Clinical and molecular aspects of a pediatric metachronous adrenocortical tumor

Aspectos clínicos e moleculares de tumor adrenocortical metacrônico pediátrico

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SUMMARY

The occurrence of metachronous adrenocortical carcinoma has rarely been described. We report a case of a child with virilizing adrenocortical metachronous tumors that, despite several metastases, presented long-term survival (15 years). We analyzed in this tumor IGF2, IGF1R and FGFR4 gene expression, and evaluated the presence of p.R337H germline p53 mutation and somatic CTNNB1 mutation. IGF2 was over-expressed in both left (Weiss score 5) and right (Weiss 7) adrenocortical tumors. IGF1R expression levels were higher in the right adrenocortical tumor. FGFR4 over-expression was also detected in the right adrenocortical tumor. In addition, this patient harbors the germline p.R337H p53 mutation and loss of heterozygosity (LOH) was detected in the tumors. No somatic CTNNB1 mutations were found in both tumors. In conclusion, we demonstrated in this unusual case the over-expression of growth signaling pathways, which are molecular mechanisms previously related to adrenocortical tumorigenesis. Furthermore, the absence of somatic CTNNB1 mutations, which is a molecular marker of poor prognosis in adults, might be related to the long-term survival of this patient.

SUMÁRIO

A ocorrência de carcinomas adrenocorticais metacrônicos é raramente relatada. Descrevemos o caso de uma criança portadora de tumor adrenocortical virilizante metacrônico que, apesar das inúmeras metástases, apresentou uma longa sobrevida (15 anos). Analisamos nesse tumor a expressão gênica de IGF2, IGF1R e FGFR4 e avaliámos a presença da mutação germinativa R337H no p53 e mutação somática no gene CTNNB1. O gene IGF2 foi hiperexpresso nos tumores adrenocorticais esquerdo (Weiss 5) e direito (Weiss 7). Os níveis de expressão de IGF1R foram maiores no tumor direito. Hiperexpressão do gene FGFR4 também foi observada no tumor adrenocortical direito. Esse paciente é portador da mutação germinativa R337H no p53, e perda de heterozigose (LOH) foi observada em ambos os tumores. Não foram encontradas mutações no gene CTNNB1 nos tumores. Em conclusão, demonstramos neste caso a hiperexpressão de vias moleculares de crescimento, que são mecanismos previamente relacionados à tumorigênese adrenocortical. Além disso, não encontramos mutações somáticas no gene CTNNB1, que é um marcador molecular de mau prognóstico em adultos e poderia estar relacionado à longa sobrevida desse paciente.
underlies the genetic predisposition in this population (1-3). Pediatric adrenocortical tumors appear to behave differently than histologically similar tumors in the adult population (4). Unlike the dismal survival statistics in adult adrenocortical carcinoma series, pediatric adrenocortical tumors with apparent poor prognosis on the basis of histopathological features may often have a better outcome (5). Unfortunately, there are no histological or molecular markers so far that can reliably distinguish benign from malignant adrenocortical tumors and define the prognosis. Clearly, the extent of surgical resection is the most important factor in patient outcome (6).

The mechanisms of adrenocortical tumorigenesis are still not fully understood, but several data suggest that malignant transformation is a multistep process (7). Molecular studies of sporadic adrenocortical tumors in the pediatric age group are limited due to the rarity of this condition, however, since children seem to have a better prognosis, it is important to determine whether the same molecular alterations described in adults are also present in children, and whether this is related to the outcome. We report the case of adrenocortical metachronous tumors presenting in a child with long-term survival, emphasizing different molecular pathways that could be involved in the adrenocortical tumorigenesis and related to the benign evolution of this patient.

**CASE REPORT**

This study was approved by the Ethics Committee of Hospital das Clínicas (São Paulo, Brazil). The parents of this child provided informed consent, including for the use of the photographs, images and the report that follows. The patient was born full term after an uncomplicated pregnancy and delivery. At 2 years and 2 months of age, the child presented with signs of virilization (pubic hair, penis enlargement, growth acceleration and aggressiveness) (Figure 1). At that time, his weight and height were 18.0 kg and 100.0 cm respectively (height SD + 3.74). At physical examination he was found to have virilization described as prepubertal testis and more recent facial acne, deepening of the voice, and increased muscle mass, but no abdominal mass was palpable. The family history was unremarkable. Laboratory evaluation demonstrated undetectable gonadotropins and abnormally elevated adrenal steroid levels: DHEA-S, 1790 ng/mL [normal range < 194 ng/mL]; androstenedione, 2.0 ng/mL [normal range < 0.3-2.9 ng/mL], and testosterone, 395 ng/dL [normal range < 19 ng/dL], characterizing isosexual precocious puberty independent of gonadotropins. Abdominal computerized tomography (CT) showed a 2.0 cm mass in the left adrenal gland (Figure 2). The patient underwent surgical procedure and an adrenal mass with 3.0 g of weight and 2.5 cm of diameter was resected.

The adrenocortical tumor had a histological Weiss score of 5 (8) and the MacFarlane modified by Sullivan staging was I (9) (Table 1). Postoperatively, the signs of virilization disappeared and all tumor markers returned to their normal ranges. At that time, the patient received no further treatment.

Two years after surgery the patient presented signs of virilization all over again and, in addition, frequent erections. Hormonal studies revealed elevated androgen levels (DHEA-S, 6,905 ng/mL [normal range, < 194 ng/mL]; androstenedione, 8.1 ng/mL [normal range, 0.3-2.9 ng/mL], and testosterone, 403 ng/dL [normal range < 19 ng/dL]). A novel right adrenal mass was discovered by an abdominal CT (Figure 3). No secondary lesions were detected by radiological studies at this time. The patient underwent surgical resection and intraoperative findings revealed a large (8.5 cm) tumor in the right adrenal gland. The mass was apparently adherent to the kidney and a freezing biopsy showed invasion of the capsule of the liver. The tumor was removed together with the right kidney and with a small portion of the right lobe of the liver. The weight of the tumor was 30.0 g and histological analysis revealed a Weiss score 7. Although no signs of invasion of the liver itself or the kidney were observed the tumor was classified as a carcinoma (MacFarlane modified by Sullivan staging III) (Table 1). Due to the poor prognosis associated with this diagnosis by the observation of invasion of the liver capsule by freezing biopsy, the patient was immediately started on mitotane. The dose was gradually increased to 1 g/d. The patient was also treated with replacement doses of cortisone acetate and fludrocortisone. Five months after tumor resection, the patient developed gynecomastia and severe neurologic side effects, and mitotane was discontinued. He continued to receive steroid replacement, and his adrenal markers remained undetectable.

At 7 years and 8 months of age, his DHEA-S levels increased to 272 ng/mL. This relapse was secondary to a pulmonary metastasis, which was treated by surgery. Histology confirmed metastatic nodule of adrenocortical carcinoma. Seven months after this surgery, a novel elevation of DHEA-S levels was observed. A Positron emission tomography-fludeoxyglucose (PET-FDG) scan and a chest CT showed a left hilar pulmonary metastasis, which was successfully removed by surgical procedure.
Pediatric metachronous adrenocortical tumor

Figure 1. Independent gonadotropins precocious puberty. Enlargement of the penis with prepubertal testis.

Figure 2. Abdominal computerized tomography showing a 2.0 cm mass in the left adrenal gland.

Figure 3. Abdominal computerized tomography showing a 8.0 cm mass in the right adrenal gland.

Table 1. Microscopic pathological assessment according to Weiss system

<table>
<thead>
<tr>
<th>First adrenal tumor</th>
<th>Second adrenal tumor</th>
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<tbody>
<tr>
<td>Left adrenal tumor with 2.5 cm of diameter and 3.0 g of weight</td>
<td>Right adrenal tumor with 8.5 cm of diameter and 30.0 g of weight (tumor + right kidney = 175.0 g)</td>
</tr>
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Cytological alterations

1) Nuclear grade 4 according to Fuhrman’s criteria
2) Mitotic rate 13 per 50 high-power fields
3) Atypical mitosis
4) Clear cells comprising 25% or less of the tumor (predominance of eosinophilic cells)
5) Diffuse architecture > 1/3 of tumor
6) Necrosis involving a confluent area of cells
7) Invasion of capsule of tumor*

Weiss score 5
Weiss score 7

* - There was tumor capsule invasion but hepatic tissue biopsy was normal.

In childhood adrenocortical tumors the criteria of Weiss classically used in the adult population have no correlation with biological behavior. In this context the first tumor was diagnosed as a tumor of the adrenal cortex with questionable prognosis. In contrast, the second tumor was clearly an adrenocortical carcinoma.

Nowadays, the patient is 17 years old with no evidence of new metastasis based on radiological imaging follow-up.

METHODS AND RESULTS

Molecular analysis

We have studied different molecular pathways related to the pathophysiology in a male pediatric case of metastatic adrenocortical carcinoma with long-term follow-up and insidious evolution. In trying to clarify the atypical tumor behavior in this case we investigated the expression of different genes in the tumor’s tissue: insulin growth factor 2 (IGF2, Taqman assay Hs01005963_m1, Applied Biosystems, Foster City, CA, USA) and its receptor (IGF1R, assay Hs00181385_m1) and fibroblast growth factor receptor 4 (FGFR4, assay Hs00242558_m1). Quantitative real-time PCR was performed as previously described (10,11). IGF2 gene was over-expressed in both left (Weiss score 5) and right (Weiss 7) adrenocortical tumors. IGF1R expression levels were higher in the right adrenocortical tumor (fold change, 7.4 vs. 3.32).

We also analyzed the presence of mutations in a particular region of genes coding for p53, a tumor su-
ppressor, and for beta-catenin (CTNNB1), a key component of Wnt pathway.

Tumor DNA was extracted according to standard procedures. Mutational analysis involving exon 3 of the CTNNB1 gene, which encodes beta-catenin, was performed using flanking intronic sequences of this exon. The primers used were 5’ TGGGTCAATGCAGCTTTTTTT 3’ and 3’TCAAAACTGCATTCTGACTTTCA 5’. PCR was performed employing TAQ DNA polymerase (Promega). The amplified product was submitted to direct sequencing on an automated sequencer (ABI 7000 sequencer detection system – Applied Biosystems). Mutations were verified in both sense and anti-sense directions. The entire exon 10 of the TP53 gene was amplified and sequenced. In addition, loss of heterozygosity (LOH) was studied in both tumors as previously described (1,2). The patient was found to be a carrier of the TP53 R337H germinal mutation in a heterozygosis pattern, which seems to be associated with a predisposition to adrenocortical tumors without prognostic implications (2). In addition, LOH was detected in the left and right tumors. Analysis of CTNNB1 exon 3 showed no alterations in both left and right tumors in comparison to wild type gene.

Immunohistochemical analysis
To investigate the role of Wnt/beta-catenin signaling in tumorigenesis, beta-catenin expression was studied by immunohistochemistry using a monoclonal mouse anti-human beta-catenin antibody at the dilution of 1:200 (code: M3539 – Dakocytomation). In addition, we performed immunohistochemistry of p53 by P53 antibody at the dilution of 1:1000 (Clone DO7 – Dakocytomation).

The usual distribution of beta-catenin at the plasma membrane was demonstrated with no nucleus/cytoplasmic staining, which is the normal pattern, since beta-catenin plays a role in the cell-cell adhesion with cadherin (12). P53 immunohistochemistry analyzes showed abnormal nuclear staining as expected, as the patient is a carrier of R337H mutation in TP53 (1,13) (Figure 4).

DISCUSSION
The diagnosis of adrenocortical tumors in children is suspected mainly based on clinical signs and symptoms of androgen hormones in excess, causing precocious pubarche. The occurrence of isolated Cushing’s Syn-

Figure 4. Immunohistochemical staining of p53. Nuclear immunoeexpression for p53 in the right adrenocortical tumor. 400 x.
ment, invasion of adjacent organs, and presence of dis-
tant metastasis (9). However, it is important to know
that is not uncommon for patients with small tumors to
experience relapses.

The pathophysiological study of the adrenocortical
carcinoma is important to improve diagnosis, prognos-
tic evaluation, and treatment. Analysis of exon 10 of
the TP53 gene revealed that our patient is a carrier of
TP53 R337H germlinal mutation as is the majority of
pediatric Brazilian patients with adrenocortical tumors.
This mutation is not related to a dismal behavior of
this disease (2). IGF2 overexpression has been consis-
tently demonstrated in adult sporadic adrenocortical
carcinomas. IGF2 exerts its mitogenic effects through
interaction with IGFR (15). Almeida and cols. (10)
reported overexpression of IGF2 in both pediatric
adrenocortical adenomas and carcinomas; on the other
hand, IGFR mRNA levels were significantly higher
in childhood adrenocortical carcinomas. Both adreno-
cortical tumors reported here had a high expression of
IGF2 and IGFR, but IGFR expression was higher in
the right tumor. Studies have demonstrated that an-
tagonizing the IGF signaling pathway with pharmaco-
logical agents results in the inhibition of in vitro and
in vivo tumor cell growth. This raises the prospect of
using target disruption of the IGFR signaling pathway
as a therapeutic agent since this targeted inhibition was
apparently more potent than the use of mitotane in xe-
nografts (10,15).

Tissier and cols. (16) reported beta-catenin ano-
malous staining at the nucleus and/or cytoplasm in a
high frequency of adult adrenocortical tumors, mainly
in carcinomas. However, the pattern differed between
these tumors. The abnormal beta-catenin immunostai-
ning was focal in most adrenocortical adenomas and
diffuse in adrenocortical carcinomas. Until now, the-
ere is little information in medical literature about the
participation of the Wnt pathway in pediatric adreno-
cortical tumorigenesis. Pusantisampan T and cols. (17)
reported a case of a child with metastatic adrenocorti-
cal carcinoma presenting a somatic mutation of beta-
catenin. Recently, somatic activating mutations of the
CTNNB1 gene were associated as a poor prognostic
factor in adult adrenocortical carcinomas (18). In the
present case, neither abnormal beta-catenin staining,
nor mutation in the exon 3 beta catenin gene was ob-
served. The absence of this somatic molecular altera-
tion might be related to the indolent behavior of the
patient’s tumors.

A previous microarray analysis of pediatric adre-
ocortical tumors demonstrated high expression of
FGFR4 (19). In fact, an overexpression of FGFR4 was
observed in the tumors of the patient reported.

Surgical resection is the treatment of choice for pa-
tients with resectable primary and even metastatic le-
sions (6). In addition, adjuvant mitotane therapy may
be given to patients in MacFarlane stage III and IV to
increase the length of time between recurrences. Mitot-
ane has a cytotoxic effect on adrenocortical cells and it
is effective in controlling steroid excess in patients with
secreting adrenocortical carcinoma; but patients often
present symptoms due to the toxicity of this drug, whi-
ch is largely related to mitotane blood levels. Mitotane
has been extensively used in adults, but there is little
knowledge of its efficacy and long-term effects in chil-
dren. The role of chemotherapy in the management of
adrenocortical carcinoma in children is not clear.

After surgical procedures the patient presented
good evolution and to date he is receiving glyccocor-
ticoid and mineralocorticoid replacement. Puberty has
developed at the expected age and the patient achieved
the target high. It is important to emphasize that the
cure achieved for this particular patient may be related
to total resection of the tumors and also of the metas-
tasis. Of note, there is a risk for recurrence even after
10-12 years and complete resection of both adrenocor-
tical tumors and metastasis. Continued surveillance is
still required. Also, we do not recommend this patient,
being a carrier of the TP53 mutation, to smoke or have
contact with substances that might damage his DNA.

Disclosure: no potential conflict of interest relevant to this article
was reported.

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