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CASE REPORT

Elevated levels of the steroidogenic factor 1 are associated with over-expression of *CYP19* in an oestrogen-producing testicular Leydig cell tumour

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Abstract

Background and objectives: Testicular Leydig cell tumours (LCTs) are rare, steroid-secreting tumours. Elevated levels of aromatase (*CYP19* or *CYP19A1*) mRNA have been previously described in LCTs; however, little is known about the mechanism(s) causing *CYP19* over-expression. We report an LCT in a 29-year-old male with elevated plasma oestradiol caused by enhanced *CYP19* transcription.
Design and methods: First, we measured the intra-tumour expression of *CYP19* and determined the use of *CYP19* promoters by qPCR. Secondly, we explored *CYP19* and promoter II (PII) for gene amplifications and activating mutations in *PII* by sequencing. Thirdly, we analysed intra-tumour expression of steroidogenic factor 1 (*SF-1* (*NR5A1*)), liver receptor homologue-1 (*LRH-1* (*NR5A2*)) and cyclooxygenase-2 (*COX2* (*PTGS2*)). Finally, we analysed *SF-1* for promoter mutations and gene amplifications.
Results: Similar to what has been recorded in normal Leydig cells, we first found the bulk of tumour *CYP19* transcripts to be PII derived, excluding promoter shift as a cause of enhanced transcription. Secondly, we excluded *CYP19* and *PII* gene amplifications, and activating mutations in *PII*, as causes of elevated *CYP19* mRNA. We found *SF-1* mRNA to be up-regulated in the tumour, while *LRH-1* and *COX2* were down-regulated. The finding of elevated *SF-1* levels in the tumour was confirmed by immunohistochemistry. The elevated level of *SF-1* was not due to promoter mutations or amplifications of the SF-1 gene.
Conclusions: Our results strongly suggest that the elevated levels of SF-1 have induced PII-regulated *CYP19* transcription in this tumour. These findings are of relevance to the understanding of CYP19 up-regulation in general, which may occur in several tissues, including breast cancer.

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Introduction

Leydig cells are found adjacent to the seminiferous tubules in the testicles. These cells constitute the main androgen-synthesising compartment in adult males and are also capable of oestrogen production (reviewed in (1, 2, 3, 4)).

Leydig cell tumours (LCTs) of the testis are rare, representing about 1–3% of all testicular tumours. They are most frequently diagnosed in pre-pubertal boys between 5 and 10 years and adult men aged 30–60 years (5, 6). While usually benign, about 10% of LCTs in adult patients reveal a malignant phenotype (7).

LCTs are steroid-secreting tumours and consequently associated with endocrine disturbances. In testosterone-secreting LCTs, boys usually present symptoms of precocious puberty, whereas excess androgen rarely causes notable effects in adults. About one-fourth of all LCTs are oestrogen secreting, and the most common symptoms include gynaecomastia in addition to sexual dysfunction and infertility in adults (6, 8, 9, 10). There have also been described cases of LCTs in pre-pubertal boys, revealing symptoms of both oestrogen and androgen production, causing gynaecomastia and precocious puberty in concert (reviewed in (4)).

Local oestrogen synthesis plays a functional role in the testis (reviewed in (11, 12)). In resemblance with other oestrogen-producing compartments, androgens are converted into oestrogens by the aromatase

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(CYP19) enzyme. CYP19 is encoded by the *CYP19A1* gene, localised at chromosome 15q21.2 (13, 14, 15). The unusually large regulatory region of this gene contains several tissue-specific promoters, and each of the promoters give rise to mRNAs with distinct untranslated first exons (16). The first exon is spliced onto a common splice junction in exon II, immediately upstream of the coding region. Thus, the different mRNA species expressed from this gene contains an identical open reading frame (exons II–X), and the translated protein is the same, regardless of the promoter used.

Elevated *CYP19* expression with subsequent high plasma oestradiol (E_2) levels has been reported in a few cases of testicular LCTs (17, 18, 19, 20, 21). The main regulator of normal testicular cell aromatization is the *CYP19* proximal promoter II (PII), which has also been reported as the main active promoter in LCTs (18, 20).

Different response elements have been identified in *PII*, and both steroidogenic factor 1 (*SF-1* (encoded by *NR5A1*)) and liver receptor homologue-1 (*LRH-1* (encoded by *NR5A2*)) have been reported to be involved in PII-regulated *CYP19* expression (22, 23, 24, 25). Moreover, several cAMP-response element (CRE)-like sequences have been identified in PII (26, 27), and cyclooxygenase-2 (*COX2* (encoded by *PTGS2*)) was recently reported to be involved in the phosphorylation of CRE-binding proteins and thus stimulate *CYP19* expression and the proliferation of Leydig tumour cells (28).

While over-expression of *SF-1* was reported in Fisher rat testicular tumours (22), so far we lack information regarding what mechanism(s) may cause *CYP19* over-expression in human testicular LCTs. Here, we report an oestrogen-producing LCT in a 29-year-old man. The finding of *SF-1* over-expression in concert with elevated levels of the PII-derived *CYP19* transcripts suggests increased *SF-1* levels to play a key role in stimulating oestrogen synthesis in these tumour cells.

Materials and methods

Patient

A 29-year-old male with severe gynaecomastia and elevated plasma E_2 levels (248 pM, normal upper range 130 pM) was referred to the Endocrine Unit. He was subsequently diagnosed with an oestrogen-producing LCT on the right testis. The tumour was not palpable but visualised on testicular ultrasound. He had normal pubertal growth, had developed normal secondary sex characteristics and was the father of one child. There was no clinical, biochemical (HCG and α-fetoprotein negative) or radiological sign of malignant disease.

The high serum E_2 levels returned to normal after surgical removal of the tumour. Although E_2, testosterone and SHBG levels normalised rapidly, LH and FSH remained elevated at follow-up, indicating impaired function of the left testicle. All serum hormone levels are summarised in Table 1. At 1-year follow-up, he felt well and the gynaecomastia had resolved.

Subject to patient consent, part of the tumour specimen obtained at orchidectomy was snap-frozen and stored in liquid nitrogen for research purposes.

Histology

A part of the testis specimen was fixed in 4% formalin and representative samples including both tumour and normal tissues were embedded in paraffin. Standard haematoxylin- and eosin (H&E)-stained sections (5 μm) were made for microscopic histology evaluation.

Immunohistochemistry

Sections (5 μm) from formalin-fixed, paraffin-embedded tumours were prepared. Immunohistochemical aromatase enzyme staining was performed using the MAB Aro677 (gift from Dr Dean Evans, Novartis AG). SF-1 was stained with the mouse antihuman MAB 434200 (Invitrogen), while the oestrogen and progesterone receptors (PRs) were stained using the MABs M7047 and M3659 (Dako, Glostrup, Denmark) respectively. For the staining procedure, the DAKO Envision HRP mouse kit (Dako) with DAB as detection method was used.

RNA extraction and cDNA synthesis

Total RNA was extracted from snap-frozen biopsies using Trizol reagent (Life Technologies) according to the manufacturer's procedure and dissolved in DEPC-treated deionised water as described by Knappskog *et al.* (29). The RNA concentration was measured on a Nanodrop ND1000 spectrophotometer and adjusted to 1 μg/μl.

Single-strand cDNA was synthesised from 4 μg total RNA using Transcriptor reverse transcriptase (Roche) according to the manufacturer's procedure. Both oligoT (16-mers) and random hexamers were used as primers in the cDNA synthesis reaction mix.

Table 1 Patient serum hormone levels (pmol/l).

	Normal	Before operation	1 Month after operation	1 Year follow-up
S-oestradiol (pmol/l)	20–130	248	112	124
S-testosterone (nmol/l)	6.7–31.9	7.8	15.2	19.0
S-SHBG (nmol/l)	13–71	50	40	41
S-LH (IE/l)	0.8–7.6	2.8	26.7	25.9
S-FSH (IE/l)	0.7–11.1	0.9	25.4	29.6

Table 2 Primers and probes.

Primers/probes	Sequences
mRNA expression	
Total *CYP19* F	5′-ATCCTCAATACCAGGTCCTGGC
Total *CYP19* R	5′-AGAGATCCAGACTCGCATGAATTCT
Total *CYP19* probe	5′-6FAM-ACCCGGTTGTAGTAGTTGCAGGCACT-BBQ
RPLP2 F	5′-GACCGGCTCAACAAGGTTAT
RPLP2 R	5′-CCCCACCAGCAGGTACAC
RPLP2 probe	5′-Cy5-AGCTGAATGGAAAAAACATTGAAGACGTC-BBQ
*CYP19*_exon1.3 F	5′-GTCTAAAGGAACCTGAGACTCTACC
*CYP19*_exon1.4 F	5′-CACTGGTCAGCCCATCAA
*CYP19*_exon1.7 F	5′-AGAAAGGGGTGAAATCAGCAA
*CYP19*_exon1 common R	5′-ACGATGCTGGTGATGTTATAATGT
*CYP19*_exon1 common probe	5′-6FAM-TCGGGTTCAGCATTTCCAAAACCAT-BBQ
*CYP19*_exonII F	5′-CCCTTTGATTTCCACAGGAC
*CYP19*_exon3 R	5′-CCCATGCAGTAGCCAGGAC
*CYP19*_exonII probe	5′-6FAM-CATGCCAGTCCTGCTCCTCA-BBQ
COX2 F	5′-ATCACAGGCTTCCATTGACC
COX2 R	5′-CAGGATACAGCTCCACAGCA
COX2 probe	5′-6FAM-CCGCAAACGCTTTATGCTGA-BBQ
SF-1 F	5′-TCATCCTCTTCAGCCTGGAT
SF-1 R	5′-AGGTACTCCTTGGCCTGCAT
SF-1 probe	5′-6FAM-ACGCTCAGGAGAAGGCCAAC-BBQ
LRH-1 F	5′-GCACAGGAGTTAGTGGCAAA
LRH-1 R	5′-CTGCTGCGGGTAGTTACACA
LRH-1 probe	5′-6FAM-AACAAGTCAATGCCGCCCT-BBQ
Gene copy number analysis	
CYP19 gDNA F1	5′-TTATGGGAGCCTGGTAGTGG
CYP19 gDNA R1	5′-AGGGTGGGGTTTTCTATTGG
CYP19 gDNA probe 1	5′-6FAM-TTGGAACATCATACCCCGGG-BBQ
CYP19 gDNA F2	5′-GGTGGCTTTTGCTGATCAAT
CYP19 gDNA R2	5′-AGAAAATGGGCACGAAACTG
CYP19 gDNA probe 2	5′-6FAM-AGCTCCTGCCAGCTCCCTTT-BBQ
CYP19 PII gDNA F1	5′-TTGGTCAAAAAAGGGGAGTTG
CYP19 PII gDNA R1	5′-ATCATCTTGCCCTTGAGTGG
CYP19 PII gDNA F2	5′-TCCACCTCTGGAATGAGCTT
CYP19 PII gDNA R2	5′-TTGCAGCATTTCTGACCTTG
CYP19 PII gDNA probes 1 and 2	5′-6FAM-CTTTCAATTGGGAATGCACG-BBQ
CYP19 PII gDNA F3	5′-GGGCTTCCTTGTTTTGACTT
CYP19 PII gDNA R3	5′-GAGGGGGCAATTTAGAGTCC
CYP19 PII gDNA probe 3	5′-6FAM-CACCCTCTGAAGCAACAGGA-BBQ
B2M gDNA F	5′-CATCCAGCAGAGAATGGAAAG
B2M gDNA R	5′-GAAAGACCAGTCCTTGCTGAA
B2M gDNA probe	5′-Cy5-TGGGTTTCATCCATCCGACA-BBQ
PCR/sequencing of *CYP19* PII and *SF-1* promoter area	
CYP19 PII PCR F1	CTCAACGATGCCCAAGAAAT
CYP19 PII PCR R1	CATGGACCAAAATCCCAAGT
CYP19 PII Seq F2	TTGGTCAAAAAGGGGAGTTG
CYP19 PII Seq R2	CTTACCTGGTATTGAGGATGTGC
CYP19 PII Seq F3	GGCAAGAAATTTGGCTTTCA
CYP19 PII Seq R3	GAGGGGGCAATTTAGAGTCC
SF-1 F2	TCTGCCTCCCAGGTTCAAGC
SF-1 R8	GGTGACTGGATCTTTTGTGTGTTCCTC
SF-1 F4	GAGGAACACACAAAAGATCCAGTCACC
SF-1 R7	CTTGGCCAGCTGGCTGTTG
SF-1 F6	TGTCCTGACTCTACTCCAATGTCCG
SF-1 R2	AACCCCTGAGAAACAAGAGCATCTG
SF-1 R9 (seq)	TGTGTGTGTTCCTCTGTTTGTCTCAC
SF-1 R10 (seq)	GGGCTTGATTTATGGGCTGCTC
SF-1 F6 (seq)	TGTCCTGACTCTACTCCAATGTCCG
SF-1 F8 (seq)	CACCAACAAAGAAGGCGAGAGG
SF-1 F9 (seq)	TCTGTCCCCCACCTGAGTTTC
SF-1 F10 (seq)	CCACTGCCACCCTCATCC

DNA extraction

Genomic DNA was extracted from snap-frozen biopsies using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's procedure.

Quantitative PCR

Transcript levels of total *CYP19*, promoter-specific *CYP19* transcript variants, *COX2*, *SF-1* and *LRH-1*, were quantified using the Lightcycler 480 instrument (Roche). Ribosomal protein P2 (*RPLP2*) mRNA level was used as reference. The amplification primers and BlackBerry-quenched hydrolysis probes used are listed in Table 2.

Amplification was performed in a 20 μl reaction solution using the LC480 Probes Master (Roche) reaction mix, 0.5 μM of each primer, 0.125 μM hydrolysis probe and 0.5 μl cDNA synthesised from 4 μg total RNA. The following thermocycling conditions were used: initial denaturation/enzyme activation at 95 °C for 5 min and then 50 cycles of 95 °C for 10 s (denaturation) and 55 °C for 20 s (annealing/elongation) before a final cooling step at 40 °C for 5 s. Negative controls (water) were included in each run.

The results were converted into relative concentrations using an in-run standard curve, and then normalised for *RPLP2* mRNA levels. All gene-specific data from the tumour sample were compared with the corresponding data from a reference sample consisting of pooled total RNA from healthy testis of five donors (BioChain, Newark, CA, USA).

The expression levels of the promoter-specific transcript variants were assessed using different primers and assay-specific standard curves (i.e. different qPCR efficiencies), and the data are therefore not directly comparable. However, an estimate of the differences in expression of these promoters can be made by assuming that all reactions run with the same efficacy (efficiency = 2) and comparing the delta crossing points of the different assays.

Gene copy number analysis (qPCR)

In order to investigate possible gene copy number changes of the *CYP19* gene and the *CYP19 PII*, levels of genomic DNA were quantified in duplex reactions with beta-2-microglobulin (*B2M*) as reference using the Lightcycler 480 instrument (Roche). Two distinct *CYP19* genomic areas and three distinct areas covering the *CYP19 PII* region were amplified using the primers and BlackBerry-quenched hydrolysis probes listed in Table 2. Amplification was performed in a 20 μl reaction solution using the LC480 Probes Master (Roche) reaction mix, 0.5 μM of each primer, 0.125 μM of each hydrolysis probe and 2 μl gDNA as template. The following thermocycling conditions were used: initial denaturation/enzyme activation of 95 °C for 5 min and then 50 cycles of 95 °C for 15 s (denaturation) and 55 °C for 20 s (annealing/elongation) before a final cooling step at 40 °C for 5 s. Negative controls (water) were included in each run. The data obtained through quantification were normalised by adjusting for *B2M* levels. These normalised values were divided by the corresponding values from a reference sample (pooled DNA from six healthy donors). The concentration of the reference was set to 1.0, and we considered the samples to have reduced copy number if the sample/reference ratio was <0.65 and increased copy number if the ratio was >1.35.

PCR amplification and sequencing

The *CYP19 PII* and *SF-1* promoter regions were amplified and sequenced using primers listed in Table 2. The *SF-1* gene has two alternative, untranslated first exons. Relative to the transcription start sites of these two exons, we sequenced the area $-1475/+1053$ (alternative 1) and $-1650/+858$ (alternative 2).

PCR amplification was performed using the Kod XL DNA polymerase system (Merck) in a 50 μl reaction mix containing 1× PCR buffer, 0.2 mM of each deoxynucleotide triphosphate, 0.2 μM of each primer, 1.25 U Kod XL DNA polymerase and 1 μl genomic DNA. The thermocycling conditions used were an initial denaturation step of 5 min at 94 °C, followed by 30 cycles of denaturation (94 °C for 1 min), annealing (*CYP19 PII*: 54.9 °C and *SF-1*: 63.2 °C for 10 s) and elongation (72 °C for 30 s) and a final elongation step of 7 min at 72 °C. Following amplification, the PCR product was treated with ExoSAP-IT (USB Products, Affymetrix, Inc., Santa Clara, CA, USA) at 37 °C for 30 min and 80 °C for 15 min according to the procedure as outlined by the manufacturer. DNA sequencing was performed in a 10 μl reaction mix containing 1×

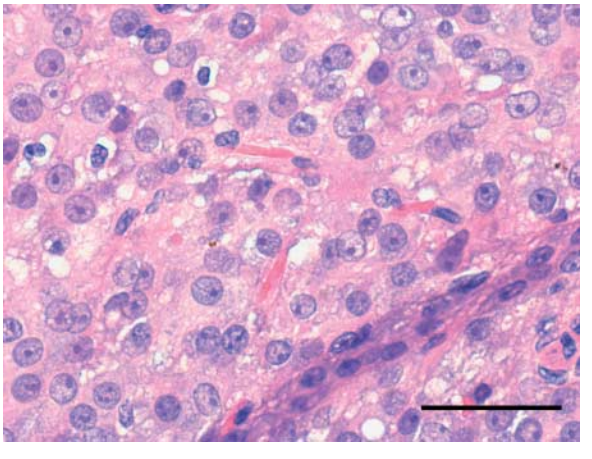

Figure 1 Standard H&E-stained section (5 μm) of testicular Leydig cell tumour. The intra-testicular tumour (largest diameter of 1.8 cm) was sharply delimited from the testicular parenchyma. The tumour cells had abundant, deeply acidophilic, finely vacuolised cytoplasm with focally deposited brownish yellow lipochrome pigment and intracytoplasmic Reinke's crystalloids. Scale bar = 50 μm.

sequencing buffer, 1 μM primer and 1× BigDye v.1.1 (Applied Biosystems, Carlsbad, CA, USA). Capillary electrophoresis was performed on an automated DNA sequencer (ABI 3730).

Multiplex ligation-dependent probe amplification

The multiplex ligation-dependent probe amplification (MLPA) method and the SALSA MLPA kit P185-B1 lot 0308 (MRC Holland, Amsterdam, The Netherlands) were used according to the manufacturer's instructions to identify deletions or amplifications in the *SF-1* gene (*NR5A1*) localised on 9q33.3. Capillary electrophoresis, data collection and peak analysis were performed on an automated DNA sequencer (ABI 3730). In the patient sample, the peak areas of all MLPA products resulting from *SF-1*-specific probes were first normalised by the average of peak areas resulting from control probes specific for locations other than on chromosome 9q33.3. A ratio was then calculated where this normalised value was divided by the corresponding value from a sample consisting of pooled DNA from six healthy individuals. A sample was scored as having a reduced copy number at a specific location if this ratio was below 0.75 and as having an increased copy number if the ratio was above 1.25.

Results

Histology

The intra-testicular tumour (largest diameter of 1.8 cm) was sharply delimited from the testicular parenchyma with a non-infiltrative 'pushing' border and a solid/nodular growth pattern. The tumour cells had abundant, deeply acidophilic, finely vacuolised cytoplasm with focally deposited brownish yellow lipochrome pigment and intracytoplasmic Reinke's crystalloids, as displayed by H&E staining in Fig. 1. No malignant histological features were observed, i.e. tumour diameter <5 cm, lack of infiltrating border, few mitoses (mean 1 per 10 high power fields), absence of necrosis, no vascular invasion and low tumour cell proliferation assessed with Ki-67/MIB-1 (1–2%). Immunohistochemically, vimentin and inhibin were strongly co-expressed and cytokeratin AE1/3 showed weak and focal positivity, typical for this entity. The epithelial marker (EMA) and germ cell markers (AFP, PLAP) were negative. PR was weakly positive in about 5% of tumour nuclei and oestrogen receptor alpha (ERα) was negative.

CYP19 *expression level in the tumour tissue*

We examined the *CYP19* expression level and found it to be strongly up-regulated (125-fold) in the tumour sample compared with normal testis tissue (Fig. 2A). Then, we determined promoter usage by quantifying *CYP19* transcript variants specific for promoter PII, 1.3, 1.4 and 1.7 in both tumour and normal tissues (Fig. 2B, C, D and E). The expression level of the PII-specific *CYP19* transcript in the tumour sample was 59-fold elevated relative to normal testis tissue. In addition, we found the transcript variants specific for promoters 1.3 and 1.7 to be up-regulated in the tumour tissue. The transcript related to promoter 1.3 displayed an 11-fold increase from normal to tumour tissue while the levels of the variant specific for promoter 1.7 became detectable in the tumour sample contrasting undetectable levels (after 50 amplification cycles) in the normal sample. Interestingly, the transcript variant specific for

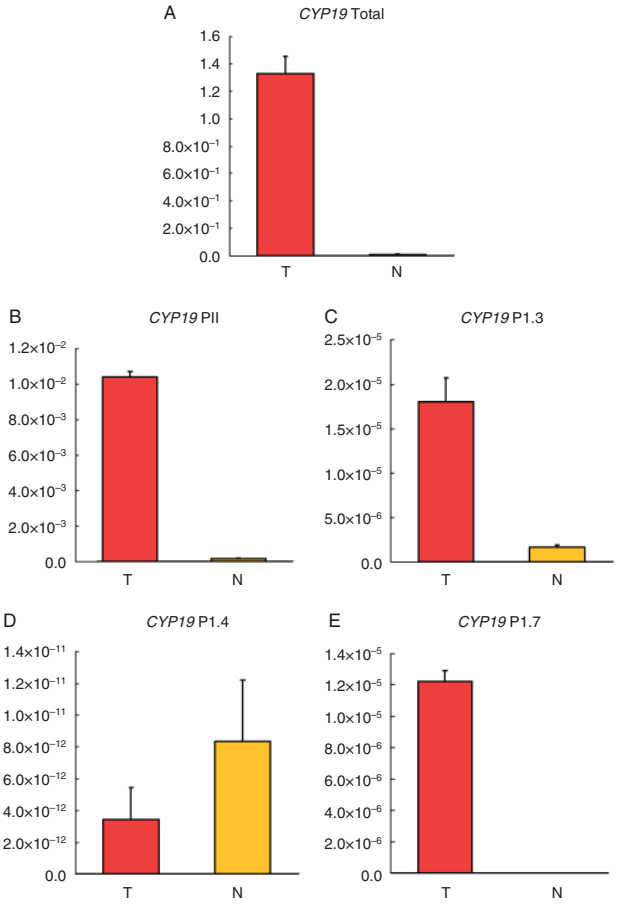

Figure 2 mRNA levels of total *CYP19* and *CYP19* promoter-specific transcript variants. Transcript levels of total aromatase (*CYP19*) and promoter-specific *CYP19* transcript variants were determined in both tumour (T) and normal (N) testes with qPCR. Triplicate runs were performed, and expression of the ribosomal protein P2 (*RPLP2*) was used as reference in all the reactions. Individual in-run standard curves were used in the five different assays and the relative concentrations as displayed on the *y*-axes are therefore not comparable between the assays A, B, C, D and E. We observed an increased transcript level of (A) total *CYP19* (125-fold), (B) promoter II-specific *CYP19* (59-fold), (C) promoter 1.3-specific *CYP19* (11-fold) and (E) promoter 1.7-specific *CYP19* (not detected in the normal sample). (D) We observed a down-regulation of promoter 1.4-specific *CYP19* transcript level (tumour to normal ratio: 0.4).

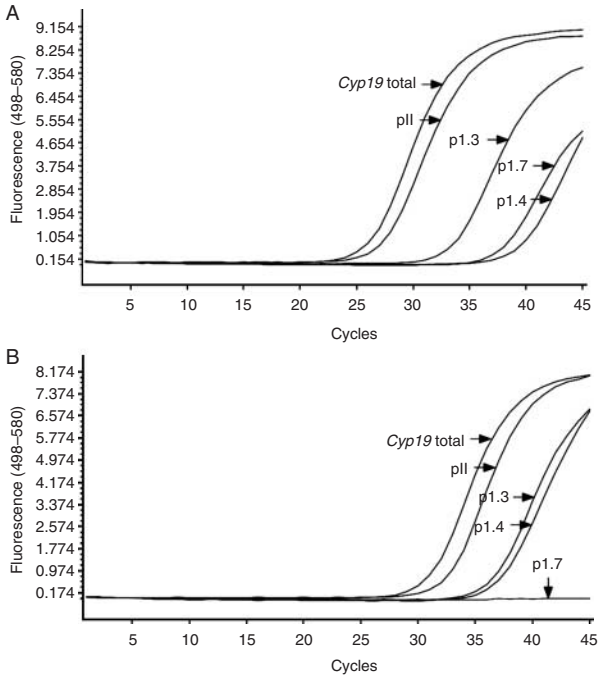

Figure 3 qPCR-crossing points (Cps) for total *CYP19* and *CYP19* promoter-specific transcript variants. The qPCR-Cp values for each of the promoters examined were compared in a common run. Three runs were performed per promoter assay, and the curves shown here are representative for these runs. All *CYP19* assays were run with *RPLP2* as a reference; however, the curves for *RPLP2* were removed from the figure for clarity. The Cps strongly indicated that the main contribution to the total *CYP19* mRNA level was from PII. (A) Tumour sample, (B) normal tissue sample.

promoter 1.4 was down-regulated in the tumour tissue (tumour to normal tissue ratio: 0.4).

Three runs were performed per promoter assay, and the curves shown in Fig. 3 are representative for these runs. The curves for *RPLP2* were removed from Fig. 3 for clarity. When assuming that the qPCRs run with the same efficacy, we found the tumour *CYP19* mRNA levels deriving from promoter PII to be 77-, 1233- and 7236-fold higher than from promoters 1.3, 1.7 and 1.4 respectively.

This finding strongly indicates that *PII*-transcribed mRNA accounts for the majority of the total *CYP19* mRNA detected. Moreover, this finding reveals PII to be the principal promoter in both normal and tumour tissue, arguing against the use of an alternative promoter as an explanation for elevated *CYP19* levels.

CYP19 *coding region and* PII *gene copy number*

Addressing the potential causes for *CYP19* over-expression, we analysed the gene copy number for *CYP19* using qPCR. No amplifications within the *CYP19* locus were observed.

In addition, due to the large contribution from PII, we speculated whether *CYP19* up-regulation could be due to either a selective amplification of the *PII* area or to an activating mutation located in this promoter area. Hence, we performed a copy number analysis specifically for the region harbouring this promoter and sequenced the *PII* region. No promoter amplification, mutations or polymorphisms were detected.

CYP19 protein staining

In order to validate the data obtained by qPCR, we analysed CYP19 at the protein level by immunohistochemistry (IHC). Anti-CYP19 (Aro 677)-stained sections revealed focal strong cytoplasmic staining of tumour Leydig cells and negative staining of normal tissue sections (Fig. 4).

The transcription factor SF-1 *is up-regulated in the tumour*

Based on the lack of copy number changes within the *CYP19* gene and PII, and/or mutations in *PII*, we hypothesised that the *CYP19* over-expression could be

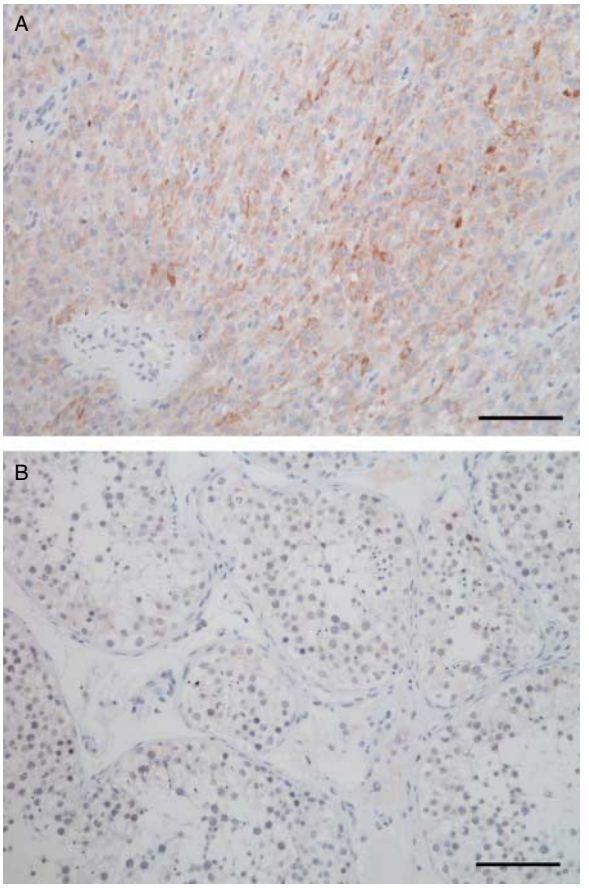

Figure 4 Anti-CYP19 staining of tumour and normal tissue. Anti-CYP19 (Aro 677) stained sections showed (A) strong cytoplasmic staining for tumour Leydig cells, and (B) negative staining in normal tissue. Scale bar: 100 μm.

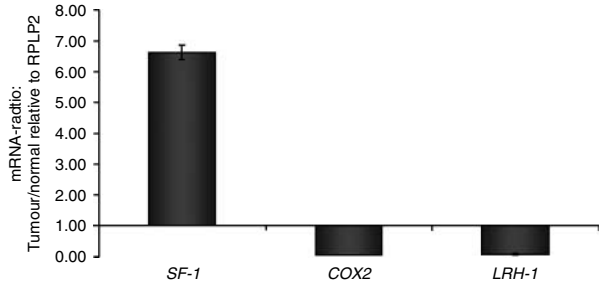

Figure 5 The mRNA levels of *SF-1*, *COX2* and *LRH-1* (tumour/normal tissue ratio) were analysed by qPCR. *COX2* and *LRH-1* were down-regulated in the tumour compared with normal tissue (tumour to normal ratios of 0.04 and 0.08 respectively). In contrast, we found the mRNA level of *SF-1* to be 6.6-fold up-regulated in the tumour tissue compared with the normal sample.

caused by increased levels of a trans-acting factor. Regarding the structure of PII and its known binding sites for different transcription factors, we analysed the mRNA levels of three potential ligand candidates: *COX2*, *LRH-1* and *SF-1*. *COX2* and *LRH-1* were down-regulated in the tumour compared with normal tissue (tumour to normal ratios of 0.04 and 0.08 respectively). In contrast, we found the mRNA level of *SF-1* to be 6.6-fold up-regulated in the tumour tissue compared with the normal sample (Fig. 5).

In order to validate the data obtained by qPCR, we analysed SF-1 at the protein level by IHC. Anti-SF-1-stained sections showed strong nuclear staining in tumour tissue and negative staining in normal tissue (Fig. 6).

Finally, we explored potential causes of *SF-1* over-expression by analysing the copy number of this gene using MLPA, as well as sequencing the promoter region for potential activating mutations. No promoter mutations, amplifications or deletions of *SF-1* were observed.

Discussion

While most LCTs reveal benign characteristics, the elevated steroid production may cause disturbing virilising or feminising symptoms.

In this report, we describe a patient with an oestrogen-producing LCT revealing elevated *CYP19* and *SF-1* mRNA levels. While some previous papers reported elevated oestrogen production in LCTs to be related to enhanced CYP19 mRNA levels (17, 18, 19, 20, 21), the underlying cause of elevated *CYP19* mRNA in these tumours has not been reported so far. The results presented here provide further insight into the mechanism(s) of CYP19 up-regulation in LCTs.

First, we ruled out gene amplification of the *CYP19* gene as a potential cause of mRNA up-regulation.

Secondly, we evaluated the use of *CYP19* promoters. While the CYP19 enzyme is coded for by a single gene, it is subject to tissue-specific regulation due to several alternative promoters (16). Further, there is evidence that *CYP19* expression may be differentially regulated in tumours compared with their normal tissue of origin. Taking breast cancer as an example, *CYP19* transcription in normal breast tissue is mainly regulated by promoter 1.4, while transcription in breast cancer tissue is regulated by the promoters II, 1.3, 1.7 and 1.4 (30). Here, we found that the majority of the *CYP19* mRNA transcripts in both normal and tumour tissue originate from PII. Thus, the elevated *CYP19* mRNA level observed in the tumour tissue was not due to alternative promoter use, in conformity with what has been described earlier in a few other cases (18, 20).

Thirdly, we measured intra-tumour mRNA levels of the ligands *SF-1*, *LRH-1* and *COX2*. We found elevated mRNA levels of *SF-1* in tumour compared with normal tissue, a finding validated by the observation of elevated SF-1 protein staining by IHC. While our results are in accordance with findings in experimental systems (22), to the best of our knowledge, *SF-1* up-regulation in LCTs has not been demonstrated in humans earlier.

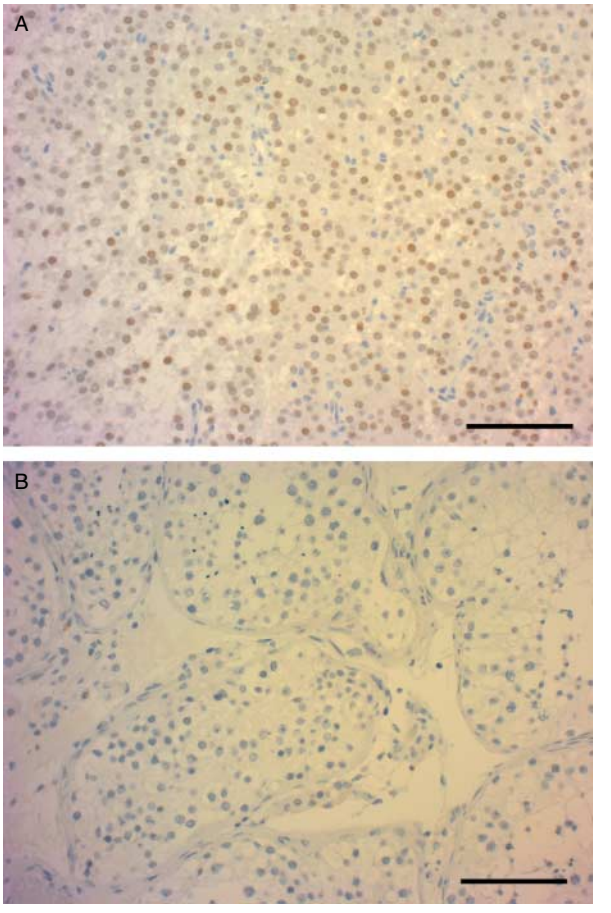

Figure 6 Anti-SF-1 staining of tumour and normal tissue. Anti-SF-1-stained sections showed strong nuclear staining in tumour tissue (A) and negative staining in normal tissue (B). Scale bar: 100 μm.

Notably, the *SF-1* mRNA up-regulation was neither due to *NR5A1* gene amplification nor due to activating mutations in the promoter area. Moreover, we found the expression of *LRH-1* and *COX2* to be significantly down-regulated in tumour tissue compared with normal tissue. LRH-1, like SF-1, has been demonstrated to bind a nuclear receptor half site in PII (31) and regulate *CYP19* expression in Leydig cells (23, 25). Our observation of *SF-1* up-regulation in concert with *LRH-1* down-regulation might indicate that there is a negative feedback loop controlling the expression of these genes.

In previous studies, local *COX2* up-regulation has been discussed as a potential mechanism of elevated oestrogen synthesis in inflammation and some cancers (reviewed in (32)), but we lack evidence confirming this *in vivo*. To the best of our knowledge, this is the first report to demonstrate a similar effect on oestrogen synthesis through local SF-1 up-regulation.

In summary, we found elevated oestrogen synthesis in an LCT to be caused by elevated PII-transcribed *CYP19* levels. Moreover, we found the enhanced *CYP19* transcription to be most likely caused by elevated levels of the transcription factor SF-1. Interestingly, CYP19 up-regulation in oestrogen-dependent breast cancer has been attributed to enhanced PII activity (30). Our findings reveal pathological SF-1 up-regulation to be a potential mechanism enhancing local oestrogen synthesis in LCTs, suggesting this mechanism to be explored as a potential cause of CYP19 up-regulation in other pathological conditions as well.

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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