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GENETICS AND EPIGENETICS OF ADRENOCORTICAL TUMORS

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Abstract

Adrenocortical tumors are common neoplasms. Most are benign, nonfunctional and clinically irrelevant. However, adrenocortical carcinoma is a rare disease with a dismal prognosis and no effective treatment apart from surgical resection. The molecular genetics of adrenocortical tumors remain poorly understood. For decades, molecular studies relied on a small number of samples and were directed to candidate-genes. This approach, based on the elucidation of the genetics of rare genetic syndromes in which adrenocortical tumors are a manifestation, has led to the discovery of major dysfunctional molecular pathways in adrenocortical tumors, such as the IGF pathway, the Wnt pathway and *TP53*. However, with the advent of high-throughput methodologies and the organization of international consortiums to obtain a larger number of samples and high-quality clinical data, this paradigm is rapidly changing. In the last decade, genome-wide expression profile studies, microRNA profiling and methylation profiling allowed the identification of subgroups of tumors with distinct genetic markers, molecular pathways activation patterns and clinical behavior. As a consequence, molecular classification of tumors has proven to be superior to traditional histological and clinical methods in prognosis prediction. In addition, this knowledge has also allowed the proposal of molecular-targeted approaches aiming better treatment options for advanced disease. This review aims to summarize the most relevant data on the rapidly evolving field of genetics of adrenal disorders.

INTRODUCTION

Adrenocortical tumors (ACT) are common neoplasms, the prevalence of which increases with age, reaching a peak of 6% after 60 years. Most are benign cortical adenomas (ACA) and some are associated with endocrine syndromes (hypercortisolism in Cushing's syndrome, hyperandrogenism in virilizing syndrome or mineralocorticoid excess in Conn's

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syndrome) (Grumbach, Biller et al. 2003, Arnaldi and Boscaro 2012). On the other hand, their malignant counterparts, adrenocortical carcinomas (ACC), are rare neoplasms with an incidence of 0.5–2/million per year (Fassnacht and Allolio 2009). ACC is usually a very aggressive disease, with a dismal prognosis, with a 5-year survival rate of 16–44% (Fassnacht and Allolio 2009). Surgical resection is the treatment of choice and the only therapeutic approach that significantly increases survival. Once ACC is not completely resectable, the available therapeutic options (which include the adrenolytic drug mitotane, systemic chemotherapy, radiation therapy, and, more recently, molecular-targeted therapies) have a small impact on survival (Fassnacht and Allolio 2009). The differential diagnosis between ACA and localized ACC can be challenging, considering that clinical, laboratory, radiological, and pathological features can overlap to some extent. The accurate distinction between ACA and ACC is very important, since treatment is radically different (Fassnacht and Allolio 2009). In recent years, considerable advances toward understanding the pathogenesis of ACT have been made. Different strategies have enabled these achievements:

1. Identification of genetic alterations in rare familial syndromes and evaluation of whether the same defects are present in sporadic tumors.
2. Investigation of signaling pathways that were proved important in other tumors types
3. Employment of high-throughput techniques such as genome wide expression profiling, methylation profiling and micro-RNA profiling to interrogate novel signaling pathways.
4. Studies with animal models with one or more genetic defects in known signaling pathways.

Here we discuss the most relevant genetic aspects of ACTs. This review summarizes our current understanding of molecular pathogenesis of ACTs.

GENETICS OF ADRENOCORTICAL TUMORS

Lessons from rare genetic syndromes

ACTs, both benign and malignant (ACC), may occur sporadically or in the setting of a heritable genetic syndrome. ACTs and adrenocortical hyperplasias are commonly a feature of multiple neoplasia syndromes (Table 1). The elucidation of the genetic basis of these syndromes has contributed to the identification of key signaling pathways that are dysregulated in sporadic ACTs. Clinical and molecular aspects of these genetic syndromes and their relationship to sporadic ACTs will be briefly discussed below.

Genetic aspects of benign adrenocortical disease

The incidence of adrenal incidentalomas has been increasing and now approaches the 8.7% in autopsy series and 4% in radiology series (Bovio, Cataldi et al. 2006, Singh and Buch 2008, Arnaldi and Boscaro 2012). Approximately 80% of the adrenocortical tumors are non functional, the remaining 20% are cortisol producing tumors and aldosteronomas (Arnaldi and Boscaro 2012). Cortisol-producing adrenocortical adenomas (CPAs) are usually sporadic, constituting the most frequent cause of endogenous ACTH-independent Cushing's syndrome. Rarely, ACTH-independent cortisol overproduction is observed in the setting of a rare genetic syndrome, such as McCune-Albright syndrome (MAS), primary pigmented nodular adrenocortical disease (PPNAD), which may be isolated or associated with Carney complex, isolated micronodular adrenocortical disease (i-MAD) and ACTH-independent macronodular adrenal hyperplasia (AIMAH) (Stratakis 2008). Considerable advances toward understanding the pathogenesis of such lesions have been made in the last two decades. A common feature of all these syndromes is the abnormal activation of protein

kinase A (PKA) signaling pathway (Figure 1). PKA is a serine/threonine kinase which is the main mediator of cAMP signaling in mammals (de Joussineau, Sahut-Barnola et al. 2012). Various physiological ligands can activate PKA-induced phosphorylation, which affects cell metabolism, proliferation, differentiation and apoptosis. In the adrenal cortex, the PKA pathway is activated when ACTH binds to the MC2R receptor, a G protein-coupled receptor, causing activation of the Gs- α subunit, which generates cyclic AMP (cAMP) from ATP (de Joussineau, Sahut-Barnola et al. 2012). The PKA holoenzyme is a tetramer composed by four distinct elements: two catalytic and two regulatory subunits. In the inactivated state, the regulatory subunits inhibit the kinase activity of the catalytic subunits. Upon activation of the pathway, cAMP binds to specific domains at the regulatory subunits, dissociating the tetramer and releasing the catalytic subunits, which will phosphorylate different intracellular targets, including the transcription factor CREB, which is translocated to the nucleus, activating the transcription of cAMP-responsive element-containing genes (Pearce, Komander et al. 2010). After the stimulus finishes, cAMP is inactivated by phosphodiesterases and the PKA tetramer is assembled again, returning to its original, inactivated state (de Joussineau, Sahut-Barnola et al. 2012). Abnormal activation of PKA pathway may be caused by mutations in different genes of the signaling cascade, as will be discussed below. In addition to PKA, MC2R signaling also activates ERK-MAPK pathway, which induces cell proliferation at the *zona fasciculata* (Gallo-Payet and Payet 2003, Roy, Pinard et al. 2011). The role of abnormal ERK-MAPK pathway activation in adrenocortical disease, however, is not clearly understood.

BENIGN CONDITIONS CHARACTERIZED BY ADRENOCORTICAL HYPERFUNCTION

Carney complex (CC; OMIM 160980)

CC is a multiple neoplasia syndrome that is inherited in an autosomal dominant pattern and is characterized by spotty skin pigmentation and several tumors, including skin tumors, myxomas, schwannomas, liver, pancreatic, breast, and endocrine neoplasms such as follicular thyroid cancer, pituitary adenomas/hyperplasia and primary pigmented nodular adrenocortical hyperplasia (PPNAD) (Carney, Gordon et al. 1985, Rothenbuhler and Stratakis 2010). Linkage analysis of affected families has associated the disease with two genetic loci: 2p16 and 17q22–24 (Rothenbuhler and Stratakis 2010). Mutations of *PRKARIA* have been identified in families with linkage at the 17q22–24 locus. This gene encodes the regulatory subunit 1A of the PKA. Studies have shown that *PRKARIA* mutations are present in approximately 60% of CC patients (Kirschner, Carney et al. 2000, Bertherat, Horvath et al. 2009). So far, no candidate gene has been identified at the 2p16 locus. The presence of inactivating *PRKARIA* mutations leads to constitutional activation of the catalytic subunits (Kirschner, Carney et al. 2000, de Joussineau, Sahut-Barnola et al. 2012). Allelic loss of the wild-type allele is frequently observed in some tumors from patients with CC, and for this reason *PRKARIA* is considered a tumor suppressor gene (Kirschner, Carney et al. 2000, de Joussineau, Sahut-Barnola et al. 2012). PPNAD can occur isolated or as a manifestation of CC. It is characterized by the formation of small (< 1 cm), dark-colored and slow growing benign nodules on the adrenal cortex bilaterally. The disease typically affects young patients and is characterized by ACTH independent cortisol production (Rothenbuhler and Stratakis 2010).

Other forms of micronodular adrenocortical hyperplasia

PPNAD and non-pigmented adrenocortical micronodular hyperplasia (i-MAD) have also been described in patients without *PRKARIA* mutations. In a subset of such patients, inactivating mutations of genes that encode phosphodiesterases (*PDE11A* and *PDE8B*) have

been identified (Stratakis 2009). These mutations impair the ability of the protein to degrade cAMP, leading to a prolonged activation of the pathway (Stratakis 2009).

ACTH-independent macronodular adrenal hyperplasia (AIMAH)

AIMAH is characterized by ACTH-independent CS associated with massive bilateral adrenocortical nodules (Lacroix 2009). Although no germline mutation has been associated with this disease so far, some clinic and epidemiological characteristics are consistent with a genetic background: 1. The disease is typically bilateral and multifocal; 2. Although sporadic cases are more common, familial cases have been described in a pattern suggestive of an autosomal dominant inheritance. The molecular “hallmark” of AIMAH is the presence of “illicit” (ectopic, aberrant) G protein-coupled receptors (GPCRs) in the adrenals, such as the gastrointestinal polypeptide receptor (GIPR), adrenergic receptors, serotonin receptors and luteinizing hormone receptor (LHR) (Lacroix 2009). Interestingly, activation of the aberrant receptors stimulates cortisol production. Pharmacological blockage of the receptors has been proposed for the management of hypercortisolemia in such cases. In addition, allelic losses of the 17q22–24 region has also been described, further suggesting that PKA dysregulation is involved in the molecular pathogenesis of this syndrome (Bourdeau, Matyakhina et al. 2006). Subsequently, whole-genome expression analyses have shown that transcriptional targets of PKA pathway are overexpressed, in agreement with previous findings. In addition, these studies have also demonstrated overexpression of Wnt signaling pathway genes, such as *WISP2*, *GSK3B* and *CTNNB1*, suggesting that Wnt overactivation may have a role in the molecular pathogenesis of this disease (Hsiao, 2009). More recently, Almeida et. al. studied samples from different nodules with different sizes from the same patients with AIMAH. Genomic gains and losses were demonstrated in several *loci* in both small and larger nodules. In addition, whole-genome expression analysis has demonstrated that while metabolic pathways are dysregulated in smaller nodules, larger nodules are characterized by dysregulation of cancer genes, such as *BCL2*, *TP53*, *E2F1*, *EGF*, *c-KIT*, *MYB*, *PRKCA* and *CTNNB1*, suggesting that in the context of a chronic proliferative stimulus imposed by polyclonal hyperplasia, multiple genomic and transcript abnormalities are further accumulated, possibly contributing to disease progression (Almeida, Harran et al. 2011).

McCune-Albright syndrome (MAS; OMIM 174800)

McCune-Albright syndrome is characterized by polyostotic fibrous dysplasia, *café-au-lait* spots, precocious puberty and hyperfunction of endocrine glands (Lee, Van Dop et al. 1986, Brown, Kelly et al. 2010). Hypercortisolism occurs in ~5% of patients, usually associated with an early-onset variation of AIMAH (Lee, Van Dop et al. 1986, Brown, Kelly et al. 2010). Post-zygotic *GNAS1* (the gene that encodes Gs-alpha) activating mutations have been identified in a pattern of mosaicism in various tissues (Weinstein, Shenker et al. 1991). In addition, activating *GNAS1* mutations have also been identified in isolated forms of AIMAH, suggesting that at least some forms of AIMAH may represent a disease in the spectrum of MAS (Fragoso, Domenice et al. 2003).

PKA abnormalities in sporadic cortisol-producing adenomas

Since virtually all the genetic defects described in ACTH-independent genetic adrenocortical disease disrupts the PKA signaling pathway (Figures 1A and 1B), it is reasonable to hypothesize that abnormal PKA activity may be also a feature of sporadic CPAs. In fact, it has been demonstrated that abnormalities of the PKA pathway are present in virtually all CPAs. Although somatic *PRKARIA* and *GNAS1* mutations have been identified only in a small subset of CPAs (Bertherat, Groussin et al. 2003, Dall'Asta, Ballare et al. 2004), other abnormalities such as LOH at 17q22–24, post-transcriptional down-

regulation of PKA regulatory subunits *PRKAR1A* and *PRKAR2B*, increased levels of cAMP, increased PKA activity and decreased total PDE activity are frequent findings in these tumors (Bertherat, Groussin et al. 2003, Vincent-Dejean, Cazabat et al. 2008, Bimpaki, Nesterova et al. 2009). A recent study further corroborated these observations. The genome-wide expression profiles of 22 ACAs (5 non-secreting, 6 subclinical cortisol production and 11 CPAs) have indicated that genes related to cortisol secretion, such as the steroidogenic enzymes, genes involved in cholesterol metabolism and glutathione S-transferases are overregulated in cortisol producing adenomas, in parallel with increased PKA activity and cAMP levels. Surprisingly, the expression levels of *PDE8B* are also elevated in these tumors, suggesting a mechanism of counter-regulation of the pathway (Wilmot Roussel, Vezzosi et al. 2013).

Genetics of mineralocorticoid excess

Primary aldosteronism is the most common cause of endocrine hypertension with an incidence in unselected patients with hypertension ranging from 3 to 20%. There are eight different subtypes of primary aldosteronism:

- Sporadic unilateral aldosterone producing adenomas
- Idiopathic non-familial bilateral adrenal hyperplasia
- Renin responsive unilateral adrenal adenomas
- Familial hyperaldosteronism type I (FH-I)
- Familial hyperaldosteronism type II (FH-II)
- Familial hyperaldosteronism type III (FH-III)
- Aldosterone producing adrenocortical carcinomas
- ACTH independent bilateral macronodular adrenal hyperplasia associated with Cushing's and hyperaldosteronism

To date, three types of familial hyperaldosteronism have been described (Quack, Vonend et al. 2010). Type I (FH-I) is also known as glucocorticoid remediable hyperaldosteronism, which is an autosomal dominant inherited disorder characterized by significant production of hybrid steroids (18-hydroxycortisol and 18-oxocortisol) and normalization of aldosterone levels and blood pressure with low doses of dexamethasone. Early onset hypertension and severe cardiovascular complications at a young age are frequent manifestations, however wide intrafamilial variability can be present. The genetic defect leading to FH-I is an unequal genetic recombination between *CYP11B1* (11-beta-hydroxylase) and *CYP11B2* (aldosterone synthase) that generates a chimeric *CYP11B* gene containing the 5' region *CYP11B1*, including the promoter, and *CYP11B2* sequences at 3' end. The resultant chimeric protein synthesizes aldosterone under the stimulus of ACTH (Miyahara, Kawamoto et al. 1992, Jackson, Lafferty et al. 2002).

Familial hyperaldosteronism type II (FH-II) is characterized by a family history of primary aldosteronism in first degree relatives caused by an aldosterone-producing adenoma (APA) or bilateral adrenal hyperplasia, clinically and biochemically indistinguishable from sporadic forms. The prevalence of FH-II ranges from ~1 – 6% (Mulatero, Tauber et al. 2012, Pallauf, Schirpenbach et al. 2012). The genetic cause is unknown, but linkage analysis of non-related families indicates a locus at chromosome 7p22 (Carss, Stowasser et al. 2011). Sequencing candidate genes located in this region, fascin 1 (*FSCN1*) and the cAMP-dependent protein kinase type I b-regulatory subunit (*PRKAR1B*), revealed no mutations (Medeau, Assie et al. 2005). No genetic abnormality has been identified in genes coding for aldosterone synthase

(*CYP11B2*), the Ang II type 1 receptor (*AT1R*), or the tumor suppressor gene p53 (Stowasser and Gordon 2003). Vertical transmission suggests an autosomal dominant inheritance.

Familial hyperaldosteronism type III (FH-III) has been described in a few families so far (Mulatero, Tauber et al. 2012). The genetic cause of this disease is an inactivating mutation of *KCNJ5*, the gene that encodes the G-protein-activated inward rectifier K⁺ channel 4 (GIRK4) (Choi, Scholl et al. 2011). The functional consequences of *KCNJ5* mutations on the channel function are loss of K⁺ selectivity, Na⁺ influx and cellular membrane depolarization, leading to increased cytosolic Ca⁺⁺ concentrations, possibly by a voltage-gated Ca⁺⁺ channel activation (Scholl, Nelson-Williams et al. 2012). The ultimate consequences are increased aldosterone production and adrenal cell proliferation. The clinical features of affected members of families with FH-III having G151R and G151E mutations illustrates strikingly different presentations. Patients with the G151E mutation showed aldosteronism early in life but had no progressive disease. These patients had remarkable responsiveness to single agent treatment with spironolactone, leading to normalization of blood pressure and K⁺ in all subjects in whom it has been used and with effectiveness persisting well into adulthood. Moreover, none of these affected subjects showed adrenal enlargement on CT at ages up to 37 years. In contrast, patients with the G151R mutation presented with clinical features similar to the family described initially with the inherited T158A mutation in *KCNJ5*. They had severe aldosteronism that was virtually unresponsive to spironolactone and that worsened with age. Among the patients with G151R mutations with follow-up, all have required the radical intervention of bilateral adrenalectomy to achieve control of hypertension and hypokalemia at very young ages (range 1–4 y). Interestingly, somatic mutations in *KCNJ5* have been identified in ~40% of sporadic APAs (Azizan, Murthy et al. 2012, Mulatero, Monticone et al. 2013). Patients with APAs harboring *KCNJ5* mutations present with severe and early onset hyperaldosteronism as well as higher lateralization index in adrenal venous sampling (Seccia, Mantero et al. 2012).

More recently, new somatic mutations in APA tumors were identified in roughly 7% of aldosterone-producing adenomas. Somatic mutations in the P-type ATPase gene family, *ATP1A1* (encoding a Na⁺/K⁺ ATPase α subunit) and *ATP2B3* (encoding the plasma membrane Ca²⁺ ATPase) were identified by exome sequencing in APA samples (Beuschlein 2013). Subsequently 308 APAs were screened for *ATP1A1* and *ATP2B3* mutations. 21 (6.8%) contained *ATP1A1* or *ATP2B3* mutations and 118 (38.3%) *KCNJ5* mutations. Concomitant *KCNJ5* and *ATP1A1* or *ATP2B3* mutations within the same tumor were not observed. The function of Na⁺/K⁺ ATPase, of which the α subunit is encoded by *ATP1A1*, involves ATP hydrolysis for the exchange of three cytoplasmic sodium ions for two extracellular potassium ions (Figures 2). The Na⁺ and K⁺ gradients created ion fluxes that generate resting membrane potential and action potentials (Figure 2A). Blockade of Na⁺/K⁺ ATPases with the specific antagonist ouabain results in dose-dependent stimulation of aldosterone release from glomerulosa cells and glomerulosa cell growth in vivo. Angiotensin II lowers Na⁺/K⁺ ATPase activity, indicating the potential contribution of this enzyme to angiotensin-dependent aldosterone release (Figure 2B). *ATP2B3* belongs to the ATPase gene family that encodes a plasma membrane Ca²⁺ ATPase that is essential to clear calcium ions from the cytoplasm (Figure 2B). The atomic structure of the plasma membrane Ca²⁺ ATPase *ATP2B3* has not yet been determined. Using the known structure of the homologous rabbit sarcoplasmic reticulum type Ca²⁺ ATPase (SERCA) it became apparent that the APA-associated deletion mutations in *ATP2B3* alter the M4 trans-membrane helix causing a major distortion of the calcium ion binding site. Functional-electrophysiological examination of primary cultured adenoma cells with different underlying mutations showed substantially higher levels of depolarization in ATPase (*ATP1A1* or *ATP2B3*)-mutant cells compared to cells from normal adjacent tissue.

GENETIC ASPECTS OF ADRENOCORTICAL CARCINOMA (ACC)

Chromosomal and sub-chromosomal alterations

Histological findings of adrenocortical tumors like nuclear aberrations, including a high nuclear grade, high mitotic counts and bizarre mitotic figures were recognized as a striking feature of ACC. With the advent of cytogenetic techniques, these gross morphological observations could be further characterized. Karyotyping studies of ACCs demonstrated that ACCs present a large number of chromosomal aberrations, including segmental duplications, rearrangements and aberrant chromosomes. Cytogenetic and flow cytometry studies further corroborated these findings, demonstrating that most ACCs exhibit aneuploidy/polyploidy, while ACAs are almost always diploid (Klein, Kay et al. 1985, Bowlby, DeBault et al. 1986, Limon, Dal Cin et al. 1987, Marks, Wyandt et al. 1992). Clonality studies have shown that, while ACCs are invariably of monoclonal origin, some ACAs can be polyclonal (Beuschlein, Reincke et al. 1994, Gicquel, Leblond-Francillard et al. 1994). Comparative genomic hybridization (CGH) studies have extended these findings to subchromosomal level: while ACAs present few regions of chromosomal gains and losses, ACCs exhibit a complex pattern of chromosomal aberrations, with multiple regions of gains and losses, but with no specific pattern among samples. It is thought that oncogenes and tumor suppressor genes are located in regions of gains and losses, respectively. In ACCs, chromosomal gains are frequently observed in regions 4q, 4p16, 5p15, 5q12–13, 5q32-qter, 9q34, 12q13, 12q24 e 19p, and chromosomal losses are observed at 1p, 2q, 17p, 22p, 22q and 1q. Microsatellite studies have identified frequent allelic losses in regions 17p13, 11q15, and 2p16 (85%, 92%, and 90% of samples, respectively) (Kjellman, Kallioniemi et al. 1996, Gicquel, Bertagna et al. 2001). Recently, an array-CGH study have identified increased copy number in chromosomes 5, 6q, 7, 8q, 12, 16q, and 20, and allelic losses in 1, 2q, 3, 6p, 7p, 8p, 9, 10, 11, 13q, 14q, 15q, 16, 17, 19q and 22q. Some of these alterations (gains in 6q, 7q, and 12q, and losses in chromosomes 3, 8, 10p, 16q, 17q, and 19q) are associated with decreased overall survival (Stephan, Chung et al. 2008). Among pediatric ACTs, CGH studies have identified frequent gains at 9q34, suggesting the presence of an oncogene at this region (Figueiredo, Stratakis et al. 1999, James, Kelsey et al. 1999). Interestingly, *SFI* (steroidogenic factor 1), a transcription factor that promotes gonadal and adrenal gland development, cell proliferation and differentiation (Morohashi, Honda et al. 1992, Luo, Ikeda et al. 1994), is located in this chromosomal region. Later studies have shown increased copy number, mRNA overexpression, and strong nuclear immunostaining of SF1 on pediatric ACTs, suggesting that it may be involved in tumorigenesis (Figueiredo, Ribeiro et al. 2000, Almeida, Soares et al. 2010). Further studies corroborated a possible role of *SFI* overexpression on adrenocortical tumorigenesis, by showing a growth-promoting and anti-apoptotic effect of *SFI* overexpression both *in vitro* and *in vivo* (Doghman, Karpova et al. 2007). Important lessons from CGH studies include:

1. ACTs are characterized by a complex pattern of chromosomal alterations, which are cumulative toward malignant transformation
2. These alterations reflect the fact that ACCs are characterized by a high degree of chromosomal instability, a well-known hallmark of cancer (Hanahan and Weinberg 2011). The elucidation of the mechanisms beyond chromosomal instability may be of interest, since therapeutic approaches targeting this phenomenon are under development (Bakhom and Compton 2012).

A consequence of the high level of chromosomal instability is the rapid accumulation of a large number of somatic mutations (a phenomenon known as *mutator phenotype*, which contributes to disease progression towards metastasis and resistance to therapy) (Loeb 2001). It is already known that many of these somatic mutations are *passenger* and does not contribute to tumor evolution but are instead a consequence of the accelerated mutagenesis

process. On the other hand, *driver mutations* are the genetic events relevant to somatic evolution of a tumor, conferring selective advantages to cellular clones in which they appear. *Driver mutations* are potential markers of malignancy and therapeutic targets. Thus, differentiating *driver* from *passenger mutations* are of key importance. In the pre high throughput sequencing era, many of the *driver mutations* relevant to molecular pathogenesis of ACTs were identified by understanding the molecular genetics of rare familial syndromes in which ACCs were a manifestation. Here, we briefly describe relevant clinical and genetic aspects of such syndromes, emphasizing the contribution of the involved genes to sporadic ACC tumorigenesis.

Li–Fraumeni syndrome (LFS; OMIM 151623)

LFS is an autosomal dominant disorder characterized by increased susceptibility to early-onset development of several types of cancer, including breast cancer, soft tissue sarcomas, brain, and hematologic cancers (Li, Fraumeni et al. 1988). ACC is a less frequent manifestation of LFS, developing in 3–10% of patients (Li, Fraumeni et al. 1988, Hisada, Garber et al. 1998, Bougeard, Sesboue et al. 2008). Germline mutations of the tumor suppressor *TP53* are found in ~70% of cases of LFS (Bachinski, Olufemi et al. 2005). *TP53* is located on 17p13 and its main functions are halting the cell cycle and/or inducing apoptosis in response to DNA damage (Hisada, Garber et al. 1998, Vogelstein, Lane et al. 2000, Suzuki and Matsubara 2011). The prevalence of germline *TP53* mutations in apparently sporadic ACT patients varies according to the age group. While in adults the prevalence is low, between 3–6% according to two recent studies (Herrmann, Heinze et al. 2012, Raymond, Else et al. 2012), among children, this proportion is significantly higher. Data from the USA and Europe indicates that the prevalence of germline *TP53* mutations in pediatric patients is ~50–80% (Wagner, Portwine et al. 1994, Varley, McGown et al. 1999). Remarkably, the incidence of pediatric ACTs in Southern Brazil is 10–18× greater than that worldwide. This fact is explained by genetic characteristics of the population of that area. A specific germline *TP53* mutation (p.R337H) is present in up to 90% of the affected patients (Latronico, Pinto et al. 2001, Ribeiro, Sandrini et al. 2001). This mutation affects the tetramerization domain of the p53 protein and has a low penetrance (DiGiammarino, Lee et al. 2002, Custódio, Komechen et al. 2012). Interestingly, all Brazilian carriers share the same haplotype, indicating a founder effect (Pinto, Billerbeck et al. 2004, Garritano, Gemignani et al. 2010). In fact, haplotype analysis indicated that, most likely, the p.R337H mutation came from a modern European ancestry (Garritano, Gemignani et al. 2010). Initially, the p.R337H mutation was thought to predispose only to childhood ACC, but it has been lately demonstrated that the carriers are at increased risk for the development of other malignancies associated with LFS, such as choroid plexus tumors and breast cancer (Custodio, Taques et al. 2011, Custódio, Komechen et al. 2012). In addition, the p.R337H mutation also predisposes to adult ACC. In an adult ACC cohort from Southeastern Brazil, the p.R337H mutation was identified in ~15% of individuals (Latronico, Pinto et al. 2001). Given the impact of identifying a germline *TP53* mutation not only to the patient but also to family members, genetic testing is recommended for any patient with ACC, even in absence of family history of cancer, since *TP53* germline mutations are known to occur *de novo* in about 25% of cases (Chompret, Brugieres et al. 2000, Chompret, Abel et al. 2001).

Somatic *TP53* mutations in ACTs

Although germline *TP53* mutations are rare in adult patients with ACC, somatic *TP53* mutations are common, being described in 25–70% of samples (Barzon, Chilosi et al. 2001, Libe, Groussin et al. 2007, Waldmann, Patsalis et al. 2012). On the other hand, the prevalence of somatic *TP53* mutations is low in ACAs, suggesting that *TP53* inactivation is a late step in tumorigenesis, a fact that is in accordance with observations from other cancers and multistep tumorigenesis models (Hollstein, Sidransky et al. 1991). It has been proposed

that *TP53* inactivation follows the classic Knudson's two-hit hypothesis for a tumor suppressor gene, in which both alleles should be inactivated. In the presence of a germline or a somatic inactivating *TP53* mutation, a second genetic event should be responsible for the inactivation of the second allele. This event could be a second somatic mutation, promoter region methylation or loss of heterozygosity (LOH) of the locus. Interestingly, it has been demonstrated that ACCs exhibit a high frequency of 17p13 LOH (~85%). However, *TP53* point mutations and 17p13 LOH do not overlap completely, suggesting that alternative mechanisms are responsible for *TP53* inactivation (Libe, Groussin et al. 2007). Recently, it has been demonstrated that the presence of somatic *TP53* inactivation has a negative clinical impact. A recent genome-wide expression study identified a molecular signature of *TP53* inactivation in tumors with poor outcome (Ragazzon, Libe et al. 2010). Somatic *TP53* mutations could be identified in the majority of these tumors (Ragazzon, Libe et al. 2010).

Beckwith–Wiedemann syndrome (BWS; OMIM 130650)

BWS is a somatic overgrowth syndrome that is characterized by prenatal and postnatal overgrowth, visceromegaly, macroglossia, neonatal hypoglycemia, ear abnormalities and abdominal wall defects. Children with BWS are at increased risk of developing childhood neoplasms, such as Wilms' tumor, hepatoblastoma, rhabdomyosarcoma, and ACC. Around 15% of cases are familial (Weksberg, Shuman et al. 2010). The molecular basis of this syndrome is complex, including genetic and epigenetic alterations at chromosomal locus 11p15, which contains the genes *CDKN1C*, *IGF2* and *H19*, structurally organized in a cluster (Figure 3). In normal individuals, these genes are expressed monoallelically, in a parent-of-origin-specific manner: *IGF2* is maternally imprinted, therefore only the paternal allele is expressed. On the other hand, the paternal alleles of *CDKN1C* and *H19* are silenced by imprinting, thus only the maternal alleles are expressed (Figure 3) (Weksberg, Shuman et al. 2010). In BWS, multiple epigenetic and structural changes at 11p15, including parent-of-origin-specific duplications, translocations/inversions, microdeletions, DNA methylation changes at regulatory regions, uniparental isodisomy, and mutations at *CDKN1C* lead to biallelic expression of *IGF2* and inactivation of *CDKN1C* and *H19* (Weksberg, Shuman et al. 2010). *IGF2* encodes a growth factor, the insulin-like growth factor 2, that is mainly expressed during fetal life and is responsible for fetal growth. *H19* does not encode any protein and acts as a transcriptional repressor of *IGF2* (Leighton, Saam et al. 1995). *CDKN1C* is a negative cell cycle regulator (Lam, Hatada et al. 1999). In addition to ACC, the adrenal phenotype observed in BWS includes adrenocortical cytomegaly, adrenal cysts and ACAs (Lapunzina 2005). Interestingly, the risk of malignancy in BWS decreases through adolescence and then remains at the level of the general population (Lapunzina 2005).

Somatic alterations at 11p15 in sporadic ACCs

In ACCs, *IGF2* overexpression and downregulation of *CDKN1C* and *H19* is observed in ~90% of cases (Gicquel, Raffin-Sanson et al. 1997, Giordano, Thomas et al. 2003, Giordano, Kuick et al. 2009). It has been demonstrated that *IGF2* overexpression is caused by somatic structural alterations of the 11p15 locus, such as paternal isodisomy (loss of the maternal allele and duplication of the paternal allele – Figure 3) and loss of imprinting because of demethylation of the maternal allele (Ogawa, Eccles et al. 1993, Rainier, Johnson et al. 1993). Parental isodisomy can be assessed by microsatellite markers within the 11p15 locus. The presence of 11p15 LOH indicates paternal isodisomy and is associated with overexpression of *IGF2* and poor outcome (Gicquel, Raffin-Sanson et al. 1997, Gicquel, Bertagna et al. 2001). The proliferative effects of *IGF2* are mediated by the insulin-like growth factor 1 receptor (IGF1R), which has also been shown to be overexpressed in ACCs, especially in pediatric cases (Logie, Boulle et al. 1999, Almeida, Fragoso et al. 2008, Doghman, El Wakil et al. 2010). In children, *IGF1R* overexpression is associated with a

worse prognosis. These facts make the IGF system an interesting target to pharmacological inhibition. Preclinical studies with pharmacological inhibitors of IGF1R have demonstrated a significant antiproliferative effect (Almeida, Fragoso et al. 2008, Barlasakar, Spalding et al. 2009). Early clinical trials with IGF1R inhibitors have demonstrated beneficial effects in some patients with advanced disease (Haluska, Worden et al. 2010). A phase 3 trial with an IGF1R inhibitor for metastatic ACC is currently under way (ClinicalTrials.gov identifier NCT00924989). More recently, *in-vitro* and *in-vivo* studies have suggested that mammalian target of rapamycin (mTOR) pathway is activated in the ACC (De Martino, van Koetsveld et al. 2010, Doghman, El Wakil et al. 2010). mTOR pathway is a downstream target of IGF1R signaling (Figure 5) and its pharmacological inhibition significantly reduces cell growth and induces apoptosis in both cell lines and xenographic models (Hay and Sonenberg 2004, Liu, Cheng et al. 2009, Doghman, El Wakil et al. 2010, De Martino, van Koetsveld et al. 2012). Despite these promising preclinical findings, a clinical trial with everolimus, a mTOR inhibitor, failed to show therapeutic activity in patients with metastatic ACC (Fraenkel, Gueorguiev et al. 2013). It has been speculated that overactivation of Akt pathway following mTOR inhibition, as demonstrated in other tumor types, is the mechanism responsible for these negative findings (O'Reilly, Rojo et al. 2006). It has been proposed that dual blockage of mTOR and upstream targets, such as PI3K and IGF1R, may enhance antitumor activity by preventing Akt overactivation. In fact, preclinical studies have demonstrated that inhibition of PI3K and mTOR with the dual inhibitor NVP-BEZ235 prevented Akt hyperphosphorylation associated with mTOR inhibitors. In addition, significant inhibitory effects were demonstrated *in-vitro* and in xenograph models, suggesting that dual inhibition of mTOR and upstream targets may be an interesting therapeutic approach (Doghman and Lalli 2012). In fact, a phase I trial of the combination of cixutumumab (a IGF1R inhibitor) and temsirolimus (a mTOR inhibitor) have demonstrated antitumor activity in metastatic ACC (Naing, Kurzrock et al. 2011). This finding was further confirmed by a recent study, in which 26 patients with advanced ACC were treated with cixutumumab and temsirolimus. Stable disease was observed in 42% of these patients (Naing, Lorusso et al. 2013).

Familial adenomatous polyposis; FAP (Gardner's syndrome; OMIM 175100)

Gardner's syndrome is characterized by the development of multiple colonic polyps with an increased risk for early onset colon cancer, congenital hypertrophy of retinal pigment epithelium, supernumerary teeth, skull osteomas, and a myriad of malignant tumors, including gastric, small intestine carcinoid, periampullary carcinoma, fibrosarcoma, astrocytoma, and papillary thyroid carcinoma (Nishisho, Nakamura et al. 1991, Half, Bercovich et al. 2009). It has been recognized that a substantial proportion of patients develop bilateral adrenocortical nodular hyperplasia, which are characteristically non-functional and benign, but ACCs have also been described (Marshall, Martin et al. 1967, Kartheuser, Walon et al. 1999). FAP is caused by germline inactivating mutations of the *APC* gene (Nishisho, Nakamura et al. 1991). *APC* is a downstream regulator of the Wnt pathway, functioning as a classic tumor suppressor gene by antagonizing Wnt activation. The Wnt pathway is a network of proteins that mediates many cellular processes that are fundamental during embryogenesis and tissue morphogenesis, such as migration, proliferation, differentiation, and survival (Moon, Kohn et al. 2004, Kim, Kim et al. 2013). This pathway also has important roles in tissue homeostasis, regulating the differentiation, self-renewal, and fate of tissue stem cells (Moon, Kohn et al. 2004). Abnormal, constitutive Wnt activation is thought to be oncogenic, as it has been frequently observed in many types of cancer (Karim, Tse et al. 2004). The activation of the Wnt pathway induces cell proliferation, cell motility, epithelial-to-mesenchymal transition, and resistance to apoptosis (Kim, Kim et al. 2013). A hallmark of Wnt pathway activation is the stabilization of cytoplasmic β -catenin, a protein that has a structural function in the *adherens junctions* of epithelial tissues as well as a transcriptional factor (Moon, Kohn et al. 2004). In the absence

of Wnt activation, a multiprotein complex (so-called “destruction complex”) constituted by the APC protein, axin, and the enzyme GSK3- β constitutively phosphorylates β -catenin at specific residues. The phosphorylated β -catenin is recognized by the proteasome complex and destroyed (Figure 4A). Once the pathway is activated, the “destruction complex” is disrupted, and β -catenin is no longer phosphorylated and escapes proteasomal degradation, accumulating on the cytoplasm. Subsequently, it is translocated to the nucleus where it acts as a transcriptional factor, regulating target genes (Figure 4B) (Kim, Kim et al. 2013). The activation status of the Wnt pathway can be assessed by immunohistochemistry. If the Wnt pathway is activated, nuclear staining for β -catenin is observed. In Gardner’s syndrome, the loss of *APC* causes a constitutional activation of the Wnt pathway, leading to tumor development in many organs, including the adrenals.

Abnormal Wnt activation in sporadic ACTs

Although somatic *APC* mutations are rare events in sporadic ACTs (Gaujoux, Pinson et al. 2010), nuclear β -catenin staining is frequently observed (~30% of cases, both ACAs and ACCs) (Tissier, Cavard et al. 2005). Activating mutations of *CTNNB1*, which affects the phosphorylation site of β -catenin and prevents its proteasomal degradation (Figure 4C), have been described in approximately 50% of such cases, suggesting that alterations in other components of the Wnt pathway may be involved (Tissier, Cavard et al. 2005, Tadjine, Lampron et al. 2008). In fact, mutations of *AXIN2*, which is part of the “destruction complex”, have been described in ACTs with positive immunostaining for nuclear β -catenin, but not in adrenocortical hyperplasias (Chapman, Durand et al. 2011). However, another study identified the same *AXIN2* somatic mutation (c2013_2024del 12) in only 1 out of 49 patients with apparently sporadic ACCs. Remarkably, this patient also had a somatic *CTNNB1* activating mutation and the *AXIN2* mutation was also identified in the germ line. In addition, *AXIN1* was also studied and no mutation was found. The authors concluded that the c2013_2024del 12 *AXIN2* mutation is probably a benign polymorphism and *AXIN* genes mutations do not have a role in Wnt activation (Guimier, Ragazzon et al. 2013). Abnormal activation of Wnt pathway has been associated with poor outcome in ACCs (Giordano, Quick et al. 2009, Ragazzon, Libe et al. 2010, Heaton, Wood et al. 2012).

Lynch syndrome (LS; OMIM 120435)

Hereditary non-polyposis colorectal cancer syndrome, also known as Lynch syndrome, is an autosomal dominant genetic condition characterized by high (~80% lifetime) risk of developing colorectal cancer. The molecular basis of the syndrome is mutations in DNA mismatch-repair genes, including *MLH1*, *MSH2*, *MSH6*, and *PMS2* (Karamurzin, Zeng et al. 2012). In addition to colorectal cancer, other malignant tumors have been described in patients with Lynch syndrome, such as carcinomas of the endometrium, ovary, small bowel, hepatobiliary system, central nervous system, renal pelvis, skin, sarcomas, melanoma, anaplastic thyroid cancer, lung adenocarcinoma, and ACCs. So far, four cases of ACCs have been reported in patients with Lynch syndrome (Karamurzin, Zeng et al. 2012). In all but one case, deficiency of *MSH2* was demonstrated immunohistochemically (Karamurzin, Zeng et al. 2012). More recently, Raymond et. al. studied the prevalence of Lynch syndrome among 94 consecutive patients with ACC who were evaluated at a specialized endocrine oncology clinic. Among these, three patients (3.2%) had a family history suggestive of Lynch syndrome. Genetic studies identified germline mutations in DNA mismatch-repair genes in all of them. In addition, the presence of ACC was retrospectively evaluated in 135 patients with known germline mutations in DNA mismatch-repair genes and two cases were identified. Four ACC samples from these patients were studied for microsatellite stability and DNA mismatch-repair genes deficiency. Three out of four had immunohistochemical evidence of DNA mismatch repair deficiency. In contrast, microsatellite instability was not identified in any sample. The authors concluded that ACC patients with a family history

suggestive of Lynch syndrome should be considered for genetic risk assessment and immunohistochemical screening in all ACC may be an effective strategy for identifying patients with Lynch syndrome (Raymond, Everett et al. 2013).

Multiple endocrine neoplasia type 1 (MEN1; OMIM 131100)

MEN1 syndrome is an autosomal dominant disease characterized by the development of tumors in tissues of endocrine origin such as parathyroid glands, pituitary gland, and neuroendocrine pancreas (Chandrasekharappa, Guru et al. 1997). This syndrome is caused by germline inactivating mutations of the *MEN1* tumor suppressor gene, on chromosome 11q13 (Chandrasekharappa, Guru et al. 1997). Approximately 40% of patients develop bilateral adrenocortical nodules (adenomas or hyperplasia), which are typically non-functional and benign (Skogseid, Larsson et al. 1992, Skogseid, Rastad et al. 1995, Gibril, Schumann et al. 2004, Waldmann, Bartsch et al. 2007). Cortisol and aldosterone-producing adenomas have also been described (Schussheim, Skarulis et al. 2001). ACCs have been reported in approximately 2.5–6% of MEN1 patients, and are considered a rare manifestation of the syndrome (Skogseid, Larsson et al. 1992, Skogseid, Rastad et al. 1995, Griniatsos, Dimitriou et al. 2011).

In sporadic ACTs, *MEN1* somatic mutations are unusual, in contrast with LOH of 11q13, which has been identified in ~83% of samples (Kjellman, Roshani et al. 1999). This fact suggests that this region may harbor other still unrecognized tumor suppressor genes involved in tumorigenesis. Alternatively, 11q13 LOH may reflect a global phenomenon of chromosomal instability, considering that large portions of the chromosome 11 are usually lost.

Other syndromes associated with ACT development

ACTs have been rarely described in patients with neurofibromatosis type 1 (Wagner, Fleitz et al. 2005), familial isolated pituitary adenoma syndrome (Toledo, Mendonca et al. 2010) and Werner syndrome (Takazawa, Ajima et al. 2004). The relative contribution of molecular defects characteristic of these syndromes to sporadic ACC has yet to be determined.

MOLECULAR PATHWAYS DYSREGULATED IN SPORADIC ACTs

Epidermal Growth Factor Receptor (EGFR)

The EGFR is a tyrosine kinase-coupled receptor, in which overexpression and activating somatic mutations have been documented in multiple human cancers, such as lung, colon, and breast (Salomon, Brandt et al. 1995). The downstream targets of EGFR include the Ras/Raf/Mek/Erk pathway, which is involved in the regulation of fundamental biological processes, such as cell fate, proliferation, survival, cell cycle control, differentiation, and motility (Figure 5) (Shields, Pruitt et al. 2000). Activating mutations of *Ras* and *BRAF* oncogenes are among the most frequently observed in human cancer (Bos 1989). In endocrine tissues, these mutations are frequently described in well-differentiated thyroid cancer (Nikiforova and Nikiforov 2008). EGFR and its downstream effectors are considered interesting therapeutic targets (Figure 5). In fact, many pharmacological inhibitors have been developed and have been shown to be valuable therapeutic agents in lung and colon cancers, especially if an activating somatic mutation is present. The contribution of this signaling pathway to ACTs has been recently assessed. Immunohistochemistry studies have shown that EGFR overexpression is rarely seen in ACAs, but is ubiquitous in ACCs (Kamio, Shigematsu et al. 1990, Edgren, Eriksson et al. 1997, Adam, Hahner et al. 2010). However, the frequency of activating somatic mutations (exons 18–21) is low. Kotoula *et al.* found such mutations in only four of 35 samples, while Adam *et al.* did not find a single activating mutation among 169 cases (Kotoula, Sozopoulos et al. 2009, Adam, Hahner et al. 2010). In

addition, activating *NRAS* mutations were identified in 12% of samples. The same study did not identify any mutations in *HRAS* and *KRAS* (Yashiro, Hara et al. 1994). However, later studies failed to reproduce these findings (Moul, Bishoff et al. 1993, Ocker, Sachse et al. 2000). The presence of activating *BRAF* p.V600E activating mutation has also been studied and was identified in only two out of 35 ACCs (Kotoula, Sozopoulos et al. 2009). More recently, Hermsem *et al.* studied the *EGFR* and downstream pathways in 47 paraffin-embedded tumor tissues from ACC samples. As expected, a low number of mutations on *EGFR* TK domain were found (just two cases). In addition, a *BRAF* activating mutation (p.V600E) was identified in a single case, but no mutations on *PIK3CA* and *KRAS* could be found (Hermesen, Haak et al. 2013). Although *EGFR* is frequently overexpressed in ACC, the low number of activating mutations suggests that pharmacological inhibition of *EGFR* with TK inhibitors would be of limited efficacy. In fact, a phase II clinical trial with erlotinib and gemcitabine for advanced ACC has shown disappointing results (Figure 5) (Quinkler, Hahner et al. 2008).

ACTH receptor (*MC2R*)

The *MC2R* is a G protein-coupled receptor, which activation induces proliferation and steroid production of *zona fasciculata* cells. Analogously to thyroid nodules, in which activating mutations of TSH receptor have been described, it has been hypothesized that activating *MC2R* mutations may contribute to adrenocortical tumorigenesis. However, such mutations have never been described in ACTs (Latronico, Reincke et al. 1995, Light, Jenkins et al. 1995). In fact, ACCs exhibit frequent allelic losses of region 18p11.2 and downregulation of *MC2R* (Reincke, Beuschlein et al. 1997, Reincke, Mora et al. 1997). On the other hand, overexpression of *MC2R* was found in cortisol-secreting ACAs, suggesting a role of this receptor in cell differentiation (Reincke, Beuschlein et al. 1997).

Vascular endothelial growth factor (VEGF)

Sustained angiogenesis is a *sine qua non* feature of cancer. Anomalous blood vessels are a characteristic of virtually all types of cancer (Hanahan and Weinberg 2000). The vascular endothelial growth factor (*VEGF*) is a chief regulator of cancer angiogenesis. Its effects are mediated through its receptors (VEGFRs) (Affara and Robertson 2004). The pharmacological inhibition of VEGFRs are considered an attractive option for cancer treatment (Figure 5) (Bagri, Kouros-Mehr et al.). Elevated VEGF levels were identified in blood samples from ACC patients (de Fraipont, El Atifi et al. 2000, Kolomecki, Stepien et al. 2001). In addition, overexpression of VEGFR type 2 in ACC samples was observed by immunohistochemistry (Wortmann, Quinkler et al.). The increased expression of *VEGF* correlates with the expression of *IGF2* (Giordano, Kuick et al. 2009). Recently, several groups utilized targeted therapeutic methods of VEGF signaling inhibition in xenograft mouse models with relative success. Marinello et al reported marked growth inhibition using sorafenib and everolimus, for VEGFR1–2 and mTOR inhibition respectively, suggesting potential antiangiogenic and antitumor effects (Figure 5) (Mariniello, Rosato et al. 2012). However, an earlier clinical trial utilizing bevacizumab, an anti-VEGF monoclonal antibody, proved to be ineffective (Wortmann, Quinkler et al. 2010). Taken together, although increased VEGF expression correlates with ACCs for potential diagnostic role, the targeted treatment lacks support for its therapeutic role.

GENOME-WIDE EXPRESSION PROFILES

Global gene expression studies aim to identify biomarkers that could provide diagnostic and prognostic utility in addition to the classic histologic analyses and hold the promise of new potential targets for therapy. ACAs and ACCs have distinct expression profiles (Giordano, Thomas et al. 2003, de Fraipont, El Atifi et al. 2005, de Reynies, Assie et al. 2009,

Giordano, Kuick et al. 2009). De Fraipont *et al.* identified a cluster of genes that could correctly discriminate ACAs and ACCs. According to their results, high expression levels of genes involved in growth factor signaling and cell proliferation characterized ACCs (the so-called IGF2 cluster), and ACAs were characterized by high expression levels of steroidogenic machinery related genes (the steroidogenic cluster). Taken together, these two clusters of genes could correctly discriminate ACCs from ACAs (de Fraipont, El Atifi et al. 2005). Similarly, Giordano *et al.* demonstrated that the expression profiles of 22 ACAs and 33 ACCs were remarkably different. Also, they identified that chromosomal regions 12q and 5q were transcriptionally activated while regions 11q, 1p, and 17p were transcriptionally repressed which mirrored and confirmed the early chromosomal studies (Giordano, Kuick et al. 2009). More recently, two large studies have correlated expression profiles in ACC with clinical outcome. Giordano *et al.* identified that tumors with high histologic grade were transcriptionally distinct from low-grade tumors and these groups had different survival rates. In the poor outcome group, cell cycle genes and “functional aneuploidy” genes were overexpressed (Giordano, Kuick et al. 2009). Similarly, De Reyniès *et al.* showed in a large cohort of ACTs that ACAs and ACCs could be clearly differentiated by cluster analysis and subgroups of ACCs with clearly distinct clinical outcomes identified. In the poor outcome group, genes related to transcriptional control and mitotic cell cycle predominated, while cell metabolism genes, intracellular transport, apoptosis, and cell differentiation genes predominated in the good outcome group. It was later determined that the poor prognosis group could be further subdivided according to three different transcriptional signatures. In one of these subgroups, a transcriptional signature of *TP53* inactivation could be identified. In the second, a transcriptional signature of Wnt activation was evident. The third exhibited a transcriptional signature yet to be characterized (Ragazzon, Libe et al. 2010). In addition, they identified a set of three genes that were highly predictive of outcome. Combining the expression levels of *BUB1B* and *PINK1*, the group identified subgroups of ACCs with clear different overall survival times, regardless of disease stage at presentation. Similarly, the combination of the expression levels of *DLG7* and *PINK1* could identify subgroups of ACCs with distinct disease-free survival times, regardless of Weiss score (de Reynies, Assie et al. 2009). These findings were validated in a different cohort of adult patients, but not in pediatric patients (Fragoso, Almeida et al. 2012).

EPIGENETICS OF ACTs

DNA methylation

DNA methylation involves the addition of a methyl group to the cytosine pyrimidine ring or adenine purine ring, occurring typically at CpG dinucleotides. In a normal cell, it acts as a regulatory mechanism for proper gene expression. There is growing evidence to suggest that DNA methylation, in addition to genetic modification may cause altered patterns of gene expression resulting in tumorigenesis (Das and Singal 2004, Wright and Gilbertson 2010). Earlier studies on DNA methylation in ACTs have focused on individual genes. Altered DNA methylation of the *H19* promoter has been shown to be involved in the abnormal expression of both *H19* and *IGF2* genes in ACC (Gao, Suppola et al. 2002). In addition, although *TP53* promoter methylation has been described in some types of cancer, it does not seem to be an important event in ACC (Sidhu, Martin et al. 2005). Two recent studies evaluated DNA methylation at a genome-wide level. Fonseca et al studied the DNA methylation levels of 27,578 CpG in 6 normal adrenal cortices, 27 ACAs and 15 ACCs. They found that tumor suppressor genes, regulators of cell cycle and apoptosis, such as *CDKN2A*, *GATA4*, *DLEC1*, *HDAC10*, *PYCARD* and *SCGB3A1* are significantly hypermethylated in ACCs. In addition, the methylation levels were inversely correlated to mRNA expression levels, as expected (Fonseca, Kugelberg et al. 2012). Barreau et al studied the methylation profiles of 51 ACCs and 84 ACAs and identified that a subset of

ACCs exhibited elevated promoter methylation levels. This group of tumors could be further divided in two subgroups with different methylation levels (low methylation and high methylation, termed CIMP-low and CIMP-high – paralleling the CIMP first described as a distinct subset of colorectal tumors (Toyota, Ahuja et al. 1999), respectively). Genes such as *H19*, *GSTP1*, *GSTM1*, *GSTT1*, *PLAGL1*, *GOS2*, and *NDRG2* exhibited high methylation levels and were transcriptionally silenced. In addition, the degree of methylation was directly correlated with poor prognosis. A total of 553 genes showed a significant association between their methylation levels and prognosis (Barreau, Assie et al. 2013). Interestingly, an overlap between previously identified transcriptional signatures of poor prognosis and high methylation patterns was verified. The subgroup of ACCs of poor prognosis that presented the molecular signature of *TP53* inactivation presented high methylation levels. In contrast, the subgroup of poor prognosis ACC with the transcriptional signature of Wnt activation did not exhibit high methylation, indicating that different mechanisms are responsible for the transcriptional dysregulation observed in these subgroups, reinforcing the concept that ACC with similar phenotypes are heterogeneous regarding the molecular mechanisms regarding tumorigenesis (Barreau, Assie et al. 2013). These observational studies provided first insights into the possible role of methylation in ACC tumorigenesis.

MicroRNAs (miRNA)

miRNAs are a class of evolutionary conserved, small, non-coding, 18–25 nucleotide RNAs that post-transcriptionally regulate gene expression by directly targeting mRNAs, affecting their stability and translation. The function of miRNAs is to direct the RNA-induced silencing complex (RISC), a multiprotein complex that include a member of the Argonaute protein family directly bound to a strand of miRNA, to the 3'UTR of the target mRNA through Watson-Crick base pairing (Pratt and MacRae 2009)(Czech and Hannon 2011). Numerous miRNA have been identified and implicated in the regulation of various cellular processes such as proliferation, apoptosis, and differentiation. In addition, dysregulation of miRNAs, such as overexpression or deletion, play an important role in disease processes including various cancers (Cimmino, Calin et al. 2005, Lujambio and Lowe 2012). The effects of miRNA dysregulation in a cancer cell include silencing of tumor-suppressor genes, activation of various oncogenes, and/or growth factors important in tumor angiogenesis, epithelial-to-mesenchymal transition, and metastasis (Lujambio and Lowe 2012). In addition, the classification of tumor samples based on their miRNA profiling may add in diagnosis and prognosis prediction and miRNAs may have a role as tumor markers, since they are more stable than mRNAs and can be directly assayed on biological fluids, such as urine or plasma (Iorio and Croce 2009, Ferracin, Veronese et al. 2010, Xiao, Yong et al. 2013). Recently, miRNA profiling studies have demonstrated that ACCs and ACAs have distinct expression profiles. Tombol et al. studied 36 adrenocortical samples (10 normal tissues, 10 non-functional adenomas, 9 cortisol-secreting adenomas, and 7 ACCs) and found differential expression of 22 miRNAs, with fourteen miRNAs preferentially expressed in ACCs. Upregulated microRNAs in ACCs included miR-184, miR-210, and miR-503. Downregulated microRNAs included miR-214, miR-375, and miR-511 (Tombol, Szabo et al. 2009). Levels of miR-184, miR-503, and miR-511 alone were able to distinguish benign from malignant ACTs (specificity 80–97%; sensitivity 100%) (Tombol, Szabo et al. 2009). A recent study of 55 adrenal samples (6 normal tissues, 22 adenomas, and 27 ACCs) similarly determined a miRNA expression signature unique to ACC (Soon, Tacon et al. 2009). The investigators identified 14 upregulated miRNAs and 9 downregulated miRNAs unique to ACC. In addition to validating the upregulation of miR-503 in ACC, the study identified a significant upregulation of miR-483 (diagnostic sensitivity of 80% and specificity of 100%) and downregulation of miR-195 and miR-335 in ACC (Soon, Tacon et al. 2009). Some of these findings were further validated by a recent study (Ozata, Caramuta

et al. 2011). Lastly, miRNA expression in 25 pediatric adrenal neoplasms (18 ACC, 6 ACA, 1 unknown) was compared with 5 normal adrenals (Doghman, El Wakil et al. 2010). Unsupervised clustering of the samples via miRNA expression resulted in clear differentiation of the tumors from the normal controls. Further differentiation between ACA and ACC could not be achieved. Similar to the adult ACCs, miR-483 was found to be significantly up regulated in the pediatric ACCs. However, a majority of the differentially expressed miRNA were down regulated in ACCs, most-notably miR-99a and miR-100. MiR-99a and miR-100 are bioinformatically predicted to target the 3'UTRs of *IGF1R*, *RPTOR*, and *FRAP1* (mTOR) and were experimentally confirmed to target several components of the IGF1 signaling pathway (Doghman, El Wakil et al. 2010). Moreover, miR-483 is located in chromosome 11p15.5, precisely within the *IGF2* locus. It is hypothesized that dysregulation of the *IGF2* locus in turn perturb the expression of miR-483 (Soon, Tacon et al. 2009, Patterson, Holloway et al. 2011). In the hepatocarcinoma cell lines HepG2, observational studies revealed the oncogenic potential of miR-483 through inhibition of apoptotic regulatory genes, *PUMA/BBC3* (Veronese, Lupini et al. 2010). These findings were recently validated in the NCI-295R cell line. Ozata et al. found that inhibition of miR-483-3p and miR-483-5p resulted in significant reduction of cell proliferation. In addition, downregulation of miR-483-3p, but not miR-483-5p, resulted in increased apoptosis. To explore the clinical and biological consequences of these findings *in-vivo*, the authors measured the PUMA protein expression levels in clinical samples and found an inversion correlation with miR-483-3p levels, suggesting a role as a proapoptotic regulator in ACC (Ozata, Caramuta et al. 2011). Finally, the role of miRNAs as prognosis markers in ACC patients was recently evaluated. Chabre et al. studied the miRNA expression profiles in six ACAs, six non-aggressive ACCs and 6 aggressive ACCs. They selected 8 miRNAs from their microarray experiments and validated in further 31 samples (10 ACAs, 9 non-aggressive ACCs, 9 aggressive ACCs and 6 normal adrenal cortices). The serum levels of five of these miRNAs were then determined in 56 subjects – 19 healthy controls, 14 ACA, 9 non-aggressive ACC and 14 aggressive ACC patients. Decreased serum levels of miR-195 and miR-335 were observed in ACC patients, relative to ACA and healthy controls. Remarkably, although tissue levels of miR-483-5p were increased in the majority of ACCs, its serum levels were elevated only in aggressive ACC patients. High serum levels of miR-483-5p and low circulating levels of miR-195 were associated with shorter recurrence-free survival and overall survival (Chabre, Libe et al. 2013).

MOLECULAR MECHANISMS OF TUMORIGENESIS AND SOMATIC EVOLUTION IN ACTs - EVIDENCE FROM CLINICAL, MOLECULAR DATA AND ANIMAL MODELS

In organs such as breast, colon, and skin, the carcinogenesis process appears to initiate from benign precursor lesions. Such lesions progressively accumulate genetic defects, culminating in malignant transformation. This fact is well demonstrated in colon carcinogenesis, in which invasive carcinomas clearly arise from tubular adenomas. For the adrenal gland, it is still a matter of debate as to whether ACCs arise from precursor benign lesions, such as ACAs or hyperplasias, or whether they arise *de novo*. The fact that ACCs have accumulated a large set of genetic changes is well accepted, but it is not totally clear whether some of these mutations were acquired in a premalignant stage. If such a premalignant lesion does exist, how would it look? How would it be diagnosed and malignant transformation anticipated? Clinical evidence of such a progression comes from a few case reports that have documented malignant degeneration of a pre-existing ACA (Bernard, Sidhu et al. 2003, Gaujoux, Tissier et al. 2008). However, since ACAs are very prevalent neoplasms and ACCs are so rare, ACAs should not be considered a premalignant lesion in the same way as a colonic tubular adenoma is considered a precursor lesion of

colorectal cancer. Recent evidence that further corroborates the multistep tumorigenesis model came from observations of ACCs arising from PPNAD in CC patients. Although PPNAD is typically a benign disease, two cases of ACCs have been described in two non-related patients with CC (Anselmo, Medeiros et al. 2012, Morin, Mete et al. 2012), suggesting that the dysregulation of the cAMP pathway may have been involved in malignant transformation. In addition, cross talk between PKA and other oncogenic pathways known to be involved in adrenocortical tumorigenesis have been demonstrated. Activation of the PKA pathway also causes activation of Wnt signaling pathway, both in-vitro and in-vivo (Horvath, Mathyakina et al. 2006, Hsiao, Kirschner et al. 2009, Roy, McDonald et al. 2009, Almeida, Muchow et al. 2010). Interestingly, somatic *CTNNB1* mutations have also been demonstrated in larger nodules of patients with PPNAD, suggesting that PKA chronic activation might cause selection pressure upon the appearance of such mutations (Gaujoux, Tissier et al. 2008).

The full spectrum of somatic alterations in different types of adrenocortical disease is represented in Figure 6. Assuming that adrenal tumorigenesis is a continuous spectrum of genetic changes, from a precursor lesion (hyperplasia or adenoma) to cancer, it would be expected that earlier genetic events would be shared by ACAs and ACCs and late events would be present only in ACCs. If this were true, abnormal PKA activation, along with Wnt pathway activation, would be early events in adrenocortical tumorigenesis, since evidence of these pathways activation is present in both ACAs and ACCs. The fact that both nuclear β -catenin immunostaining and activating *CTNNB1* mutations are present in ACAs as well as in ACCs suggests that Wnt activation may be an early step in adrenocortical tumorigenesis, which precedes malignant transformation, assuming that ACCs may arise from ACAs. A recent study on mouse models corroborates this hypothesis (Heaton, Wood et al. 2012). Mice with constitutive activation of the Wnt signaling pathway obtained by adrenal-specific *Apc* knockout develop adrenal hyperplasia and adenomas by 30 weeks of life. On the other hand, no adrenal phenotype is observed in the adrenal-specific *Igf2* overexpression mouse model. However, when the *apc* KO model was crossed with the adrenal-specific *Igf2* overexpression mouse model, early onset adrenal nodular hyperplasia evolving to large tumors later in life (including an invasive cortical tumor similar to an ACC) was observed, suggesting that both pathways may have synergistic effects on adrenocortical tumorigenesis (Heaton, Wood et al. 2012). This study was further validated in a similar model of adrenocortical-specific β -catenin stability and IGF2 over-expression (Drelon, Berthon et al. 2012). Clinical and epidemiological observations suggest that later events toward malignant transformation include *TP53* inactivation, *CDKN2A* silencing and telomerase over-activation (Pilon, Pistorello et al. 1999, Else, Giordano et al. 2008).

CONCLUSION REMARKS

In conclusion, much has been learned about the genetics and molecular classification of adrenocortical tumors over the past decade. Many of the key genes and pathways have been elucidated and are the current focus of therapeutic intervention (Figure 5). It is expected that pangenomic and other global analyses that will be done in the upcoming years, will advance our understanding of adrenocortical tumorigenesis to a higher level. We believe that, in the near future, molecular markers will guide the choice for specific therapeutic agents.

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Highlights

abnormal activation of ligands/receptors/channels and associated signaling pathways drives

adrenocortical neoplastic transformation

Figure 1. The cAMP signaling pathway in the adrenal cortex

A) ACTH binds to its specific seven-domain transmembrane receptors (MC2R), leading to activation the G_{α_s} protein, which will activate adenylyl cyclase (AC) and, therefore, cAMP production. After binding of cAMP to PKA regulatory subunits, the catalytic subunits are released and activated. The free catalytic subunit (C) then phosphorylates CREB at serine 133, which is translocated to the nucleus and activates the transcription of target genes. cAMP is inactivated by phosphodiesterases, allowing the PKA tetramer to reassembled, terminating its activity.

B) Anomalous activation of cAMP pathway in adrenocortical disease. At cell membrane level: 1. activating mutations of protein G_{α_s} lead to constitutive activation; 2. expression of "illicit" G protein-coupled receptors cause pathway activation in response to unusual ligands. Inactivating mutations of PKA regulatory subunit PRKARIA or LOH at the gene locus lead to constitutive catalytic subunits activation. Inactivating mutations of phosphodiesterases (PDE) lead to an increase in cytoplasmatic cAMP levels, augmenting PKA activity.

Figure 2. Physiology of aldosterone secretion in the normal adrenal cortex

A) In the absence of stimulus, the activity of the ATP1A1 Na^+/K^+ ATPase and the KCNJ5 potassium channel create an electrical gradient between the inner surface and the outer surface of the plasma membrane. The electrical gradient (polarization) closes the L-type and T-type voltage-gated calcium channels. In addition, the activity of the ATP2B3 calcium pump keeps the cytosolic levels of calcium very low.

B) The activation of the angiotensin receptor (ATR) by angiotensin 2 (AT2) inhibits the activity of the ATP1A1 Na^+/K^+ ATPase and decreases the K^+ permeability of the KCNJ5 channel. The resulting depolarization of the plasma membrane will open the voltage-gated calcium channel, causing an inward calcium flow, elevating its cytosolic levels. As a result, a cytosolic calcium-sensitive protein kinase (CAMK) will be activated, promoting the phosphorylation of transcription factors such as CREB, NURR1, NGFIB and ATF1, which in turn will promote CYP11B2 (aldosterone synthase) transcription.

C) Molecular mechanisms beyond aldosterone overproduction in aldosterone-producing adenomas. A permanent increase in cytosolic calcium levels causes constitutive activation of CAMK and downstream pathways, leading to aldosterone overproduction. The increased cytosolic calcium may be the result of a permanent depolarization of plasma membrane caused by somatic KCNJ5 mutations (which will result in loss of selectivity of the channel to K^+ ions, allowing an influx of Na^+) or by inactivating ATP1A1 Na^+/K^+ ATPase mutations. In addition, high cytosolic calcium levels may be also resultant of inactivating mutations of the ATP2B3 Ca^{+2} ATPase.

Normal Cell

Tumor Cell

Figure 3.

Molecular mechanism beyond *IGF2* overexpression in ACC. *IGF2* is located in chromosome 11p12, downstream to the cell cycle regulator *CDKN1C* and upstream to the *H19* transcription regulator. In normal cells, *IGF2* is imprinted on the maternal allele and *CDKN1C* and *H19* are imprinted on the paternal allele. In ACC, the maternal copy of these genes is lost and the paternal copy gets duplicated, resulting in biallelic *IGF2* expression and in transcriptional silencing of *CDKN1C* and *H19*.

Figure 4. Wnt signaling pathway in normal and cancer cells

A) In the absence of the ligand, cytosolic β -catenin is continuously phosphorylated by the «destruction complex». This phosphorylation targets β -catenin to proteasomal (PR) degradation.

B) After binding of Wnt to Frizzled and LRP5/6, GSK3 and CK1 phosphorylate LRP5/6, which in turn recruits axin. The ultimate consequence is the dissociation of the «destruction complex» and accumulation of unphosphorylated β -catenin on the cytosol which is translocated to the nucleus and activates the transcription of target genes.

C) One of the mechanisms by which the Wnt pathway is constitutively activated in cancer cells is the presence of an activating somatic mutation on *CTNNB1* gene. As a result of an abnormal phosphorylation site, the mutant β -catenin is no longer phosphorylated by the «destruction complex», being accumulated at the cytosol and translocated to the nucleus, where it activates the transcription of target genes.

Figure 5.

Tyrosine-kinase coupled receptors (TKs) that are abnormally activated in ACC and downstream signaling pathways. The effects of pharmacological inhibition of some of these molecules have been evaluated in pre-clinical studies and clinical trials. The pharmacological compounds and specific targets are indicated.

Figure 6. Molecular findings in the whole spectrum of nodular adrenocortical disease. Some alterations are shared by benign disease (hyperplasias and ACA) and ACC and are thought to be early events in the tumorigenesis process. Alterations that are exclusive of ACC are thought to be late events.

TABLE 1

Genetic Syndromes Associated with Adrenal Hyperplasia/Neoplasia

Syndrome	Heritage	Locus	Gene	Clinical features	Adrenal manifestations	Comments
Multiple endocrine neoplasia type 1	Autosomal dominant	11q13	<i>MEN1</i>	Primary hyperparathyroidism, gastric, pancreatic, and duodenal neuroendocrine tumors, pituitary adenomas, thymic carcinoid tumors	Non-functioning macronodular hyperplasia in up to 40% of patients. ACCs rarely described	Somatic <i>MEN1</i> mutations are rarely described in sporadic ACCs, in spite of the high frequency of 11q LOH
Carney's complex	Autosomal dominant	17q22–24	<i>PRKAR1A</i>	Cutaneous lentigines, pituitary adenomas, cardiac myxomas, pancreatic, and cutaneous tumors	Micronodular pigmented adrenal hyperplasia	Somatic <i>PRKAR1A</i> have been described in functioning ACAs; 17q LOH frequently described in ACTs
McCune–Albright syndrome	Sporadic (post-zygotic somatic mosaicism)	20q13.3	<i>GNAS1</i>	Polyostotic bone dysplasia, gonadotropin-independent precocious puberty, café-au-lait spots, pituitary adenomas	Cortisol-producing bilateral nodular hyperplasia	Activating <i>GNAS1</i> mutations have been described in cortisol-producing ACAs
Gardner's syndrome	Autosomal dominant	5q21–q22	<i>APC</i>	Familial adenomatosis polyposis, increased risk for colon cancer, thyroid tumors, osteomas of the skull	Bilateral adrenocortical hyperplasia in 7–13%	Somatic <i>APC</i> mutations have not been described in sporadic ACTs. Abnormal nuclear β -catenin staining has been described in one-third of ACCs and ACAs
ACTH-independent macronodular hyperplasia (AIMAH)	Sporadic/a utosomal dominant	?	?/ overexpression of GPCRs of different classes in adrenal nodules		Bilateral nodular enlargement of adrenal glands associated with Cushing's syndrome	Overexpression of GPCRs has also been documented in ACAs
Li–Fraumeni syndrome	Autosomal dominant	17p13	<i>TP53</i>	Increased risk for sarcomas, hematologic malignancies, lung tumors, breast tumors	ACCs in 5%	Germline inactivating <i>TP53</i> mutations are very frequent in pediatric ACCs but rarely seen in adults. Somatic inactivating <i>TP53</i> mutations are present in 30% of samples
Beckwith–Wiedemann syndrome	Autosomal dominant/ sporadic	11p15	<i>IGF2</i>	Organomegaly, omphalocele, microcephalia, mental retardation, fetal neoplasms (Wilm's tumor, hepatoblastoma, ACC)	ACT in 1.5%	<i>IGF2</i> overexpression and structural abnormalities of 11p15 are present in up to 90% of sporadic ACCs.
Neurofibromatosis type 1	Autosomal dominant	17q11.2	<i>NF1</i>	Café au lait spots, cutaneous neurofibromas, nerve sheath tumors, pheochromocytoma	ACTs describes in at least 4 cases, including 2 children	
FIPA	Autosomal dominant	11q13.3	<i>AIP</i>	Familial pituitary tumors (somatotropinomas)	ACC described in one case, in which AIP LOH could be verified	<i>AIP</i> inactivation leads to abnormal PKA activity;