the link between estrogens and the tumoral process is scarcely known in human testicular tumors originating from the gonadal stroma. However, some studies on transgenic rodents suggested a role of estrogens in the development of Leydig cell hyperplasia and Leydig cell tumor (18, 19).

The aim of the present work was to investigate the expression of aromatase and ER isoforms, ER $\alpha$ , ER $\beta_1$ , and ER $\beta_2$ , in testes from two patients with a Leydig cell tumor, a rare neoplasm deriving from the interstitial cells.

### Materials and methods

#### Patients

The investigation was performed on formalin-fixed and paraffin-embedded testis tissues from two male patients with Leydig cell tumors (aged 31 and 33 years). Control testicular tissues were obtained from two male patients (aged 29 and 35 years) showing testes with a sarcoidosis granulomatous-like lesion. The archival cases were provided by the Department of Medicine and Medical Specialties, University of Modena and

**Table 1** Hormonal and seminal patterns in Leydig cell tumor patients.

	Patient	Before orchi- dectomy	Two months later	Normal values
LH (mUI/ml)	I	5.8	12	1.4–8.9
	II	3.8	6.9	
FSH (mUI/ml)	I	3.3	6	1.7–6.9
	II	3.1	5.8	
PRL (ng/ml)	I	12	10	2.1–17.7
	II	11	12	
T (ng/dl)	I	298	498	260–780
	II	287	576	
E <sub>2</sub> (pg/ml)	I	108	30	0–52
	II	127	42	
E <sub>2</sub> /T × 100	I	0.36	0.06	/
	II	0.4	0.07	
Sperm count (×10 <sup>6</sup> /ml)	I	3.25	8	> 20
	II	4.75	5.5	
Sperm motility (%)	I	20	50	≥ 50
	II	20	60	

Reggio Emilia, Policlinico, Italy. The patients showed gynecomastia, normal follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), and testosterone (T) serum levels but high estradiol-17 $\beta$  (E<sub>2</sub>) levels, as well as oligoasthenozoospermia, before therapeutic orchidectomy. After orchidectomy, sperm motility and E<sub>2</sub> levels of patients were in the normal ranges (see collected data in Table 1).

The ethical committee members of the University of Calabria approved the investigation program.

### Morphological analysis

Paraffin-embedded sections, 5  $\mu$ m thick, were deparaffinized, dehydrated, and stained by hematoxylin–eosin.

### Antibodies

Anti-aromatase primary antibody was mouse monoclonal MCA2077 (Serotec, Oxford, UK), which recognizes epitope mapping at the C-terminus region of the human native P450arom. Anti-ER $\alpha$  primary antibody was mouse monoclonal F-10 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), which recognizes epitope mapping at the C-terminus region of the human native ER $\alpha$ . Anti-ER $\beta$ <sub>1</sub> primary antibody was mouse monoclonal MCA1974S (Serotec), which recognizes epitope mapping at the C-terminus regions of human native ER $\beta$ <sub>1</sub>. Anti-ER $\beta$ <sub>2</sub> primary antibody was mouse monoclonal MCA2279S (Serotec), which recognizes epitope mapping at the C-terminus regions of human native ER $\beta$ <sub>2</sub>. Rabbit polyclonal anti- $\beta$ -actin (Santa Cruz Biotechnology) was also used as a loading control. Biotinylated goat anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA, USA) and goat anti-mouse horseradish peroxidase-conjugated IgG (Amersham) were used as secondary antibodies.

### Immunohistochemical analysis

Paraffin-embedded sections, 5  $\mu$ m thick, were mounted on slides precoated with poly-lysine, and then they were deparaffinized and dehydrated (7–8 serial sections). Immunohistochemical experiments were performed after heat-mediated antigen retrieval (20). Hydrogen peroxide (3% in distillate water) was used, for 30 min, to inhibit endogenous peroxidase activity, while normal goat serum (10%) was utilized, for 30 min, to block the nonspecific binding sites.

Immunodetection was carried out using mouse monoclonal anti-aromatase (1:50), anti-ER $\alpha$  (1:50), anti-ER $\beta$ <sub>1</sub>, and anti-ER $\beta$ <sub>2</sub> (1:100) primary antibodies at 4 °C overnight. Then, a biotinylated goat anti-mouse IgG was applied (1:600) for 1 h at RT, followed by the avidin–biotin/horseradish peroxidase complex (ABC/HRP; Vector Laboratories). Immunoreactivity was visualized by using the diaminobenzidine chromogen (DAB; Zymed Laboratories, San Francisco, CA, USA). P450arom sections were also counterstained with hematoxylin. The primary antibody was replaced by normal rabbit serum in negative control sections. Absorption controls have utilized primary antibodies preabsorbed with an excess (5 nmol/ml) of the purified respective blocking peptides (Santa Cruz Biotechnology), at 4 °C for 48 h.

Rat ovary was used as a positive control for P450arom and ER $\alpha$ , while control human testis was used as a positive control for both ER $\beta$ <sub>1</sub> and ER $\beta$ <sub>2</sub>.

**Scoring system** The immunostained slides were examined by light microscopy using the 'Allred score' (21), which combines a proportion score and an intensity score. A proportion score was assigned representing the estimated proportion of positively stained tumor cells (0, none; 1, < 1/100; 2, 1/100 to < 1/10; 3, 1/10 to < 1/3; 4, 1/3 to 2/3; 5, > 2/3). An intensity score was assigned by the average estimated intensity of staining in positive cells (0, none; 1, weak; 2, moderate; 3, strong). Proportion and intensity scores were added to obtain a total score that ranged from 0 to 8.

### Protein extraction

Protein extraction from formalin-fixed paraffin-embedded sections was carried out according to Ikeda (22). Briefly, 50  $\mu$ m testis sections were deparaffinized in xylene, dehydrated in graded ethanol, immersed in distilled water, and air dried. Then, the neoplastic area was recovered from the glass slides, cut into small pieces and placed in Eppendorf tubes. Two hundred microliters of RIPA buffer, pH 7.6 (1 M NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 154 mM NaCl, 1% Triton X-100, 12 mM C<sub>24</sub>H<sub>39</sub>O<sub>4</sub>Na, 0.2% Na<sub>3</sub>N, 0.95 mM NaF, 2 mM PMSE, 50 mg/ml aprotinin, 50 mM leupeptin; Sigma Chemical) containing 0.2% SDS, were added to each tube and the contents were

incubated at 100 °C for 20 min, followed by incubation at 60 °C for 2 h. After incubation, tissue lysates were centrifuged at 15 000 *g* for 20 min at 4 °C and the supernatants were stored at –80 °C until biochemical analysis.

### Western blot analysis

Tissue lysates were quantified using the Bradford protein assay reagent (23). Equal amounts of protein (50 µg) were boiled for 5 min, separated under denaturing conditions by SDS–PAGE on 10% polyacrylamide Tris-glycine gels and electroblotted onto nitrocellulose membranes. Nonspecific sites were blocked with 5% nonfat dry milk in 0.2% Tween-20 in Tris-buffered saline (TBS-T) for 1 h at room temperature and incubated overnight with anti-aromatase (1:500), anti-ER $\alpha$  (1:500), anti-ER $\beta_1$  (1:500), anti-ER $\beta_2$  (1:500), and anti-actin (1:1000) primary antibodies. The antigen–antibody complexes were then detected by incubation of the membranes for 1 h at 22 °C with the horseradish peroxidase-conjugated secondary antibody (1:7000). The bound secondary antibodies were located with the ECL Plus western blotting detection system (Amersham) according to the manufacturer's instructions. Each membrane was exposed to the film for 2 min.

Protein extracts from a rat ovary and control human testis were used as positive controls as in immunohistochemical analysis. Negative controls were prepared using tissue lysates, where antigens were previously removed by preincubation with specific antibodies (1 h at room temperature) and subsequently immunoprecipitated with protein A/G agarose.

## Results

### Histological study

H–E staining showed similar cell arrangement of neoplastic Leydig cells in the two testis specimens with unilateral Leydig cell tumors (sized 1.4×1.1 cm and 4×4 cm). Tumoral samples revealed large, vacuolated Leydig cells arranged in multiple clusters with pseudotubular appearance. Mild nuclear pleomorphism and occasional mitotic figures were observed (Fig. 1A and B).

Control testis H–E staining is shown in Fig. 1C.

### Immunohistochemistry

**P450arom** The cytoplasm of vacuolated neoplastic Leydig cells has shown a strong P450arom immunoreactivity (total score 8) in both the samples, while immunonegative nuclei displayed only the blue counterstaining (Fig. 1A1 and B1). Control testes have shown an intense P450arom immunoreactivity exclusively in interstitial Leydig cells (total score 8), while the cells of

tubular compartment were immunonegative (Fig. 1C1). Negative controls (not shown) and the absorption controls (inserts) were all immunonegative.

### Estrogen receptors

Neoplastic Leydig cells of both samples showed a moderate ER $\alpha$  immunoreactivity in the nuclei (total score 5) and a weak immunostaining (total score 4) was also observed in the cytoplasm (Fig. 1A2 and B2). Control testis sections were all ER $\alpha$  immunonegative (Fig. 1C2).

A strong nuclear ER $\beta_1$  (total score 7) immunoreactivity was observed in neoplastic Leydig cells of both samples as well as a weak (total score 4) signal has been detected in their cytoplasm (Fig. 1A3 and B3). The control testis showed a strong ER $\beta_1$  immunostaining (total score from 5 to 7) in the cells of both the tubular and interstitial compartments (Fig. 1C3).

Neoplastic Leydig cells show a very intense ER $\beta_2$  immunoreactivity (total score 8) in the nuclei, while their cytoplasm was moderately stained (total score 5; Fig. 1A4 and B4). In the control testis, ER $\beta_2$  signal (total score from 5 to 8) was mainly revealed in Leydig cells, Sertoli cells, spermatogonia, and spermatocytes (Fig. 1C4).

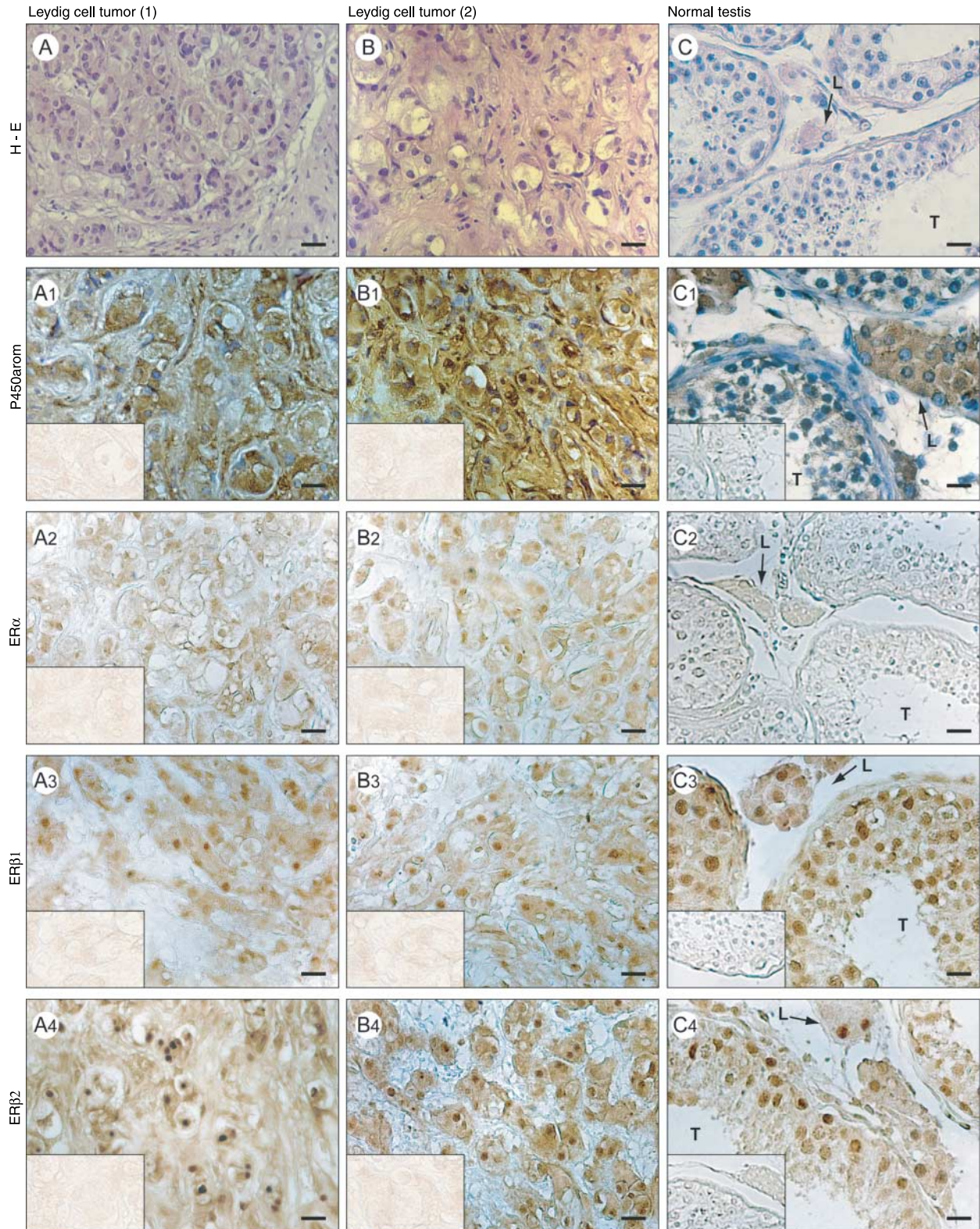
Negative controls (not shown) and the absorption controls (inserts) were all immunonegative.

### Western blot analysis

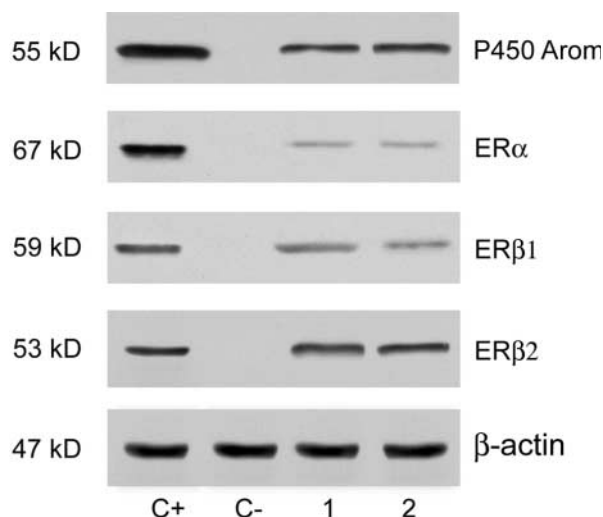
Using an antibody against P450arom, the western blot of tumoral tissues revealed a single band corresponding to a molecular mass of ~55 kDa (Fig. 2: lanes 1 and 2) consistent with the human P450arom size and co-migrating with the ovary extract band (positive control; Fig. 2: C+). Furthermore, using an antibody specific to ER $\alpha$ , the western blot of neoplastic tissues showed a single band at ~67 kDa (Fig. 2: lanes 1 and 2), with the same mobility of ovary extract (positive control; Fig. 2: C+). Conversely, no protein band was observed in the western blot of control testes (Fig. 3: lanes 1 and 2) except for ovary extract (positive control; Fig. 3: C+). In addition, using antibodies specific to ER $\beta_1$  and ER $\beta_2$ , two bands were detected, at ~59 kDa and ~53 kDa respectively (Fig. 2: lanes 1 and 2). The two bands migrated as in control testis extracts (Fig. 2: C+). All the negative control lanes were unlabeled (Fig. 2: C–).

## Discussion

The biological significance of estrogen-induced testicular tumorigenesis has been suggested by the *in vivo* model overexpressing aromatase transgenic mice (19). Half of these males were infertile and some of them showed larger than normal testis and Leydig cell hyperplasia/Leydig cell tumor. Furthermore, aromatase was



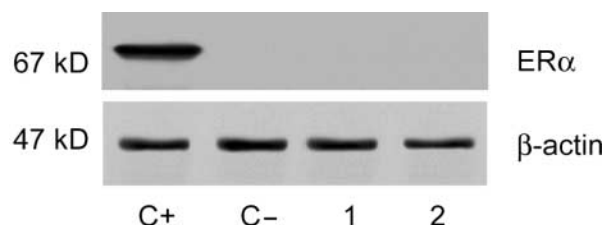
**Figure 1** Morphology and immunolocalization of aromatase and estrogen receptors in Leydig cell tumors (A and B) and control testis (C). A–C: Hematoxylin–eosin staining. A1–C1: Aromatase immunolocalization. A2–C2: ER $\alpha$  immunoreactivity. A3–C3: ER $\beta$ <sub>1</sub> immunodetection. A4–C4: ER $\beta$ <sub>2</sub> immunolocalization. L, Leydig cells; T, seminiferous tubule. Scale bars=12.5  $\mu$ m (A, B, C, A1, B1, A2, B2, C2, A3, B3, A4, B4); 5  $\mu$ m (C1, C3, C4).



**Figure 2** Immunoblots of aromatase and estrogen receptors from protein extracts of Leydig cell tumors and control tissues. P450arom: rat ovary (C+), negative control (C-), Leydig cell tumors (lanes 1 and 2). ER $\alpha$ : rat ovary (C+), negative control (C-), Leydig cell tumors (lanes 1 and 2). ER $\beta_1$ : control human testis (C+), negative control (C-), Leydig cell tumors (lanes 1 and 2). ER $\beta_2$ : control human testis (C+), negative control (C-), Leydig cell tumors (lanes 1 and 2).  $\beta$ -Actin serves as a loading control. Numbers on the left-hand side correspond to molecular weights of detected proteins. All the negative controls were prepared as described in Material and Methods.

markedly immunolocalized in the cytoplasm of interstitial cells, and its immunoreactivity appeared to be strongest in the testes with more advanced stages of neoplasia. The same transgenic animals exhibited circulating estrogen levels at least twice higher than those of control animals, and the levels of aromatase mRNA in their testicular tissues were fourfold higher when compared with controls. It is worth mentioning how ER $\alpha$  protein in testicular tissue of aromatase transgenic animals was very high with respect to the undetectable levels of control animals. So the authors suggest how an enhanced synthesis of estrogens in tumoral tissues led to an upregulation of ER $\alpha$  expression.

Human Leydig cell tumor is a rare testicular neoplasm where the estrogen involvement in tumorigenesis process has scarcely been investigated. In our patients, a strong



**Figure 3** Immunoblot of ER $\alpha$  from protein extracts of control testes. Control testes (lanes 1 and 2), rat ovary (C+), negative control (C-).  $\beta$ -Actin serves as loading control. Numbers on the left-hand side correspond to molecular weights of the detected protein. All the negative controls were prepared as described in Material and Methods.

aromatase expression in tumoral tissues was revealed by immunostaining and western blotting. This finding agrees with a single previous report (24) showing the aromatase immunolocalization in Leydig cell tumors. Furthermore, aromatase expression in control human testicular tissue confirmed Turner's report in normal testes (25). The enhanced endogenous synthesis of estrogens by Leydig cell tumors was reflected in both patients by a dramatic increase of circulating estrogen levels, resulting in a more than twofold higher level than those of normal adult male, and by the low testosterone levels (at the lower limit of normal range).

We may reasonably assume how the ratio between the free fraction levels of the two steroids is furthermore increased in the target tissues. The diminished sperm count and motility of both patients may not only be related to altered testicular tropism, parenchymal compression and increased local temperature ipsilateral to the tumor (26), but also to the detrimental effects of high circulating estrogen levels on the counter-lateral gonad activity. In the normal adult male, 80% of the plasma estradiol originates from aromatization of testosterone and androstenedione in fat, striated muscle, and other tissues including bone and brain, while 20% in the circulation is secreted by the testis. So, it is reasonable to argue that the excessive increase of estradiol circulating levels, observed in the two patients with Leydig cell tumor, is the consequence of an enhanced rate of testicular secretion. This is confirmed by the evidence that estradiol, as well as circulating E<sub>2</sub>/T ratio levels, drops dramatically following surgical treatment, while for one of the two patients the persistence of a conspicuous bilateral gynecomastia led to bilateral mastectomy.

Following orchidectomy, the two patients exhibited a moderate increase of sperm count and a remarkable augmentation of sperm motility. The latter event may be reconducted to the restored circulating testosterone levels likely affecting the entire male genital tract.

The expression of ER isoforms in Leydig cell tumors is, to date, unknown. In fact, only a single work showed the ER immunolocalization in cryostat sections of Leydig cell tumors (24), but these data were obtained before the ER $\beta$  discovery.

Immunohistochemical and western blot analysis of tumoral tissues revealed the expression of ER $\alpha$  and of the two ER $\beta$  isoforms, ER $\beta_1$  and ER $\beta_2$ , in the neoplastic Leydig cells of both patients. So, the pattern of ERs expression in tumoral cells appears different from that of control Leydig cells, exhibiting only ER $\beta_1$  and ER $\beta_2$  as previously reported (8, 27).

There is a growing body of evidence suggesting that ER $\alpha$  and ER $\beta$  can be expressed together in the same cell type and independently expressed in another. Therefore, homodimers (ER $\alpha$ -ER $\alpha$ /ER $\beta$ -ER $\beta$ ) or heterodimers (ER $\alpha$ -ER $\beta$ ) can be formed (28). The binding affinity of ER $\alpha$ -ER $\alpha$ /ER $\alpha$ -ER $\beta$  dimers for a consensus DNA estrogen response element is reported to be higher than that of the ER $\beta$ -ER $\beta$  homodimer (29). Thus, the presence of ER $\alpha$  could reinforce the estradiol-induced tumor cell proliferation.

Finally, the present report demonstrates that neoplastic Leydig cells are potential estrogen biosynthesis sites and display a modified ER expression pattern. Therefore, it appears reasonable to suggest that the high estrogen levels, measured in the two patients, could play a role in the neoplastic transformation of Leydig cells, while the exclusive presence of ER $\alpha$  in tumoral cells could amplify E<sub>2</sub> signaling, contributing to the tumor cell growth and progression.

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## References

- Huseby RA. Leydig cell neoplasia. In *The Leydig Cell*, pp 555–572. Eds AH Payne, MP Hardy & LD Russell. Vienna: Cache River Press, 1996.
- Zarrilli S, Lombardi L, Paesano C, Di Somma C, Colao A, Mirone V & De Rosa M. Hormonal and seminal evaluation of Leydig cell tumour patients before and after orchidectomy. *Andrologia* 2000 **32** 147–151.
- Alonso Dominguez FJ & Osorio Acosta VA. Leydig cell tumor presenting as precocious pseudopuberty in a 4-year-old boy. *Archivos Espanoles de Urologia* 2004 **57** 426–428.
- Kim I, Young RH & Scully RE. Leydig cell tumor of the testis. A clinicopathological analysis of 40 cases and review of the literature. *American Journal of Surgical Pathology* 1985 **9** 177–192.
- McCluggage WG, Shanks JH, Arthur K & Banerjee SS. Cellular proliferation and nuclear ploidy assessment augment established prognostic factors in predicting malignancy in testicular Leydig cell tumours. *Histopathology* 1998 **33** 361–368.
- Pelletier G & El-Alfy M. Immunocytochemical localization of estrogen receptors  $\alpha$  and  $\beta$  in the human reproductive organs. *Journal of Clinical Endocrinology and Metabolism* 2000 **85** 4835–4840.
- Taylor AH & Al-Azzawi F. Immunolocalization of oestrogen receptor  $\beta$  in human tissues. *Journal of Molecular Endocrinology* 2000 **24** 145–155.
- Saunders PTK, Sharpe RM, Williams K, Macpherson S, Urquart H, Irvine DS & Millar MR. Differential expression of oestrogen receptor  $\alpha$  and  $\beta$  proteins in the testes and male reproductive system of human and non-human primates. *Molecular Human Reproduction* 2001 **7** 227–236.
- Brodie A, Inkster S & Yue W. Aromatase expression in human male. *Molecular Cellular Endocrinology* 2001 **178** 23–28.
- O'Donnell L, Robertson KM, Jones ME & Simpson ER. Estrogen and spermatogenesis. *Endocrine Reviews* 2001 **22** 289–318.
- Blobel GA & Orkin SH. Estrogen-induced apoptosis by inhibition of the erythroid transcription factor GATA-1. *Molecular and Cellular Biology* 1996 **16** 1687–1694.
- Mabuchi S, Ohmichi M, Kimura A, Nishio Y, Arimoto-Ishida E, Yada-Hashimoto N, Tasaka K & Murata Y. Estrogen inhibits paclitaxel-induced apoptosis via the phosphorylation of apoptosis signal-regulating kinase 1 in human ovary cancer cell lines. *Endocrinology* 2004 **145** 49–58.
- Weir HK, Marrett LD, Kreiger N, Darlington GA & Sugar L. Prenatal and peri-natal exposure and risk of testicular germ cell cancer. *International Journal of Cancer* 2000 **87** 438–443.
- Dieckmann KP, Endsins G & Pichlmeier U. How valid is the prenatal estrogen excess hypothesis of testicular germ cell cancer? A case control study on hormone-related factors. *European Urology* 2001 **40** 677–683.
- Strohsnitter WC, Noller KL, Hoover RN, Robboy SJ, Palmer JR, Titus-Ernstoff L, Kaufman RH, Adam E, Herbst AL & Hatch EE. Cancer risk in men exposed *in utero* to diethylstilbestrol. *Journal of the National Cancer Institute* 2001 **93** 545–551.
- Fleming LE, Bean JA, Rudolph M & Hamilton K. Cancer incidence in a cohort of licensed pesticide applicators in Florida. *Journal of Occupational and Environmental Medicine* 1999 **41** 279–288.
- Ohlson CG & Hardell L. Testicular cancer and occupational exposure with a focus on xenoestrogens in polyvinyl chloride plastics. *Chemosphere* 2000 **40** 1277–1282.
- Li X, Nokkala E, Yan W, Streng T, Saarinen N, W rri A, Huhtaniemi I, Santti R, M kel  S & Poutanen M. Altered structure and function of reproductive organs in transgenic male mice overexpressing human aromatase. *Endocrinology* 2001 **142** 2435–2442.
- Fowler KA, Gill K, Kirma N, Dillehay DL & Tekmal RR. Overexpression of aromatase leads to development of testicular leydig cell tumors: an *in vivo* model for hormone-mediated testicular cancer. *American Journal of Pathology* 2000 **156** 347–353.
- Shi SR, Key ME & Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue section. *Journal of Histochemistry and Cytochemistry* 1991 **39** 741–748.
- Allred DC, Harvey JM, Berardo M & Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Modern Pathology* 1998 **11** 155–168.
- Ikeda K, Monden T, Kanoh T, Tsujie M, Izawa H, Haba A, Ohnishi T, Sekimoto M, Tomita N, Shiozaki H & Monden M. Extraction and analysis of diagnostically useful proteins from formalin-fixed, paraffin-embedded tissue sections. *Journal of Histochemistry and Cytochemistry* 1998 **46** 397–403.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 1976 **72** 248–254.
- Due W, Dieckmann KP, Loy V & Stein H. Immunohistological determination of oestrogen receptor, progesterone receptor, and intermediate filaments in Leydig cell tumours, Leydig cell hyperplasia, and normal Leydig cells of human testis. *Journal of Pathology* 1989 **157** 225–234.
- Turner KJ, Macpherson S, Millar MR, McNeilly AS, Williams K, Cranfield M, Groome NP, Sharpe RM, Fraser HM & Saunders PT. Development and validation of a new monoclonal antibody to mammalian aromatase. *Journal of Endocrinology* 2002 **172** 21–30.
- Ho GT, Gardner H, Mostofi K, De Wolf WC, Loughlin KR & Morgentaler A. The effect of testicular nongerm cell tumors on local spermatogenesis. *Fertility and Sterility* 1994 **62** 162–166.
- Saunders PT, Millar MR, Macpherson S, Irvine DS, Groome NP, Evans LR, Sharpe RM & Scobie GA. ER $\beta$ 1 and the ER $\beta$ 2 splice variant (ER $\beta$ cx/ $\beta$ 2) are expressed in distinct cell populations in the adult human testis. *Journal of Clinical Endocrinology and Metabolism* 2002 **87** 2706–2715.
- Moore JT, McKee DD, Slentz-Kesler K, Moore LB, Jones SA, Horne EL, Su JL, Kliewer SA, Lehmann JM & Willson TM. Cloning and characterization of human estrogen receptor  $\beta$  isoforms. *Biochemical and Biophysical Research Communications* 1998 **247** 75–78.
- Cowley SM, Hoare S, Mosselman S & Parker MG. Estrogen receptors alpha and beta form heterodimers on DNA. *Journal of Biological Chemistry* 1997 **272** 19858–19862.

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