

Immunophenotypic differences between neoplastic and non-neoplastic androgen-producing cells containing and lacking Reinke crystals

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Abstract We performed a detailed morphologic, immunophenotypic, and endocrine characterization of neoplastic and non-neoplastic lesions of androgen-producing cells known to harbor or lack Reinke crystals (RCs) with an aim to provide further insight into the nature of these cells and crystals. Study cases were selected from the files of participating hospitals and subclassified according to current classifications: 20 with Leydig cell tumors (LCTs), 2 with testicular adrenal rest tumors (TARTs), 2 with testicular tumors of adrenogenital syndrome (TTAGS), and 2 with androgen insensitivity syndrome (AIS). An extensive immunophenotypic panel including markers used in sex cord-stromal cell tumors, androgen hormones, enzymes, and receptors was applied to the cases and 10 non-tumoral adrenal glands. Non-tumoral tissues were scored separately. RCs were present in 90 % of LCT cases and all cases with normal Leydig cells; RCs stained specifically with calretinin and 3 β -hydroxysteroid dehydrogenase (3BHSD) and were present only in cells with high concomitant expression of both proteins, a phenotype unique to Leydig cells and LCTs. Leydig cells from AIS cases lack

RCs due to decreased expression of 3BHSD. Calretinin is decreased in testicular adrenal-like tumors and absent in normal adrenocortical cells, which explain why they lack RCs. Calretinin expression in androgen-producing cells is independent from androgen receptors and androgen synthesis. RCs represent for the most part, if not exclusively, crystallized forms of a 3BHSD/calretinin complex. Androgen-producing cells containing and lacking RCs differ mainly in the level of expression of these proteins and androgen receptors.

Keywords Reinke crystals · Leydig cell · Adrenal rest tumor · Gonadal steroid hormones · Immunohistochemistry

Introduction

In a recent study, we showed that Reinke crystals (RCs) are invariably present in a subset of Leydig cells from a normal testis, that the crystals dissolve rapidly in formalin fixative and slowly in alcohol, and that they stain specifically with antibodies against 3 β -hydroxysteroid dehydrogenase (3BHSD). Contrary to previous assertions, RCs lack expression of nestin and are not products of degradation [1–3]. We have now studied neoplastic and non-neoplastic examples of androgen-producing cells known to harbor or lack RCs. The goal of this study was to determine if consistent immunophenotypic differences exist between these morphologically and biologically closely related subgroups and if these differences provide further insight on the nature of RCs.

Materials and methods

The pathology databases from five hospitals affiliated with the University of Minnesota Medical School were searched for

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Table 1 Clinicopathologic features

No.	DX	Age	Size cm	Met/Rec	FU-Mo	Presenting symptom	Crystals LC LCT		Mitoses (10 HPF)
1	LCT	33	0.1	N/A	N/A	Seminoma	+++	+	0
2	LCT	38	0.1	No	30	Incidental	+++	++	0
3	LCT	76	0.3	No	151.4	Incidental	+++	++	0
4	LCT	46	0.2	No	239.8	Incidental	+++	++	0
5	LCT	37	1.5	No	46.0	Gynecomastia	+++	+++	3
6	LCT	42	2.2	No	110.8	Pain	+++	+++	0
7	LCT	45	0.8	No	8.5	Pain	+++	+++	0
8	LCT	68	0.5	No	15.8	Incidental	+++	+++	0
9	LCT	56	1.7	No	149.7	Incidental	N/A	+++	0
10	LCT	68	0.7	No	78.7	Pain	N/A	+++	1
11	LCT	63	1.2	No	11.4	Incidental	+++	+++	0
12	LCT	65	1.2	No	18.4	Incidental	N/A	+++	0
13	LCT	38	3.4	No	46.0	N/A	N/A	+++	1
14	LCT	56	1.4	No	13.3	N/A	++	+++	0
15	LCT	40	0.7	N/A	N/A	Pain	++	+	0
16	LCT	49	0.7	No	32.0	Pain	++	++	0
17	LCT	44	0.6	N/A	N/A	N/A	+	+	0
18	LCT	50	1.8	No	29.2	Incidental	+++	+	0
19	ALCT	43	0.8	No	76.6	Incidental	+++	–	0
20	ALCT	66	4.2	No	4.2	Mass	+++	–	1
21	TART	30	0.4	No	30.0	Incidental	+++	–	0
22	TART-L	48	1.0	N/A	N/A	Incidental	+	–	0
23	TTAGS	30	3.5	N/A	N/A	Infertility	N/A	–	1
24	TTAGS	6	1.2	N/A	N/A	Precocious puberty	N/A	–	0
25	AIS	17	No tumor	N/A	N/A	Amenorrhoea	–	N/A	N/A
26	AIS	15	No tumor	N/A	N/A	Amenorrhoea	–	N/A	N/A

Scoring: – (absent), + (scant), ++ (moderate), and +++ (abundant)

DX diagnosis, Met/Rec metastases/recurrence, FU-Mo follow-up in months, HPF high-power field, LC non-tumoral Leydig cells, LCT Leydig cell tumor, ALCT atypical Leydig cell tumor, TART testicular adrenal rest tumor, TART-L TART with lipomatous component, TTAGS testicular tumor of adrenogenital syndrome, AIS androgen insensitivity syndrome. N/A not available/not applicable

cases with testicular sex cord-stromal cell tumors (SCSCTs), testicular specimens from cases with androgen insensitivity syndrome (AIS), and cases with adrenogenital syndrome (AGS). Microscopic slides of potential study cases were reviewed. Confirmed cases with available paraffin-embedded blocks were included in the study. Participating institutions use 10 % buffered formalin for tissue fixation. Sertoli cell lesions were excluded from the analysis. The lesions were subclassified using the current World Health Organization and Armed Forces Institute of Pathology classifications [4, 5]. The number of RCs per slide was semiquantified as follows: absent, scant (less than 5), moderate (5 to 20), and abundant (>20). Crystals were semiquantified separately in tumoral and non-tumoral entities. Immunohistochemical studies included cytokeratin cocktail (CK) (AE1/AE3+8/18 cocktail, Biocare Medical, catalog # CM162B, Concord CA; dilution 1:900), epithelial membrane antigen (EMA) (Cell Marque, catalog # 247M-95, Rocklin, CA; dilution 1:1000), inhibin A (Novacastra, catalog # NCL-L-Inhibin A, Newcastle upon Tyne, UK; dilution 1:1500), calretinin (Novacastra, catalog # NCL-L-CALRET-566, Newcastle upon Tyne, UK; dilution 1:1200), CD117 (Cell Marque, catalog # 117R-14, Rocklin, CA; dilution 1:300), synaptophysin (Novacastra, catalog # NCL-SYNAP-299,

Newcastle upon Tyne, UK; dilution 1:100), CD56 (Biocare Medical, Concord, CA; catalog # CD164A, dilution 1:100), vimentin (Cell Marque, Rocklin, CA; catalog # 347M-14, dilution 1:250), S100 (Novacastra, catalog # CL-L-S100P, Newcastle upon Tyne, UK; dilution 1:1600), testosterone (GeneTex, Irvine, CA; product # GTX72779; dilution, 1:125), androstenedione (ASD) (US Biological, Salem, MA; product # 151581; dilution, 1:100), dehydroepiandrosterone (DHEA) (US Biological; product # 140041; dilution, 1:50), 3BHS (Santa Cruz Biotechnology, Dallas, TX; product # sc-100466; dilution, 1:250), 17 α -hydroxylase/17,20-lyase (CYP17A1) (Santa Cruz Biotechnology; product # sc-46084; Dallas, TX; dilution 1:110), 17 β -hydroxysteroid dehydrogenase (17BHS) (Abcam Inc., Boston, MA; [EP1682Y] ab51045; dilution, 1:50), androgen receptor (Bio SB, Santa Barbara, CA, catalog # BSB 6074), PAX2 (Bio SB, Santa Barbara, CA, catalog # BSB 2566, pre-dilute), and PAX8 (Cell Marque, Rocklin, CA; catalog # 363M-14, pre-dilute). Immunohistochemistry was performed on a Leica BOND-III automated stainer (Leica Biosystems, Melbourne, Australia) using citrate and EDTA buffer antigen retrieval protocols. Dilutions and retrieval methods were optimized in preliminary studies. The full immunohistochemical panel was performed in 17 testes with SCSCTs, 11 of these had lesions

included in this study; in the remaining six cases which contained Sertoli cell lesions, only the non-neoplastic cells were evaluated. The panel was also applied to 10 non-tumoral adrenal glands from radical nephrectomy cases. A second panel selected because of its discriminative diagnostic power was applied to all 26 study lesions and 10 non-tumoral adrenal glands. The second panel included androgenic hormones, enzymes, and receptors, as well as calretinin, inhibin, and cytokeratin cocktail. For the evaluation of the immunohistochemical stains, a numerical system for grading the intensity of expression was used (1 = weak, 2 = moderate, and 3 = strong), relative to the strongest and negative internal control cells in each preparation. In cases with partial expression of a marker, the percentage of positive cells was recorded. All parameters were evaluated in tumoral and non-tumoral androgen-producing cells present on each study slide. Electronic medical records of identified cases were reviewed for retrieving demographic data, after approval from the respective institutional review boards from the participating institutions.

Results

Clinicopathologic features

The clinicopathologic features are summarized in Table 1. Twenty-six cases met the inclusion criteria: 20 with Leydig cell tumors (LCTs), 2 with testicular adrenal rest tumors

(TARTs), 2 with testicular tumors of adrenogenital syndrome (TTAGS), and 2 with AIS. Two of the LCT cases showed atypical morphology: one showed extensive myxoid and/or clear cell change and one showed prominent myxoid change and cystic degeneration. One of the TART cases showed a prominent lipomatous component (adrenolipoma). None of the cases showed histologic features associated with malignancy. Segregated by diagnostic categories, the mean age at diagnosis for LCT was 50 years (range 30–76), TART 39 (range 30–48), TTAGS 18 (range 6–30), and AIS 16 (range 15–17). The mean size of LCT was 1.2 cm (range 0.1–4.2), TART 0.7 cm (range 0.4–1), and TTAGS 2.4 cm (range 1.2–3.5). Follow-up (FU) was available in 69 % (18/26) of the cases, and the mean FU time was 60.7 months (range 4.2–239.8); none of the cases developed recurrences or metastases. Among the LCT cases with available clinical information, 63 % (12/19) were discovered incidentally and 26 % (5/19) consulted for pain, one for a testicular mass, and one for gynecomastia. The TART cases were found incidentally. In the TTAGS group, the presenting symptoms were precocious puberty and infertility, and in the AIS group, the presenting symptom was primary amenorrhea.

Immunohistochemical studies

Table 2 summarizes the results of a panel of markers commonly utilized in the work-up of SCST performed in a

Table 2 Panel 1: markers commonly used in sex cord-stromal tumors

IHC	LC (<i>n</i> = 17)	LCT (<i>n</i> = 9)	TART (<i>n</i> = 1)	TTAGS (<i>n</i> = 1)	Adrenal cortex (<i>n</i> = 10)		
					Zona reticularis	Zona fasciculata	Zona glomerulosa
Vimentin	Positive Intensity = 1–3	Positive Intensity = 2–3	Variable 5 % Intensity = 1	Positive Intensity = 1–2	Negative	Negative	Positive Intensity = 1–3
S100	Variable = 2/17 0–50 % Intensity = 1	Negative	Negative	Negative	Negative	Negative	Negative
EMA	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Synaptophysin	Variable = 4/17 0–60 % Intensity = 1	Variable = 3/9 Rare cells Intensity = 1	Variable 0–10 % Intensity = 1	Rare cells Intensity = 1	Variable 0–10 % Intensity = 1	Variable 0–10 % Intensity = 1	Variable 0–20 % Intensity = 1
C-Kit	Negative	Variable = 1/9 0–40 % Intensity = 1	Negative	Negative	Negative	Negative	Negative
CD56	Positive Intensity = 2–3	Positive Intensity = 1–3	Positive Intensity = 1–3	Positive Intensity = 1–3	Negative	Variable 10–30 % Intensity = 1–2	Positive Intensity = 3
PAX2/PAX8	Negative	Negative	Negative	Negative	Negative	Negative	Negative

n = number of cases; scoring: 1 (weak), 2 (moderate), and 3 (strong)

LC normal Leydig cells, LCT Leydig cell tumor, TART testicular adrenal rest tumor, TTAGS testicular tumor of adrenogenital syndrome, EMA epithelial membrane antigen

subset of the cases and Table 3 the expanded endocrine panel performed in all cases. Inhibin is consistently and strongly positive in normal Leydig cells, Leydig cells from AIS (LC-AIS), LCTs, TTAGS, and TARTs. In the adrenal cortex, inhibin is variably expressed only in the androgen-producing zona reticularis. Vimentin expression is strong in normal Leydig cells, LCTs, and LC-AIS; it is

weak to moderate in TTAGS and TARTs. In the adrenal cortex, vimentin is negative in the zonas reticularis and fasciculata; it is patchy, with variable intensity in the zona glomerulosa. Calretinin is strongly positive in normal Leydig cells, LCTs, and LC-AIS; its expression is markedly decreased or absent in TTAGS and TARTs and is absent in normal adrenocortical cells (Fig. 1). CD56 is

Table 3 Panel 2: panel for androgen-producing cells

IHC	LC (<i>n</i> = 17)	LC-AIS (<i>n</i> = 2)	LCT (<i>n</i> = 18)	TART (<i>n</i> = 2)	TTAGS (<i>n</i> = 2)	Adrenal cortex (<i>n</i> = 10)		
						Zona reticularis	Zona fasciculata	Zona glomerulosa
Testosterone	Positive Intensity = 1–3	Positive Intensity = 3	Variable = 8/ 18 0–100 % Intensity = 1–3	Positive Intensity = 1–3	Positive Intensity = 1–3	Positive Intensity = 1–3	Negative	Negative
DHEA	Positive Intensity = 2–3	Positive Intensity = 3	Variable = 7/ 18 0–100 % Intensity = 1–3	Positive Intensity = 1–3	Positive Intensity = 1–3	Positive Intensity = 1–3	Negative	Negative
ASD	Variable = 15/ 17 0–100 % Intensity = 1–3	Positive Intensity = 3	Variable = 2/ 18 0–100 % Intensity = 1–3	Positive Intensity = 1–3	Positive Intensity = 1–3	Negative	Negative	Negative
3BHSD	Positive Intensity = 2–3	Positive Intensity = 1	Positive Intensity = 1–3	Positive Intensity = 1	Positive Intensity = 1–2	Positive Intensity = 2–3	Positive Intensity = 2–3	Positive Intensity = 2–3
CYP17A1	Positive Intensity = 3	Positive Intensity = 1	Positive Intensity = 1–3	Positive Intensity = 3	Positive Intensity = 3	Positive Intensity = 3	Positive Intensity = 3	Negative
17BHSD	Variable = 15/ 17 0–30 % Intensity = 1–3	Negative	Variable = 13/ 18 0–100 % Intensity = 1–3	Variable 50–100 % Intensity = 1–3	Negative	Negative	Negative	Negative
Androgen receptor	Positive Intensity = 2	Negative	Positive Intensity = 2	Variable 10 % Intensity = 1	Variable = 1/ 2 0–60 % Intensity = 1	Negative	Rare cells Intensity = 1	Variable 20 % Intensity = 1
Calretinin	Positive Intensity = 3	Positive Intensity = 2–3	Positive Intensity = 2–3	Variable 1/2 0–100 % Intensity = 1–2	Variable 1/2 0–100 % Intensity = 1	Negative	Negative	Negative
Inhibin	Positive Intensity = 3	Positive Intensity = 2–3	Positive Intensity = 2–3	Positive Intensity = 3	Positive Intensity = 3	Positive Intensity = 1–3	Negative	Negative
Pankeratin	Variable 4/17 0–100 % Intensity = 1–3	Rare cells Intensity = 1	Rare cells Intensity = 1–3	Negative	Rare cells Intensity = 1–3	Rare cells Intensity = 1–3	Rare cells Intensity = 1–3	Rare cells Intensity = 1–3

n = number of cases; scoring: 1 (weak), 2 (moderate), and 3 (strong)

DHEA dehydroepiandrosterone, ASD androstenedione, 3BHSD 3 β -hydroxysteroid dehydrogenase, CYP17A1 cytochrome P450 17A1, 17BHSD 17 β -hydroxysteroid dehydrogenase, LC normal Leydig cells, LC-AIS Leydig cells from androgen insensitivity syndrome, LCT Leydig cell tumor, TART testicular adrenal rest tumor, TTAGS testicular tumor of adrenogenital syndrome

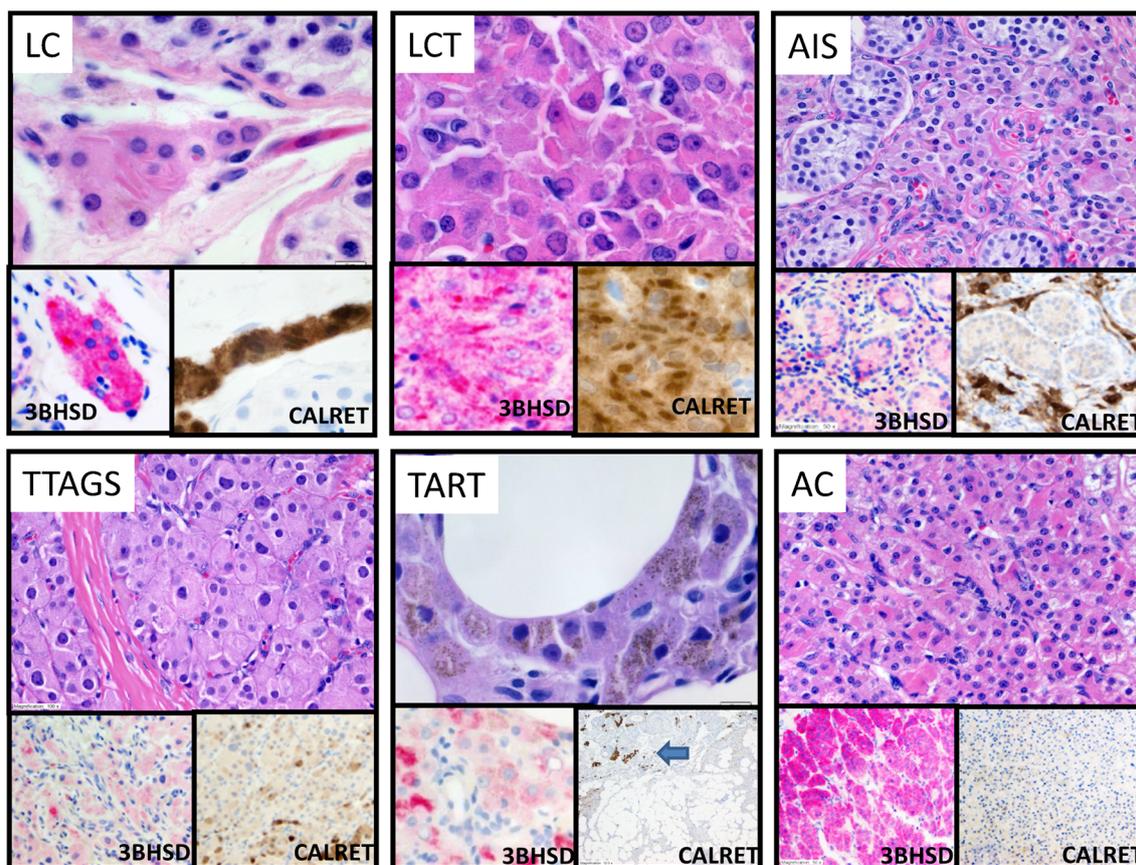


Fig. 1 Expression of 3BHSD and calretinin in steroid-producing cells. *3BHSD* 3 β -hydroxysteroid dehydrogenase, *LC* Leydig cell, *LCT* Leydig cell tumor, *AIS* adrogen insensitivity syndrome, *TTAGS* testicular tumor of adrenogenital syndrome, *TART* testicular adrenal rest tumor, *AC* adrenal cortex, *SC* Sertoli cell. LC and LCT are the only cells with Reinke

crystals and strong coexpression of both markers. LCs from AIS show decreased 3BHSD (no difference between LC and SC) but preserved calretinin. TART, TTAGS, and AC show minimal or absent expression of calretinin; the TART case shows preserved calretinin expression in extratumoral LC (*arrow*)

positive in normal Leydig cells, LCTs, LC-AIS, TTAGS, and TARTs. In adrenocortical cells, CD56 expression is strong in the zona glomerulosa, moderate in the exterior layers of the zona fasciculata, and negative in the internal zona fasciculata and zona reticularis. Pankeratin was diffuse and strong in one of the LCT cases with atypical morphology. In normal Leydig cells, LCTs, LC-AIS, TTAGS, and TARTs, pankeratin expression is non-uniform and highly variable. In the adrenal cortex, pankeratin expression is also non-uniform and highly variable, being more common in the zona glomerulosa, followed by the zonas reticularis and fasciculata. S100 showed partial weak reactivity in normal Leydig cells in a few cases (2/17), but it was negative in the remaining normal Leydig cells, LCTs, LC-AIS, TTAGS, TARTs, and adrenal cortex cells. Synaptophysin showed focal weak staining in a small subset of cells in all the study groups. C-Kit showed weak partial staining in one of the atypical LCT cases, but it was negative in all the remaining groups. EMA, chromogranin, PAX2, and PAX8 were consistently negative in normal Leydig cells, LCTs, LC-AIS, TTAGS, TARTs, and adrenocortical cells.

Endocrine profile

Figure 2 and Table 3 summarize the results of these studies. Normal Leydig cells, LC-AIS, TARTs, and TTAGS show consistent expression of testosterone and DHEA, and variable ASD. Most LCTs have no or decreased expression of the three hormones. In the adrenal cortex, only the zona reticularis is positive for testosterone and DHEA but negative for ASD. Normal Leydig cells, LCTs, and all layers of the adrenal cortex show strong and consistent expression of 3BHSD; by contrast, expression is weak in TARTs, TTAGS, and LC-AIS. Expression of CYP17A1 is strong and consistent in all groups except for the zona glomerulosa of the adrenal cortex. Expression of 17BHSD is variable in normal Leydig cells, LCTs, and TARTs and negative in LC-AIS, TTAGS, and adrenocortical cells. Androgen receptors (ARs) are consistently positive in normal Leydig cells and LCTs; expression in TARTs was weak and focal (~10%). In TTAGS, the younger patient (age 6) had a complete loss of AR expression, while the older patient (age 30) retained weak expression in ~60% of the cells. In the adrenal cortex, a small subset of cells in the zona glomerulosa (~20%) and in the zona fasciculata (~5%)

Cell Type	TESTO	DHEA	ASD	3BHSD	CYP17A1	17BHSD	CALRET	INH	CK
LC	Positive moderate/strong	Positive moderate/strong	Negative to strongly positive	Positive moderate/strong	Positive moderate/strong	Negative to strongly positive	Positive moderate/strong	Positive moderate/strong	Negative to strongly positive
LCT	Negative to weakly positive	Negative to weakly positive	Negative to weakly positive	Positive moderate/strong	Positive moderate/strong	Negative to strongly positive	Positive moderate/strong	Positive moderate/strong	Negative to strongly positive
LC-AIS	Positive moderate/strong	Positive moderate/strong	Positive moderate/strong	Positive weak	Positive moderate/strong	Negative to weakly positive	Positive moderate/strong	Positive moderate/strong	Negative to strongly positive
TART	Positive moderate/strong	Positive moderate/strong	Positive moderate/strong	Positive weak	Positive moderate/strong	Negative to weakly positive	Negative to weakly positive	Positive moderate/strong	Negative
TTAGS	Positive moderate/strong	Positive moderate/strong	Positive moderate/strong	Positive weak	Positive moderate/strong	Negative to weakly positive	Negative to weakly positive	Positive moderate/strong	Negative to strongly positive
AC-Ret	Positive moderate/strong	Positive moderate/strong	Negative to strongly positive	Positive moderate/strong	Positive moderate/strong	Negative to weakly positive	Negative	Positive moderate/strong	Negative to strongly positive

Fig. 2 Endocrine profile of steroid-producing cells and visual scale of level of expression. *TESTO* testosterone, *DHEA* dehydroepiandrosterone, *ASD* androstenedione, *3BHSD* 3 β -hydroxysteroid dehydrogenase, *CYP17A1* cytochrome P450 17A1, *17BHSD* 17 β -hydroxysteroid dehydrogenase, *CALRET* calretinin, *INH* inhibin, *CK* cytokeratin cocktail, *LC* Leydig cell, *LCT* Leydig cell tumor, *AIS* androgen insensitivity

syndrome, *TART* testicular adrenal rest tumor, *AC-Ret* adrenal cortex, zona reticularis. LCTs have decreased expression of androgens compared to the other groups. LC and LCT are the only groups showing strong coexpression of 3BHSD and calretinin. LC-AIS show weak 3BHSD expression but strong calretinin. TART, TTAGS, and AC-Ret have weak or negative expression of calretinin

show weak AR expression, but the androgen-producing zona reticularis was negative.

Reinke crystals

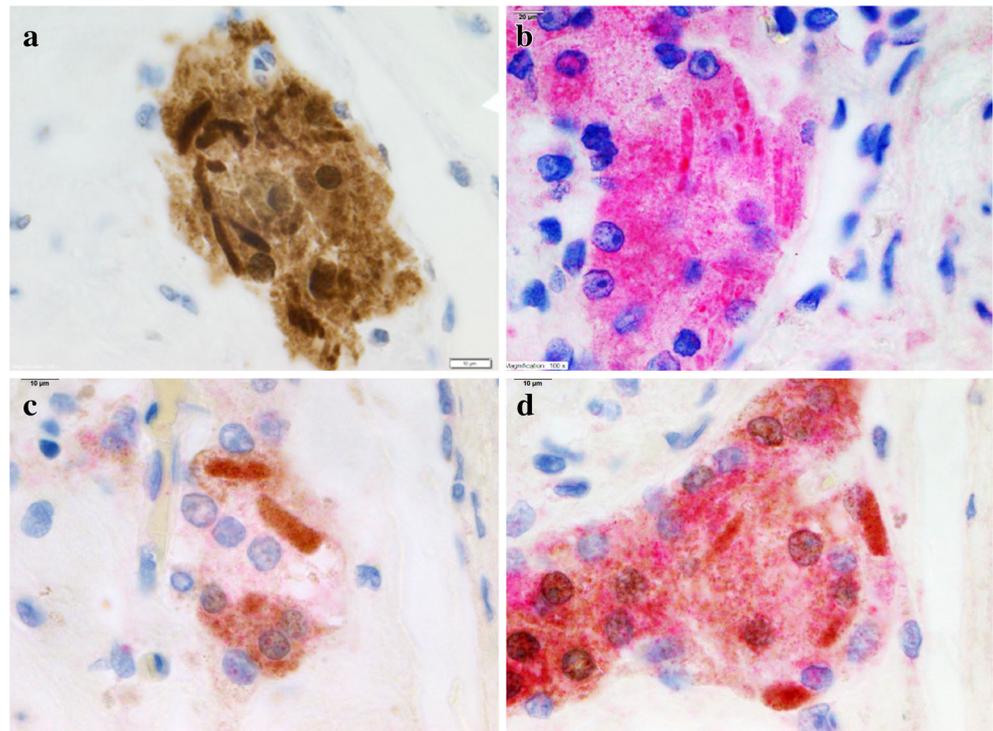
RCs were identified in 18/20 (90 %) LCT cases; RCs were not observed in the two cases with atypical morphology. RCs were abundant in 10, moderate in four, and scant in four cases. Non-tumoral Leydig cells were present in adjacent testicular tissue in 16/20 of the specimens with LCTs, and RCs were identified in all cases (100 %): 12 abundant, 3 moderate, and 1 scant. RCs were present in the non-tumoral Leydig cells of the two cases of TART. The two cases of TTAGS did not have normal Leydig cells for evaluation. Crystals were absent in the two cases of AIS. RCs were consistently positive for calretinin and 3BHSD and negative for all the remaining markers. The staining pattern with both antibodies was grumous and discontinuous. Because on immunostained slides the crystals were frequently obscured by cytoplasmic staining, we performed several dilutions and combinations of calretinin and 3BHSD antibodies to improve the crystal to cytoplasmic contrast with success (Fig. 3); however, the crystals continued to appear as grumous sausage-shaped formations instead of the crisp elegant structures observed on hematoxylin and eosin.

Discussion

RCs, originally described in 1896, are unique to Leydig cells and LCTs and help separate them from their close relatives TARTs, TTAGS, and adrenal cortex cells. We recently clarified several misconceptions about RCs: we demonstrated that

RCs are almost invariably present in a subset of Leydig cells in normal testes as originally described by Friedrich Reinke, but their number is variable; we showed that RCs are amphiphilic: they dissolve rapidly in formalin and slowly in alcohol; we demonstrated that RCs stain specifically with antibodies against 3BHSD and disproved the previous assertion that the crystals are positive for nestin, and finally, we demonstrated that they do not represent waste/degenerative products as previously reported [2, 3]. Embryologically, the SCSCs of the gonads and the adrenal cortex share a common origin from the cells of the adrenogenital primordium [6]. This explains the close immunomorphologic resemblance between all these cell groups [7]. While Sertoli cells develop highly specialized functions directed at providing an optimal environment for regulation of spermatogenesis [6–8], Leydig cells, testicular adrenal-like cells, and adrenocortical cells remain to be primary endocrine/paracrine cells [9]. In the current study, we found RCs in all cases containing normal Leydig cells and in 90 % of LCT cases, a percentage that is much higher than the one third of cases described in reference books [4, 5]. This discrepancy may be explained by fixation times and the time and effort dedicated to this task. By immunohistochemistry, in addition to 3BHSD, RCs are also positive for calretinin. Among steroid-producing cells, the main difference between cells containing and lacking RCs was the level of coexpression of 3BHSD and calretinin. Strong coexpression of both proteins was unique to normal Leydig cells and LCTs. Figure 1 shows the different expression levels of 3BHSD and calretinin in all the study groups. Our results indicate that RCs represent for the most part, if not exclusively, crystallized forms of a 3BHSD/calretinin complex. RCs did not contain androgens; however, given the hydrosoluble properties of the crystals, it is

Fig. 3 Reinke crystals in normal Leydig cells. **a** Calretinin, 1:1200 dilution. **b** 3 β -Hydroxysteroid dehydrogenase (3BHSD), 1:250 dilution. **c** Combined calretinin/3BHSD, 1:1800/1:300 dilutions. **d** Combined calretinin/3BHSD, 1:1800/1:250 dilutions. The crystals stain strongly with calretinin and variably with 3BHSD. The combination of both diluted antibodies improves the contrast with the cytoplasm; however, the grumous pattern of staining persists



possible that the presence of lipophilic hormones in the cytoplasm may be necessary or contributes to the crystallization process. Leydig cells from AIS cases express high calretinin but low 3BHSD, TTAGS and TARTs show decreased expression of both 3BHSD and calretinin, and adrenocortical cells show high 3BHSD but absent calretinin, while the expression of androgens is maintained in all these groups. All these groups lack RCs, suggesting that a high concentration of both proteins is necessary for the crystallization process. The rare cases of adrenal neoplasms containing RCs [10] can probably be explained by the frequent aberrant expression of calretinin by adrenal neoplasms [11], which is not present in normal adrenocortical cells. If concurrent high coexpression level of 3BHSD/calretinin occurs in any given steroid-producing tumor, crystallization may happen. Crystalline structures resembling RCs were observed by Magalhães in periendothelial spindle cells in the adrenal gland of two thirds of patients who underwent adrenalectomy for Buerger's disease [12]. The crystals were absent on the histologic preparations and were only identified by electron microscopy. These spindle cells lacked the ultrastructural features of adrenocortical cells and resembled fibroblasts. In our study, we did not identify any population of spindle cells coexpressing 3BHSD/calretinin in the 10 studied normal adrenal glands. Despite their intra-abdominal location, Leydig cells from AIS cases lack RCs, and this dispels the notion that formation of RCs is caused by an increased core body temperature [3]. The lack of ARs in LC-AIS correlated with markedly reduced expression of 3BHSD but did not affect calretinin. This indicates that

under physiologic conditions, ARs either upregulate or prevent the downregulation of 3BHSD but do not affect calretinin. Calretinin is not present in androgen-producing adrenal cortex cells, indicating that it does not participate in androgen synthesis and that it is independent from adrenocorticotrophic hormone receptor (ACTH-R). The origin of testicular adrenal rest tumors, TARTs, and TTAGS is not clear; it has been hypothesized that they represent ectopic adrenal tissue or that they arise from pluripotent perivascular cells; however, experimental support is lacking [13–16]. Our work shows that TARTs and TTAGS show a unique phenotype between Leydig cells and androgen-producing adrenocortical cells. All these groups show similar expression of inhibin and androgens; however, TARTs/TTAGS show reduced expression of 3BHSD compared to Leydig and adrenocortical cells, and the expression of calretinin and androgen receptors is intermediate between Leydig and adrenocortical cells, in which these proteins are either strongly expressed or negative, respectively. In contrast to what has been previously reported, synaptophysin and CD56 immunostains lacked a discriminative power for separating testicular adrenal-like lesions from LCTs; expression of CD56 is consistent in both lesions, while expression of synaptophysin is erratic [13–15]. We tried to determine if differences in the expression of ACTH-Rs exist between LCs, testicular adrenal-like lesions, and adrenocortical cells as suggested by the pathophysiology of adrenogenital syndrome; however, after testing several commercially available antibodies, we did not find one with adequate specificity for performing these studies. Finally, LCTs compared to

normal Leydig cells and testicular adrenal-like lesions showed decreased expression of androgens. It is not clear if this is the result of decreased production, decreased cytoplasmic storage, or increased secretion of these hormones.

Conclusions

We found RCs in 90 % of LCT cases, a percentage that is much higher than what has been described in reference books. We also demonstrated consistent immunophenotypic differences between the different groups of normal and neoplastic androgen-producing cells and between androgen-producing cells containing and lacking RCs. The main differences among these groups were in the level of expression of 3BHSD, calretinin, and AR. We found that RCs stain specifically for 3BHSD and calretinin, indicating that they represent crystallized forms of a 3BHSD/calretinin complex. The main difference between androgen-producing cells containing or lacking RCs is the level of expression of these two proteins; crystals are only present in cells with strong concomitant expression of both proteins, a feature unique to Leydig cells and LCTs. Our findings clarify previously unknown aspects of normal and neoplastic androgen-producing cell groups with overlapping morphologic and biological features and the composition of Reinke crystals. It would have been important to determine if differences in expression of ACTH-Rs exist between these cell groups; unfortunately, we were not able to find a reliable antibody for performing these studies.

Compliance with ethical standards The Institutional Review Board (IRB) of the Minneapolis VA Health Care System approved this project on 11 December 2015 (IRB # 4615B).

Conflict of interest The authors declare that they have no conflicts of interest.

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