Case report

Precocious pseudo-puberty in a 2-year-old girl, presenting with bilateral ovarian enlargement and progressing to unilateral juvenile granulosa cell tumour

Short title: Juvenile Granulosa Cell Tumour in a 2-yr-old girl

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What is known about this subject?

- Causes of feminising precocious pseudo-puberty of ovarian origin include follicular cysts, McCune-Albright syndrome (MAS) and Juvenile Granulosa Cell tumour (JGCT)
- MAS and JGCT are associated with mutations in the GNAS and AKT1 genes respectively, but GNAS mutations have also been found in JGCT

Ovarian involvement is usually unilateral in JGCT, and unilateral or bilateral in MAS

What does this study add?

- We present a case in which feminizing precocious pseudo-puberty was initially associated with bilaterally enlarged, cystic ovaries.
 - Shortly after presentation, the signs of feminisation escalated and repeat imaging showed a histologically proven JGCT in one ovary, with return of the other ovary to normal.
- Although a molecular genetic cause has not yet been identified, with normal GNAS, AKT1 exon 3 and FOXL2 sequencing, this unique observation shows how a unilateral JGCT was preceded by enlargement and cystic change in both ovaries.

Abstract

Ovarian causes of precocious pseudo-puberty (PPP) include McCune-Albright syndrome (MAS) and juvenile granulosa cell tumour (JGCT). We describe a case of PPP in which bilateral ovarian enlargement with multiple cysts progressed to unilateral JGCT. A girl aged 2.17 years presented with 3 months of breast development, and rapid growth. Examination showed tall stature, height + 2.6 standard deviations, Tanner stage B3P2A1. A single café au lait patch was noted. Bone age was advanced at 5 years. Pelvic ultrasound showed bilaterally enlarged ovaries (estimated volumes 76 ml on the left, 139 ml on the right), each containing multiple cysts. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) values before/after gonadotrophin administration were 0.43/0.18 and < 0.1/<0.1 mUI/ml, serum estradiol 130 pg/ml, (prepubertal range < 20). PPP of ovarian origin was diagnosed, and Tamoxifen 20 mg daily started. However, after only 7 weeks height velocity escalated and breast development increased to B3-4 with menorrhagia. Basal/stimulated LH and FSH were still suppressed at 0.13/0.25 and <0.1/<0.1 mUI/ml, serum estradiol 184 pg/ml. Repeat imaging now showed normal right ovary (volume 1.8 ml) and a large left-sided vascular solid/cystic ovarian tumour which was excised (weight 850g). Histology showed JGCT, FIGO stage IA. DNA from tumour tissue showed no mutation in GNAS, exon 3 of AKT1 (which contains a mutational hotspot) or FOXL2. The observation that bilateral ovarian activity progressed to unilateral development of JGCT in this patient is novel. This case highlights current uncertainties in the ontology of JGCT, and its possible relationship with MAS.

Keywords: feminizing pseudoprecocious puberty; ovary; juvenile granulosa cell tumour; McCune-Albright syndrome

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Introduction

Premature sexual development can be broadly divided into true, or central, precocious and early puberty arising from activation of the hypothalamo-pituitary axis; and precocious pseudo-puberty in which there is either autonomous secretion of sex steroids by the gonads or adrenal glands, or from exogenous sources [1].

Causes of ovarian precocious pseudo-puberty include autonomous follicular cysts, which are usually self-limiting [2], the McCune Albright syndrome (MAS), sex cord tumours arising from granulosa and thecal cells, and from germ cell tumours. MAS usually results from a somatic gain-of-function mutation in the *GNAS1* gene which encodes the G-protein alpha subunit – GNAS, causing a mosaic pattern involvement of various tissues, particularly skin, bone and ovary [3]. Principal features are café au lait patches, precocious pseudo-puberty due to ovarian involvement and polyostotic fibrous dysplasia [4]. Renal phosphate wasting may accompany the latter [5]. Rarely, MAS occurs in association with Cushing's syndrome [6], gigantism/acromegaly [7] and hyperthyroidism [8]. The diagnosis can be made clinically when there is multisystem involvement, but isolated monostotic bone lesions may require confirmation by histology and molecular analysis is indicated in atypical cases with single organ involvement [9].

Granulosa cell tumours belong to the group of sex cord-gonadal stromal tumours and account for 5% of all malignant ovarian tumours [10]. Adult granulosa cell tumours are caused by mutations in *FOXL2*, the gene encoding Forkhead box protein L2 [11], usually present after 30 years of age and account for only <1% granulosa cell tumours in prepubertal children [12]. Juvenile granulosa cell tumours, which represent 5-12% of ovarian tumours in childhood, usually present before 30 years of age with feminizing precocious pseudo-puberty in children, disturbance of menstrual cycle with or without signs of hyperandrogenism in women, and abdominal mass at any age (13). In some cases, torsion of the annexe or tumour rupture with haemo-peritonitis result in presentation with an acute abdomen. Biological markers such as Inhibin B and Anti-Müllerian Hormone levels are raised and are of value in tumour monitoring. Juvenile granulosa cell tumour has been associated in 60% of cases with mutations of the *AKT1* gene, which encodes the RAC-alpha serine/threonine-protein kinase enzyme [14]. Of note, *GNAS* mutations have been described in some cases of juvenile granulosa cell tumour [15].

We present a case in which feminizing precocious pseudo-puberty was associated with bilateral ovarian enlargement initially, but which then progressed to juvenile granulosa cell tumour with normal appearances of the contralateral ovary.

Methods

Clinical assessment, biochemical investigations and diagnostic imaging

These were carried out in Military Hospital of Tunis. Height and weight were measured using standardized equipment, and values converted to standard deviations (SD) for corresponding ages using the normative French data of Sempe and Pedron [16]. Skeletal maturity ("bone age") was assessed by the method of Greulich and Pyle [17]. Luteinising hormone (LH), follicle stimulating hormone (FSH) were sampled before and 15, 30 and 60 minutes after stimulation with 100 mcg of luteinizing-hormone releasing hormone (LHRH) and measured using chemiluminescence immunoassay (UniCel DxI 600 Access Immunoassay System, Beckmann Coulter). Estradiol was measured using enzyme-linked fluorescent immunoassay (VIDAS, Biomérieux). Laboratory reference ranges for LH during the follicular phase, mid-cycle, and luteal phase were 1-7, 6-73 and 0.5-10 mIU/ml. For FSH the equivalent ranges were 3-8, 4-18 and 2-8 mIU/ml and for estradiol < 266, 118-255, and 26-165 pg/ml. For prepubertal girls, mean $\pm$ SD for LH was 0.03 ± 0.03 mIU/ml and for FSH 2.16 ± 1.14 . Prepubertal range for estradiol was < 20 pg/ml. Limit of detection was <0.1 mIU/ml for LH and FSH, and <10 ng/ml for estradiol.

Ultrasound imaging was carried out in the radiology department of the Military Hospital of Tunis using a General Electric Logiq S7 model featuring a 3,6-15 MHz transducer.

Magnetic resonance imaging (MRI) was carried out using MRI MAGNETOM (Verio-Siemens 3 Tesla). Since only two of three dimensions – length, width, and depth – were available for the initial ultrasound, approximate volumes were calculated using the prolate ellipsoid formula and assuming that width and depth were the same on the sagittal view and that length was at least the same as width and depth on the transverse view.

Bone imaging was carried out according to a standardised protocol using a gamma camera (Siemens), and scanning the whole skeleton two hours after injecting 99m technetium-labelled hydroxymethyl diphosphonate

Tumour histopathology, immune staining and genetic studies

Paraffin blocks of tumour were sent first to the Royal National Orthopaedic Hospital in London where histopathology findings were corroborated and mutational analysis for *GNAS1* were performed. DNA was successfully extracted, and the sample was tested for the hotspots R201H, R201C and Q227L, as previously described [18].

The paraffin blocks were then sent on to the François Jacob Institute of Biology in Paris, where mutational analysis of exon 3, a mutational 'hot spot' of the *AKT1* gene, and *FOXL2*-C134W status were carried out. DNA was extracted from paraffin-embedded tumor material using xylene. Tissue was then rehydrated with successive baths of ethanol, then vortexed and centrifuged, and the supernatant removed. After further ethanol rehydration steps, tissue was digested with proteinase K and DNA extraction performed with the Nucleospin DNA rapid Lyse kit (Macherey-Nagel). Given the poor quality of the DNA extracted from paraffin-embedded tissue, amplification of exon 3 of *AKT1* required a semi-nested PCR, using three primers. The central part of *FOXL2* was amplified by conventional PCR. PCR was performed with the Herculase II Fusion DNA Polymerase (Agilent) according to the manufacturer protocol. Sanger sequencing was performed by Eurofins according to their in-house procedures.

Finally, since the original histology report from Tunisia had suggested the presence of Call-Exner bodies, which are associated with adult rather than juvenile granulosa cell tumour, tissue was sent from Paris to the pathology department at Glan Clwyd Hospital in Wales, United Kingdom for further review.

Consent for genetic analysis and publication of the case was obtained from the parents.

Case study

A girl was admitted aged 2.17 years for assessment of premature sexual development which took the form of rapid growth and breast development over a 3-month period.

Delivery was by Caesarean section at 38 weeks' gestation, birth weight 3650 grams, birth length 50 cm, head circumference 34.5 cm. There was no relevant family history, and parents were unrelated. The mother's menarche was at 12 years.

On examination, the child was well, height 94 cm (+2.6 SD), weight 13.2 kg (+1 SD) compared with a mid-parental height of -0.4 SD (mother's height 160 cm, father's height 174 cm). Bone age was 5.0 years (chronological age 2.2 years). Pubertal stage according to Tanner was B3P2A1. The abdomen was supple and non-tender with no palpable masses. Skin examination showed a single café au lait patch situated on the antero-lateral border of the left thigh, 3 cm in its longest axis, with irregular outline. There were no lentiginos, haemangiomas or subcutaneous tumours, no hepato-splenomegaly, and no bony tenderness or deformity.

Biochemical investigations before and after LHRH stimulation showed basal/peak LH values of 0.43/0.18 mIU/ml, FSH values < 0.1/<0.1 mIU/ml. Serum estradiol was 130 pg/ml, (prepubertal range < 20 pg/ml).

Pelvic ultrasound examination showed uterine length 3.6 cm with fundus-cervical ratio 1.3 and pubertal configuration, 4 mm of endometrial thickness. The images of each ovary, recorded at the time of examination, are of relatively inferior quality but show enlargement with multiple cysts and echogenic stroma (Figure 1a and b). The left ovary measured 63 x 48 mm in sagittal plane, estimated volume 76 ml, right ovary 66 x 61 mm in transverse plane, estimated volume 139 ml. Each ovary contained both discrete and coalescent cysts (see Figure 1a and b).

MAS was considered a possibility in the light of the pelvic ultrasound and skin findings. Further investigations to assess parathyroid, growth hormone, adrenal, thyroid and skeletal status were therefore carried out, with normal results: serum calcium 2.36 mmol/L, phosphate 1.59 mmol/L (reference range 1.2-2.0 mmol/L), IGF-1 103 µg/L (age-related reference range 82-166 µg/L), urine free cortisol 160 nmol/L (reference range 100 -300 nmol/24 hours), free thyroxine 13.5 pmol/L, TSH 2.37 µIU/ml. Bone scintigraphy of the whole skeleton was also carried out to exclude fibrous dysplasia and no bony lesion was identified.

In view of the pseudo-precocious puberty, Tamoxifen 20 mg orally daily was started at 2.3 years but after only 7 weeks, aged 2.45 years the features of premature sexual development escalated with an increase in height of 4 cm, progression of breast development, Tanner stage now B3-4P2A1, and development of menorrhagia. Bone age had advanced further to 6.5 years. A second LHRH test, carried out to exclude secondary activation of the hypothalamo-pituitary axis still showed gonadotrophin suppression with basal/peak LH 0.13/0.25 and FSH <0.1/<0.1 mIU/ml. Serum estradiol was 184 pg/ml.

Repeat pelvic ultrasound now demonstrated a left-sided vascular solid/cystic ovarian tumour measuring 10x8x6 cm lying postero-lateral to the bladder. The right ovary was normal in appearance, volume 1.83 ml.

The left ovarian mass was confirmed by MRI scan (Figure 2) which showed a well-defined solid-cystic abdominal-pelvic mass of left ovarian origin, measuring 12 x 10 x 5 cm, extending to aorta and kidney with no evidence of local invasion. The right ovary was normal. Tumour markers were normal: α fetoprotein 4.2 IU/L (reference range <10 ng/ml), human chorionic gonadotrophin <0 (reference range <2 ng/ml), and ACE: 2.3 ng/ml (reference range <5 ng/ml). CA-125 was slightly raised at 43 IU/ml (reference range <35 nIU/ml). Inhibin B assay was not available.

After a week, the child developed symptoms related to torsion of the ovarian annex, requiring urgent laparotomy with removal of left ovary and annexectomy. At surgery, the tumour capsule of tumour was intact, tumour weight 850g. No malignant cells were found on peritoneal lavage and there was no macroscopic evidence of infiltration of capsule, and no spread to the Fallopian tubes.

Histopathology review showed a tumour with nodular architecture, the nodules being encircled by fibrous tissue forming septae (Figure 3a). There were necrotic foci in some nodules (Figure 3b). High power imaging showed that the tumour cells had abundant pale eosinophilic cytoplasm, with oval, irregular vesicular nuclei, rarely showing nuclear grooves, and with rare mitoses (Figure 3c). Reticulin staining showed fibres surrounding groups of granulosa cells (Figure 3d). No follicles or pseudopapillary architecture was noted and no Call-Exner bodies were seen. Features of germ cell tumour or gonadoblastoma were not identified. There was no capsular infiltration.

Immunohistochemistry showed strongly positive staining for inhibin B but α -fetoprotein and anti-CD30 were not detected. The findings were consistent with a juvenile granulosa cell tumour, graded as FIGO stage IA in view of its confinement within the tumour capsule. Post-operative progress showed immediate regression of the pubertal signs and menorrhagia.

At review aged 5.7 years height was 113 cm (+0.9 SD), weight 17.2 kg (0 SD), pubertal stage B1P1A1, bone age 7.0 years. Basal gonadotrophins showed LH 2.49 mIU/ml, FSH 2.52 mIU/ml, oestradiol 13 pg/ml. Abdominal and pelvic ultrasound were normal.

Thereafter, the girl has remained prepubertal. At last review aged 8.1 years, height was 125 cm (-0.53 SD), Tanner stage B1P1A1, bone age now only slightly advanced at 8.7 years. However, basal LH has continued to show mild elevation despite breast stage and estradiol levels remaining prepubertal. Thus LH/FSH and estradiol values at 6.5, 7.0 and 8.1 years were: 0.87/3.78 and 11 pg/ml; 1.24/1.62 mIU/ml and 12 pg/ml; 1.46/566 mIU/ml and 13 pg/ml.

GNAS1 gene mutation analysis by PCR and restriction enzyme digestion of DNA extracted from paraffin blocks of the tumour was negative for the common hot-spot mutations (R201C, R201H and Q227L). Sequencing of exon 3 in the *AKT1* gene in the tumour tissue did not show a somatic mutation. Finally, sequencing of *FOXL2* was carried out to exclude an adult granulosa cell tumour, and the C134W *FOXL2* pathogenic variant was not found.

Discussion

Although the feminising precocious pseudo-puberty in this case was clearly of ovarian origin, its precise aetiology remains unclear. At presentation with premature sexual development, the morphology of the ovaries reflected bilateral activity with cysts, gonadotrophin suppression and a single café au lait patch on the thigh. Although this pattern is evocative of McCune-Albright syndrome there were no other features of MAS such as bony fibrous dysplasia, with no bony lesion being found on scintigraphy, no abnormality of renal phosphate transport, no disturbance of the GH / IGF-1 axis, Cushing's syndrome, or hyperthyroidism. Moreover, the ovarian volumes – estimated at 76 ml on the left and 139 ml on the right – were extraordinarily large, far greater than in the study of 8 patients with MAS described by Foster et al in whom mean ovarian volumes overlapped with those seen in girls with central precocious puberty [19]. Also, *GNAS1* analysis in the tumour was negative. However, *GNAS1* mutations are not found in the ovarian tissue of every patient with this MAS. It therefore remains possible that our patient has MAS and may show additional signs of this syndrome as she grows older although she remains well with no additional signs six years after initial presentation.

While it was not possible to diagnose MAS with certainty, it was clear that our patient had precocious pseudo-puberty of ovarian origin, as opposed to true precocious puberty, at presentation and she was therefore treated with Tamoxifen, a medication which exerts an anti-estrogen effect through competitive inhibition of binding of estradiol with its receptors. The clinical situation then changed dramatically in less than 2 months, with development of a large tumour in a single ovary. The tumour was limited to one side with intact and tumour-free capsule on the surface of the ovary. According to the International Federation of Gynecology and Obstetrics (FIGO) classification this corresponds to stage IA, where the tumour is localized to the organ of origin. Histological review showed the tumour to be a granulosa cell tumour of juvenile type. In keeping with this, reticulin staining showed fibres surrounding groups of granulosa cells, contrasting with thecoma and fibroma tumours in which fibres surround individual granulosa cells. Moreover, Call-Exner bodies which are a feature of adult granulosa cell tumour, were not present while mutational analysis of *FOXL2* was negative. The diagnosis of juvenile granulosa cell tumour is therefore secure.

Just as a diagnosis of MAS in our patient cannot be supported, neither can her case be fully explained by the diagnosis of unilateral juvenile granulosa tumour since there was documented evidence of bilateral ovarian activity and enlargement on ultrasound at initial presentation. The present diagnosis therefore is purely descriptive – precocious pseudo-puberty of ovarian origin, with progression to juvenile granulosa cell tumour, in which the underlying mechanism remains unclear. We speculate that some disorder of cell-signalling resulted initially in the bilateral ovarian enlargement with cyst formation, and that the cysts were estrogen-secreting. Following this, the process seems to have spontaneously resolved in one ovary, with transformation, perhaps involving one of the cysts, into juvenile granulosa cell tumour.

We believe that ours is a unique observation. Although bilateral juvenile granulosa cell tumour has been reported (12), presentation with bilateral ovarian enlargement evolving towards unilateral regression and contralateral tumour formation has not. The clinical and pathological features of our case are consistent with previous reports, including the recent paper from Ye et al [20].

There is an intriguing overlap between MAS and JGCT juvenile granulosa cell tumour. Mutational analysis using nested PCR is reported to have a 54% sensitivity for detecting a *GNAS* mutation in suspected MAS [21]. The same group also found an activating *GNAS* mutation in 9 of 30 patients with JGCT [15]. Bessière et al found in 10 of 16 juvenile granulosa cell tumour patients in-frame duplications in exon 3 of the oncogene *AKT1* which lead to its activation [14]. Given that analysis of exon 3 in the *AKT1* was negative in our case, whole gene sequencing would be the next diagnostic step in seeking a molecular genetic cause for juvenile granulosa cell tumour. However, given the time and expense involved, this measure would be difficult to justify in an individual patient.

To investigate a putative link between MAS and juvenile granulosa cell tumour, we have examined gene expression in adrenal tissue bearing activating *GNAS1* mutations (Geo Dataset GSE33694 ref PMID: 22259056), and in ovarian tissue from patients with juvenile granulosa cell tumour [22]. Interestingly, both the tissues studied showed significant enrichment in genes which are significantly dysregulated by overexpression of the proto-oncogene *B-Raf* (adjusted p-value 0.002 for the directly expressed McCune-Albright genes and <0.001 for the directly expressed JCGT genes). *B-Raf* is known to be phosphorylated by the *AKT1* gene and also to be differentially regulated by cAMP-dependent protein kinase A activation, which is itself dependant on *GNAS1* activity. Further work examining the interplay between *B-Raf*, *GNAS1* and *AKT* genes might clarify a possible link between MAS and juvenile granulosa cell tumour.

In practical terms, our patient requires continuing follow up in case other features of MAS develop, paying special attention to the morphology of her remaining ovary. Critical attention is also needed to growth rate and pubertal status given the risk of developing true central precocious puberty through previous exposure to raised sex steroid levels – the phenomenon of “priming”. Fortunately, more than five years since the tumour was successfully removed our patient remains prepubertal, height now in the lower half of the normal centile range, and only marginal bone age advance.

Finally, a puzzling aspect of our case for which we have no explanation is the mild, but consistent, elevation of basal LH observed throughout, with a paradoxical drop from 0.43 to 0.18 mIU/ml after LHRH administration at initial presentation. Since successful surgery, mild basal LH elevation has persisted at a level suggestive of true central puberty. Despite this, the patient has remained prepubertal clinically since surgery, with normal oestradiol values for age.

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Disclosure Statement

The authors have no conflict of interest to declare.

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Author Contributions

The patient's clinical care was administered by Dr Barakizou and Professor Gannouni, and the paper was written by these authors, Professor Kamoun and Dr Donaldson. Transport and analysis of tumour blocks for *GNAS* mutation was undertaken by Dr Huma and Dr Amary in London. After transportation of tumour tissue to Paris, Professor Veitia and Dr Todeschini sequenced exon 3 in the *AKT1* gene as well as sequencing *FOXL2*. Dr Mehdi reviewed the tumour histology, created the images for Figure 3 and wrote the histopathological elements of the text.

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Legend to Figures

Figure 1a & b. Ultrasound images of left (1a) and right (1b) ovaries in a 2-year-old girl with feminizing precocious pseudo-puberty and suppressed gonadotrophins. Both ovaries are very enlarged (see text) with echo-dense stroma. Both ovaries contain multiple cysts, some discrete and others coalescent as shown by the arrows.

Figure 2. MRI image of right ovary showing solid/cystic tumour, histology of which showed juvenile cell granulosa tumour. B = bladder, C=cystic tumour, S = solid tumour.

Figure 3a-d. Histology of juvenile granulosa cell tumour with labelled features showing: a) low power haematoxylin and eosin (H&E) image showing cellular neoplasm with nodules divided by fibrous septae; b) low power H&E showing necrotic areas within some nodules and intact ovarian capsule; c) high power H&E showing abundant eosinophilic cytoplasm with round to oval tumour cells with vesicular chromatin, only a few nuclei showing groove formation, and rare mitoses; and d) medium power reticulin stain showing fibres surrounding groups of granulosa cells.

a)

b)

c)

d)