REVIEW

Genetics of tumors of the adrenal cortex

Fideline Bonnet-Serrano^{1,2} and Jérôme Bertherat^{1,3}¹Institut Cochin, INSERM U1016, CNRS UMR8104, Paris Descartes University, Paris, France²Hormonal Biology Laboratory, Assistance Publique Hôpitaux de Paris, Hôpital Cochin, Paris, France³Department of Endocrinology, Assistance Publique Hôpitaux de Paris, Hôpital Cochin, Paris, FranceCorrespondence should be addressed to J Bertherat: jerome.bertherat@aphp.fr

Abstract

This review describes the molecular alterations observed in the various types of tumors of the adrenal cortex, excluding Conn adenomas, especially the alterations identified by genomic approaches these last five years. Two main forms of bilateral adrenocortical tumors can be distinguished according to size and aspect of the nodules: primary pigmented nodular adrenal disease (PPNAD), which can be sporadic or part of Carney complex and primary bilateral macro nodular adrenal hyperplasia (PBMAH). The bilateral nature of the tumors suggests the existence of an underlying genetic predisposition. PPNAD and Carney complex are mainly due to germline-inactivating mutations of *PRKAR1A*, coding for a regulatory subunit of PKA, whereas PBMAH genetic seems more complex. However, genome-wide approaches allowed the identification of a new tumor suppressor gene, *ARMC5*, whose germline alteration could be responsible for at least 25% of PBMAH cases. Unilateral adrenocortical tumors are more frequent, mostly adenomas. The Wnt/beta-catenin pathway can be activated in both benign and malignant tumors by *CTNNB1* mutations and by *ZNRF3* inactivation in adrenal cancer (ACC). Some other signaling pathways are more specific of the tumor dignity. Thus, somatic mutations of cAMP/PKA pathway genes, mainly *PRKACA*, coding for the catalytic alpha-subunit of PKA, are found in cortisol-secreting adenomas, whereas *IGF-II* overexpression and alterations of p53 signaling pathway are observed in ACC. Genome-wide approaches including transcriptome, SNP, methylome and miRome analysis have identified new genetic and epigenetic alterations and the further clustering of ACC in subgroups associated with different prognosis, allowing the development of new prognosis markers.

Key Words

- ▶ adrenal tumors
- ▶ adrenal cortex
- ▶ Cushing' syndrome
- ▶ PKA
- ▶ ARMC5

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Introduction

Adrenocortical tumors can be unilateral or bilateral, secreting or non-secreting various types of adrenal steroids depending on tumor type. Unilateral adrenocortical tumors are frequent in the general population; most of them are incidentally discovered on medical imaging. The prevalence of such incidentalomas ranges from 1 to 7% depending on the series and increases with ageing (Bertherat *et al.* 2002, Grumbach *et al.* 2003, Fassnacht

et al. 2016). Most of them are benign adrenocortical adenomas (ACA), which can be non-secreting but can also be responsible for cortisol excess (Cushing syndrome) in 5–47% of cases and more rarely for aldosterone excess (Conn adenomas) in 1.6–3.3% of cases (Mansmann *et al.* 2004, Fassnacht *et al.* 2016). In contrast, adrenocortical carcinomas (ACC) are rare tumors with an estimated annual incidence of 0.7–2 cases by year and a global

prevalence of 4–12 cases per million. Their prognosis is poor with a 5-year survival rate inferior to 35% in most series. However, prognosis varies according to tumor stage with a 5-year survival rate of respectively 82% for stage I tumors, 58% for stage II tumors, 52% for stage III tumors and finally 18% for stage IV tumors (Fassnacht *et al.* 2009). They are responsible for steroid excess in 60–70% of cases (Mansmann *et al.* 2004, Kebebew *et al.* 2006, Kerkhofs *et al.* 2013). However, if clinically significant symptomatic Cushing syndrome or androgens excess can be observed, steroidogenesis is often altered in tumor cells, leading to asymptomatic steroid precursors accumulation (Arlt *et al.* 2011). Mineralocorticoids and estrogens excess have also been reported.

Bilateral adrenocortical tumors are less common. Two different entities can be distinguished depending on nodules size and aspect: primary bilateral macro-nodular adrenal hyperplasia (PBMAH) and bilateral micro-nodular adrenal hyperplasia (MiAH). PBMAH is defined by the presence of several nodules, whose diameter is superior to 10mm, and which are generally bilateral and more rarely confined to one adrenal. MiAH is rare and primary pigmented nodular adrenal disease (PPNAD) is its most frequent form. PPNAD is characterized by a specific macroscopic aspect of adrenals with bilateral pigmented micro-nodules widespread in adrenal cortex. PPNAD is usually diagnosed in patients with Cushing syndrome while PBMAH can cause variable degree of cortisol excess.

The molecular mechanisms of tumorigenesis are not the same in these different types of adrenal tumors. The first molecular alterations were identified by the study of rare familial tumor syndromes, including adrenocortical tumors due to an identified germline mutation or epigenetic alteration (Libé & Bertherat 2005). Most of these alter a tumor suppressor gene. According to the Knudson model, in this context, tumors result from the clonal proliferation of a cell, in which a somatic second ‘hit’ has occurred on the germline wild-type allele. Alterations of these candidate genes were searched in sporadic tumors at the somatic level and allowed the identification of TP53 mutation (Li–Fraumeni syndrome) in ACC (Reincke *et al.* 1994), various 11p15 locus alterations leading to insulin growth factor 2 (IGF2) overexpression (Beckwith–Wiedemann syndrome) in sporadic ACC (Gicquel *et al.* 1997) and protein kinase A regulatory subunit 1- α (PRKAR1A) (Carney complex) in sporadic cortisol-secreting ACA (Bertherat *et al.* 2003). However, this candidate gene approach is limited and the recent development of genome-wide tools with the emergence of next-generation sequencing (NGS) has allowed at a much

faster rate identification of new molecular alterations at the somatic levels in the different types of adrenal tumors.

This review aims to describe the reported molecular alterations in the different types of tumors of the adrenal cortex, a topic which has already been addressed in previous reviews (Åkerström *et al.* 2016, Lodish 2017). The choice was made to exclude Conn adenomas to allow a specific focus on the many recent advances made in the genomic characterization of PPNAD, PBMAH, cortisol-secreting ACA and ACC.

Benign adrenocortical cortisol-producing tumors

Bilateral cortisol-producing tumors

Bilateral adrenocortical tumors are characterized by the presence of several nodules in both adrenals and responsible for Cushing syndrome to varying degrees. Their bilateral nature suggests a possible genetic predisposition, relying on the existence of a germline mutation or epigenetic alteration. Furthermore, families with several members presenting PPNAD or PBMAH had been reported.

Primary pigmented nodular adrenal disease (PPNAD)

PPNAD, the most common form of MiAH, is a rare cause of ACTH-independent hypercorticism, most often diagnosed in children and young adults. Its name comes from the particular macroscopic aspect of adrenals in this pathology, with pigmented micro-nodules (diameter inferior to 10mm) widespread in the cortex of both adrenals. PPNAD is most often observed in patients with Carney complex (CNC), a familial tumor predisposition syndrome, responsible for several types of tumor, including other endocrine lesions: somatotroph pituitary adenomas, testicular Sertoli cell-calcified tumors and thyroid benign or malignant lesions but also non-endocrine manifestations, mainly, cardiac myxomas, pigmented skin lesions (lentiginosis and naevi) and melanocytic schwannomas. The prevalence of PPNAD in patients with CNC is approximately 60% (Stratakis 2000, Bertherat *et al.* 2009).

CNC is inherited in an autosomal dominant manner. Linkage studies allowed the identification of 2 different loci associated with the disease: one in the short arm of the chromosome 2 (2p16), a region including proopiomelanocortin (POMC) gene and DNA-mismatch repair gene MSH2, 2 genes further excluded

(Stratakis *et al.* 1996) and the other on the long arm of chromosome 17 (17q22–24) (Casey *et al.* 1998). Deleterious germline mutations of *PRKARIA* gene, coding for one of the regulatory subunit of PKA, were identified at the 17q22–24 locus and are responsible for more than two-thirds of Carney complex index cases, from 37% in the sporadic forms to 80% in the typical familial forms (Bertherat *et al.* 2009). The first germline mutations identified led to frameshift, usually causing a premature stop codon (Kirschner *et al.* 2000). They were often associated with loss of heterozygosity (LOH), involving somatic loss of the wild-type allele in CNC tumors. Expression of PKA regulatory subunit R1A was therefore completely lost in tumor cells.

This is a clear example of alteration of the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway in endocrine tumors. Physiologically, in adrenocortical cells, adrenocorticotrophic hormone (ACTH) binds to its 7-transmembrane G protein-coupled receptor MC2R, resulting in Gs protein activation, further activating adenylyl cyclase resulting in enhanced cAMP production (Fig. 1). PKA is a hetero tetramer, composed of 2 regulatory subunits and 2 catalytic subunits. The binding of 4 cAMP molecules to PKA regulatory subunits dimer, allows the release and subsequent activation of the 2 catalytic subunits, which will further phosphorylate several targets, including the transcription factor CREB (cAMP response element-binding protein), responsible for the stimulation of cAMP-dependent genes transcription. Phosphodiesterases, involved in cAMP degradation, act as negative regulators of this pathway. ACTH is known to stimulate both adrenocortical cell growth and cortisol synthesis, it is then easy to understand how constitutive activation of cAMP/PKA pathway can lead to both tumorigenesis and Cushing syndrome.

Indeed, inactivation of *PRKARIA* results in constitutive activation of the cAMP/PKA pathway. *PRKARIA* is considered as a potential tumor suppressor gene in tissues where tumors occur in Carney complex patients. Very few hotspot mutations were identified in the *PRKARIA* gene. Reported mutations are distributed along the whole coding sequence and to a smaller extent (about 20%) noncoding sequence, intronic mutations affecting splicing. Only 2 mutations were found with a higher prevalence in a large study, including 353 patients with CNC and/or PPNAD: the c.709-7del6 in intron 7, mostly reported in cases of isolated PPNAD and the c.491-492delTG in exon 5, significantly associated with cardiac myxoma, lentigines and thyroid tumors when compared with other *PRKARIA* defects. About 80% of

these mutations are subject to mRNA nonsense-mediated decay (NMD), coding for an mRNA, which is not translated into protein. The 20% of mutations, escaping NMD, lead potentially to the expression of an alternative protein and are probably responsible for a more aggressive form of the disease, including a higher total number of CNC manifestations (Bertherat *et al.* 2009). A dominant-negative effect was described for at least one *PRKARIA* mutation escaping NMD, an intronic base substitution in intron 6 (exon 6 IVS+1G>T)-inducing exon 6 skipping and expression of a shorter protein. Indeed, 5 different CNC tumors (left and right adrenal nodules, pancreatic adenocarcinoma, schwannoma and hepatoma) from a patient carrying this specific mutation were screened for LOH at *PRKARIA* locus. The loss of the wild-type allele was only found in the most aggressive tumor, the pancreatic adenocarcinoma, suggesting that this event was not necessary for cell proliferation in the other tissues. The mutant R1A had necessarily a dominant-negative effect, responsible for R1A downregulation in the initial tumor stages (Groussin *et al.* 2002). Other mutations, leading to frameshift and creation of a new stop codon into the 3' untranslated open-reading frame (3'-UTR), escape NMD and are expected to generate a longer protein. However, the resulting elongated proteins are directly addressed to proteasome and precociously degraded (Patronas *et al.* 2012), leading to haploinsufficiency. Mouse models confirmed the role of *PRKARIA* alteration in adrenal tumorigenesis and excessive cortisol production. Indeed, mice lacking *PRKARIA* specifically in the adrenal cortex (AdKO) developed pituitary-independent Cushing's syndrome with increased PKA activity. The loss of R1A in adrenocortical cells led to autonomous steroidogenic genes expression, deregulated differentiation, increased proliferation and resistance to apoptosis, mimicking the phenotype observed in human PPNAD (Sahut-Barnola *et al.* 2010).

Genetic alterations of other actors of the cAMP/PKA pathway were further described in patients with PPNAD. They affect in particular the genes coding for phosphodiesterases *PDE11A* and *PDE8B*, which are involved in cAMP degradation. Genome-wide single-nucleotide polymorphism (SNP) array, focusing on cAMP-related genes, compared ten tetrads of samples, each tetrad containing leucocyte DNA from the proband and its 2 parents plus adrenal tumor DNA from the proband. This allowed identification of the 2q31–2q35 locus as the largest LOH region in the dataset. Located in this locus, *PDE11A* appeared as an obvious candidate gene because of its inclusion in identified allelic losses

Figure 1

The cAMP/PKA signaling pathway in the adrenal cortex. (A) The cAMP/PKA signaling pathway in normal adrenocortical cell. Adrenocorticotrophic hormone (ACTH) binds to its G protein-coupled receptor MC2R, resulting in Gs protein activation, which further activates adenylyl cyclase (AC), resulting in enhanced cAMP production. PKA is a hetero tetramer, composed of 2 regulatory subunits (R) and 2 catalytic subunits (C). The binding of 4 cAMP molecules to PKA regulatory subunits dimer allows the release and subsequent activation of the 2 catalytic subunits. The free catalytic subunits will then phosphorylate several targets, including the transcription factor CREB (cAMP response element-binding protein), responsible for the stimulation of cAMP-dependent genes transcription. Phosphodiesterases (PDE), involved in cAMP degradation, act as negative regulators of this pathway. (B) cAMP/PKA signaling pathway genetic alterations in PPNAD 1: *PRKAR1A* inactivation (germline mutation + somatic second-hit); 2: phosphodiesterases (*PDE11A* or *PD8B*) inactivation (germline mutation + tumoral LOH); 3: *PRKACA* duplication, *PRKACB* triplication (C) cAMP/PKA signaling pathway genetic alterations in PBMAH 1: Illegitimate G-protein-coupled receptors expression 2: Phosphodiesterases (*PDE11A* or *PD8B*) inactivation (germline mutation + tumoral LOH) 3: *PRKACA* duplication 4: Activating mutation of *GNAS1* (MacCune Albright syndrome) 5: Activating mutation of *MC2R* (D) cAMP/PKA signaling pathway genetic alterations in cortisol-producing adenomas 1: Activating mutations of *PRKACA* (hotspot L206R) 2: *PRKAR1A* inactivation 3: *GNAS1*-activating mutation, apart from McCune-Albright syndrome context.

and its known function and expression pattern. Three germline deleterious variants in *PDE11A* were indeed identified in 16 patients with isolated MiAH/PPNAD and no mutations of *PRKAR1A* nor other Carney complex tumors (Horvath *et al.* 2006). Furthermore, *PDE11A* could also act as a modifier gene in patients with CNC secondary to *PRKAR1A* mutation (Libé *et al.* 2011). *PDE11A* gene was entirely sequenced in 150 CNC patients carrying a germline mutation of *PRKAR1A*, allowing the identification of single-nucleotide variations (SNV) in the heterozygous state in 38 cases (25.3%): missense substitutions (in 31 patients) or premature stop codon and nonsense mRNA (in 7 patients). In comparison, *PDE11A* SNVs were only identified in 19 of 279 control subjects (6.8%) and consisted of 4 previously reported missense variants and no nonsense mutations. Some of the variants, identified in CNC patients, were shown to have decreased enzymatic activity *in vitro* (Horvath *et al.* 2006) and could thus participate to genetic predisposition to PPNAD. Interestingly, *PDE11A* variants were also significantly more frequent in CNC patients with PPNAD than in those without PPNAD.

After the *PDE11A* locus, *PDE8B* locus had the second highest linkage score in the previously described genome-wide association (WGA) study (Horvath *et al.* 2006, 2008). A single base substitution c.914A>C/p.His305Pro was identified in a young girl with PPNAD, who had presented Cushing's syndrome at the age of 2 years, decreased activity of the mutant *PDE8B* being confirmed *in vitro* in HEK293 cells (Horvath *et al.* 2008). The functional role of *PDE8B* in the adrenal cortex was confirmed in mouse models, *PDE8B*-knockout mice exhibiting elevated urinary corticosterone as a result of adrenal hypersensitivity toward ACTH (Tsai *et al.* 2011).

Notably, *PDE11A* and *PDE8B* alterations were not specific of PPNAD and were further described in other types of adrenocortical tumors. Thus, a heterozygous-inactivating mutation in *PDE11A* was found in a non-secreting adrenocortical adenoma (Libé *et al.* 2008), and heterozygous missense variants were reported in PBMAH (24%), in ACA (19%) and in ACC (16%), with a higher frequency than in control subjects (5.7%). *In vitro* study confirmed that 2 *PDE11A* variants, only found in PBMAH (and not in controls) had decreased enzymatic activity in comparison to wild-type *PDE11A* (Vezzosi *et al.* 2012). It is speculated that these *PDE11A* variants play a role in the genetic predisposition to develop PBMAH as a secondary modifying gene and not as a 'causal' gene on its own. Concerning *PDE8B*, the screening of 216 adrenocortical tumors negative for mutations in *PRKAR1A*, *PDE11A* and

GNAS, identified 6 different variants of *PDE8B* in a total 7 patients (1 ACC, 1 PPNAD, 2 PBMAH, 2 secreting ACA and 1 non-secreting ACA). The deleterious effect of the genetic alteration was confirmed for at least two of them (Rothenbuhler *et al.* 2012).

More recently, the catalytic subunits of the PKA enzyme were reported to be involved in MiAH. Indeed, a comparative genomic hybridization (CGH) study including 35 patients with bilateral adrenal hyperplasia and overt Cushing syndrome, with no *PRKAR1A*, *PDE11A* and *PDE8B* germline mutations, identified 5 patients (4 kindreds) with duplication of 19p region, including *PRKACA*. As expected, tumor samples of these patients exhibited higher expression of *PRKACA* at mRNA and protein level, resulting in higher basal and stimulated PKA activity. Their phenotype was quite heterogeneous, including both micro-nodular and macro-nodular forms and various degrees of Cushing syndrome severity (Beuschlein *et al.* 2014). Recently, a 1.6Mb triplication of 1p31.1 chromosome region, including *PRKACB* gene, was found in a genome-wide study in a young woman with Carney complex who did not carry either *PRKAR1A* or *PRKACA* mutation (Forlino *et al.* 2014). Her Carney complex manifestations associated acromegaly, pigmented spots and myxomas but no PPNAD.

Primary bilateral macronodular adrenal hyperplasia (PBMAH)

PBMAH is typically characterized by the presence of several bilateral adrenal macro-nodules (diameter superior to 10mm). More rarely, PBMAH can also be asymmetric and in some case apparently unilateral. Diagnosis is made in patients, between 40 and 65 years of age, presenting with Cushing syndrome and low plasma ACTH levels or often nowadays after the investigation of an adrenal incidentaloma. This pathology is slightly more prevalent in women. The severity of Cushing syndrome is extremely variable and can even be completely asymptomatic. PBMAH has long been referred to as 'ACTH-independent macronodular adrenal hyperplasia' (AIMAH), but the recent discovery of an intraadrenal ACTH synthesis responsible for local stimulation of cortisol production made the former appellation obsolete. This ectopic ACTH is secreted by adrenocortical cells, localized in the sub-capsular region and in hyperplastic nodules which express both pro-convertase 1 (PC1), the ACTH-precursor cleavage enzyme, and usual enzymatic resources of cells capable of steroidogenesis. The regulation of this ectopic ACTH is very different from that of pituitary ACTH (Louisset *et al.* 2013). The great originality in PBMAH resides in

the cortisol response to non-physiological stimuli, giving birth to the concept of illegitimate membrane receptors in adrenocortical cells. Various stimulating ligands, binding to protein G-coupled receptors were described, including glucose-dependent insulintropic peptide (GIP) responsible for food-dependent Cushing syndrome (Reznik *et al.* 1992), LH/HCG responsible for Cushing syndrome during pregnancy and after menopause (Lacroix *et al.* 1999), vasopressin, catecholamines, serotonin 5-HT, angiotensin II (ang II) and glucagon (Imöhl *et al.* 2002, Miyamura *et al.* 2002, Lee *et al.* 2005, Vezzosi *et al.* 2007, Gagliardi *et al.* 2009, Libé *et al.* 2010, Hofland *et al.* 2013). The prevalence of such illegitimate membrane receptors in PBMAH is high, varying from 77% to 87% among studies (Libé *et al.* 2010, Hofland *et al.* 2013). However, despite whole-genome approaches (Lampron *et al.* 2006), genetic alterations responsible for this ectopic expression have not been elucidated yet. Adrenal tumorigenesis and excessive cortisol secretion are again secondary to cAMP/PKA pathway activation in the context of PBMAH, most of these illegitimate G protein-coupled receptors stimulating adenylyl cyclase.

Genetic alterations of other actors of cAMP/PKA pathway were also reported in PBMAH. As described in the previous section, *PDE11A* variants were found with a prevalence of 24–28% in PBMAH patients (Libé *et al.* 2008, Vezzosi *et al.* 2012), some of them being associated with decreased enzymatic activity confirmed *in vitro* (Vezzosi *et al.* 2012). Hypersensitivity to ACTH, secondary to mutations of ACTH receptor *MC2R*, was also described in an isolated case of bilateral adrenal hyperplasia. In this particular case, the association of 2 mutations (responsible for loss of ligand binding and responsiveness) on the same allele of *MC2R* resulted in significant elevation in its constitutive activity and subsequent autonomous cortisol secretion (Swords *et al.* 2004). The last actor of cAMP/PKA pathway involved in PBMAH is the alpha-subunit of Gs protein (encoded by *GNAS1* gene) in the context of McCune-Albright syndrome (Fragoso *et al.* 2003). This syndrome associates polyostotic fibrous dysplasia, ‘café au lait’ spots, precocious puberty and hyperfunction of multiple endocrine glands. Somatic activating mutations of *GNAS1* occur early in embryogenesis, giving birth to a mosaic population of wild-type and mutant gene-bearing tissues, underlying the clinical manifestations. Again, these activating mutations result in constitutive activation of cAMP/PKA pathway and autonomous cortisol secretion (Weinstein *et al.* 1991).

Few rare cases of PBMAH are part of other genetic tumors predisposition syndrome including multiple

endocrine neoplasia type 1 (*MEN1*), familial adenomatous polyposis (*APC*) and hereditary leiomyomatosis (fumarate hydrogenase, *FH*) (Matyakhina *et al.* 2005, Hsiao *et al.* 2009, Gatta-Cherifi *et al.* 2012, Shuch *et al.* 2013). However, these particular alterations only explain a very small proportion of PBMAH cases and are by nature associated with other tumors.

Genome-wide approaches allowed considerable progress in understanding PBMAH pathogenesis with the recent identification of a new tumor suppressor gene involved in this disease. Blood and tumor DNA samples were collected from 33 patients with PBMAH, who had undergone adrenal surgery for Cushing's syndrome and/or very large adrenal (Assié *et al.* 2013). LOH was detected at 16p locus by SNP array in the tumors of 8/33 patients (24%). Whole-genome sequencing in 5 paired tumor and leucocyte DNA samples identified only one gene affected in more than one tumor sample, *ARMC5*, mapping to 16p11.2. Direct sequencing of tumor DNA further identified *ARMC5* mutations in a total of 18/33 patients. All tumor DNA samples of these 18 patients harbored 2 genetic alterations in *ARMC5* locus, whereas their leucocyte DNA samples carried only one of the 2 alterations identified in the tumor, confirming the role of *ARMC5* as a tumor suppressor gene in PBMAH. Interestingly, tumor DNA was sequenced in more than one tumor in a few patients. In each case, the germline *ARMC5* mutation was associated with a second nodule-specific, somatic *ARMC5* alteration (Assié *et al.* 2013). This extensive genetic variance in the second event leading to tumorigenesis was confirmed by the analysis of 20 adrenal nodules from one patient with PBMAH. In addition to what appeared to be the germline mutation p.Trp476*, sixteen of the 20 nodules harbored a second somatic *ARMC5* variant, each of them being unique and specific to one nodule. Allelic losses were identified in two of them (Correa *et al.* 2015).

The prevalence of *ARMC5* damaging germline mutations in PBMAH was further estimated from 21% to 26% in the following studies (Faucz *et al.* 2014, Espiard *et al.* 2015). The majority of index case patients carrying *ARMC5* deleterious mutations had clear adrenal Cushing syndrome. Cortisol hypersecretion in this subgroup was more severe in comparison to index case patients without *ARMC5* mutation or with *ARMC5* variant predicted to be benign. This difference had an anatomical substratum, adrenals in patients carrying *ARMC5* deleterious mutation were indeed larger and harbored a higher number of nodules (Espiard *et al.* 2015, Albiger *et al.* 2017). An *ARMC5*-damaging mutation or deletion was found to

segregate in several families of PBMAH (Suzuki *et al.* 2015, Bourdeau *et al.* 2016). Interestingly, adrenal hyperplasia was associated with meningioma in one family carrying (p. A110fs*9) *ARMC5* mutation (Elbelt *et al.* 2015). A second 'hit' on *ARMC5* gene was found in the meningioma DNA, suggesting an additional role of *ARMC5* in the predisposition to meningiomas. Expression of the four known *ARMC5* isoforms was recently investigated in 46 normal human tissues (Berthon *et al.* 2017a): only one isoform was ubiquitously expressed throughout the body, whereas only 7 tissues expressed the four isoforms, including adrenals and brain.

ARMC5 is a cytosolic protein with no enzymatic activity, containing 7 armadillo domains and one BTB domain toward its C-terminus, which allows its di- or trimerization (Hu *et al.* 2017). More than 240 proteins from yeast to human are known to contain armadillo domains, including the well-known beta-catenin. Armadillo-containing proteins are involved in many different functions, including T-cell, neural tube and adrenal cortex development as well as tumor suppression. First functional investigations showed that inactivation of *ARMC5* in H295R cells by siRNA led to a decrease in expression of steroidogenesis enzymes and in cortisol synthesis. Furthermore, *ARMC5* missense mutations or deletions were not able to induce apoptosis in H295R and HeLa cells in the contrary of wild-type *ARMC5* (Assié *et al.* 2013, Espiard *et al.* 2015). Despite the reduced capacity of each cell, excess in cortisol secretion in PBMAH could therefore result from the increased number of adrenocortical cells, underlying the insidious installation of Cushing syndrome (Assié *et al.* 2013). Recently, an *ARMC5*-KO mouse model was developed to investigate *ARMC5* function. *ARMC5*-KO was first associated with a high embryonic lethality and growth retardation. In old age (>15 months), *ARMC5*-KO mice presented enlarged adrenals (without any identifiable nodule) and increased glucocorticoids serum level (Berthon *et al.* 2017b, Hu *et al.* 2017). These animals were immunodeficient, with altered T-cell proliferation and differentiation into Th1 and Th17 cells and increased T-cell apoptosis. *ARMC5*-binding partners were further investigated by yeast-2-hybrid assay, and identified actors in pathways regulating both cell cycling (for example, CUL3) and apoptosis (for example, DAPK1) as well as *ARMC5* itself, suggesting multimerization (Hu *et al.* 2017).

Finally, other possible driver genes of PBMAH were reported in a small number of cases. Thus, whole exome sequencing (WES) in 7 patients with PBMAH allowed

the identification of *DOT1L* and *HDAC9* mutations in respectively 2 patients and 1 patient. *DOT1L* codes for a histone H3 lysine methyl-transferase and *HDAC9* for a histone deacetylase, both being involved in histone modifications, chromatin organization and therefore gene transcription regulation (Cao *et al.* 2014). A single study reported a single mutation in *Endothelin Receptor type A EDNRA* gene, but this has not been confirmed in any other sequencing studies of large series of patients with adrenal hyperplasia. *EDNRA* belongs to G protein-coupled proteins and was also reported to play a role in cardiovascular and polycystic kidney disease (Zhu *et al.* 2013). The frequency of the alterations of these genes has not been investigated in large series.

Unilateral cortisol-producing adenomas

As for germline genetic alterations identified in bilateral adrenal tumors, somatic alterations of actors of the cAMP/PKA pathway were found in sporadic cortisol-secreting adenomas.

The most prevalent defect alters the PKA catalytic alpha-subunit (*PRKACA*). WES (Beuschlein *et al.* 2014) allowed the identification of somatic *PRKACA* mutations in 8 of 10 patients with cortisol-producing adenomas, the same hotspot mutation (c.617A>C/p.Lys206Arg) being reported in seven patients. Twenty-two *PRKACA* mutations were further identified in a total of 59 ACA with overt Cushing syndrome, no mutations being found either in patients with subclinical Cushing syndrome or with other adrenal tumors. Clinical phenotype was more severe in patients whose tumor carried *PRKACA* mutation. The hotspot mutation (c.617A>C/p.Lys206Arg) was further confirmed in four following studies (Cao *et al.* 2014, Di Dalmazi *et al.* 2014, Goh *et al.* 2014, Sato *et al.* 2014) with a prevalence ranging from 28 to 50%. This mutation is located within the active site-cleft of the PKA catalytic subunit to which the inhibitory domain of the regulatory PKA subunit binds, mimicking a substrate for the catalytic subunit. Lysine substitution by an arginine in this position prevents *PRKACA* binding to PKA regulatory subunit 1 alpha, increasing basal PKA activity, further promoting PKA substrate phosphorylation and target genes expression (Beuschlein *et al.* 2014, Calebiro *et al.* 2014, Cao *et al.* 2014). Apart from this specific site, some rare other mutations, leading to activation of *PRKACA* have been reported, including c.595_596insCAC/p.Leu199_Cys200insTrp (Beuschlein *et al.* 2014), c.639 C>G+c.638_640insATTATCCTGAGG/p.Ser213Arg+p.

Leu212_Lys214insIle-Ile-Leu-Arg (Di Dalmazi *et al.* 2014), c.589A>G/p.Trp197Arg and c.731_745del/p.245_248.del (Ronchi *et al.* 2016), all found respectively in one patient and c.600_601insGTG/p.Cys200_Gly201insVal reported in three patients (Di Dalmazi *et al.* 2014). All these alterations occurred in a region of PRKACA affecting its interaction with regulatory subunit 1 alpha (Di Dalmazi *et al.* 2014, Ronchi *et al.* 2016).

Besides, somatic alterations of *PRKAR1A* were also identified in sporadic adrenocortical tumors. LOH in *PRKAR1A* locus 17q was found in 7 of 29 adenomas. Three different damaging mutations were further found in 3 other adenomas, all subject to nonsense mRNA decay and therefore undetectable on the protein level (Bertherat *et al.* 2003). Interestingly, the three adenomas carrying these mutations were responsible for an overt Cushing syndrome and appeared pigmented on pathological examination. Moreover, they shared additional clinical features with PPNAD, including cyclic hypercortisolism, paradoxical increase in cortisol secretion during dexamethasone administration and small adenoma size (Bertherat *et al.* 2003). Another case of *PRKAR1A* mutation was further described in a WES study, including a total of 39 cortisol-producing adenomas (Cao *et al.* 2014). The exact prevalence of *PRKAR1A* somatic alteration in sporadic cortisol-producing adenomas is yet difficult to estimate, given the small number of cases reported in the different series but is probably below 5%.

Finally, apart from McCune-Albright syndrome, somatic-activating mutations of *GNAS1*, the gene coding for Gs protein alpha-subunit, were also reported in few cases of ACA (Kobayashi *et al.* 2000, Libé & Bertherat 2005, Goh *et al.* 2014, Ronchi *et al.* 2016) with an exact prevalence also difficult to estimate, ranging from 4.5 to 11%.

Even if all these alterations affect cAMP/PKA pathway, they seem to activate different downstream signaling pathways. Thus, in a whole genome expression profile study, comparing normal adrenal tissue to 3 *GNAS*-mutant tumors and 3 *PRKAR1A*-mutant tumors, both types of tumors shared overexpression of MAPK and p53 signaling pathways in comparison to normal tissue but differed substantially on others. Indeed, extracellular matrix receptor interaction and focal adhesion pathways (involving *NFKB*, *NFKB1A* and *TNFRSF1A* genes) were overexpressed in *GNAS*-mutant tumors while genes related to Wnt signaling pathway (*CCND1*, *CTNNB1*, *LEF1*, *LRP5*, *WISP1* and *WNT3*) were higher in *PRKAR1A*-mutant tumors (Almeida *et al.* 2012).

Wnt/beta-catenin signaling pathway activation in benign cortisol-producing tumors

Familial adenomatous polyposis (FAP) is a model of activation of Wnt/beta-catenin in oncogenesis and is characterized by the development of tens to thousands of adenomas in the rectum and colon during the second decade of life, leading to colorectal cancer development generally one decade after the appearance of the polyps. FAP may present with some extraintestinal manifestations such as osteomas, dental abnormalities (unerupted teeth, congenital absence of one or more teeth, supernumerary teeth, dentigerous cysts and odontomas), congenital hypertrophy of the retinal pigment epithelium (CHRPE), desmoid tumors and extracolonic cancers (thyroid, liver, bile ducts and central nervous system). A less-aggressive variant of FAP, Gardner syndrome, is characterized by fewer colorectal adenomatous polyps, later age of adenoma appearance and lower cancer risk. Classic FAP is inherited in an autosomal dominant manner and results from a germline mutation in the adenomatous polyposis (*APC*) gene (Half *et al.* 2009), encoding APC protein, which belongs to beta-catenin degradation complex, therefore acting as a negative regulator of Wnt/beta-catenin signaling pathway. More than 50 cases of adrenocortical tumor (ACT) in FAP have been reported, many of them being asymptomatic and discovered at autopsy (Beuschlein *et al.* 2000, Berthon *et al.* 2012). In retrospective studies, occurrence of adrenocortical tumors was significantly increased in FAP patients (7–13%) (Marchesa *et al.* 1997) in comparison to non-FAP patients, although it should be kept in mind that these values might be overestimated because of increased imaging prescription in FAP. All ACT tumors in FAP patients harbored biallelic inactivation of *APC*, in favor of both germline and somatic mutations according to Knudson's two-hit model, leading to inappropriate cytoplasmic and/or nuclear accumulation of beta-catenin (Gaujoux *et al.* 2010).

Activation of the Wnt/beta-catenin pathway in sporadic adrenocortical tumors was from the initial study by our group in 2005 not only reported in ACC but also in ACA (Tissier *et al.* 2005). Later, in a study including 9 PPNADs (5 with macro nodules), 3 sporadic cortisol-secreting ACA with *PRKAR1A* somatic mutation and 1 heterogeneous tumor with an ACC developing within an ACA, beta-catenin accumulation was found in all tumor types. Furthermore, somatic mutations in *CTNNB1*, the gene coding for beta-catenin were identified in 2 of the 5 macronodular PPNAD, 1 ACA and in the malignant

part of the last heterogeneous tumor. These mutations seemed to be associated with a more aggressive phenotype (Gaujoux *et al.* 2008). This result was confirmed in another study, which reported 2 somatic *CTNNB1* mutations in 18 patients with PPnAD (Tadjine *et al.* 2008), these mutations were again associated with larger nodules. Beta-catenin accumulation might also be caused by cAMP/PKA pathway activation. GSK3- β , phosphorylated by PKA on Ser 9, is unable to participate in Axin-APC complex and to phosphorylate beta-catenin. Beta-catenin thus escapes degradation and accumulates in both nuclear and cytoplasmic compartments. Besides, beta-catenin by itself can be directly phosphorylated by PKA on Ser 675, inhibiting its degradation or promoting its binding to its transcriptional coactivator cAMP response element-binding protein (CREB) (Gaujoux *et al.* 2008, Berthon *et al.* 2012). Beta-catenin accumulation was also found in 10 of 26 ACA without specific somatic mutation of *PRKARIA*. Seven of them harbored a somatic mutation in *CTNNB1*. Most of these mutations affected Ser 45 in the exon 3 of *CTNNB1*, in the consensus GSK3- β phosphorylation site and led to subsequent activation of T-cell factor-dependent transcription in functional studies (Tissier *et al.* 2005). Interestingly, in a large study including 100 ACA, beta-catenin mutations were more frequent in non-secreting ACA (61%) than in subclinical cortisol-secreting ACA (22%) and cortisol-secreting ACA (16%). Mutated tumors were also significantly larger than non-mutated lesions (Bonnet *et al.* 2011).

Adrenocortical carcinomas (ACC)

ACC is a rare tumor with a poor prognosis, which can be diagnosed by the investigation of endocrine signs of steroid excess, compression symptoms due to tumor growth or simply on adrenal incidentaloma. Most tumors demonstrate steroid over secretion, often associating glucocorticoids and androgens. Despite an overall low 5-year survival rate (below 30% in most series), some tumors will not show recurrence after surgical removal and others will have a relatively slow metastatic progression. This heterogeneity long remained not understood. However, the recent discovery of genetic alterations underlying ACC tumorigenesis led to the identification of ACC molecular subgroups and molecular prognostic factors, underlying the clinical outcome heterogeneity. As in benign adrenocortical tumors, germline mutations responsible for familial syndromes predisposing to ACC, allowed the identification of signaling pathways involved in cancer development, somatic alterations of these

pathways being further screened in sporadic cases of ACC (Libè *et al.* 2007, Espiard & Bertherat 2015).

Signaling pathways involved in adrenal malignant carcinogenesis

p53 signaling pathway

Li-Fraumeni syndrome (LFS) is a rare autosomal dominant cancer predisposition syndrome, including soft tissue sarcoma and osteosarcoma, breast cancer, brain cancer, leukemia and ACC (Malkin *et al.* 1990, Bougeard *et al.* 2008, Mai *et al.* 2016). Clinical diagnostic criteria for the 'classic' LFS include an individual with sarcoma diagnosed before 45 years of age, with a first-degree relative with any cancer diagnosed before 45 years of age and another first-degree or second-degree relative with a sarcoma diagnosed at any age or any cancer diagnosed before 45 years of age. Less stringent criteria were further established to define Li-Fraumeni-like (LFL) syndrome, the proband's cancer type being extended to childhood cancers, namely brain cancers and ACC and the relatives' age at the time of diagnosis to <60 years. Germline mutations in *TP53*, the underlying molecular basis of LFS, were identified in 70% of 'classic LFS' families and 40% of LFL families. De novo mutations of *TP53* are not rare with a prevalence ranging from 7 to 20% among studies (Wasserman *et al.* 2012, Mai *et al.* 2016). In the National Cancer Institute's Li Fraumeni Study cohort, including 286 *TP53*⁺ individuals from 107 families, the cumulative cancer incidence was 50% by the age of 31 years among females and by the age of 46 years among males (Mai *et al.* 2016). One hundred and ninety-three patients (67%) of the cohort had a first cancer diagnosis. Interestingly, ACC was the first cancer in 5 cases, diagnosis being always made before the age of 17 years. Among these 193 patients, 50% developed a second cancer, which was an ACC in only one case (Mai *et al.* 2016). Prevalence of *TP53* germline mutations is very different between pediatric and adult ACC. The first pediatric series of ACC were biased by the geographic origin of included patients. Indeed, the incidence of pediatric ACC is remarkably high (10–15 times the world-wide occurrence) in Southern Brazil because of the segregation of the *TP53* hotspot mutation p.R337H. In this region, 90% of pediatric ACC cases harbor this mutation. A free screening of 171,649 newborns in the state of Parana identified the mutation with a prevalence of 0.27% (Faria & Almeida 2012, Custódio *et al.* 2013). A recent study including 88 unrelated children with ACC who were not specifically originating from Southern Brazil, reported 50% of *TP53* germline mutations, this high prevalence

declined with age (Wasserman *et al.* 2015). Indeed, *TP53* germline mutations are far less frequent in the context of adult ACC, ranging from 3.9 to 5.8% among studies (Espiard & Bertherat 2015). Furthermore, some *TP53* SNPs seem to be associated with the risk of ACC development (3 of 4 SNPs tested were significantly different between control subjects and patients with ACC) and the overall survival (patients harboring at least one SNP among the four tested had a poorer prognosis) (Heinze *et al.* 2014) (Fig. 2).

TP53 alterations were also identified in ACC at the somatic level. The protein p53 is known as the 'guardian of genome' and is involved in cellular response to stress. In normal conditions, p53 is degraded by an E3 ubiquitin ligase mdm2. Under stress conditions, the association between p53 and mdm2 is abolished, leading to an increase in intracellular p53. p53 then undergoes subsequent modifications allowing its activation as a transcriptional factor, leading to the transcription of many genes. This process can have two different results: cellular division stop, DNA repair or resolution of other abnormalities and cellular division recovery or apoptosis according to the severity of the alterations. Its gene is located at the 17p13 locus. LOH at this locus are very frequent (85%) in adult sporadic ACC (Gicquel *et al.* 2001). However, *TP53* mutations and intragenic LOH were only found in respectively, 33% and 44% of tumors with LOH at the 17p13 locus, suggesting that *TP53* might not be the only tumor suppressor gene at this locus (Libè *et al.* 2007).

TP53 mutations' prevalence in a large cohort of adults ACC ranged between 16 and 21% in two recent genome-wide studies (Assié *et al.* 2014, Zheng *et al.* 2016). *TP53*-mutant tumors often exhibit a strong nuclear immunoreactivity, which can be used as a diagnostic tool. Their phenotype is more severe than non-mutant tumors with larger tumors, more advanced stage of tumor progression and shorter disease-free survival (Libè *et al.* 2007).

Alterations of other actors of the p53 signaling pathway were also been reported. Overexpression of *PTTG1*, encoding a securin, a negative regulator of *TP53* was reported in 84% of ACC and appeared to be a marker of poor survival (Demeure *et al.* 2013). Loss of the retinoblastoma protein pRb in immunohistochemistry, secondary to deleterious mutation or allelic loss was found in 27% of ACC, all these tumors being aggressive (Ragazzon *et al.* 2014). Mutations of *RB1* were identified in 7% of ACC in two different large cohorts (Assié *et al.* 2014, Zheng *et al.* 2016). Additional inactivating mutations or homozygous deletions of *CDKN2A* were found in 11–16% of ACC, while high-level amplifications of *CDK4* and *MDM2* were both reported in 2–7% of cases, leading to an alteration of p53 signaling pathway in 33–45% of ACC (Assié *et al.* 2014, Zheng *et al.* 2016).

Wnt/beta-catenin signaling pathway

As previously described, beta-catenin activation is observed in ACA. However, as determined by

Figure 2

p53 signaling pathway. (A) p53 signaling pathway in physiology. In normal conditions, p53 is degraded by an E3 ubiquitin ligase mdm2. Under stress conditions, the association between p53 and mdm2 is abolished, leading to p53 activation as a transcriptional factor. This process can have two different results: cell cycle arrest or apoptosis. Cell cycle arrest involves p21 (encoded by *CDKN1A*) activation by p53, leading to *cdk4* inhibition. *Cdk4* usually inactivates Rb by phosphorylation, liberating E2F, acting as a transcription factor for target genes involved in cell cycle progression. *CDKN2A* codes for different proteins including ARF, which prevents p53 degradation by mdm2. (B) Genetic alterations of p53 signaling pathway in ACC. 1: Inactivating mutations of *TP53*; 2: Inactivating mutations of *CDKN2A*; 3: Inactivating mutations of *RB1*; 4: High-level amplification of *MDM2* and 5: High-level amplification of *CDK4*.

immunohistochemistry, it is more dramatic in ACC in which inappropriate cytoplasmic and/or nuclear accumulation of beta-catenin is very frequent with a reported prevalence between 39% and 84% (Tissier *et al.* 2005, Gaujoux *et al.* 2010). Somatic mutations in *APC* are rare with a reported prevalence of 2–3% (Assié *et al.* 2014, Zheng *et al.* 2016), suggesting the importance of somatic alteration of other actors of the Wnt/beta-catenin pathway in ACC. Indeed, activating somatic mutations of beta-catenin itself were reported with a prevalence of about 16% in large cohorts of ACC (Gaujoux *et al.* 2011, Assié *et al.* 2014, Zheng *et al.* 2016). Screening of others genes coding for proteins involved in the multiprotein complex leading to beta-catenin degradation (*WTX*, *AXIN2*, *AXIN1*) did not identify any deleterious mutations in these genes (Gaujoux *et al.* 2011, Guimier *et al.* 2013). Indeed, a 12pb-in frame deletion in exon 7 of *AXIN2* was reported in one of the 6 ACC (Chapman *et al.* 2011) and was associated with cytoplasmic/nuclear beta-catenin accumulation, but the tumor also harbored an activating mutation of beta-catenin. This deletion was further found in only one of 49 ACC (Guimier *et al.* 2013), and in the cell lines H295 and H295R. It was always associated with an activating

mutation of beta-catenin, and no correlation was found between *AXIN2* expression level and nuclear beta-catenin staining, making a functional consequence of this deletion unlikely. Transcriptome studies have shown that Wnt/beta-catenin target genes, including *BIRC5*, *ENC1*, *PTTG1* and *TWIST1*, were overexpressed in ACC. Moreover, beta-catenin alterations segregate in a subgroup of aggressive ACC, also identified by transcriptome-based classification. Positive transcriptional target genes such as *CLDN1*, *AXIN2* and *LGR5* were also overexpressed in this aggressive subgroup (Assie *et al.* 2012). Taken together, these results suggest that Wnt/beta-catenin activation is a driver of molecular alteration in a subgroup of ACC. Interestingly, p53 and beta-catenin mutations in the aggressive subgroup are almost mutually exclusive (Ragazzon *et al.* 2010, Assie *et al.* 2012, Assié *et al.* 2014, Zheng *et al.* 2016). The role of the Wnt/beta-catenin pathway in adrenal tumorigenesis is supported by mouse models. Constitutive activation of beta-catenin in the adrenal cortex of transgenic mice resulted in progressive steroidogenic and undifferentiated spindle-shaped cells hyperplasia, which could lead to macroscopic adenomas development. Malignant characteristics such

Figure 3

Wnt/β-catenin signaling pathway (A) Regulation of Wnt/β-catenin signaling pathway (*in the absence of Wingless Wnt ligand*). 1: ZNRF3 acts as a negative regulator of Wnt/β-catenin signaling pathway. It prevents complex formation between Wnt receptor Frizzled and its coreceptor low-density lipoprotein LRP5/6, leading to their internalization and further degradation. 2: In the absence of Wnt ligand, β-catenin is segregated in the cytoplasm, phosphorylated by GSK3β, which belongs to the degradation complex of scaffolding proteins, including also APC, AXIN and CK1, and is finally targeted by an E3-ubiquitin ligase before proteosomal degradation. (B) Physiological activation of Wnt/β-catenin signaling pathway (*in the presence of Wnt ligand*). 1: Wnt ligand binding to its Frizzled-LRP5/6 receptors dimer, leads to dishevelled (DVL) protein recruitment and disruption of the degradation complex. 2: In the absence of functional degradation complex, the active form of β-catenin accumulates in the cytoplasm and translocates to the nucleus where it can play its role of transcriptional regulator, activating particularly the transcription of LEF/TCF transcription factor, which will further stimulate the transcription of target genes. (C) Pathological activation of Wnt/β-catenin signaling pathway in adrenocortical carcinoma (ACC) Wnt/β-catenin signaling pathway can be altered at different levels in ACC with: 1: Inactivating mutation of *ZNRF3* in 19–21% of cases; 2: Activating mutation of *CTNNB1* in 16% of cases; 3: Inactivating mutation of *APC* in 2–3% of cases.

Figure 4

11p15 locus. (A) IGF-II and the 11p15 locus in physiology. The 11p15 locus includes 2 imprinted regions ICR1 and ICR2, inversely methylated on maternal and paternal allele. On the maternal allele, the absence of methylation on ICR1 allows CTF binding on a sequence located between *IGF-II* and *H19* genes, and further acting like an insulator. Thus, the enhancer (E) is only able to activate the transcription of *H19* and not that of *IGF-II*. On the contrary, on the paternal allele, the methylation of this specific sequence prevents CTF binding, allowing the enhancer (E) to activate *IGF-II* transcription while repressing that of *H19*. Methylation pattern is the opposite on ICR1, with methylation of the maternal allele and no methylation on the paternal one. This results in opposite expression of *CDKN1C* and *KCNQ1* (only expressed from the maternal allele) and *KCNQ1QT* (only expressed from the paternal allele). *Expressed genes are represented in red and silent genes in green.* (B) 11p15 locus in Beckwith-Wiedemann syndrome (BWS): 1: Loss of maternal methylation on ICR2. 2: Gain of methylation on maternal ICR1. 3: Paternal isodisomy. *Expressed genes are represented in red and silent genes in green.* (C) 11p15 locus in adrenocortical carcinoma (ACC): 1: High-IGF-II ACC: Paternal isodisomy. 2: Low-IGF-II ACC: Paternal isodisomy and possible loss of methylation on ICR1. *Expressed genes are represented in red and silent genes in green.*

as uncontrolled neovascularization and loco-regional metastatic invasion appeared only lately in this context, suggesting the necessity of other associated genetic alterations (Berthon *et al.* 2010, Heaton *et al.* 2012) (Fig. 3).

More recently, a new actor of the Wnt/beta-catenin pathway involved in adrenal tumorigenesis was identified. *ZNRF3* encodes a cell-surface transmembrane E3 ubiquitin ligase and acts as a negative feedback regulator of Wnt signaling. *ZNRF3* is associated with the Wnt receptor complex and inhibits Wnt signaling

pathway by promoting the turnover of frizzled and LRP6. It is regulated by R-spondin protein that promotes the association of *ZNRF3* with leucine-rich repeat-containing G protein-coupled receptors LGR4, resulting in membrane clearance of *ZNRF3* and activation of Wnt/beta-catenin pathway. Recently, *ZNRF3* was found to be the most frequently altered gene in 2 large cohorts of ACC investigated by integrated genomics, with a prevalence of respectively 21% (Assié *et al.* 2014) and 19% (Zheng *et al.* 2016). These alterations consisted of biallelic

homozygous deletions or heterozygous mutations, most often associated with LOH. Transcriptome of *ZNRF3*-mutant tumors also showed activation of beta-catenin target genes but to a lesser extent than *CTNNB1*-mutant tumors, both alterations being mutually exclusive (Assié *et al.* 2014). Downregulation of *ZNRF3* has been described in other types of cancer, including colorectal carcinoma (Bond *et al.* 2016) and papillary thyroid carcinoma (Qiu *et al.* 2016).

Insulin-growth-factor II (IGF-II) locus

Beckwith–Wiedemann syndrome (BWS) is an overgrowth and tumor predisposition syndrome and is clinically heterogeneous. It is characterized by visceromegaly including macroglossia and hemi-hyperplasia, malformations (wall defect, umbilical hernia, ear abnormalities) and predisposition to embryonal malignancies. ACC belongs to BWS tumor spectrum, which also includes Wilms tumor, hepatoblastoma, rhabdomyosarcoma and neuroblastoma with an overall risk for tumor development in children estimated at 7.5%, most of the tumors occurring in the first 8–10 years of life. Dysregulations at the imprinted 11p15.5 locus, including both genetic and epigenetic alterations, are responsible for the disease. This locus includes 3 main genes: *H19*, *IGF-II* and *CDKN1C*. *H19* codes for a long 2.3kb-noncoding RNA, which has been associated with several cancers and is physiologically only expressed by the maternal allele while *IGF-II* is only expressed by the paternal allele. Expression of both the genes is regulated by the imprinting center IC1, which is methylated on the paternal allele and unmethylated on the maternal one. *CDKN1C*, a growth suppression gene, is under the control of another imprinting center IC2. BWS is etiologically heterogeneous arising from dysregulation of one or both imprinting centers and/or imprinted growth regulatory genes on chromosome 11p15.5. Most BWS cases are sporadic and result from loss of maternal methylation at IC2, gain of maternal methylation at IC1 or paternal uniparental disomy; some cases of copy number variations in the 11p15.5 chromosome region were also described. Hereditary forms of BWS were attributed to mutations in *CDKN1C*. These different alterations have been described in Libè *et al.* (2007). Regardless of the causative molecular defect, these alterations all result in *IGF-II* overexpression (biallelic instead of monoallelic expression) and decrease in *H19* expression and/or decrease in *CDKN1C* expression. Gain of maternal methylation at IC1 or paternal uniparental disomy are associated with a higher risk of

tumor development than the other forms suggesting a role of *IGF-II* overexpression or *H19* downregulation in the tumorigenic process (Fig. 4) (Weksberg *et al.* 2010, Baskin *et al.* 2014).

At the somatic level, *IGF-II* overexpression is one of the first molecular abnormalities which has been described in sporadic adult ACC, with a very high prevalence of about 90%. This increase in expression was associated with DNA demethylation at *IGF-II* locus, paternal isodisomy in most of cases (Gicquel *et al.* 1997). Further transcriptome studies confirmed that *IGF-II* is the most upregulated gene in ACC. *IGF-II* overexpression is observed in more than 85% of ACC. Because of such a high prevalence, *IGF-II* overexpression could not be responsible for the ACC classification derived from transcriptome data by unsupervised clustering, IGF-II-high tumors clustering with IGII-low tumors (De Reyniès *et al.* 2009). No significant differences in clinical, biological and transcriptomic traits were found between IGF-II-high and IGF-II-low tumors (Guillaud-Bataille *et al.* 2014). *IGF-II* was also overexpressed in pediatric adrenocortical tumors, both ACC and adenomas, and the expression of IGF1-receptor (*IGF1-R*), which mediates IGF-II effects *in vivo*, was more discriminant between malignant (overexpression) and benign tumors in children (Almeida *et al.* 2008). IGF1-R kinase inhibitor has therefore been developed and were shown to have antitumor effects in both adult and pediatric adrenocortical tumor cell lines (Almeida *et al.* 2008). However, phase I trials evaluating the feasibility of IGF1-R (Figitumumab and OSI-906) and insulin receptor (OSI-906) inhibition were quite disappointing, only one of 25 patients showing a partial response by RECIST criteria (Haluska *et al.* 2010, Drelon *et al.* 2013). This result is concordant with mouse models in which *IGF-II* overexpression is not sufficient to promote tumorigenesis, even in association with beta-catenin activation (Heaton *et al.* 2012, Drelon *et al.* 2013). Overexpression of *IGF-II* might therefore not be a driver force of adrenal tumorigenesis but maybe simply the reflection of other genetic alterations at the 11p15.5 locus. Dysregulation of noncoding RNA *H19* was described in various types of cancers, including Bcr-Abl-induced leukemia (Guo *et al.* 2014). *H19* promoter was shown to be highly methylated in ACC, leading to a decrease in *H19* expression, suggesting a role of *H19* in adrenal tumorigenesis (Gao *et al.* 2002).

Other genetic alterations in ACC

The MEN1 (multiple endocrine neoplasia) is an autosomal dominant tumor predisposition syndrome, affecting

multiple endocrine and non-endocrine tissues. The three main endocrine lesions are primary hyperparathyroidism (95%), entero-pancreatic neuroendocrine tumors (50%) and pituitary adenomas (40%). Tumors are caused by a germline heterozygous mutation ('first hit') of the *MEN1* gene, located at the 11q13 locus, followed by a somatic 'second-hit', leading to a complete loss of menin protein function (Agarwal 2013). Adrenal hyperplasia was reported in 20% of patients with MEN1, among which 10% had an authentic adrenal tumor. Tumors were associated with hormonal hypersecretion in only 15% of cases, ACC accounted for 14% of tumors (Gatta-Cherifi *et al.* 2012). Somatic mutations of *MEN1* in sporadic ACC were recently identified with a prevalence of 7% in two large cohorts by exome analysis (Assié *et al.* 2014, Zheng *et al.* 2016). Menin function is not well characterized, recent studies suggest interaction with transcription regulating factors like JunD or Smad 3.

Lynch syndrome, or hereditary nonpolyposis colorectal cancer (HNPCC) syndrome, is another autosomal dominant tumor predisposition syndrome, secondary to germline heterozygous mutation of DNA-mismatch repair genes (*MSH2*, *MSH6*, *MLH1* and *PMS2*). Tumors are usually characterized by the loss of expression of one of these genes, caused by somatic 'second-hit' and microsatellite instability phenotype (Karamurzin *et al.* 2012). Apart from colorectal cancer, incidence of endometrium, ovaries and urinary tracts cancer is significantly increased in Lynch syndrome. The prevalence of Lynch syndrome in a large cohort of 114 ACC was found to be around 3% (Raymond *et al.* 2013), but no somatic mutations of MMR genes have been reported yet, contribution of this molecular alteration to adrenal tumorigenesis remaining unclear.

A very small number of ACC cases were also reported in the context of Carney complex (Anselmo *et al.* 2012, Morin *et al.* 2012). The first case was associated with a novel *PRKARIA* mutation c.95_96delAA/p.Lys32Argfs*12, subject to nonsense-mediated mRNA decay, and resulting in haploinsufficiency at the protein level. The second case was associated with c.439A>G/p.S147G *PRKARIA* mutation, which belongs to the small group of expressed mutant *PRKARIA*, not associated with LOH and thought to exert a negative dominant effect. The very low frequency of malignant adrenal tumors in Carney complex might however suggest the coexistence of additional germline factors predisposing to malignancies (Bertherat 2012). Interestingly, 7 heterozygous inactivating mutations and 3 homozygous deletions of *PRKARIA* were identified at the somatic level in a large cohort of sporadic ACC, resulting in increased MEK and BRAF proteins expression (Zheng *et al.* 2016).

At the somatic level, alterations of another pathway of 'DNA preservation', telomere maintenance, were also recently identified. Thus, 73% of tumors harbored shorter telomeres in comparison to their matched control samples in a large cohort of ACC. (Zheng *et al.* 2016) *TERT* (encoding for a telomerase) amplification was reported with a prevalence of about 15% (Juhlin *et al.* 2015, Zheng *et al.* 2016) while mutations in *DAXX* and *ATRX*, two genes associated with the alternative lengthening of telomeres (ALT) phenotype, were found with a global prevalence of about 10% (Assié *et al.* 2014, Zheng *et al.* 2016).

Genome-wide approaches and identification of prognosis factors

Transcriptome

Unsupervised transcriptome-based tumor classification (De Reyniès *et al.* 2009, Giordano *et al.* 2009) unraveled two distinct groups of ACC, reflecting tumor proliferation (measured by mitotic counts and cell cycle genes): one group of aggressive proliferating tumors (C1A), associated with a poor prognosis and one group of less aggressive tumors (C1B), associated with a better outcome. Multivariate analysis including tumor stage and mitotic rate as variables showed that transcriptome data contained independent prognosis information. One of these studies (De Reyniès *et al.* 2009) also used the transcriptome data to define a two genes predictor of malignancy, using disease-free survival as end point and for malignant tumors, a two genes predictor of survival, using overall survival as end point. The combined expression of *DLG7* and *PINK1* was the best predictor of disease-free survival, overcoming the uncertainties of intermediary Weiss score, while the combined expression of *BUB1B* and *PINK1* was the best predictor of overall survival in malignant tumors. Unsupervised clustering (Ragazzon *et al.* 2010) further identified 3 different subgroups in the group of ACC of poor prognosis: one was enriched in *TP53* mutations (C1A/p53), the second in beta-catenin cytoplasmic and nuclear accumulation (C1A/beta-catenin) and/or mutations and the third presented neither of these two alterations (C1A/x). The delineation of these 3 subgroups relied on different tumorigenesis processes but they were equally associated with a poor prognosis (Fig. 5).

Chromosome alterations

Chromosome alterations are very frequent in tumor cells, especially in malignancies. In ACC, they were confirmed by different approaches, including conventional CGH

Figure 5

Integrated genomic characterization of ACC. According to transcriptome, ACC can be clustered in 2 main groups C1A and C1B, predicting further prognosis of the tumor. In C1A group, associated with poor prognosis, alterations of Wnt/ β -catenin and p53 signaling pathway were found, being mostly mutually exclusive; a third subgroup of C1A tumors did not harbor any of these two alterations. In C1B group, associated with a better prognosis, alterations of Wnt/ β -catenin signaling pathway could be found in a small subgroup of tumors and no p53 signaling pathway alterations were further identified. No difference of IGF-II expression level was found between C1A and C1B tumors, IGF-II-high tumors accounting for more than 85% of the tumors in the 2 groups. Interestingly, ACC clustering based on methylation pattern and miRNAs expression seemed to be linked with transcriptome-based clustering. Thus, CIMP-High phenotype was only found in the more aggressive C1A group, CIMP-Low and Non-CIMP phenotype being found in both C1A and C1B tumors but with a large majority of Non-CIMP tumors in C1B group. Concerning miRNAs-based classification, Mi3 tumors were only found in C1A group as well as Mi1 tumors were only found in C1B group; in the contrary, Mi2 tumors could be found in both C1A and C1B groups (Assié *et al.* 2014, Zheng *et al.* 2016).

(Kjellman *et al.* 1996, Zhao *et al.* 1999), CGH array (Stephan *et al.* 2008, Szabó *et al.* 2010, Barreau *et al.* 2012) and more recently SNP array (Ronchi *et al.* 2013, Assié *et al.* 2014). Copy number variations (CNVs) are different and are far more frequent in ACC than in benign adrenal tumors (Kjellman *et al.* 1996, Zhao *et al.* 1999, Barreau *et al.* 2012, Ronchi *et al.* 2013). The most constant alterations in ACC reported in these different studies include gains in chromosomes 5, 7, 12, 16, 19 and 20 and deletions in chromosome 22. Copy-neutral LOH was reported with a prevalence of more than 90% in ACC (Ronchi *et al.* 2013). SNP array analysis in a large European cohort (ENSAT: European network for the Study of Adrenal Tumors) (Assié *et al.* 2014) unraveled several recurrent gene amplifications or deletions in ACC, including high-level amplifications of *TERT* and *CDK4* and homozygous deletions of *CDKN2A*, *RB1*, *ZNRF3*, 3q13.1 and 4q34.3 loci. Both loci include a long noncoding RNA, respectively, LOC285194 and LINC00290, whose implication in cancer is increasingly being appreciated. Concerning prognosis marker identification, a precise genetic pattern (i.e. large gains at chr 5, 7, 12 and 19 and losses at chr 1, 2, 13, 17, and 22) was associated with a better prognosis (Ronchi *et al.* 2013). An international large cohort of ACC, investigated by the TCGA program, further analyzed chromosomal alterations in ACC. By a detailed bioinformatics analysis, the TCGA study revealed a subgroup of ACC with numerous chromosomal alterations (the 'noisy' phenotype) and presenting a poor prognosis. This analysis also revealed a quite unique

feature of ACC by comparison with other solid cancers investigated by the same program with a sequential development going from hypodiploidy to polyploidy via whole genome doubling in a subset of ACC (Zheng *et al.* 2016).

Methylome

DNA methylation is the most investigated epigenetic modification, regulating gene expression and stable gene silencing. DNA methyltransferases allow covalent addition of a methyl group on cytosine within CpG dinucleotides, which are clustered in specific promoter regions, called 'CpG islands'. Alterations in methylation pattern have been associated with cancer development. Inactivation of certain tumor suppressor genes was reported to occur as a consequence of promoter hypermethylation and genomic instability as a consequence of global hypomethylation, both being responsible for cell transformation (Kulis & Esteller 2010). The first genome-wide study of adrenal tumor DNA methylation compared methylation levels of 27,578 CpG sites in adrenocortical normal tissue samples, adenomas and carcinomas and identified a total of 212 CpG islands significantly hypermethylated in ACC, responsible for a decrease in expression of genes involved in cell cycle and apoptosis regulation (*CDKN2A*, *GATA4*, *DLEC1*, *HDAC10*, *PYCARD*, *SCGB3A1/HIN1*) (Fonseca *et al.* 2012). Another study reported a global hypomethylation pattern in ACC associated with hypermethylation of specific promoter regions responsible for downregulation

of the expression of a certain number of genes (Rechache *et al.* 2012). A third study reported a negative correlation between methylation and expression for a total of 1741 genes of 12,250 studied, among the top genes were found *H19* and other tumor suppressor genes. This last study further used unsupervised clustering of DNA methylation profiles, unraveling two groups of carcinomas, one with an elevated methylation level, in favor of a CpG island methylator phenotype (CIMP) and the other with a methylation level only slightly increased in comparison to adenomas (non-CIMP). CIMP group could be further divided into 2 subgroups with two different methylation levels (CIMP high and CIMP low). Hypermethylation was associated with a poor survival (Barreau *et al.* 2013). This was further confirmed in a recent study, which showed that tumor methylation status was a significant prognostic factor of disease-free survival (DFS) and overall survival (OS) (Jouinot *et al.* 2016). Jouinot and coworkers identified a strong methylation marker, taking into account mean methylation of 4 genes (*PAX5*, *GSTP1*, *PYCARD* and *PAX6*). More interestingly, they showed that this biomarker remained a significant prognostic factor for DFS and OS in multivariate analysis including ENSAT stage and Ki67. This biomarker seems particularly attractive, most of the previous reported molecular markers being only investigated in univariate analysis of survival (Jouinot *et al.* 2016). Integrative approaches confronting transcriptome and methylome data found that CIMP-high phenotype was only found in C1A/x and C1A/p53 tumors while non-CIMP phenotype was observed in C1A/beta-catenin and C1B tumors (Barreau *et al.* 2013, Assié *et al.* 2014). For a review of methylation dysregulation in ACC, see Jonker *et al.* (2017).

miRome

MicroRNAs (miRNAs) are small noncoding RNAs of approximately 22 nucleotides, regulating gene expression at the posttranscriptional level by targeting mRNAs for cleavage or translation repression. They are involved in the pathogenesis of several neoplasms, their deregulation being responsible for both oncogene activation and tumor suppressor genes silencing. Their profile of expression can serve as diagnostic and/or prognosis markers, their possible detection in blood samples making them easily accessible. They also open the way for new therapeutic perspectives (Lujambio & Lowe 2012). Deregulation of several miRNAs were described in ACC, the most frequently reported being downregulation of miR-335 and miR-195 and upregulation of miR-483-5p (Soon *et al.* 2009, Özata *et al.* 2011, Patterson *et al.* 2011, Schmitz *et al.*

2011, Chabre *et al.* 2013, Patel *et al.* 2013). Some of these alterations, including downregulation of miR-195 and upregulation of miR-483-5p and also of miR-503, miR-1202 and miR-1275 were associated with a poor DFS (Soon *et al.* 2009, Özata *et al.* 2011). Upregulation of miR-483-3p was also reported in ACC leading to a decrease in protein expression of PUMA (p53-upregulated modulator of apoptosis), a proapoptotic factor (Özata *et al.* 2011). Other miRNAs were reported to be deregulated in childhood ACC, including miR-99a and miR-100. Functional analysis in ACT cell lines showed that they coordinately regulate the expression of insulin-like growth factor-mTOR-raptor signaling pathway through binding in their 3'-UTR. Everolimus, a pharmacological agent inhibiting mTOR pathway, was reported to reduce cell growth *in vitro* and *in vivo* (Doghman *et al.* 2010). Interestingly, some circulating serum miRNAs are detectable and can be used as diagnosis or prognosis biomarkers. Thus, detectable miR-483-5p in serum was specific of ACC and high levels of circulating miR-483-5-p associated with low levels of circulating miR-195 were associated with poor prognosis (Chabre *et al.* 2013). Hierarchical clustering analysis of miRNAs expression profile identified 3 stable tumor clusters associated with different prognosis (Özata *et al.* 2011, Assié *et al.* 2014). Cluster Mi1 tumors showed the largest differences of miR expression with normal adrenal tissue. Cluster Mi3 tumors were associated with a worse prognosis. In an integrative point of view, C1A tumors included almost all Mi3 tumors, whereas C1B tumors belonged to Mi1 or Mi2 clusters (Assié *et al.* 2014).

Conclusion

Development of genome-wide approaches allowed considerable progress in adrenocortical tumorigenesis understanding, identifying alterations in different signaling pathways. This opens several perspectives including the development of diagnosis and prognosis biomarkers and the identification of new therapeutic targets. These advances are particularly important in the context of ACC whose prognosis remained very poor.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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