Abundance of DLK1, differential expression of CYP11B1, CYP21A2 and MC2R, and lack of INSL3 distinguish testicular adrenal rest tumours from Leydig cell tumours

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Abstract

Objective: Testicular adrenal rest tumours (TARTs) are a common finding in patients with congenital adrenal hyperplasia (CAH). These tumours constitute a diagnostic and management conundrum and may lead to infertility. TART cells share many functional and morphological similarities with Leydig cells (LCs), and masses consisting of such cells are occasionally misclassified as malignant testicular tumours, which may lead to erroneous orchiectomy in these patients.

Design: In this study, we aimed to investigate the potential of LC developmental markers and adrenal steroidogenic markers in the differential diagnosis of TARTs and malignant LC tumours (LCTs).

Methods: We investigated mRNA and protein expression of testicular steroidogenic enzymes; CYP11A1 and HSD3B1/2, markers of adrenal steroidogenesis; CYP11B1, CYP21A2 and ACTH receptor/melanocortin 2 receptor (MC2R), and markers of LC maturation; and delta-like 1 homolog (DLK1) and insulin-like 3 (INSL3) in testicular biopsies with TART, orchiectomy specimens with LCTs and samples from human fetal adrenals.

Results: Expression of testicular steroidogenic enzymes was observed in all specimens. All investigated adrenal steroidogenic markers were expressed in TART, and weak reactions for CYP11B1 and MC2R were observed at the protein level in LCTs. TART and fetal adrenals had identical expression profiles. DLK1 was highly expressed and INSL3 not detectable in TART, whereas INSL3 was highly expressed in LCTs.

Conclusions: The similar expression profiles in TART and fetal adrenals as well as the presence of classical markers of adrenal steroidogenesis lend support to the hypothesis that TART develops from a displaced adrenal cell type. Malignant LCTs seem to have lost DLK1 expression and do not resemble immature LCs. The different expression pattern of DLK1, INSL3 and most adrenocortical markers adds to the elucidation of the histogenesis of testicular interstitial tumours and may facilitate histopathological diagnosis.

Introduction

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder with impaired function of the adrenal cortex, characterised by decreased synthesis of adrenal glucocorticoids and often also mineralocorticoids, combined with increased synthesis of adrenal androgens. CAH is predominantly (80–95% of cases) caused by mutations in the CYP21A2 gene encoding the enzyme steroid 21-hydroxylase (cytochrome P-450c21) (1, 2).
Adult male patients with CAH often suffer from infertility and testicular adrenal rest tumours (TARTs) are observed in up to 96% of these patients (3). TART lesions have been observed in boys with CAH as young as age 7.5 (4), and their presence seems to depend on the specific CYP21A2 mutation. TART is not an invasive tumour, but may eventually cause a testicular degeneration by obstruction of the rete testis and destruction of the surrounding testicular tissue (5). The current consensus among researchers is that TARTs are derived from ectopic adrenal cells that have migrated to the gonad in early fetal life (6, 7, 8). This hypothesis is in agreement with the findings of the expression in TART of mRNAs that are normally expressed in the adrenal cortex, and that TART cells are able to produce steroid hormones characteristic of the adrenal cortex and Leydig cells (LCs) in the testis (9, 10).

TART cells share morphological and functional similarities with the steroid-producing LCs, and it is very difficult to distinguish TART from true LC tumours (LCTs), which are the most common neoplasms of the testicular interstitial compartment. The factors used to differentiate TART from LCT include: the high frequency of bilateralism of TART (83%) in contrast to LCT (3%), the hormone profile with high 17-hydroxyprogesterone and adrenal androgens, and the regression of TART following dexamethasone treatment and a previous diagnosis of CAH. A correct diagnosis is essential, as TART and LCT require different management strategies; TART may respond well to medical treatment (11), although testis-sparing surgical resection has been proven possible (12). By contrast, LCTs require surgery, most often testis-sparing surgery, but orchiectomy may be needed in rare malignant LCTs (8). The coexistence of TART and a true LCT is rare, but complicates the differential diagnosis in such patients (13, 14, 15). In most patients, the CAH diagnosis is established shortly after birth, but in milder forms of CAH the presence of a testicular mass later in life may be the first clinical sign. TARTs in such patients are in high risk of being misclassified, resulting in erroneous removal of the entire testis (16, 17).

Our previous work has shown that insulin-like 3 (INSL3) and delta-like 1 homolog (DLK1) are differentially expressed in LCs during development, with the expression pattern suggesting that DLK1 is a marker of progenitor or immature LCs (18). In contrast, INSL3 is a specific marker of mature LCs (19). DLK1, also known as fetal antigen 1 (FA1), is not specific for LCs; it is expressed broadly in endocrine tissues and its expression has also been found in cells of the fetal and adult adrenal cortex, and in adrenal gland tumours (20, 21, 22). We therefore hypothesised that TART would also express DLK1, and that TART would be unlikely to express INSL3. We also wished to examine at the protein level the expression of classical adrenal steroidogenic markers, such as CYP11B1, CYP21A2 and the adrenocorticotropic hormone/melanocortin 2 receptor (MC2R). Hence, in this study, we investigated the above-mentioned markers and specifically addressed the question whether the expression pattern of DLK1 and INSL3 could be used in differential diagnosis of TART and LCT.

Materials and methods

Tissue collection and preparation

Patients with TARTs and LCTs were recruited from Copenhagen University Hospital, Rigshospitalet, Denmark. Informed consent from patients was obtained before sample collections. Testicular specimens containing TART were obtained at the time of a diagnostic biopsy. Two LCTs were collected at the time of surgical removal or orchiectomy, and four additional LCTs were obtained from the archive at the pathology department. Fetal adrenals were obtained from elective-induced abortions performed at gestational week (GW) 7–11. Following surgical excision, the samples were immediately fixed in formalin, or modified Stieve’s fixative, dehydrated and embedded in paraffin. Samples for gene expression analysis were snap frozen on dry ice or embedded in O.C.T. compound (Sakura Fintek Europe, Zoeterwonde, The Netherlands) and snap frozen at −80°C in isopentan (TART and fetal adrenals) and stored at −80°C. A total of 16 fixed tissue specimens – eight TARTs, five LCTs and five fetal adrenals – were included in the immunohistochemical investigations. For analysis of gene expression by reverse transcriptase (RT)-PCR, some cell populations were microdissected from specimens with heterogeneous histology (see next paragraph), and the analysis was performed in a panel of nine samples: two microdissected TARTs, one LCT, one normal adult testis, one adult testis with severe LC hyperplasia and two each microdissected samples of fetal adrenal permanent zone and fetal zone.

Microdissection of TART and fetal adrenals

Pools of TART cells and cells from the fetal adrenal permanent and fetal zones were isolated by laser capture microdissection. Briefly, 6–10 µm (fetal adrenals) and 16–18 µm (TART) sections were cut on a Microm HM550 Cryotome (Thermo Scientific, Kalamazoo, MI, USA), collected on nuclease and nucleic acid-free membrane.
slides (Molecular Machines and Industries, Zurich, Switzerland), immediately fixed in 75% EtOH for 10 min and stored in 100% EtOH at –80°C. All procedures were performed using burned glassware and diethyl pyrocarbonate (DEPC) (Sigma Chemical Co.)-treated water. Before microdissection, the sections were stained with Meyer’s haematoxylin, dehydrated in a series of EtOH solutions (75–100%) in DEPC-treated water and air dried at room temperature. TART cells and fetal adrenal zones were identified by location and morphology and were microdissected at room temperature using the LMD CellCut and SmartCut systems (Olympus/Molecular Machines and Industries). The collected cells were immediately dissolved in 100 μl lysis solution provided in the RNAqueous micro Kit (Ambion, Austin, TX, USA) and stored at –80°C.

**Gene expression analysis by RT-PCR**

RNA was isolated from microdissected samples of TART and fetal adrenals using the RNAqueous–Micro Kit (Ambion) and subsequently amplified using the MessageAmp II aRNA Amplification Kit (Ambion). RNA from frozen specimens of LCTs and testis with normal spermatogenesis was purified using the Nucleospin RNA II Kit (Macherey-Nagel, Duèren, Germany), including DNase I treatment, as described by the manufacturers. The RNA concentration was quantified using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

One microgram of RNA was reverse-transcribed with AMV reverse transcriptase (USB, Cleveland, OH, USA) using a dT20 primer and random hexamers, as described by the manufacturer. PCR experiments were performed thrice with Taq DNA polymerase (GE Healthcare, Buckinghamshire, UK) in a total volume of 30 μl, using 1 μl of cDNA as template or water as a negative control.

Specific intron-spanning primers (Table 1) were based on human sequences from Ensembl (www.ensembl.org), except primers for MC2R, which were identified in a previous published paper (23). PCR conditions were: 95°C for 3 min, 40 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 60 s, and then a final extension at 72°C for 5 min. PCR products were separated by gel electrophoresis, and DNA was extracted using the Nucleospin Extract II Kit (Macherey-Nagel) and sequenced (Eurofins MWG Operon, Ebersberg, Germany). Sequences were verified by Ensembl BLAT analysis (www.ensembl.org/Homo_sapiens/blastview).

**Table 1** RT-PCR primers used in this study.

<table>
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<tr>
<th>Gene symbol</th>
<th>Primer sequence (forward)</th>
<th>Primer sequence (reverse)</th>
<th>Amplicon (bp)</th>
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<td>DLK1</td>
<td>TGGCCACGCCACCTATGG</td>
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<td>INSL3</td>
<td>AGCCGAGGAGCGCTGCCAC</td>
<td>CAGCCGCACTGAGCCTAGT</td>
<td>198</td>
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<tr>
<td>CYP11A1</td>
<td>TTGGATCGTGCTGCGGACTT</td>
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<td>CYP11B1</td>
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<td>CYP21A2</td>
<td>GAGTTCTGTAGCCGATGAG</td>
<td>GAATCACGTCCACAATTGGAT</td>
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<tr>
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<td>CCTGCCGTTGGAATAGCGGAGG</td>
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<td>RPS20</td>
<td>AGACTTTGGAATCACTACAAGA</td>
<td>ATCTGCAATGCTCTGCCAC</td>
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**Table 2** Primary antibodies used in this study.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Concentration (Stieve’s/Formalin)</th>
<th>Buffer</th>
<th>Source</th>
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<tr>
<td>Insulin-like 3 (INSL3)</td>
<td>1:750/1:1500/TEG</td>
<td>Urea/TEG</td>
<td>Dr Charlotte Harken Jensen, University of Southern Denmark, Denmark</td>
</tr>
<tr>
<td>Cytochrome P450, family 11, subfamily B, polypeptide 1 (CYP11B1)</td>
<td>1:50/1:250</td>
<td>Citrate</td>
<td>HPA028615, Atlas Antibodies AB, Sweden</td>
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<tr>
<td>Cytochrome P450, family 21, subfamily A, polypeptide 2 (CYP21A2)</td>
<td>1:100/1:200</td>
<td>TEG</td>
<td>sc-374096, Santa Cruz Biotechnology, Inc., TX, USA</td>
</tr>
<tr>
<td>Melanocortin 2 receptor (MC2R) Smooth muscle actin (SMA) Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 and 2 (HSD3B1 + 2)</td>
<td>1:2500/1:500</td>
<td>Urea</td>
<td>sc-13107, Santa Cruz Biotechnology, Inc., TX, USA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TEG</td>
<td>ab7817, Abcam, UK</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Citrate</td>
<td>Prof. Ian Mason, University of Edinburgh Centre for Reproductive Biology, Scotland</td>
</tr>
</tbody>
</table>
Histology and immunohistochemistry

The histology was evaluated using haematoxylin and eosin (HE)-stained sections. The immunohistochemical staining was performed using a standard indirect peroxidase method and a commercial Kit (Zymed Histostain Kit, San Francisco, CA, USA), as described previously (18). Primary antibodies used in this study are listed in Table 2. For negative control, the primary antibody was replaced with Tris-buffered saline.

All sections were first examined under a light microscope (Reichert-Jung Polyvar, Depew, NY, USA) and subsequently scanned on NanoZoomer HT 2.0 and evaluated using NDPview software version 1.2.36 (Hamamatsu Photonics, Herrsching am Ammersee, Germany).

Ethical considerations

Patient material was obtained and handled in accordance with the Helsinki declaration and approved by the ethics

Figure 1

Histological comparison of TART (A1, A2 and A3) and malignant LCT (B1, B2 and B3). Haematoxylin and eosin (HE)-stained sections from testicular biopsies with TART or LCT shown in different magnifications. Note the abundance of fibrous tissue surrounding the nodule and scattered in the tissue of TART (see arrows). The prominent vascularisation in TART was demonstrated by IHC analyses for SMA expression (C1 and C2). Bars correspond to 250 \( \mu m \) in A1 and A2, B1 and B2, to 50 \( \mu m \) in A3, B3 and to 200 \( \mu m \) in C.
committees of the Capital Region of Denmark (protocol number H-2-2009-37).

Results

Histological features of TART and LCT

A histological comparison of TART and LCT is illustrated in Fig. 1. The general features were similar, with dense borders of fibrous tissue, particularly surrounding islands of TART cells (Fig. 1A and B). We found the most prominent vascularisation in TARTs compared with LCTs, as demonstrated by the immunohistochemical staining for smooth muscle actin (SMA) in vascular epithelial cells (Fig. 1C1 and C2).

Gene expression of DLK1, INSL3, CYP11A1, CYP11B1, CYP21A2 and MC2R

DLK1 transcripts were detected in TART and in the permanent and fetal zones of fetal adrenals, but not in the LCT (Fig. 2). DLK1 was also expressed in adult testis with ongoing spermatogenesis, where its expression seemed to be increased in samples with LC hyperplasia. The markers of adrenal steroidogenesis, cytochrome P450, family 11, subfamily B, polypeptide 1 (CYP11B1), cytochrome P450, family 21, subfamily A, polypeptide 2 (CYP21A2) and melanocortin 2 receptor (MC2R), showed a pattern similar to DLK1, with a signal in TART and fetal adrenals, but not in LCT.

In contrast, INSL3 gene expression was detected in LCT and testis with normal spermatogenesis, but not in TART or fetal adrenals. CYP11A1 expression was observed in all samples investigated.

Protein expression of markers of LC maturation and of adrenal and testicular steroidogenesis

Having observed a mutually exclusive gene expression pattern of DLK1 and markers of adrenal steroidogenesis vs INSL3, we examined protein expression by immunohistochemistry. In LCTs, the expression of DLK1 and CYP21A2 was very low or non-detectable, and when expressed, the two proteins were only observed in a small subset of the tumour cells (Fig. 3 A1 and E1). CYP11B1 and MC2R showed a weak but more consistent expression in LCTs (Fig. 3 D1 and F1). In contrast to LCTs, DLK1 was highly expressed in all samples with TART; five out of seven samples expressed DLK1 in all TART cells, while the remaining two samples expressed DLK1 in the majority of TART cells, with only a few DLK1-negative cells (Fig. 3A2). All steroidogenic markers were also present in TART, but the intensity of staining was less pronounced than DLK1 (Fig. 3 D2, E2 and F2). The INSL3 protein was not observed in TART samples (Fig. 3B2). Conversely, INSL3 was expressed in varying degrees in the LCTs (Fig. 3B1); it was observed in all tumour cells (n=2), or in a subset of tumour cells (n=2). We also examined markers of testicular steroidogenesis, and all TARTs and LCTs expressed CYP11A1 (Fig. 3 C1 and C2) and HSD3B1/2 (data not shown). The expression profile in TART was similar to that observed in fetal adrenals (Fig. 3 column 3). The immunohistochemical expression of all markers is summarised in Table 3.

Figure 2

Gene expression of DLK1, INSL3, CYP11A1, CYP11B1, CYP21A2, and MC2R transcripts in fetal adrenal permanent zone (FA-PZ) and fetal zone (FA-FZ), TART, LCT, testis tissue with normal spermatogenesis (NT) and testis tissue with decreased spermatogenesis and severe LC hyperplasia (NT-LC Hyp). The expression of RPS20 was used as a loading control. (The water sample was run on the same gels as the other samples, but three lanes apart from the NT-LC Hyp sample.).
Figure 3

Immunohistochemical expression of DLK1 (A1, A2 and A3), INSL3 (B1, B2 and B3), CYP11A1 (C1, C2 and C3), CYP11B1 (D1, D2 and D3), CYP21A2 (E1, E2 and E3) and MC2R (F1, F2 and F3) in testicular specimens of LCT (column 1), TART (column 2) and fetal adrenals at gestational week 10 (column 3 A–C) or gestational week 11 (column 3 D–E) analysed by IHC. Bars correspond to 50 μm.
we have detected the transcripts in TART and fetal insensitivity of our mRNA expression analysis, although in the Leydig tumours. This could be explained by the MC2R proteins, but we have not detected any transcripts some immunohistochemical reaction of CYP11B1 and detectable or expressed at low levels in LCTs. We observed steroidogenic enzymes, except CYP11A1, were non-

Interestingly, INSL3, a marker of mature LCs, was expressed in TART (10) point towards a high plasticity and close relationship of all steroidogenic cell types, but this needs more studies to be confirmed and fully understood.

Immunohistochemical analysis of a panel of markers described in this study may improve differential diagnosis of these conditions and prevent erroneous orchiectomy, which still occasionally happens (16). We propose that using only two biologically relevant markers, DLK1 and INSL3, is informative because the two proteins display a mutually exclusive expression pattern and robust antibodies are available.

LCTs and LC hyperplasia with cells grouped in large micronodules are the primary conditions interfering with the diagnosis of TART. The malignant tumours are fortunately rare, but LC micronodules are very common in men with subfertility or testicular failure. LC hyperplasia with micronodules is always benign and may be distinguished from TART by the absence of encapsulating fibrous tissue, and the presence of remnant seminiferous tubules within and adjacent to the LC nodules. Despite the fact that DLK1 is expressed in both benign conditions, it is strikingly abundant in TART, whereas the typical pattern of DLK1 expression in LC hyperplasia is very heterogeneous (18).

Our previous observation of DLK1 expression in immature and progenitor LCs is consistent with the presence of DLK1 in undifferentiated cells in various endocrine tissues (26, 27, 28). The high expression of DLK1 in TART may suggest that these nodules develop from an immature or even fetal cell type. The similar expression profiles of TART and fetal (this study) and adult (21) adrenal cortical cells lend support to the hypothesis that TART derives from an adrenal-like cell type, possibly misplaced in the gonad during early embryonic development (6, 7, 8). Moreover, the absence of INSL3 expression in TARTs is at odds with the development of TART from a less mature cell type. However, it does not exclude the possibility that TART derives from the DLK1-positive/INSL3-negative LC population, which predominantly exists during fetal life, and is also observed in the adult testis with signs of dysgenesis (18). It is also not clear what cell type is the progenitor of malignant

<table>
<thead>
<tr>
<th>DLK1</th>
<th>TART (n=8)</th>
<th>LCTs (n=5)</th>
</tr>
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<tbody>
<tr>
<td>INSL3</td>
<td>+ + + a</td>
<td>Neg to +</td>
</tr>
<tr>
<td>CYP11A1</td>
<td>+ + to + + +</td>
<td>+ to + + +</td>
</tr>
<tr>
<td>HSD3B1/2</td>
<td>+ + +</td>
<td>+ to + + +</td>
</tr>
<tr>
<td>CYP11B1</td>
<td>+ + to + + +</td>
<td>+ to + + +</td>
</tr>
<tr>
<td>CYP21A2</td>
<td>+ + b</td>
<td>Neg to +</td>
</tr>
<tr>
<td>MC2R</td>
<td>+ to + + +</td>
<td>Neg to +</td>
</tr>
</tbody>
</table>

Staining score: + + +, positive in all cells; + + , positive in the majority of cells; +, positive in a few cells; Neg, negative.

aA few cells negative in 2/8 specimens.
bCYP21A2 was only investigated in one TART sample due to incompatibility of the antibody with the fixative of the other TART samples.

Discussion and conclusions

Young adult or adolescent patients presenting with a testicular enlargement represent a difficult diagnostic dilemma. Besides testicular germ cell tumours, which are relatively easy to diagnose due to the distinct clinical features and availability of diagnostic serum markers, TART and LCT are the primary cause of management considerations. Especially in patients with milder forms of CAH, which are often recognised late and not properly treated, a management decision is often made during explorative surgery. Surgery is also suggested if an LC neoplasm is suspected, as the majority of LCT cases in this age group are benign. Few studies investigated immunohistochemical profiles of TART and LCT, and the currently available markers include synaptophysin, CD56 (both stronger in TART than in LCT) and the androgen receptor (absent in TART but positive in most cases of LCT) (24, 25).

A recent study, published after our study had been submitted for publication, investigated classical markers of steroidogenesis at mRNA level, and found most markers expressed in TART (10).

In this study, we investigated markers of testicular and adrenal steroidogenesis and more specific markers of LC maturation, at both the mRNA and protein levels. The results revealed a marked differential expression of DLK1 and classical adrenal steroidogenesis enzymes vs INSL3.

Interestingly, INSL3, a marker of mature LCs, was expressed in LCTs, at both the mRNA and protein levels, but not in TART nodules. DLK1 and most of the steroidogenic enzymes, except CYP11A1, were non-detectable or expressed at low levels in LCTs. We observed some immunohistochemical reaction of CYP11B1 and MC2R proteins, but we have not detected any transcripts in the Leydig tumours. This could be explained by the insensitivity of our mRNA expression analysis, although we have detected the transcripts in TART and fetal

adrenals. An alternative explanation is a possible unspecific cross-reaction of the antibodies with related proteins. A similar discrepancy but in the opposite direction was observed in the study by Smeets et al. (10), who detected low levels of the INSL3 mRNA transcripts but no protein in TART samples. Observations of the low expression of adrenal steroidogenic markers in LCTs (this study) and of classical LC markers in TARTs (10) point towards a high plasticity and close relationship of all steroidogenic cell types, but this needs more studies to be confirmed and fully understood.

Our previous observation of DLK1 expression in immature and progenitor LCs is consistent with the presence of DLK1 in undifferentiated cells in various endocrine tissues (26, 27, 28). The high expression of DLK1 in TART may suggest that these nodules develop from an immature or even fetal cell type. The similar expression profiles of TART and fetal (this study) and adult (21) adrenal cortical cells lend support to the hypothesis that TART derives from an adrenal-like cell type, possibly misplaced in the gonad during early embryonic development (6, 7, 8). Moreover, the absence of INSL3 expression in TARTs is at odds with the development of TART from a cell population within one of the LC lineages. However, it does not exclude the possibility that TART derives from the DLK1-positive/INSL3-negative LC population, which predominantly exists during fetal life, and is also observed in the adult testis with signs of dysgenesis (18). It is also not clear what cell type is the progenitor of malignant

| Table 3  Immunohistochemical analysis of TART and LCTs. |
|---------------------------|------------|------------|
|          | TART (n=8) | LCTs (n=5) |
| DLK1     |             |            |
| INSL3    |             |            |
| CYP11A1  |             |            |
| HSD3B1/2 |             |            |
| CYP11B1  |             |            |
| CYP21A2  |             |            |
| MC2R     |             |            |

Staining score: + + +, positive in all cells; + + , positive in the majority of cells; +, positive in a few cells; Neg, negative.

aA few cells negative in 2/8 specimens.
bCYP21A2 was only investigated in one TART sample due to incompatibility of the antibody with the fixative of the other TART samples.
LCTs, but these cells have lost DLK1 expression and do not resemble immature LCs. Smeets et al. (10) proposed that TART may be derived from an ‘embryonic totipotent’ cell type and suggested that a fetal LC may be such a cell type. However, additional markers of different types of steroid cell differentiation are needed to further elucidate the aetiology of TART and to assess possible differential stages of TART cells.

In conclusion, a careful histological evaluation of tumour biopsy is essential for correct diagnosis of TART and LCTs, and the diagnosis is greatly aided by immunohistochemical analysis of markers of adrenocortical steroidogenesis and LC maturation, especially DLK1 and INSL3. The differential expression pattern of these markers adds also to the elucidation of the histogenesis of testicular interstitial tumours.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
G Lottrup, A Juul, N E Skakkebæk, and E Rajpert-De Meyts conceived and designed the study. G Lottrup and J E Nielsen developed and performed the experiments and analysed the data. G Lottrup and E Rajpert-De Meyts prepared the manuscript, which was amended and approved by all authors.

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