Pediatric adrenocortical tumors: what they can tell us on adrenal development and comparison with adult adrenal tumors

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DYNAMICS OF HUMAN ADRENOCOR TICAL MORPHOLOGY AND HORMONE SECRETION DURING DEVELOPMENT AND POSTNATAL LIFE

The adrenal gland is a continuously evolving endocrine organ from the developmental to the elder age. In humans, adrenal gland development begins at 3–4 weeks of gestation by a condensation of the coelomic epithelium lining the abdominal cavity, followed at 4–6 weeks of gestation by proliferation and migration of coelomic epithelial cells, and subsequent differentiation of fetal adrenal cortical cells into two distinct zones (inner fetal zone and outer definitive zone) at 8–10 weeks of gestation, while neural crest-derived cells start to infiltrate the gland at 7–8 weeks of gestation to give origin to adrenomedullary cells (1). Starting from around the ninth week of gestation, the embryonal adrenal is surrounded by the adrenal capsule formed by mesenchymal cells. Fetal adrenal cells, which are large and rich in lipids, express the steroidogenic enzyme CYP17, which enables them to produce high levels of DHEA and its sulfoconjugate DHEAS, which play a key role for the maintenance of pregnancy, being metabolized into estrogens by the placenta (1, 2). By the end of the second trimester of gestation, a distinct zone (transitional zone) differentiates between the definitive and fetal zones, which express HSD3B2, this way starting cortisol synthesis in the fetus. Close to birth, HSD3B2 is expressed in the definitive zone, which acquires the capacity to synthesize the mineralocorticoid hormone aldosterone. Cell proliferation in the fetal adrenal is mainly localized in the outer definitive zone, followed by centripetal migration and differentiation into fetal zone cells, which subsequently die from apoptosis in the center of the gland. This streaming process of adrenocortical cell differentiation continues during the whole life, as shown by studies in the mouse (3–5).

Starting shortly after birth, a rapid, dramatic remodeling of adrenal cortex structure takes place, with massive shrinkage of the gland due to apoptosis of the fetal zone and progressive differentiation of the glomerulosa, fasciculata, and reticularis zona, which are the hallmark of the adult adrenal (1). Defects in this process may cause the cytomegalic form of adrenal hypoplasia congenita, a syndrome of adrenal insufficiency due to altered postnatal adrenocortical differentiation due to mutations in the NR0B1 (DAX-1) gene [reviewed in Ref. (6)]. Studies in pre-term neonates have shown that parturition itself is the cause for fetal adrenal involution (7), suggesting a crosstalk between placenta and fetal adrenal in reciprocal maintenance. Remarkably, postnatal adrenal remodeling also takes place in the mouse adrenal cortex, where an inner zone adjacent to the medulla termed zone X, that lineage tracing experiments have shown to be derived from the fetal adrenal (8), regresses after puberty in males and after the first pregnancy in females.

After being suppressed following the regression of the fetal zone, adrenal production of DHEA/DHEAS starts to progressively...
increase again by around 8 years of age. This phenomenon is termed adrenarche and is concomitant with full differentiation of the reticularis zone, which expresses CYP17 but not HSD3B2. Moreover, in the zona reticularis, CYP17 has an increased ratio of 17,20-lyase to 17α hydroxylase activity (which favors DHEA production) compared to the zona fasciculata, probably due to increased serine phosphorylation and increased abundance of cytochrome b5 (CYB5), which allosterically stimulates 17,20-lyase activity of CYP17 (9). DHEA/DHEAS levels continue to increase until adulthood and then progressively decline (adrenopause) reaching pre-adrenarche levels by the ninth decade, correlating with progressive atrophy of the zona reticularis (10).

ADRENOCORTICAL TUMORS IN CHILDREN AND ADULTS: SIMILARITIES AND DIFFERENCES

Adrenocortical tumors (ACT) are among the most common neoplasms in humans and are frequently detected by hazard during diagnostic procedures for other medical issues (incidentalomas), in the great majority of cases remaining clinically silent and having a completely benign prognosis. In contrast, adrenocortical malignancies (adrenocortical carcinomas or ACC) are very rare, with a general incidence of 0.7–2 cases/million/year, with a maximum between 40 and 50 years of age and a higher frequency in women than in men (11). They become clinically evident with signs and symptoms due to hormone excess (Cushing’s syndrome, androgen excess) and/or local symptoms (pain, abdominal discomfort). The prognosis of ACC is still poor, with an average 5-year overall survival around 40%, which is influenced to a great extent in the fetal adrenal where it has an important role to regulate proliferation and steroid production (1). Conversely, IGF2 overexpression and abnormalities in the 11p15 region are a marker of malignancy in ACT of adults (21, 22). In mouse models, Igf2 overexpression in the adrenal induces tissue hyperplasia but is not able to induce malignant tumorigenesis per se (23, 24).

(2) Adrenocortical tumors are a distinctive feature of Li-Fraumeni syndrome (LFS), a multiple cancer syndrome due to germline mutations in the TP53 tumor suppressor gene (25) encoding p53, a transcription factor that has a pivotal role in preserving genome integrity and activating apoptosis of cells bearing irreparable DNA damage (27). It has been shown that in LFS, excessive DNA copy number variation exists in the patients’ germline, which may predispose to cancer (28). Due to its rarity and its characteristic association with LFS, discovery of an ACT in a child is an absolute indication for researching TP53 mutations in the proband and in his/her parents as well indicative for genetic counseling. Conversely, germline TP53 mutations are much less common in adults with ACC (29, 30) (Table 1). The high incidence of children ACT in LFS suggests that normal p53 function is required for the physiological process of postnatal fetal adrenal regression (Figure 1). In the absence of p53, genetic alterations may accumulate in the adrenal driving proliferation [such as NRS5A1 overexpression, (31, 32); see below section on Whole Genome Studies in Children and Adult ACT] revealing important drivers for tumorigenesis and LOH of 11p15 leading to Igf2 overexpression (18–20)] of specific cellular clones. This increased proliferative capacity may favor the emergence of further genetic alterations ultimately leading to clonal expansion and tumorigenesis [reviewed in Ref. (33)].

In classical LFS, due to TP53 mutations that completely abolish protein function, the lifetime incidence of cancer in carriers is close to 100%. However, low-penetrance mutated TP53 alleles exist that can increase the risk of developing cancer only in a fraction of carriers (34). A remarkable example of that situation exists in southern Brazil. In that geographical region, children ACT prevalence is at least 15-fold higher than in the rest of the world (10). This is related to a specific germline TP53 mutation (R337H) (35, 36), whose prevalence is very high (0.3%) in the population but whose penetrance to produce ACT in children has been estimated at only about 2% (37). However, the TP53 R337H mutation has also been reported to be associated to other cancers in the Li-Fraumeni spectrum (38–41) and so its overall penetrance is still unknown. R337 is a conserved arginine residue in the C-terminal tetramerization domain of p53 whose mutation to histidine destabilizes p53 tetramer formation in conditions of elevated temperature and pH (42). It has been shown that a founder effect is responsible for the spreading of the TP53 R337H mutation in the population of southern Brazil (43, 44). An about 0.5 Mb identical by descent haplotype in 17p13 encompassing the TP53 gene carrying the R337H mutation is
Table 1 | Distinctive features and common characteristics of ACT in children and adults.

| Feature                                    | Children ACT                                                                 | Adult ACC                                                                 |
|--------------------------------------------|------------------------------------------------------------------------------|----------------------------------------------------------------------------|
| Peak age at diagnosis                      | 3–4 years                                                                   | 40–50 years; peak extending into the seventh decade                       |
| Clinical presentation                      | Most often virilization; may be associated with Cushing’s syndrome         | Cushing’s syndrome or hypertension; may be associated with virilization   |
| Prevalence                                 | Worldwide: 0.3 cases/million/year; southern Brazil: 3.4–4.2 cases/million/year | 0.7–2/million/year for ACC                                                 |
| Most common genomic alterations            | 11p15 LOH; 9q34 gain; 4q34 loss                                             | Complex pattern                                                           |
| Genetic syndromes                          | Overall LFS >50%                                                           | Sporadic germline TP53 mutations                                            |
|                                            | Endemic germline TP53 R337H (Brazil) >93%                                    | <20%                                                                       |
|                                            | Beckwith-Wiedemann syndrome                                                 | Uncommon                                                                  |
|                                            | FAP                                                                          | Yes                                                                        |
|                                            | MEN1                                                                         | Uncommon                                                                  |
|                                            | Lynch syndrome                                                               | Uncommon                                                                  |
|                                            | NF1                                                                          | Uncommon                                                                  |
| Prognostic relevance of Pathological (Weiss) score | Low                                                                          | High                                                                      |
|                                            | Ki-67 index                                                                  | Unknown                                                                   |
|                                            | Prognostic relevance of TP53 mutations                                       | No (germline)                                                             |
|                                            | IGF2 overexpression                                                         | Yes (somatic)                                                             |
|                                            | NOV down-regulation                                                         | Yes                                                                        |
|                                            | SF-1 overexpression                                                         | Yes                                                                        |
|                                            | HLA class II down-regulation                                                 | No                                                                         |
|                                            | DLGAP5-PINK1 expression                                                      | Yes                                                                        |
|                                            | BUB1B-PINK1 expression                                                       | Yes                                                                        |
| Molecular pathways involved                | IGF2                                                                        | Yes (TP53 mutations)                                                      |
|                                            | p53/Rb                                                                       | Yes (TP53/CDKN2A/ RB1 mutations)                                           |
|                                            | Beta-catenin                                                                 | Yes (CTNNB1 mutations)                                                    |
|                                            | Chromatin remodeling                                                        | Yes (ATRX mutations)                                                      |
|                                            |                                                                               | Yes (MEN1/DAXX/ATRX/MED12/TERT mutations)                                  |

conserved in all carriers of the mutation (45, 46). A newborn screening and surveillance program of the TP53 R337H mutation carriers in the state of Paraná has proven to be successful to detect ACT in children at an early stage and to treat it with better therapeutic results compared to children who did not undergo surveillance (37).

Apart from rare cases of germline TP53 mutations, as mentioned before (29, 30), ACC in adults may also be associated to other hereditary conditions in some uncommon cases [reviewed in Ref. (47)]: familial adenomatous polyposis (FAP) (48), multiple endocrine neoplasia type 1 (MEN1) (49), Lynch syndrome (50), and neurofibromatosis type 1 (NF1) (51) (Table 1).

**GENOME-WIDE STUDIES IN CHILDREN AND ADULT ACT**

**DISTINCT PATTERNS OF CODING GENES EXPRESSION IN CHILDREN VS. ADULT ACT**

Children ACT can be readily differentiated from age-matched normal adrenals by unsupervised clustering based on their gene expression profiles (19). As reported before, IGF2 is the single gene that is most highly up-regulated in children ACT, while genes in the 11p15 region expressed from the maternal allele (KCNQ1, CDKN1C) are among the most strongly down-regulated transcripts. These data are consistent with the systematic LOH of 11p15 in those tumors, with conservation of the paternal allele and loss of the maternal allele (18, 20). Genes belonging to growth factor receptor and mitogen-activated kinase pathways are also dysregulated in children ACT. This suggests that those signaling pathways may be targets for therapeutic intervention. Furthermore, HSD3B2, a steroidogenic enzyme involved in the synthesis of aldosterone and cortisol and expressed in the glomerulosa and reticularis zones of the adult adrenal cortex and its transcriptional regulators NR4A1 and NR4A2 are strongly down-regulated in children ACT, lending further support to the hypothesis of their derivation from the fetal adrenal. This is also suggested by the finding that global gene expression profiles of children ACT are significantly correlated with those present in the fetal adrenal. Another
FIGURE 1 | A hypothesis for TP53 role during physiological adrenocortical remodeling in early postnatal life and pathogenesis of children ACT. A window of sensitivity (with an early peak; shaded in grey) of human adrenal to defective p53 function exists during the first years after birth when its physiological involution takes place. Mutant p53 may favor genomic instability, which in some cells may cause LOH of 11p15 and gain/amplification of NR5A1, which confer those cells a selective advantage for growth and lead to tumor formation. Additional genetic alterations arising in this mutation-prone background may cause malignancy. Combined adrenal weight is indicated with black circles. Relative adrenal weight in relationship to body weight is indicated with white circles. Adapted in part from Ref. (1) with permission from Endocrine Society Press.

strongly down-regulated gene in children ACT is NOV, encoding a secreted multimodular protein that has a pro-apoptotic function on adrenocortical cancer cells (52). In the study by West et al. (19), a set of 52 differentially expressed genes between adrenocortical adenomas and carcinomas (as distinguished by histological parameters) was identified. It is noteworthy that those included some transcripts encoding HLA class II molecules. Down-regulation of class II expression may represent a mechanism to escape immune surveillance, which could contribute to malignancy. Since malignancy markers are dramatically lacking for children ACT, it will be very important to confirm those data in larger series of patients. However, a recent immunohistochemical study failed to detect consistent HLA class II immunoreactivity in children ACT, both benign and malignant (53).

Unsupervised clustering of gene expression profiles of adult ACT allowed to distinguish two groups, termed C1 and C2 in the study by de Reyniès et al. (54). The C1 group could be further subdivided into C1A and C1B, which correspond to unfavorable and favorable outcome, respectively. Those results were confirmed by another independent study (55), which also confirmed that IGF2 overexpression is associated to malignancy in adult ACC. From gene expression data, de Reyniès and collaborators identified both a signature for malignancy based on the analysis of the expression of two genes (DLGAP5/PINK1) and a two-gene (BUB1B/PINK1)
molecular predictor of overall survival for patients with ACC (54). Remarkably, those molecular markers were confirmed to be valid prognostic indicators in adult but not in children ACT in a study on patients from southern Brazil (56) (Table 1). Further studies showed that tumors classified in the CIA group could be further divided into two subgroups each one bearing either TP53 or CTNNB1 (beta-catenin) mutations and in a third subgroup with no other known mutation (57). The importance of the activation of the beta-catenin pathway for adrenocortical tumorigenesis is also shown by studies in mouse models [(23, 24, 58); reviewed in Ref. (59)].

**MicroRNA sets differentially expressed in children and adult ACT**

In the only study published to date investigating microRNA expression profiles in children ACT, a distinct subgroup of miRNA was found to be differentially expressed in tumor samples compared to age-matched normal adrenal cortex (60). This subgroup included miR-99a and miR-100, which are down-regulated in children ACT and are able to down-regulate expression of IGF-1R (the receptor for IGF2), mTOR, and its associated protein raptor in adrenocortical cell lines. These proteins are up-regulated in children ACT and their pharmacological blockade is able to significantly decrease adrenocortical cancer cell proliferation (60–63). These results show that miR-99a and miR-100 have an important role in children ACT by the modulation of growth factor signaling through the IGF-1R-mTOR pathway.

On the other hand, several studies reported data on microRNA expression profiles in adult ACT. Those studies show in general only limited overlap [reviewed in Ref. (64)]. Nevertheless, most studies detected overexpression in ACC of miR-483-5p and/or -3p, whose gene is situated in an intron of IGF2 and may have an independent oncogenic function (65). miR-483-3p was also found up-regulated in children ACT in the study by Doghman et al. (60). Other miRNAs that display similar differential regulation in children and adult ACT are miR-503 (up-regulated), miR-195, miR-214, and miR-375 (down-regulated). A recently published integrative analysis of genomic alterations in adult ACC (66) found up-regulation of miRNAs belonging to the miR-506-514 cluster on chromosome Xq27 and down-regulation of the expression of the DLK1-MEG3 miRNA cluster on chromosome 14q in one subgroup of samples with favorable prognosis (C1B; see below section on Whole Genome Studies in Children and Adult ACT Reveal Important Drivers for Tumorigenesis). There is of great interest for the potential use of circulating miRNAs as biomarkers of malignancy in ACC (67–69).

**Whole genome studies in children and adult ACT reveal important drivers for tumorigenesis**

The first studies analyzing children ACT genome copy number alterations by comparative genomic hybridization (CGH) reported patterns of recurrent gains and losses (70–72). In particular, one of the most common alterations found in almost all cases of children ACT investigated was the gain/amplification of 9q34. Gains in this region were also reported in some studies of chromosomal alterations in adult ACT (73, 74). In close proximity to this chromosomal region (9q33) is situated the gene (NR5A1) encoding the transcription factor SF-1, a master regulator of adrenocortical and gonadal development [reviewed in Ref. (75, 76)]. Further studies showed that the NR5A1 gene is amplified and the SF-1 protein is overexpressed in the large majority of Brazilian children ACT (31, 77, 78). Interestingly, the SF-1 protein was overexpressed even in cases lacking gene amplification (31, 78), suggesting that mechanisms in addition to copy number gain may also account for SF-1 overexpression. The dosage-dependent effect of SF-1 in boosting adrenocortical cell proliferation was shown by studies in human cell lines and in transgenic mice (32) by regulation of a fairly large set of dosage-dependent target genes far exceeding its classical steroidogenic targets [(79); reviewed in Ref. (80)]. In children ACT, SF-1 overexpression appears to be a widespread finding, with no relationship with malignancy [(31, 78); see Figure 1]. Conversely, SF-1 overexpression in adult ACT is less common than in children (78) and is an unfavorable prognostic marker (81, 82) (Table 1). Remarkably, SF-1 transcriptional regulatory activity can be pharmacologically targeted leading to a decrease of adrenocortical cancer cell proliferation (83), suggesting that this transcription factor may represent a novel therapeutic target in ACT.

A subsequent SNP array study on both Brazilian and non-Brazilian ACT cases precisely defined recurrent genomic alterations in children ACT (46), the most frequent being loss of 4q34, gain of 9q33-q34 and 19p, and LOH of the whole chromosome 17 (harboring TP53) and 11p15 (harboring IGF2).

Remarkably, a number of focal deletions were detected at 4q34, defining a common deleted region surrounding the non-coding RNA LINC00290 gene. It is also noteworthy that the extent of the peak region of gain in 9q33–q34 suggests that other genes lying in a telomeric position with respect to NR5A1 may also be important for ACT pathogenesis. In addition, focal amplifications and homozygous deletions comprising well-known oncogenes (MYC, MDM2, PDEGRA, KIT, MCL1, BCL2L1) and tumor suppressors (TP53, RB1, RPH3AL) were identified. Although genomic profiles in non-Brazilian tumors with a mutated TP53 (other than R337H) were similar to Brazilian tumors, those with a wild-type TP53 displayed distinct genomic alterations, harboring significantly fewer rearrangements. Remarkably, 50% of TP53 wild-type tumors investigated in this study displayed as sole rearrangement a copy-neutral LOH of the imprinted region at 11p15, providing further evidence for a major role of this region in ACT development.

The landscape of genomic alterations in a worldwide series of children ACT enrolled at IPACTR (84) has been more precisely defined by a very recent study integrating whole exome, whole genome, and RNA-sequencing data (85). This work confirmed LOH in the 11p15 region in the large majority of cases and systematic overexpression of IGF2, together with frequent TP53 mutations, widespread 9q copy number gain, and 4q34 loss. By comparing the mutant allele fraction of SNV in copy-neutral LOH regions to allelic imbalance values, it was possible to establish that in most cases copy-neutral LOH of chromosomal 11p and 17 occurred early during tumorigenesis, suggesting that those events drive tumor formation. Additional recurrent genetic alterations in children ACT were somatic mutations in the ATRX (a DNA helicase) and CTNNB1 genes. Intriguingly, some tumors
bore integration of human herpesvirus-6 (HHV6) in the telomeric region of chromosome 11p. A poor outcome was predicted by concomitant \textit{TP53/ATRX} mutations and associated genomic abnormalities, including massive structural variations and a high background mutation rate (Table 1).

In adult ACT, earlier CGH studies showed a significantly increased prevalence of genomic imbalances in carcinomas compared to adenomas and sometimes contrasting patterns of gain and losses (73, 74, 86–88). CGH array studies evidenced a set of chromosomal aberrations in ACC associated with survival in a fashion dependent on their accumulation (89). Carcinomas were confirmed to harbor a higher number of chromosomal alterations than adenomas (90–92). Recently, activating mutations of the PKA catalytic subunit were shown to be associated with cortisol-secreting adrenocortical adenomas in adults (93–95). In general, gains had a higher impact than losses on gene expression profiles (91). A comparison between genome alterations in children ACT and adult ACC is shown in Figure 2. Methylome studies were also performed in adult ACC (97–99). According to their DNA methylation levels, malignant tumors could be divided into two groups, one displaying low and the other one elevated levels of methylation in CpG islands (CpG island methylator phenotype, CIMP). This hypermethylated tumors group could in turn be subdivided into two subgroups (CIMP-high and CIMP-low), which had prognostic relevance, with the CIMP-high phenotype clearly being associated to worse prognosis (99).

A study integrating transcriptome, miRNome, copy number alterations, methylome, and whole exome sequencing data in adult ACC was recently published (66), showing that major pathways involved by mutation or homozygous deletion include beta-catenin (\textit{CTNNB1} and \textit{ZNRF3}), p53/Rb signaling (\textit{TP53, CDKN2A, and RB1}), and chromatin remodeling (\textit{MEN1, DAXX, ATRX, MED12, and TERT}) (Table 1). In addition, recurrent homozygous deletions were found in 4q34, similarly to children ACT. This study also showed that a substantial overlap exists among the different omics classifications of ACC: the previously identified gene expression profile clusters (C1A, C1B, and C2; see section on Distinct Patterns of Coding Genes Expression in Children vs. Adult ACT) (54) are strongly correlated with subgroups based on DNA methylation and miRNA expression, mutation rate, and alteration of key molecular pathways.

**PERSPECTIVES**

Children ACT represent a distinct pathological entity compared to tumors in adults concerning their origin, clinical manifestations, molecular alterations, and prognostic evolution. Important fields of investigation in the future will be the search for genetic and environmental factors that modulate penetrance of ACT in carriers of germline \textit{TP53} mutations in order to orient screening procedures to detect disease at an early stage, the identification of robust biomarkers of malignancy, which are still lacking, and the clinical testing of targeted therapies against the major molecular pathways that are altered in this disease (100).

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