ENDOCRINE TUMOURS

The genomics of adrenocortical tumors

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Abstract

The last decade witnessed the emergence of genomics, a set of high-throughput molecular measurements in biological samples. These pan-genomic and agnostic approaches have revolutionized the molecular biology and genetics of malignant and benign tumors. These techniques have been applied successfully to adrenocortical tumors. Exome sequencing identified new major drivers in all tumor types, including *KCNJ5*, *ATP1A1*, *ATP2B3* and *CACNA1D* mutations in aldosterone-producing adenomas (APA), *PRKACA* mutations in cortisol-producing adenomas (CPA), *ARMC5* mutations in primary bilateral macronodular adrenocortical hyperplasia (PBMAH) and *ZNRF3* mutations in adrenocortical carcinomas (ACC). Moreover, the various genomic approaches – including exome sequencing, transcriptome, miRNome, genome and methylome – converge into a single molecular classification of adrenocortical tumors. Especially for ACC, two main molecular groups have emerged, showing major differences in outcomes. These ACC groups differ by their gene expression profiles, but also by recurrent mutations and specific DNA hypermethylation patterns in the subgroup of poor outcome. The clinical impact of these findings is just starting. The main altered signaling pathways now become therapeutic targets. The molecular groups of diseases individualize robust subtypes within diseases such as APA, CPA, PBMAH and ACC. A revised nosology of adrenocortical tumors should impact the clinical research. Obvious consequences also include genetic counseling for the new genetic diseases such as *ARMC5* mutations in PBMAH, and a better prognostication of ACC based on targeted measurements of a few discriminant molecular alterations. Identifying the main molecular groups of adrenocortical tumors by extensively gathering the molecular variations is a significant step forward towards precision medicine.

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Invited Authors' profile

Guillaume Assie is a Professor in the Department of Endocrinology, Cochin Hospital, Assistance Publique Hôpitaux de Paris, Paris Descartes University. He specialized in General Endocrinology, including adrenal and pituitary diseases, especially Cushing syndromes. His research activities are mainly centered on bioinformatics, working at Institut Cochin, INSERM, CNRS, leading a team of four people analyzing the genomics of adrenocortical tumors.

Simon Faillot is currently a PhD student in computational biology, studying the genomics of adrenocortical tumors in Institut Cochin, Paris.

Introduction

Adrenocortical tumors gather a large panel of diseases, mainly differing by their pathology – e.g. benign or malignant, focal tumor or diffuse hyperplasia – and by their secretion types and levels. Adrenocortical adenomas (ACA) are common and differ mainly by their secretion profiles. Indeed, the aldosterone-producing adenomas (APA) are responsible for primary aldosteronism, the cortisol-producing adenomas (CPA) are responsible for Cushing syndrome, while a majority of ACAs are non-secreting adenomas. In-between are the subclinical Cushing adenomas responsible for subtle hormonal alterations but no overt clinical Cushing syndrome (1). The adrenocortical hyperplasia includes several pathological forms. The primary bilateral macronodular adrenocortical hyperplasia (PBMAH) is probably the most common, potentially responsible for Cushing syndrome. The primary pigmented nodular adrenal dysplasia (PPNAD) is much rarer, but quite well individualized in terms of pathology, and responsible for overt Cushing. Other forms exist, with more intermediate features (2). Finally adrenocortical carcinomas (ACC) are rare but overall aggressive tumors. However, the behavior of ACC differs between patients (3). Indeed a few carcinomas are of limited aggressiveness and can be cured by surgery, while others will not be cured by surgery, despite complete resection, even when localized to the adrenal gland.

A general hypothesis is that the variable presentations of adrenocortical tumors are related to tumor variability, at the molecular level. To test this hypothesis, new generations of tools have been recently used, able to catch extensively molecular variability. These tools, also called ‘genomics,’ were mostly developed during the last decade. Genomics is a set of ever improving high-throughput measurement techniques, measuring variability at different levels: at the gene expression level (transcriptome), at the miRNA expression level (miR-Nome), at the DNA methylation level (methylome), at the chromosomal structure level (copy-number alterations, loss of heterozygosity caught by SNP and CGH arrays) and at the DNA sequence level (mutations). All these genomic approaches have been recently applied to adrenocortical tumors, providing an agnostic molecular classification, leading to better classification and better understanding of pathophysiology.

The aim of this review is to show how genomics changed our knowledge of adrenocortical tumors and to present the clinical perspectives of this new knowledge.

Methods

The published literature on the genomics of adrenocortical tumor was screened using the following search terms on PubMed between 2009 and October 2015: ‘(adrenal OR adrenocortical) AND (hyperplasia OR adenoma OR dysplasia OR cancer OR carcinoma).’ Review articles were also considered, and their reference lists were screened for additional relevant publications.

Exome sequencing of adrenocortical tumors: it's raining genes!

Before the genomic era, after two decades of candidate gene approaches, half a dozen of driver genes were identified for different adrenocortical tumor types. These drivers include mainly *TP53* inactivating mutations in ACC, *CTNNB1* activating mutations in ACA and ACC and mutations activating the cAMP/protein kinase A (PKA) pathway in ACA and adrenocortical dysplasia/hyperplasia – including *PRKARIA* inactivating mutations, *PDE8B* and *PDE11A* inactivating mutations, *GNAS* activating mutations (4) – (Table 1, Fig. 1B). Since the recent rise of next generation sequencing and in particular exome sequencing, it took only 4 years to identify a new dozen of driver genes in these tumors (Table 1).

APA: mutations activating the calcium signaling pathway

Primary aldosteronism is either related to an APA, or to a bilateral disease referred to as adrenal hyperplasia.

The first exome sequencing reported for an adrenal tumor was in APA, identifying recurrent mutations of *KCNJ5* in 2011 (5). Mutations in this gene, encoding a potassium channel GIRK4, have been subsequently confirmed in larger series, occurring in about 26–40% of APA, with higher prevalence in Japanese population (6). Subsequently, other mutations were found in APA, implying active ion transporters regulating the plasma membrane potential (*ATP2B3* (7) and *ATP1A1* (7, 8)), and the voltage-dependent calcium channels *CACNA1D* (8, 9) and *CACNA1H* (10).

All these mutations share a common consequence of increasing intra-cellular calcium. Intra-cellular calcium is the main second messenger regulating aldosterone production in physiology. Indeed, both the angiotensin and the raise of extracellular potassium induce calcium signaling pathways in glomerulosa cells, through the activation of type 2 angiotensin receptor – with

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Table 1 Mutations in adrenocortical tumors.

Gene symbol	Reference	Gene function/type of alteration	Specific phenotype	Compartment (% mutations)
APA				
<i>KCNJ5</i>	(5)	Potassium channel coupled with G protein. A hotspot mutation induces an abnormal sodium flux inward	More common in women	Somatic (30–50%) (germline)
<i>ATP1A1</i>	(8)	Encodes Na ⁺ /K ⁺ ATPase α 1 subunit. Inactivating mutations induce a plasma membrane depolarization		Somatic (< 10%)
<i>ATP2B3</i>	(7, 8)	Ca ²⁺ ATPase, calcium transporter. Mutations decrease calcium efflux from cytosol		Somatic (< 10%)
<i>CACNA1D</i>	(8, 9)	Encodes Cav1.3, alpha subunit of voltage-dependent calcium channel. Mutations increase calcium entrance into glomerulosa cells		Somatic (< 10%) germline
<i>CACNA1H</i>	(10)	Encodes Cav3.2, alpha subunit of voltage-dependent calcium channel. Mutations may increase calcium entrance into glomerulosa cells		Germline
<i>CYP11B1/CYP11B2</i>	(75)	Gene fusion of two steroidogenic enzymes, implicated in cortisol and aldosterone synthesis	Glucocorticoid-remediable aldosteronism	Germline
CPA, SCA and NSA				
<i>PRKACA</i>	(15, 16, 17, 18)	Catalytic subunit of protein kinase A, key effector of the cAMP/PKA pathway. L205R activating hotspot mutation induces constitutive PKA activation	Overt Cushing	Somatic (20–50% of CPA)
<i>GNAS</i>	(20)	Alpha subunit of protein Gs; hotspot gain of function activates adenylate cyclase, independently of any MC2R activation by ACTH	Overt Cushing	Somatic
<i>PRKAR1A</i>	(21)	Negative regulatory subunit of protein kinase A. Inactivating mutations activate PKA through derepression	Overt Cushing	Somatic
<i>CTNNB1</i>	(22)	Encodes β -catenin, key regulator of the Wnt/ β -catenin pathway. Activating mutations induce abnormal activation of this pathway	Low secretion (SCA and NSA) and larger size	Somatic (25%)
Hyperplasia				
<i>ARMC5</i>	(26)	Encodes armadillo-repeat containing 5. Inactivating mutations	PBMAH $\pm$ meningiomas	Germline, with a somatic second hit
<i>PRKAR1A</i>	(76)	Negative regulatory subunit of protein kinase A. Inactivating mutations activate PKA through derepression	PPNAD $\pm$ Carney complex	Germline
<i>PDE8B, PDE11A</i>	(77, 78)	Encodes a phosphodiesterase, catalyzing the degradation of cAMP. Inactivating mutations activate the cAMP/PKA pathway by slowing cAMP degradation		Germline
<i>GNAS</i>	(79)	Alpha subunit of protein Gs; hotspot gain of function activates adenylate cyclase, independently of any MC2R activation by ACTH	$\pm$ McCune–Albright syndrome	Somatic mosaicism
<i>PRKACB</i>	(80)	Catalytic subunit of protein kinase A, effector of the cAMP/PKA pathway. Activating mutation induces constitutive PKA activation		Germline
<i>MEN1</i>	(81)	Regulates transcription by coordinating the chromatin remodeling. Inactivating mutations	MEN1	Germline
<i>FH</i>	(82)	Mitochondrial enzyme implicated in Krebs cycle. Catalyzes fumarate to malate transformation. Inactivating mutations	$\pm$ HLRCC	Germline
ACC				
<i>ZNRF3</i>	(32, 33, 34)	E3 ubiquitin ligase, negatively modulates the Wnt/ β -catenin pathway by promoting LRP5/frizzled receptor turn-over. Inactivating mutations and homozygous deletions induce an activation of the Wnt/ β -catenin pathway		Somatic (20%)
<i>CTNNB1</i>	(22)	Encodes β -catenin, key regulator of the Wnt/ β -catenin pathway. Activating mutations induce abnormal activation of this pathway		Somatic (15–20%)

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Table 1 Continued.

Gene symbol	Reference	Gene function/type of alteration	Specific phenotype	Compartment (% mutations)
<i>TP53</i>	(32, 33, 34, 38)	Encodes p53, key positive regulator of apoptosis, cell-cycle arrest and DNA repair. Inactivating mutations	± Li Fraumeni syndrome	Somatic (15–20%) (germline)
<i>CDKN2A</i>	(32, 33, 34, 36, 83)	Tumor suppressor genes encoding two proteins, acting through the activation of p53 and pRB. Inactivating mutations and homozygous deletions		Somatic
<i>CDK4</i>	(32, 33, 34, 36)	Oncogene, inhibiting pRB (encoded by RB1) by phosphorylation. Activated by high-level amplifications		Somatic
<i>TERT</i>	(32, 33, 34, 36)	Reverse transcriptase of telomerase complex. Need for maintaining the chromosome length in cancer cells. Activated by high-level amplification		Somatic
<i>RB1</i>	(32, 33, 34, 39)	Encodes pRB, negative regulator of cell-cycle. Inactivating mutations and homozygous deletions	(MEN1)	Somatic
<i>MEN1</i>	(32, 33, 34)	Regulates transcription by coordinating the chromatin remodeling. Inactivating mutations		Somatic (germline)
<i>DAXX</i>	(32, 33, 34)	Implicated in chromatin remodeling, in alternative lengthening of telomere and in apoptosis. Inactivating mutations		Somatic
<i>ATRX</i>	(32, 33, 34)	Implicated in chromatin remodeling, and in alternative lengthening of telomere. Inactivating mutations		Somatic
<i>MDM2</i>	(32, 33, 34, 38)	E3 ubiquitin ligase, negatively regulates p53 protein by leading it to its proteasomal degradation. High-level amplification		Somatic
<i>PRKAR1A</i>	(33)	Negative regulatory subunit of protein kinase A. Inactivating mutations activate PKA through derepression		Somatic
<i>RPL22</i>	(33)	Encodes 60S ribosomal protein L22. Inactivating mutations		Somatic
<i>APC</i>	(84)	Important negative regulator of Wnt/β-catenin pathway. Inactivating mutations induce abnormal activation of this pathway	Familial adenomatous polyposis	Germline

APA, aldosterone producing adenoma; CPA, cortisol producing adenoma; SCA, subclinical Cushing adenoma; NSA, non-secreting adenoma; ACC, adrenocortical carcinoma; HLRCC, hereditary leiomyomatosis renal clear cell carcinoma; PBMAH, primary bilateral macronodular adrenal hyperplasia; PPNAD, primary pigmented nodular adrenal dysplasia; MEN1, multiple endocrine neoplasia type 1.

subsequent Gq/phospholipase C signaling – and the plasma membrane depolarization – with subsequent opening of calcium voltage-dependent channels – respectively (6). In APA, the new mutated genes also demonstrate the central role of intra-cellular calcium for autonomous aldosterone production. This intra-cellular calcium raise is either related to mutations in ion transporter genes (*KCNJ5*, *ATP2B3* and *ATP1A1*) inducing a plasma membrane depolarization with subsequent opening of voltage-dependent calcium channels (5, 7, 8), or to mutations in voltage-dependent calcium channels (*CACNA1D* and *CACNA1H*) inducing the opening at lower thresholds of membrane depolarization (8, 9) (Fig. 1D).

The majority of these mutations were identified in the somatic compartment, by comparing the APA (tumor tissue DNA) exome to the germline (leucocyte DNA) exome. In the germline compartment, rare instances of mutations were also identified for *KCNJ5*, *CACNA1D* and

CACNA1H, mainly in families with primary aldosteronism, and as *de novo* mutations (5, 9, 10).

The molecular alterations in the *zona glomerulosa* may be more complex. Indeed recently Fernandes-Rosa *et al.* (11) identified different somatic mutations in the adrenals of patients presenting an APA with multiple nodules. In addition, Nishimoto *et al.* (12) reported somatic mutations in *CACNA1D*, *ATP1A1* and *ATP2B3* in ‘normal adrenals’ – e.g. from patients not included as presenting a primary aldosteronism. These mutations were identified in clusters of glomerulosa cells with elevated expression of CYP11B2, and different mutations could be found in different such clusters within the same adrenal (12).

Beyond the calcium signaling, the Wnt/β-catenin seems to be another major player in APA (Fig. 1). Berthon *et al.* (13) recently reported Wnt/β-catenin activation in a majority of APA, related to the downregulation of a negative regulator of this pathway, named SFRP2 – this

receptor; AT1R, angiotensin 2 type 1 receptor; DVL, dishevelled; in red, activated proteins; in light-blue, inactivated proteins.

In 2014, four independent research teams identified activating mutations of *PRKACA* – the alpha subunit of PKA – in CPA (15, 16, 17, 18). As for APA, CPA exome was compared with the germline exome, leading to the

identification of a hotspot mutation transforming a Lysine into an Arginine at position 205 (L205R). These studies and subsequent ones identified this mutation in 22–50% of CPA associated with overt Cushing (15, 16, 17, 18).

In physiology, activation of the cAMP/PKA pathway is central in *zona fasciculata* cells for the cortisol production. This pathway is activated by the binding of ACTH to its receptor MC2R, subsequently activating Gs, then adenylyl cyclase and finally PKA. In CPA with the L205R mutation, the autonomous cortisol production results from direct activation of PKA (19). Indeed, the *PRKACA* L205R mutation was shown to increase PKA activity (15, 17). These *PRKACA* mutations add to previously reported *GNAS* (20) and *PRKARIA* (21) mutations, identified in rare instances of CPA and also activating the cAMP/PKA pathway (Fig. 1).

In addition to these mutations activating the cAMP/PKA pathway, mutations of *CTNNB1* – activating the Wnt/ β -catenin pathway – have also been reported in adrenocortical adenomas, found in about 25% of cases (22). Adenomas with *CTNNB1* mutations are bigger and secrete less cortisol than their WT counterparts (23). Of note, the CPA with *PRKACA* mutations reported recently do not often harbor *CTNNB1* mutation (15, 16, 17, 18). Are these mutations somehow mutually exclusive? Is there any interplay between the Wnt/ β -catenin and the AMPc/PKA pathway? These points remain to be clarified.

ARMC5, a new gene predisposing to PBMAH

Though being bilateral and a global adrenal cortex disease, adrenal hyperplasia has not clearly been deemed as genetic diseases until recently, at least in its most common macronodular subtype. Indeed only rare families have been reported, and mutations in several genes (*PRKARIA*, *PDE11A*, *PDE8B*, *GNAS*, *PRKACB*, *MEN1* or *FH* among others; review in (4, 24, 25)) have been identified only in rare instances and/or specific subtypes of these diseases – such as *PRKARIA* mutations in PPAD.

In 2013, we identified *ARMC5* mutations in 50% of PBMAH patients requiring an operation (26). This gene was identified by comparing the macronodules to germline DNA. A SNP array approach first identified a somatic copy-neutral loss of heterozygosity in chromosome 16p in 25% of patients. An additional whole-genome sequencing identified mutations of *ARMC5*, located in 16p. *ARMC5* is a typical tumor suppressor gene, with two hits identified in each macronodule: a first germline hit – found in all the tissues and all the adrenal macronodules of each

patient – and a second somatic hit – specific of each adrenal macronodule (26).

Subsequent studies identified a prevalence of 25–50% of *ARMC5* mutations in PBMAH (27, 28, 29). Patients with *ARMC5* mutations seem to present a more severe Cushing syndrome and a more nodular adrenal shape than their WT counterparts (29). Alencar *et al.* (27) also reported a large family with *ARMC5* mutations, giving important insight on the penetrance of the disease.

ARMC5 is a ‘new gene,’ poorly characterized in terms of function. *ARMC5* inactivating mutations were shown to decrease steroidogenesis and to reduce apoptosis in adrenocortical cells (24). Its precise role remains to be established. Does *ARMC5* play a role in the recently reported intra-adrenal ACTH production (30) or with the aberrant GPCR expression profiles (31)? Further studies are needed.

The mutation landscape of ACC: a reduced number of genes with recurrent mutations

In 2014, we reported the first ACC genomic landscape based on exome sequencing and SNP arrays – the latter technique is convenient for detecting homozygous deletions and high-level amplifications – comparing the tumor to germline DNA (32). The cancer genome atlas (TCGA) consortium has also recently presented the mutational landscape on a large cohort of ACC, also comparing the tumor and the germline exome (33). Juhlin *et al.* (34) also reported a few months ago another analysis of the ACC exome, using a similar strategy. These studies converge on a reduced list of recurrent ACC drivers (Table 1). Two other studies used next generation sequencing to characterize the mutations in ACC, analyzing the genetic variation of a limited number of cancer-related genes, and restricting the analysis to the tumor DNA (35, 36).

Among these drivers, *ZNRF3* appeared as the most commonly altered gene in ACC, found in more than 20% of ACCs (32, 33, 34). This is also the first report of *ZNRF3* alterations in cancer. *ZNRF3* is an E3 ubiquitin ligase, with a negative regulator function of the Wnt/ β -catenin pathway (37). In ACC, *ZNRF3* is inactivated, mainly by homozygous deletions, but also by mutations (32, 33, 34). This suggested that *ZNRF3* inactivation induces an activation of the Wnt/ β -catenin pathway (Fig. 1). The Wnt/ β -catenin pathway can also be activated directly in ACC by *CTNNB1* activating mutations (22) – encoding the β -catenin – also commonly found in ACC – ~15% of ACC (32, 33, 34). Interestingly and logically,

ZNRF3 and *CTNNB1* mutations are mutually exclusive in ACC (32, 33, 34).

Other recurrently mutated genes are related to the cell-cycle regulation. Among these, *TP53* mutations are found in ~15% (32, 33, 34). Other genes regulating the cell-cycle and commonly altered in cancer are altered in ACC, including the tumor suppressors *CDKN2A* and *RB1*, and the oncogenes *MDM2* and *CDK4* (32, 33, 34, 36, 38, 39) (Table 1). These alterations globally unbrake the cell cycle, one of the key elements of cancer proliferation (Fig. 1A).

Other pathways emerging from the genetic landscape of ACC include the chromatin remodeling – with mutations of *MEN1*, *DAXX* and *ATRX* – and the chromosome maintenance – with *TERT* and *TERF2* amplifications (32, 33, 34, 36).

A few other recurrently mutated genes have been identified, including *PRKAR1A* and *RPL22* (33). *PRKAR1A* mutations had been previously identified in PPNADs and in some CPA (see previous paragraph). These instances of ACC with *PRKAR1A* mutations raise the question of the potential role of the cAMP/PKA pathway in some ACC, and subsequently of the potential predisposition to ACC in the benign adrenal tumors with activated cAMP/PKA. Further studies are needed to clarify this point.

Finally other genes are altered, with no clear recurrence among ACC ('private' mutations). The global importance of these genes in ACC pathophysiology remains to be clarified.

Of note, none of these alterations oriented obviously towards the use of any targeted cancer therapy commonly available and used in other cancers.

Integrated genomics of ACC identify distinct molecular groups with different outcomes

Several teams focused on the genomics of ACC (Table 2), showing major differences between ACC and ACA, and identifying molecular subgroups of ACC, with different outcomes.

Common molecular features of ACC

ACCs share common molecular characteristics (Table 2). At the transcriptome level, thousands of genes are deregulated, in comparison to ACA. A large proportion of these genes is implicated in the cell-cycle regulation, chromosomal maintenance, survival, inflammation, immunity and the global modulation of gene expression, as part of a global 'malignancy signature' (40). This type of

gene expression signature is shared by many different cancer types, independently of the tissue of origin. In addition to these unspecific malignancy features, the ACC specifically show a global decrease in expression of genes related to steroidogenesis, reflecting the dedifferentiation of ACC cells (41, 42, 43, 44, 45, 46, 47, 48, 49). Finally massive *IGF2* over-expression is one of the most striking differences between ACC and ACA, observed in >85% of ACC (41). This was known before genomics, first observed in congenital Beckwith–Wiedemann syndrome (50). *IGF2* over-expression is considered as an early tumorigenesis event, activating the IGF1 receptor (51).

miRNAs are small untranslated transcripts, regulating the gene expression level by RNA-interference. Several hundreds of miRNA have been identified in humans. The miRNome of ACC shows globally several miRNA differentially expressed compared with ACA. The most consistent one is the overexpression of miR-483-5p (52, 53, 54), in which gene is located in *IGF2* gene locus, and probably deregulated simultaneously with *IGF2*. Other deregulated miRNAs include downregulation of miR335 and miR195 (47, 52, 53, 54, 55, 56) among others. There is currently no precise determination of the global impact of these miRNA deregulations on gene expression in ACC.

In terms of DNA methylation, ACC are globally hypomethylated compared with ACA (57). This hypomethylation is mainly related to hypomethylation in intergenic regions of DNA, probably contributing to the genome instability, as seen in cancer in general (58). Beyond this global hypomethylation, a subset of ACC shows specific patterns of methylation, with hypermethylation in CpG islands of gene promoter regions (59). This point will be detailed in the next paragraph.

Finally the ACC genome tends to be altered compared with ACA, with extended chromosomal gains, losses and losses of heterozygosity in a majority of ACC (60, 61, 62, 63).

Genomics identify different molecular groups of ACC

The major finding of the integrated genomics of ACC is the existence of distinct molecular subgroups. Remarkably, these distinct groups are strongly associated with the patient's outcome.

At the transcriptome level, two main distinct subgroups have been defined, strongly associated with prognosis (33, 42, 64). At the miR level, 2–3 subgroups are reported, also associated with different outcomes (32, 33, 54, 55).

Table 2 Principal genomics publications on adrenocortical tumors.

Reference	Disease	Summary
Transcriptome		
Giordano <i>et al.</i> (41)	ACC and ACA	ACC and ACA differ in their gene expressions; 91 genes are differentially expressed between ACC and ACA (threefold difference), including IGF2
De Fraipont <i>et al.</i> (43)	ACC and ACA	Two genes clusters, IGF2 and steroidogenesis, predict malignancy; the expression of 14 genes predicts recurrence
Velazquez Fernandes <i>et al.</i> (44)	ACC and ACA	Gene expression-based unsupervised classification separates ACC from ACA; 571 genes are differentially expressed between ACC and ACA, included IGF2- and IGF-related genes
Slater <i>et al.</i> (45)	ACC and ACA	In total, 42 genes are differentially expressed (> fourfold) between ACC and normal adrenal tissue, 21 genes between ACC and ACA
Lombardi <i>et al.</i> (85)	ACC and ACA	Four genes are differentially expressed (> 1.5-fold) between ACC vs ACA
Fernandez-Ranvier <i>et al.</i> (86)	ACC and ACA	In total, 25/314 genes of 11q13 locus are differentially expressed between ACC and ACA ($P < 0.005$, > twofold)
Fernandez-Ranvier <i>et al.</i> (46)	ACC and ACA	Transcriptome separates ACC from ACA, 37 genes are differentially expressed between ACC and ACA (> eightfold)
Tombol <i>et al.</i> (47)	ACC and ACA	In total, 614 genes show expression difference in ACC vs ACA and normal adrenal (twofold, $P > 0.05$), including IGF2 and TOP2A overexpressed in ACC
Soon <i>et al.</i> (48)	ACC and ACA	Unsupervised classification separated ACC from ACA; 177 genes are differentially expressed between ACC and ACA
Giordano <i>et al.</i> (64)	ACC and ACA	In total, 1890 genes are differentially expressed between ACC and ACA (twofold). Unsupervised classification of ACC according to their gene expression reveals two groups of distinct ACC, which differs in prognosis and mitotic count
De Reyniès <i>et al.</i> (42)	ACC and ACA	Unsupervised classification separates ACC from ACA, but also reveals two types of ACC with different outcome. Two qRT-PCR predictors are proposed: DLG7-PINK1 for disease-free survival and BUB1B-PINK1 for overall survival
Laurell <i>et al.</i> (49)	ACC and ACA	Clustering analysis discriminates ACC from ACA and reveals two subtypes of ACC with different prognosis
Lenzini <i>et al.</i> (87)	APA	Transcriptome profiling of adenomas in the setting of primary aldosteronism identify different tumor types
Wang <i>et al.</i> (88)	APA	In total, 14 genes significantly overexpressed in APA vs normal adrenal. CYP11B2 is upregulated in APA, AKR1C3, CYB5 and CYP17 are downregulated
Azizan <i>et al.</i> (89)	APA	Gene expression separates APA from normal adrenal samples. CYP17A1 and CYP11B1 expressions seem to define <i>zona fasciculata</i> -like APA
Monticone <i>et al.</i> (90)	APA	Gene expression shows fourfold expression difference between <i>KCNJ5</i> APA mutant vs normal adrenal for <i>CYP11B2</i> , which is upregulated in <i>KCNJ5</i> compared with WT APA
Willmot-Roussel <i>et al.</i> (19)	ACA	By unsupervised classification, transcriptome separates CPA from a mixed group of SCA, NSA and a few CPA. Steroidogenic genes, cholesterol metabolism genes and glutathione S transferases are positively correlated with cortisol production
Methylome		
Rechache <i>et al.</i> (57)	ACC and ACA	Methylome distinguishes normal adrenal, ACA, ACC and metastatic tissue samples. ACC are hypomethylated in intergenic regions. Identification of 52 genes both hypermethylated and downregulated
Fonseca <i>et al.</i> (91)	ACC and ACA	In total, 212 CpG island in promoters genes are recurrently hypermethylated in ACC compared with ACA, including genes implicated in cell-cycle regulation and apoptosis; 6 of these genes, including CDKN2A and GATA4, are shown to be down-regulated in ACC
Barreau <i>et al.</i> (59)	ACC	Methylation of CpG island regions distinguishes two types of ACC according to the presence or absence of a hypermethylation (CIMP); CIMP tumors have a lower outcome; 1741 genes present a negative correlation between their expression and methylation, including H19
Howard <i>et al.</i> (92)	APA	Global hypomethylation of CpG island in APA compared with normal adrenal. CYP11B2 upregulated and hypomethylated, PRKCA and AVPR1A downregulated and hypermethylated
Murakami <i>et al.</i> (93)	APA	Global hypomethylation of CpG island in APA compared to normal adrenal; 34 genes upregulated and hypomethylated, among them CYP11B2, MC2R and HPX implicated in aldosterone production

Table 2 Continued.

Reference	Disease	Summary
miRNome		
Soon <i>et al.</i> (53)	ACC and ACA	In total, 23 miRNA are differentially expressed between ACC and ACA; ACC with high miR-483-5p and low miR-195 show a lower survival
Tombol <i>et al.</i> (47)	ACC and ACA	Six miRNAs are differentially expressed in ACC vs ACA and normal adrenal. Combining level expression measures of miR-511 and miR-503 predicts accurately malignancy
Patterson <i>et al.</i> (52)	ACC and ACA	miRNA expression distinguishes ACC from ACA; 23 miR differentially expressed between ACC and ACA, including miR-483-5p, located within the parentally imprinted IGF2 locus. IGF2 and miR-483-5p expression are correlated
Schmitz <i>et al.</i> (56)	ACC and ACA	miRNA expression distinguishes ACC from ACA; 248 miRNAs are differentially expressed between ACC and ACA, including miR-675 and miR-335. Low expression of these miRs is associated with malignancy
Ozata <i>et al.</i> (55)	ACC and ACA	miRNA expression distinguishes ACC from ACA; 72 miRNAs are differentially expressed between ACC and ACA, including miR-503, miR-1202 and miR-1275. High expression of these miRNAs is associated with a poor prognosis
Chabre <i>et al.</i> (54)	ACC and ACA	In total, 12 miRNAs are differentially expressed between ACC and ACA. 29 miRNAs are differentially expressed between recurring/metastatic ACC and non-recurring ACC, including miR-483-5p and miR-195 which can be detected in patient serum. These circulating miRNAs have a diagnostic and prognostic value
Glover <i>et al.</i> (94)	ACC and ACA	In total, 85 long non-coding (lnc)RNA are differentially expressed between ACC and ACA, 66 are associated with recurrence and the lncRNA PRINS is associated with metastatic disease at presentation
Szabo <i>et al.</i> (95)	ACC and ACA	Difference of expression of circulating miRNAs is found in ACC patients' plasma vs ACA patients' plasma; 5 miR, including miR-483-5p, are candidate circulating biomarkers for malignancy diagnosis
Patel <i>et al.</i> (96)	ACC and ACA	Difference of expression of circulating miRNAs is found in ACC patients' plasma vs ACA patients' plasma. miR-483-5p et miR 34a are candidate biomarkers for diagnosis
Robertson <i>et al.</i> (97)	APA and CPA	miR24 is differentially expressed in APA than normal adrenal, presents binding sites on CYP11B1/CYP11B2 enzymes and regulate their activity
Velazquez Fernandes <i>et al.</i> (98)	ACA	miRNA discriminates CPA from APA. Specific miRNAs are associated respectively with CPA, APA and non-hyperfunctioning adenomas
Chromosomal alterations		
Zhao <i>et al.</i> (38)	ACC	MDM2 and CDK4 amplifications in ACC
Stephan <i>et al.</i> (60)	ACC	Several recurrent gains (5, 7, 12, 16q and 20) and losses (1, 3p, 10q, 11, 14q, 15q, 17 and 22q) are identified in ACC. Several isolated CGH probes scattered among the genome are associated with survival. The combination of these probes identifies three groups of ACC with different survival
Szabo <i>et al.</i> (61)	ACC	Several recurrent gains (5, 7, 12 and 19q) are identified in ACC
Barreau <i>et al.</i> (62)	ACC and ACA	A larger proportion of the genome is altered in ACC vs ACA. A qPCR-based diagnostic tool is proposed. The chromosome alterations also contain prognostic information
Ronchi <i>et al.</i> (63)	ACC and ACA	A larger part of the genome is altered in ACC vs ACA in terms of copy number alterations, but also in terms of loss of heterozygosity. A specific pattern is associated with the survival
De Martino <i>et al.</i> (36)	ACC	Several recurrent gains (5, 7, 12, 19, 20) and losses (1, 22) are reported. Recurrent amplification of CDK4 and deletion of CDKN2A and CDKN2B are identified
Ronchi <i>et al.</i> (99)	CPA	SNP array analyses identify recurrent copy-numbers variations in CPA; 46 recurrent alterations affecting only one gene have been described, including CYP11B1 and CTNNB1 alterations
Integrated genomics		
Assie <i>et al.</i> (32)	ACC	Transcriptome, miRNome, methylome and exome are convergent and define molecular subgroups of ACC with different outcome
Lerario <i>et al.</i> (33)	ACC	Unsupervised pan-genomics classification (RNA/miRNA sequencing, copy-number alteration and methylation profiling identified three different subgroups of ACC with different outcome)

At the DNA methylation level, an important variability occurs when focusing on the methylation of gene promoter regions, in regions enriched in 'CG' bases (called CpG islands) (57, 59). Indeed some ACC are hypermethylated in these regions, evoking a CpG island methylator phenotype (CIMP), as described in other malignancies such as colon cancer (65). In ACC, CIMP is associated with a poor outcome (59).

As stated in the previous paragraph, recurrent somatic mutations have been found in ~50% of ACC (32, 33, 34). These ACC are associated with a poorer outcome.

These omics classifications have been extensively generated in two independent international cohorts of ACC: one from Europe (European Network for the Study of Adrenal Tumors) (32, 33) and one from the TCGA ACC Group from America, Europe and Australia (32, 33). A global molecular classification has emerged from these studies, highly consensual (32, 33). Indeed, a strong agreement was found between the different omics

classifications, defining two main molecular groups. Interestingly these two groups are associated with a quite different outcome. Finally a more granular classification can isolate a third molecular subgroup, associated with intermediate survival (Fig. 2). The TCGA cohort should be published soon, warranting a more detailed comparison with the ENSAT cohort.

The molecular group of ACC with better outcome

In terms of transcriptome, the molecular group of ACC with better outcome shows a higher expression of genes intervening in cell metabolism, intracellular transport, cell differentiation and in apoptosis (42). These tumors have a low mutation rate, and scarcely harbor mutations in the recurrently mutated genes described in the previous paragraph (32, 33). Instead private mutations can be observed. Finally in terms of DNA methylation, these tumors do not show any CIMP (32, 33) (Fig. 2).

Figure 2

Classification of adrenocortical tumors according to pathology, secretion, prognosis, genomics and pathways. ACC, adrenocortical carcinoma; ACA, adrenocortical adenoma; APA, aldosterone producing adenoma; CPA, cortisol producing adenoma;

SCA, subclinical cortisol producing adenoma; NSA, non-secreting adenoma; PBMAH, primary bilateral macronodular adrenal hyperplasia; PPNAD, primary pigmented nodular adrenal dysplasia; GPCR, G protein coupled receptors.

Though being of better outcome, with few recurrences after surgery, these tumors do correspond to malignant tumors in terms of molecular profiling, and not to benign tumors. Indeed the transcriptome and miRNome profiles of these tumors are much closer to the transcriptome of other ACCs, the aggressive ones, compared with ACAs. In addition the chromosomes of these tumors are extensively altered in a majority of tumors, a feature not observed in ACAs (60, 62, 63). Finally, in our study, a majority of these tumors have an elevated number of malignancy features as defined by pathology, scored by the Weiss score, whereas almost all the tumors defined as benign by the transcriptome show evident pathological features of ACA with Weiss scores of 0 or 1 (42). Interestingly in this study seven tumors show borderline pathology, corresponding to Weiss scores of 2 or 3. Six out of seven are classified as malignant by the transcriptome, including four in the molecular group of better outcome (42). This suggests that adrenal tumors with borderline pathology are probably malignant in terms of molecular profile. Larger cohorts are needed to confirm this finding.

The molecular group of aggressive ACC

In terms of transcriptome, the molecular group of aggressive ACC shows a higher expression of cell-cycle related genes (42, 64). In terms of mutations, these tumors accumulate alterations on the recurrent ACC drivers reported in the previous paragraph (32, 33, 34) (Table 1).

In terms of pathology, Giordano *et al.* (64) suggested that this group of ACC is of 'higher grade,' as defined by increased proliferation index – e.g. high Ki67 immunopositivity and high mitotic count. The transcriptome signature is indeed in agreement with the notion of increased proliferation. However, despite a significant difference in terms of proliferation index between the two main molecular types of ACC, this criterion is not sufficient on its own to discriminate. Indeed in this study, some low grade ACC are observed in the transcriptome group of poor outcome, and some high-grade ACC are observed in the transcriptome group of better outcome (64). This relationship between proliferation index and molecular classification deserves further studies on larger cohorts. If these discrepancies are confirmed, it will be of major interest to specifically determine the prognosis of ACC with discrepant proliferation index and molecular group. Finally Ki67 reproducibility and mitotic count are not absolute (66). Of note, the reproducibility of the discriminant molecular measures within a single ACC has not been tested yet.

Within the group of aggressive ACC, a subset of tumors shows hypermethylation on CpG islands in genes promoters (CIMP). This hypermethylation is associated with a poorer prognosis compared with tumors with no hypermethylation (32, 33). Therefore methylation probably further divides the group of aggressive ACC into one subgroup of poorer outcome, and another subgroup of intermediate outcome. Further studies are needed to confirm this observation.

None of the driver genes is known to impact directly DNA methylation, and the specific methylation pattern associated with a poor outcome – e.g. CIMP – is not explained yet in terms of underlying mechanisms. The link between driver mutations and transcriptome can probably be partially explained: mutations in drivers such as TP53 or CDKN2A probably contribute to the cell-cycle signature identified in the subgroup of poor outcome, as observed in other cancer types, though the precise transcriptome signature induced by each driver mutation in adrenal cells is not precisely established. Finally, concerning the link between methylation and transcriptome, the CIMP is potentially responsible for modulating the expression level of 15% of genes (59).

Clinical implications of the genomic knowledge

The genomics findings have clinical implications for adrenocortical tumors.

Towards an improved nosology of adrenocortical tumors?

Before genomics, gold standards for classifying the tumors were based on a combination of pathology, hormonology and clinical outcome. Genomics provide agnostic molecular classifications. Can these molecular classifications help to better define the different diseases and their subtypes? Possibly.

For primary aldosteronism, can we properly discriminate a true APA, from an adrenal incidentaloma associated with a diffuse or multifocal *zona glomerulosa* disease? The adrenal vein sampling performed before surgery warrants somehow this problem by demonstrating the asymmetry of aldosterone secretion between the two adrenal glands. However the thresholds are often discussed and asymmetric forms of hyperplasia have been reported (67). 'Functional pathology' has emerged as a potential way to confirm the diagnosis of APA. The strategy is to ascertain the expression of aldosterone synthase (CYP11B2) – the

key aldosterone producing enzyme – in the adenoma. This was first achieved by *in situ* hybridization and more recently by immunohistochemistry (see (6) for review). Genomics has brought up the knowledge of somatic mutations in APA (affecting *KCNJ5*, *ATP2B3*, *ATP1A1* and *CACNA1D*), and finding such a mutation may also be a way to ascertain the true nature of an APA. The potential benefit of this genotyping in clinical practice remains to be established. For instance will it be possible to use molecular markers for preoperative diagnosis, such as detecting the somatic mutations in the patient's plasma from the cell-free tumor DNA, or detecting circulating miRNA signing the presence of an APA?

Bilateral adrenal hyperplasia include a wide spectrum of pathological aspects – including mainly macronodular and micronodular hyperplasia, hyperplasia with no nodule, and PPNAD – and are associated with different levels of cortisol secretion (2). Are these diseases different, or are they variations of a single disease? Genomics finding help to clarify this question, especially with the discovery of driver genes defining specific diseases. The most obvious example is the *PRKAR1A* mutation in PPNAD. In PPNAD there is globally a good agreement between the genotype and the pathological phenotype – micronodules, adrenal atrophy between the nodules and pigmented spots. However some PPNAD do show macronodules (68) (often harboring a somatic *CTNNB1* mutation), and reversely, no *PRKAR1A* mutation is found in ~30% of PPNAD. Are these PPNAD a different disease? With genomics another gene, *ARMC5*, was recently discovered. *ARMC5* mutations seem to be related to a phenotype with several macronodules, and in the index cases more often an overt Cushing syndrome (29). Meningiomas may also be associated with the disease (69). However more than 50% of these hyperplasias are not related to *ARMC5*. Are these different diseases? How many diseases? Associated with which phenotype? With which secretion level? Identifying the molecular drivers of these diseases could help to better classify these diseases.

Genetic counseling for the new genetic diseases

Some adrenal tumors are hereditary genetic diseases. From the genomic studies, drivers have been identified, opening new possibilities for genetic counseling. In PBMAH patients with *ARMC5* mutations, a genetic screening can be proposed to the relatives. The first estimation of the penetrance arises from the large Brazilian family reported by Alencar *et al.* (27). This genetic screening is important because the long term consequences of hypercortisolism,

progressively starting subclinical, should be prevented in *ARMC5* mutations carriers, through early and aggressive correction of mild complications – hypertension, diabetes, osteoporosis – and early medical treatment or adrenalectomy if required.

In primary aldosteronism, familial forms with *KCNJ5* mutations have been reported (6). Here again, screening the relatives should help to identify the patients at risk, and diagnose and treat early their hypertension. However these familial forms seem very rare compared with the vast majority of sporadic diseases related to somatic mutations.

A better diagnosis and a better determination of prognosis for ACC patients

Genomic studies clearly discriminate benign from malignant carcinomas. There is probably no need for sophisticated molecular tool when the pathological diagnosis is obvious, either for benign tumors with Weiss scores of 0 or 1, or for malignant tumors with Weiss scores of 4 or more. However in several situations, molecular diagnosis may be relevant. First, tumors with borderline pathology – especially those with Weiss scores of 2 or 3 – may be properly classified with molecular tools. As stated previously, in our study a majority of these tumors are most often malignant in terms of molecular profile (42). Confirmation is necessary on larger series. Another question is about the inter- and intra-observer variability of the pathological criteria from the Weiss scoring system (66). Can molecular diagnosis help to limit the impact of this variability? To which extent is a molecular assessment reproducible? This remains to be determined. Finally diagnosing malignancy may not be trivial in specific subtypes such as the oncocytic variants of adrenocortical tumors. A specific scoring has been proposed by Biseglia *et al.* (70). Would a molecular diagnostic tool be relevant for such tumors? This remains to be determined.

Another question is about the potential malignant evolution of an ACA. Does it exist? Is there an ACA to ACC sequence? Epidemiology does not plead in favor of this sequence, considering the contrast between the high prevalence of adrenal ACA and low prevalence of ACC. However, rare cases of ACC in patients with ACA or hyperplasia have been reported (discussed in (63)). Genomics also brings some information. The most remarkable is the high proportion of *CTNNB1* mutations in both ACA and ACC. In addition, *CTNNB1*-mutated ACA are bigger and secrete less cortisol (23): is it a premalignant state? In addition somatic mutations of *PRKAR1A* have been reported in ACC (33), a gene mutated

in PPNAD and in rare instances of adrenal adenomas. No extensive exome sequencing of ACA has been performed so far. Such data would help to better define the full spectrum of mutated genes shared between ACC and ACA. Concerning other omics, it is hard to draw any conclusion about any potential ACA to ACC sequence from transcriptome, miRNome and methylome. Finally a few chromosomal alterations common to ACA and ACC have been identified (63), but no definitive conclusion can be drawn either.

Among ACC, distinct molecular subgroups have emerged from genomics, associated with different outcomes. This information is of major importance, especially if it is possible to discriminate ACC potentially curable by a complete surgery from ACCs with later recurrences despite a complete (R0) surgery. There is here a unique opportunity to classify the ACC patients in distinct mechanistic and prognostic groups (Fig. 2). We proposed a tool to discriminate the two transcriptome groups of ACC, and could recapitulate the whole transcriptome information with the expression of two genes, *BUB1B* and *PINK1*. This tool was further validated by Frago *et al.* (71), and is currently prospectively tested in a routine clinical laboratory at our institution. As stated in the previous paragraphs, other omics help to classify the ACC, such as DNA methylation and recurrent drivers sequencing. This information could also be important for optimal oncogenetic classification. However it will be necessary to promote additional studies to confirm the predictive value of each of these molecular markers, precisely determine the interactions between these markers, and assess the benefits compared with the already existing prognostic markers – especially the proliferation index.

The success of molecular tools in clinical practice will also depend on the simplicity, the robustness and the price of the molecular tools. As stated before, gene expression, but also miRNAs can be conveniently assayed by quantitative RT-PCR. Concerning methylation, the CIMP can be recapitulated by focal measurements of methylation, using methylation-specific multiplex ligation probe assay, a technique widely available in oncogenetic departments (59). Finally targeted sequencing of driver genes can be performed by next generation sequencing (35, 36). In theory all these techniques are easily applicable.

Molecular findings from genomics should also open the way to liquid biopsies in a close future. These include a set of measurements in the blood reflecting the molecular features of the tumor. The most straightforward application is the detection of cell-free tumor DNA in patients' plasma by catching in plasma a specific tumor mutation,

used as a proxy of tumor DNA (72). We should be able to use *CTNNB1* mutations, mutated in ~15% of ACC, and corresponding usually to a limited number of mutation hotspots. Such hotspots should be detectable prior to surgery. After surgery, sequencing one patient's tumor should help to identify specific mutations that could be later used as proxies for monitoring the remaining tumor DNA, and therefore the remaining tumor tissue. Another application is the detection of circulating miRNAs signatures, also reflecting the diagnosis, and potentially the prognosis of an ACC (54).

Towards new treatments of adrenocortical tumors?

In APA, many patients present a somatic mutation increasing the intracellular calcium by depolarizing the plasma membrane implying the opening of voltage-dependent calcium channels. These patients may benefit from calcium channel blockers, beyond the antihypertensive effect of these treatments (see (6) for review).

For ACC patients the medical therapies currently available are of limited efficiency, and there is a crucial need for new treatments. Genomics may help to identify new therapeutic targets. However the exome sequencing of ACC did not identify directly drugable-molecular alterations, considering the drugs currently commonly available. Especially, no tyrosine kinase mutation could be recurrently found (32, 33, 34). However the genomic analyses have identified and/or confirmed the implication of a few cardinal signaling pathways. These include mainly the Wnt/ β -catenin signaling and p53/Rb signaling. These pathways are commonly deregulated in cancer, and important effort is now put to try and develop drugs specifically targeting these pathways in cancer, without altering too much these essential pathways in all other cells. For instance, several inhibitors of the Wnt/ β -catenin pathway have been identified (73). In contrast, successes in targeting p53/Rb pathway have been limited so far, despite wide efforts. Hopefully this field of research will benefit to ACC patients in a close future.

Genomics may also help ACC patients through new generations of treatment. One example is the RNA interference therapy, a promising strategy for targeting molecular alterations. The team of Sidhu proposed to target these tumors using miR-7 (74). The genomic classification may also help to propose a new algorithm for treating ACC patients, based on a better risk stratification. Indeed, ACC showing the molecular features associated with a better outcome could probably be proposed a simple follow up as far as a complete surgery

could be performed, and in case of recurrence the preferred use of repeated loco regional treatments over systemic treatments. On the opposite, ACC with molecular features associated with a poor outcome could be proposed systemic therapies in priority, even adjuvant systemic therapies after an apparently complete surgery. Such clinical recommendations based on the molecular classification, would need to be validated by prospective clinical trials.

Declaration of interest

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

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