REVIEW

Pathobiology and genetics of adrenocortical carcinoma

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Abstract

Adrenocortical carcinoma (ACC) is a rare malignancy with an incidence worldwide of 0.7–2.0 cases/million/year. Initial staging is the most important factor in determining prognosis. If diagnosed early, complete surgical resection +/- adjuvant treatment can lead to 5-year survival of up to 80%. However, often it is diagnosed late and in advanced disease, 5-year survival is <15% with a high recurrence rate even after radical surgery. The mainstay of adjuvant treatment is with the drug mitotane. Mitotane has a specific cytotoxic effect on steroidogenic cells of the adrenal cortex, but despite this, progression through treatment is common. Developments in genetic analysis in the form of next-generation sequencing, aided by bioinformatics, have enabled high-throughput molecular characterisation of these tumours. This, in addition to a better appreciation of the processes of physiological, homeostatic self-renewal of the adrenal cortex, has furthered our understanding of the pathogenesis of this malignancy. In this review, we have detailed the pathobiology and genetic alterations in adrenocortical carcinoma by integrating current understanding of homeostasis and self-renewal in the normal adrenal cortex with molecular profiling of tumours from recent genetic analyses. Improved understanding of the mechanisms involved in self-renewal and stem cell hierarchy in normal human adrenal cortices, together with the identification of cell populations likely to be co-opted by oncogenic mutations, will enable further progress in the definition of the molecular pathways involved in the pathogenesis of ACC. The combination of these advances eventually will lead to the development of novel, effective and personalised strategies to eradicate molecularly annotated ACCs.

Key Words

- ▶ adrenal cortex
- ▶ cancer
- ▶ molecular genetics
- ▶ stem cell

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Introduction

Adrenocortical carcinoma (ACC) is a rare malignancy with an incidence worldwide of 0.7–2.0 cases/million/year (Kebebew *et al.* 2006, Kerkhofs *et al.* 2013). It can occur at any age and has a bimodal incidence, predominantly affecting adults in their 40–50s and children aged 1–5 years (Wajchenberg *et al.* 2000). ACC is a heterogeneous condition with a varied presentation. In adults, 40–60%

of patients present with signs and symptoms of adrenal steroid hormone excess. In those without clinical evidence of hormonal excess, approximately half will present with features of abdominal mass effect and half will be discovered incidentally through imaging for unrelated medical conditions (Fassnacht & Allolio 2009, Else *et al.* 2014). In children, hormonal excess is a more

common presentation, occurring in up to 90% of cases (Michalkiewicz *et al.* 2004).

Initial staging is the most important factor in determining prognosis. The staging system proposed by the European Network for the Study of Adrenal Tumors is widely used and is reliable in predicting prognosis (Fassnacht *et al.* 2009, Lughezzani *et al.* 2010). Tumours that are confined to the adrenal gland are defined as stage 1 (if <5 cm) and stage 2 (if >5 cm). Stage 3 disease has features of local disease spread, including infiltration of surrounding tissue, local vascular tumour thrombus and positive regional lymph nodes. Stage 4 disease refers to patients with distant metastatic spread.

Five-year survival is as high as 81% in those presenting with stage 1 disease. However, approximately half of the patients present after the disease has breached the adrenal capsule and 5-year survival drops to 50% in stage 3 and as low as 13% in those with stage 4 (Fassnacht *et al.* 2009, Else *et al.* 2014).

The histopathological diagnosis of ACC, and importantly its distinction from benign adrenal adenomas, cannot be made on single morphological parameters alone. The Weiss score has widely been used since its introduction in 1984 to diagnose ACC (Weiss 1984). It consists of nine histopathological parameters relating to tumour invasion, tumour cell properties, tumour structure and mitotic rate to predict the malignant potential of the tumour. It has subsequently been modified to the Weiss Revised Index, consisting of only five parameters with greater weight on mitotic rate and tumour cell type (Aubert *et al.* 2002). Despite the overall good predictive value of this scoring method, it performs less well in cases with borderline features, tumours of different cell types (myxoid, oncocytic and sarcomatoid) (Pohlink *et al.* 2004, Papotti *et al.* 2011) as well as in children, where the Wieneke criteria are more predictive of the clinical outcome (Wieneke *et al.* 2003). In order to address this, the Helsinki Score has been developed which in addition to the morphological parameters of the Weiss score, includes immunohistochemical evidence of proliferation in the form of Ki67 staining (Pennanen *et al.* 2015). Ki67 has been independently shown to be a good predictor of malignant potential and have a major prognostic role in ACC (Beuschlein *et al.* 2015). The Helsinki Score has also been validated as a marginally superior prognostic tool for ACC compared to Weiss score with notably a more useful role in myxoid variant tumours (Duregon *et al.* 2017).

In addition to Ki67, there has been a lot of interest in defining new immunohistochemical markers to aid in the prognosis of ACC (reviewed in Lalli & Luconi 2018).

Most recently, the cytoskeletal remodelling factor VAV2 has been shown to improve prognostic stratification in ACC when combined with Ki67 (Sbiera *et al.* 2017). Increased VAV2 expression is independently correlated with shorter disease-free and overall survival. It may have an advantage over Ki67 as its expression is more homogenous in tissues studied. VAV2 expression is increased by steroidogenic factor-1 (SF1) in a dose-dependent manner and drives cell invasion in ACC (Ruggiero *et al.* 2017). SF1 is another immunohistochemical marker whose increased expression in ACC is correlated with worse disease-free and overall survival (Sbiera *et al.* 2010, Duregon *et al.* 2013).

Currently, surgical resection offers the only hope of a curative intervention in ACC. The outcomes are far improved when this is undertaken in specialist centres with extensive experience of these operations (Bilimoria *et al.* 2008). Complete resection of the primary tumour (resection status R0) is predictably associated with a better prognosis and patients in whom there is microscopic or macroscopic involvement of the tumour margins, 5-year survival is as low as 20 and 10% respectively (Bilimoria *et al.* 2008). However, even in patients with R0 resection, recurrence and disease progression is still commonly seen, so that adjuvant therapy is frequently recommended.

The mainstay of adjuvant treatment is with the drug mitotane, a derivate from the insecticide dichlorodiphenyltrichloroethane. The adrenolytic nature of this drug has been well known since 1950s but its method of action has been poorly understood. Recently, the exact molecular target of mitotane has been identified as the enzyme sterol-o-Acyl transferase 1 (SOAT1/ACAT). SOAT1 is inhibited by mitotane, causing accumulation of fatty acids, oxysterols and free cholesterol (and concomitant reduction of cholesterol esters) in cells with high steroidogenic activity, leading to cell death via the endoplasmic stress response (Sbiera *et al.* 2015). SOAT1 is a mitochondria-associated membranes (MAM) protein and further evidence has shown that the disruption on MAM proteins may be important in the action of mitotane (Hescot *et al.* 2017).

Retrospective studies have shown that mitotane treatment improves recurrence-free survival in those with radically resected ACC compared to matched controls who underwent surgery alone (Terzolo *et al.* 2007, Else *et al.* 2014, Berruti *et al.* 2017). Data regarding overall survival are not clear-cut but trend towards mitotane shows benefit. The ADIUVO trial is a prospective randomised controlled trial looking at the effect of mitotane in patients with low/intermediate risk of recurrence and is currently ongoing (ClinicalTrials.gov Identifier: NCT00777244).

Currently, the only other adjuvant treatment shown to have effect is radiotherapy which two out of three studies have shown to improve rates of local recurrence however overall survival was not improved (Fassnacht *et al.* 2006, Sabolch *et al.* 2011, Habra *et al.* 2013).

Mitotane is currently the only medication approved by the U.S. Food and Drug Administration and European Medicine Executive Agency in advanced ACC. Systemic chemotherapy in the form of EDP-M (etoposide, doxorubicin, cisplatin and mitotane) has been shown in a randomised control trial to improve progression-free survival and objective response rate; however, no significant overall survival benefit is reported when compared to treatment with mitotane and streptozotocin (14.8 and 12 months respectively) (Fassnacht *et al.* 2012).

Through better classification of disease in the form of scoring and staging as detailed above, management of ACC has improved over the last 40 years; however, the fact remains that for most, it is a life-limiting diagnosis. Although there have been no dramatic improvements in the management of this condition in clinical practice off late, the molecular understanding of adrenal self-renewal and adrenocortical tumourigenesis has improved exponentially. The advancement of genetic analysis in the form of next-generation sequencing and aided by bioinformatics has helped in the molecular characterisation of these tumours and opened up new avenues for investigation to improve understanding and ultimately clinical outcomes for patients with this condition.

Self-renewal and signalling in the adrenal cortex

As we have described earlier, ACC is a heterogeneous condition. The drug mitotane clearly has a role in the adjuvant setting, however, often fails to prevent progression especially in higher-risk disease. We now know that the molecular target of mitotane is the enzyme SOAT1, which is highly expressed in steroidogenic cells of the adrenal cortex (Sbiera *et al.* 2015). The same study also showed that SOAT1 expression was associated with a better response to mitotane treatment in terms of time to progression. However, despite the specific cytotoxicity of mitotane to steroidogenic cells of the adrenal cortex, progression frequently occurs. This has led to the theory that the presence of undifferentiated/poorly steroidogenic cells in the adrenal cortex, which are not affected by mitotane, may have a role in the development and chemoresistance of this malignancy.

Dysregulation of signalling pathways involved in the organogenesis and homeostasis of the adrenal cortex is implicated in many diseases, and it is theorised that corruption of these intricate processes are integral in the pathogenesis of ACC. Our understanding of the development and self-renewal of the adrenal cortex throughout life is informed mostly by rodent studies and by monogenic conditions affecting the development of the adrenal cortex. In addition, animal models of adrenal oncogenesis exist with heterogeneous penetrance of tumour formation (Basham *et al.* 2016, Leccia *et al.* 2016).

It has long been thought that the capsule and subcapsule are important sources of steroidogenic cells in the adrenal cortex and therefore a reservoir of stem/progenitor cells. This was first discovered in rats through the regeneration of the adrenal cortex from these layers after adrenal enucleation (Ingle & Higgins 1938). Cell-labelling studies have indeed identified that adrenocortical cells progress centripetally throughout life from the capsule towards the medulla where they undergo apoptosis and/or senescence (Vinson 2003). Since then, further studies using transgenic animals have identified the capsule/subcapsule as the site of stem and progenitor cells in the adult rodent and have delineated some of the pathways involved in the maintenance of stem cell populations and also in the differentiation of functional adrenocortical cells (Walczak & Hammer 2015).

Sonic hedgehog signalling pathway

Sonic hedgehog (Shh) is a glycoprotein molecule and secreted ligand whose signalling pathway is known to have a key role in organogenesis (Bambakidis & Onwuzulike 2012). It has been shown to have important paracrine effects in the development and self-renewal of the adrenal cortex (Walczak & Hammer 2015). Shh expression is seen in the subcapsular regions of mouse and rat adrenals starting from e12.5 and e13.5 respectively (King *et al.* 2009, Guasti *et al.* 2011). In rats, this occurs as part of the undifferentiated zone (ZU) whereas in mice this occurs in discrete clusters in the zona glomerulosa (ZG), similar to the pattern seen in human adrenals (Boulikroun *et al.* 2011). Cells expressing Shh appear to have steroidogenic potential as they co-express steroidogenic factor-1 (SF1), a master regulator of adrenocortical differentiation and function. However, they are not functional, differentiated cells as they do not express the enzymes required for synthesis of mineralocorticoid or glucocorticoid hormones (King *et al.* 2009, Huang *et al.* 2010).

Lineage-tracing studies have shown that capsular cells expressing the transcription factor Gli1, of the zinc finger glioma-associated oncogene family, delaminate into the cortex under Shh signalling. These cells lose their responsiveness to Shh and become fully mature steroidogenic cells forming the distinct histological and functional layers of the ZG (secreting aldosterone and expressing CYP11B2/aldosterone synthase) and zona fasciculata (ZF, secreting glucocorticoids and expressing CYP11B1/11 β hydroxylase) (King *et al.* 2009). Capsular Gli1 cells are therefore a source of steroidogenic progenitor cells. Most Gli1 progenitor cells also express Wilms tumour suppressor (Wt1) and Gata4. Wt1 lineages can also generate mature steroidogenic cells (Bandiera *et al.* 2013).

In rats, capsular Gli1 expression is tightly regulated by the protein delta-like homologue 1 (Dlk1) (Guasti *et al.* 2013). Dlk1 is a cleavable single-pass transmembrane protein and a member of the Notch/Delta/Serrate family (Falix *et al.* 2012). It is maternally imprinted and is expressed in embryological development and in immature cells (Floridon *et al.* 2000). In neuronal cells, Dlk1 expression maintains an undifferentiated phenotype and promotes clonogenicity, findings suggestive of cancer stem cell-like functionality (Kim *et al.* 2009). In the rat adrenal cortex, Dlk1 is co-expressed with Shh cells in the ZU (Guasti *et al.* 2013). Its expression is markedly modulated by remodelling of the gland triggered by the activation/inactivation of the renin-angiotensin-aldosterone axis, a process likely involving the recruitment/latency of stem/progenitor cells. Moreover, Dlk1 itself functionally interacts with Shh in activating capsular Gli1 expression in a β 1integrin-dependent fashion (Guasti *et al.* 2013). Further study of Dlk1 in adrenal self-renewal and tumour development is ongoing.

Wnt/ β -catenin signalling pathway

Another important pathway in adrenal development, self-renewal and homeostasis is canonical Wnt/ β -catenin signalling. The pathway is characterised by Wnt ligand binding to the Frizzled cell-surface receptor leading to the stabilisation and translocation of β -catenin to the nucleus, where it activities transcriptional targets through interaction with TCF/LEF transcription factors. In the absence of Wnt ligand, β -catenin-mediated gene transcription is inhibited by a destruction complex, which includes amongst others, the scaffold protein Axin, glycogen synthase kinase 3 β (GSK3 β), casein kinase 1 α (CK1 α) and the tumour suppressor adenomatous

polyposis coli (APC). The destruction complex binds to and phosphorylates cytoplasmic β -catenin leading to degradation (reviewed by Kahn 2014).

Wnt/ β -catenin signalling plays a critical role in embryonic adrenocortical development and maintenance as an adult. This has been demonstrated experimentally in mice through targeted disruption of Wnt/ β -catenin signalling in cells expressing SF1. This results in adrenal aplasia in embryos by e18.5 and causes a progressive adrenal wasting in adults histologically consistent with adrenal failure (Kim *et al.* 2008). It is proposed that this pathway is integral in maintaining adult adrenal stem cell reserve, a theory furthered by the finding that most Shh+ and ZG cells in mice are Wnt responsive as determined by TCF/LEF transcriptional activity (Walczak *et al.* 2014).

Canonical Wnt signalling also has a role in zonation and steroidogenesis. This appears to be most important in the development and maintenance of ZG. Wnt signalling has been shown to be almost entirely restricted to ZG. Experimental activation of β -catenin signalling leads to upregulation of CYP11B2 and AT1R, two determinants of ZG identity (Berthon *et al.* 2010, 2014). Signalling through the Wnt4 ligand appears to be particularly important in ZG development, with homozygous Wnt4 loss in mice leading to decreased aldosterone secretion at birth (Heikkila *et al.* 2002). Recently, it has been proposed that Wnt4 signalling acts as a local relay for the R-spondin ligand pathway in the development of the ZG (Vidal *et al.* 2016). R-spondin ligands Rspo3 and Rspo1 act upon the leucine-rich repeat containing G protein-coupled receptor 5 (LGR5) in the ZG of humans and mice causing amplification of Wnt signalling (Shaikh *et al.* 2015, Vidal *et al.* 2016). This amplification is partly due to sequestration of zinc and ring finger 3 (ZNRF3) and ring finger 43 (RNF43), which are negative feedback regulators of Wnt/ β -catenin signalling (Hao *et al.* 2012, Zebisch *et al.* 2013). Rspo3 is expressed in Gli1-expressing cells in mice from e14.5 onwards. Knock out of Rspo3 in mice causes *Shh* and *Gli1* loss in the embryonic adrenal. It also causes loss of CYP11B2 and therefore aldosterone production. In adult mice, Rspo3 loss also causes loss of Shh expression and thinning of ZG (Vidal *et al.* 2016). It has been postulated that Rspo3 and Wnt canonical signalling therefore are important in maintaining the stem/progenitor cell population in the adrenal cortex, more so than Shh signalling alone as the degree of ZG differentiation loss and thinning is more profound in Rspo3 knock out compared to Shh knock out in mice (Huang *et al.* 2010, Vidal *et al.* 2016).

Fibroblast growth factors

In addition to Shh and Wnt signalling, there is an established role for fibroblast growth factors (FGFs) in the development of many organs including the adrenal (Ornitz & Itoh 2001, Guasti *et al.* 2013). In mice, knock out of the IIIb isoform of FGF receptor 2 (Fgfr2 IIIb) results in reduced cortical proliferation and adrenal hypoplasia. Cells expressing this receptor are found in the subcapsule of the mouse, in cells that express Shh. In Fgfr2 IIIb knock out models, there is an increase in Gli1+ cells in the capsule suggesting FGF may negatively regulate Shh signalling. Dlk1 signalling is also lost in this transgenic model, further suggesting a role for Dlk1 in maintaining an undifferentiated cell phenotype in the adrenal cortex (Guasti *et al.* 2013).

Insulin-like growth factor 2 signalling

Another important growth factor in adrenal development is the paternally expressed insulin-like growth factor 2 (IGF2). IGF2 is the only insulin-like growth factor ligand that is highly expressed in human foetal adrenal (Rainey *et al.* 2001). IGF2 binds to IGF1 receptor (IGF-1R) and the insulin receptor isoform A (INSR-A) to activate MAPK and PI3K-AKT signalling pathways. This causes promotion of cell growth and cell survival. IGF2 appears important in the embryological rodent adrenal as mice with loss of IGF signalling fail to develop adrenal cortices (reviewed by Dupont & Holzenberger 2003). IGF2 expression in human adrenals decreases quickly after birth (Belgorosky *et al.* 2009) furthering the hypothesis that it plays a more important role in the embryological development of the adrenal and establishing the cellular architecture for the stem/progenitor pool rather than maintaining it.

cAMP/PKA signalling pathway

The subcapsule and ZG are the sites for the majority of the signalling pathways highlighted above, although cells of the ZF also originate from Gli1+ and Shh+ lineage (King *et al.* 2009). As we know, the functional layers of the adrenal cortex are populated developmentally and as part of a self-renewal process throughout life centripetally from stem/progenitor cells in the subcapsule (King *et al.* 2009, Freedman *et al.* 2013). Lineage-tracing experiments have shown that this likely occurs through initial differentiation into ZG phenotype and then subsequent lineage conversion to ZF cells (Freedman *et al.* 2013). A direct conversion of cortical Shh+ cells to ZF cells could

also be possible. The cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signalling pathway is essential for the development and maintenance of functional ZF. Binding of adrenocorticotrophic hormone (ACTH) to the melanocortin 2 receptor (MC2R) activates the cAMP/PKA pathway resulting in progression of cells into ZF phenotype (Chida *et al.* 2007, Gorrigan *et al.* 2011). Recently, it has been shown that activation of this pathway inhibits canonical Wnt signalling. This leads to decrease in Wnt4-mediated ZG differentiation and facilitates lineage conversion to ZF, suggesting an intricate interplay between cAMP/PKA and Wnt/ β -catenin signalling in the development and maintenance of the functional adrenocortical zones (Drelon *et al.* 2016a).

It is plausible to speculate that physiologically normal cell populations involved in self-renewing developmental pathways (thus regulating stem to progenitor to partially differentiated steroidogenic cells) might be corrupted by oncogenic processes leading to a clinically manifesting ACC. The current understanding of zonation and factors affecting self-renewal as detailed above has been schematised in Fig. 1.

Genetic alterations in ACC

Developments in high-throughput genomic assays have led to a detailed molecular characterisation of adrenocortical tumours. Integration of these results with the current understanding of physiological adrenal cortical development and homeostasis has improved understanding of the molecular pathogenesis of ACC.

Insulin-like growth factor 2 signalling

An important study looking at the gene expression profile of ACC compared with other benign adrenal pathology was published in 2003. Using Affymetrix microarrays, Giordano and colleagues compared the gene expression of 11 ACC, four adrenocortical adenomas (ACA), three normal adrenal cortices and one macronodular hyperplastic adrenal. This study identified 91 genes with significantly increased expression in ACC compared to the other samples. This included clusters of genes associated with cell proliferation, notably *IGF2* which was increased in 10/11 ACC. Few transcriptional changes were noted between normal adrenal cortices and the benign cortical processes and none they deemed statistically significant (Giordano *et al.* 2003).

IGF2 mRNA had previously been recognised as being significantly upregulated in ACC compared to other

Figure 1

Schematic representation of the pathways involved in mouse adrenocortical self-renewal and zonation. Capsular Gli1 cells respond to Shh signalling, secreted by discrete clusters of subcapsular cortical cells, by delaminating and migrating into the cortex. The activation of canonical Wnt signalling is concentrated in the ZG and is dependent on the secretion of Rspo3 by Gli1 cells (Shh positive) in the rat. Zonal specific secreted factors/morphogens/transcription factors/pathways, which have been implicated in adrenocortical self-renewal, are indicated in blue on the left. Newly generated ZG steroidogenic cells can transdifferentiate into ZF cells under cAMP/PKA signalling (although a Shh to ZF conversion is also possible). Steroidogenic cells are characterised by centripetal migration and undergo apoptosis/senescence at the cortical/medullary boundary. Genes known to be mutated in ACC and part of the Wnt/ β -catenin pathway are identified in red. The nature of cells co-opted by other known oncogenic mutations (*TP53*, *CDKN2A*, *MEN1* etc.) is unknown.

adrenocortical pathology (Ilvesmäki *et al.* 1993, Gicquel *et al.* 1994). Increased IGF2 expression is also implicated in one of the familial genetic conditions that predispose patients to ACC, Beckwith-Wiedemann syndrome. This syndrome is associated with overgrowth, anterior abdominal wall defects and macroglossia. In 10% of cases, there is an increased risk of tumour development, predominantly Wilms tumours, rhabdomyosarcomas, hepatoblastomas and adrenocortical tumours. In this condition, there is altered expression of imprinted genes in the region 11p15.4–p15.5, including *IGF2* (Li *et al.* 1997).

IGF2 expression is increased in up to 85% of adult ACC (Heaton *et al.* 2012, Zheng *et al.* 2016). In most cases, this is due to loss of heterozygosity at 11p15 locus, leading to loss of maternal imprinting and increased expression (Bouille *et al.* 1998, Gicquel *et al.* 2001). IGF2 does not appear to be a driver of ACC; however, it has been shown to have a role in disease prognostication. Early studies showed that overexpression of IGF2 was associated with a shorter disease-free survival (Gicquel *et al.* 2001). A further study using cDNA microarray analysis of 230 candidate genes in 33 ACA and 24 ACC identified a cluster of IGF2-associated genes whose increased expression was a good predictor of malignancy in adrenocortical tumours. They also identified a cluster of genes related to steroidogenesis whose increased expression was associated with a lower

risk of malignancy (de Fraipont *et al.* 2005). In paediatric ACC, IGF2 is increased in both ACA and ACC and does not have the same value in predicting malignancy (West *et al.* 2007).

Prognostic clustering in ACC

The molecular profile of ACC was further defined through two large studies looking at whole genome expression in ACC and ACA (de Reyniès *et al.* 2009, Giordano *et al.* 2009). A group from France and a group from USA both identified that in addition to being able to differentiate ACC from ACA, ACC in turn could be clustered into two different prognostic groups depending on transcriptome expression. In the high-risk cluster (C1A), there was increased enrichment of genes involved with transcription, mitotic cell cycle and functional aneuploidy. The low-risk cluster (C1B) had enrichment of genes pertaining to cell differentiation, cell metabolism and apoptosis (de Reyniès *et al.* 2009, Giordano *et al.* 2009). These studies also both highlighted the heterogeneity of ACC compared to ACA as the variability was much higher in ACC than in ACA or normal cortex. The French study went on to highlight that the two clusters of ACCs can be more simply identified by combined expression of *PINK1* (PTEN induced putative kinase 1) with *DLG7* (discs, large homologue 7) or *BUB1B* (budding uninhibited by benzimidazoles 1 homologue

beta). Combined expression of *PINK1* and *DLG7* is the best predictor of disease-free survival and combined expression of *PINK1* and *BUB1B* is the best predictor of overall survival (de Reyniès *et al.* 2009). *PINK1* and *BUB1B* combined expression has been independently validated as the best predictor of disease-free survival in a paediatric ACC cohort from Brazil (Fragoso *et al.* 2012).

Wnt/ β -catenin signalling pathway

The identification of molecularly annotated clusters of ACC has been validated and refined further by three independent, international studies performing pan-genomic analyses of 122, 41 and 91 patients with ACC respectively (Assié *et al.* 2014a, Juhlin *et al.* 2015, Zheng *et al.* 2016). These studies have enabled the identification of driver genes in ACC (Table 1). In all of these studies, the most commonly altered gene is *ZNRF3* (in 12–21% of cases). As detailed above, *ZNRF3* is a negative feedback regulator of Wnt signalling and its inactivation by mutations or homologous deletion leads to activation of canonical Wnt signalling. The same effect is also a result of activating mutations in *CTNNB1*, encoding β -catenin (in 9.8–16% of cases). These mutations are mutually exclusive in all three studies suggesting their function is through a common pathway in Wnt signalling (Assié *et al.* 2014a, Juhlin *et al.* 2015, Zheng *et al.* 2016). Mutations in *APC*, encoding part of the β -catenin destruction complex, were also recognised leading to increased activation of Wnt/ β -catenin signalling. Germline inactivating mutations in this gene are responsible for familial adenomatous polyposis 1 and its variant Gardner's syndrome. This condition, characterised by the development of numerous colorectal adenomatous polyps and cancer, is associated with a higher incidence of developing ACC (Barlaskar & Hammer 2007, Kerr *et al.* 2013).

p53 apoptosis/Rb1 cell cycle pathway

Across these studies, Wnt signalling was the most commonly affected pathway (in 39–66% of cases). The next most commonly affected pathway was that involving the p53 apoptosis/Rb1 cell cycle (in 19.5–44.9% of cases). *TP53* inactivating mutations are implicated in more than 50% of human cancers (Leroy *et al.* 2014). *TP53* encodes the transcription factor p53, which has a crucial tumour suppressor role in cells. DNA damage and cellular stress signals result in p53 activation leading to DNA repair or apoptosis/senescence where damage is irreparable. p53 activation occurs under the influence of a pathway starting with the tumour suppressor gene *CDKN2A*. This gene encodes two proteins, p16^{INK4a} and p14^{ARF}. p14^{ARF} production in response to cell damage causes disinhibition of p53 production through inhibition of MDM2, which is responsible for p53 degradation. p16^{INK4a} also plays a tumour suppressor role by causing increased expression of E2F-dependent genes, blocking G1/S cell cycle progression. This occurs through inhibition of the inactivation of pRb by phosphorylation under the influence of CDK4. E2F-dependent gene expression also leads to increased p14^{ARF} production and in turn p53 production (reviewed in Pacifico & Leone 2007).

Similar to other malignancies, inactivation of p53 activity has long been recognised in ACC. Somatic *TP53* mutations have previously been reported in approximately 30% of sporadic ACCs (Reincke *et al.* 1994, Sidhu *et al.* 2005, Libe *et al.* 2007). In addition, loss of heterozygosity at the *TP53* locus, 17p13, is reported in as many as 80% of cases (Libe *et al.* 2007). Further analysis of the original ACC clusters C1A and C1B identified by de Reyniès in 2009 revealed that *TP53*-mutated tumours were associated with the more aggressive phenotype (de Reyniès *et al.* 2009, Ragazzon *et al.* 2010). The same study also showed that activating β -catenin mutations were also associated

Table 1 Most common genetic alterations in adrenocortical carcinoma.

Pathway affected	Gene	Frequency alteration in series (122 cases) (Assié <i>et al.</i> 2014a) (%)	Frequency alteration in series (41 cases) (Juhlin <i>et al.</i> 2015) (%)	Frequency alteration in series (91 cases) (Zheng <i>et al.</i> 2016) (%)
Wnt/ β -catenin signalling	<i>ZNRF3</i>	21	12	19
	<i>CTNNB1</i>	16	10	16
p53 apoptosis/Rb1 cell cycle	<i>TP53</i>	16	20	21
	<i>CDKN2A</i>	11	–	15
Chromatin remodelling/maintenance	<i>TERT</i>	6	22	14
	<i>MEN1</i>	7	–	7
cAMP/PKA signalling	<i>PRKAR1A</i>	–	–	11

Table detailing the most commonly altered genes in adrenocortical carcinoma identified by the three pan-genomic analyses. A cut-off of 7% in two or more studies was used. When not stated, the frequency was not published in that study.

with the more aggressive phenotype and were mutually exclusive from *TP53* mutations in this cohort (Ragazzon *et al.* 2010). This finding, however, was not repeated in two of the pan-genomic studies, which showed that *TP53* and *CTNNB1* mutations can co-exist in ACC (Assié *et al.* 2014a, Zheng *et al.* 2016).

Germline *TP53* mutations are found in 50–75% of children with ACC (Pinto *et al.* 2015, Wasserman *et al.* 2015). These often occur in the context of Li-Fraumeni or Li-Fraumeni-like syndromes. Li-Fraumeni is a syndrome diagnosed clinically, associated with early-onset bone and soft tissue sarcomas and other early onset malignancies, including ACC in 10% of patients (Li & Fraumeni 1969, Birch *et al.* 2001, Olivier *et al.* 2003). A specific *TP53* mutation, c.1010G>A (p.R337H), has been identified as being highly prevalent in southern Brazil where childhood incidence of ACC is increased by a factor of 10–15 (Achatz *et al.* 2007). This mutation, where arginine is replaced by histidine at codon 337 in exon 10, results in failure of oligodimerisation between two p53 monomers leading to decreased transactivation of downstream target genes (DiGiammarino *et al.* 2002).

The three large pan-genomic studies recently have further demonstrated the involvement of *TP53* mutations in ACC (in 16–21.5% of cases) (Assié *et al.* 2014a, Juhlin *et al.* 2015, Zheng *et al.* 2016). In addition, they have identified other alterations in parts of the tumour suppressor and cell cycle regulation pathway, notably inactivation of *CDKN2A* (11–15% of cases) (Assié *et al.* 2014a, Zheng *et al.* 2016). Inactivation of *RB1* and high-level amplification of *CDK4* and *MDM2* were also noted in both studies leading to a functional loss of tumour suppression ability (Assié *et al.* 2014a, Zheng *et al.* 2016). *CCNE1* activation, leading to decreased Rb action, has also been identified as a driver mutation affecting this pathway (Zheng *et al.* 2016).

Chromatin remodelling/maintenance

Another consistent finding across two of these studies was mutations in genes associated with chromatin remodelling, including *DAXX*, *ATRX* and more prominently *MEN1* (in 7% of cases) (Assié *et al.* 2014a, Zheng *et al.* 2016). *MEN1* encodes the tumour suppressor, menin, which is a tissue-specific protein and has been reported to interact with histone methyltransferases (e.g. *MLL*, *MLL2*) and with transcriptional factors, including notably β -catenin, which has been shown to directly interact with and downregulate through degradation *in vitro* (Wu & Hua 2008, Kim *et al.* 2017). Mutations in the

genes encoding histone modification *MLL*, *MLL2* and *MLL4* were identified in the most recent pan-genomic analysis, which described a cumulative incidence of driver mutations in histone modification and chromatin remodelling genes (excluding *MEN1* – which they included as part of Wnt signalling) in 22% of cases – suggestive of a role of epigenetic dysregulation in the pathogenesis of ACC (Zheng *et al.* 2016).

In addition, the histone methyltransferase *EZH2* has been shown to be upregulated both in histological samples and in gene expression data from three cohorts (Ip *et al.* 2015, Drelon *et al.* 2016b). *EZH2* functions as a transcriptional repressor causing nucleosomal H3 methylation resulting in chromatin condensation and gene silencing (Cao *et al.* 2012). Increased *EZH2* expression is a result of deregulated P53/RB/E2F pathway activity and is associated with more aggressive disease phenotype and worse prognosis (Drelon *et al.* 2016b).

Genes affecting chromatin maintenance have also been identified as drivers in ACC. *TERT* encodes telomerase reverse transcriptase, an enzymatic protease that prevents the telomerase shortening associated with normal cell division and replication associated with ageing and senescence (Greider & Blackburn 1987). High-level amplifications of this gene were noted across the studies (in 7–14% of cases) (Assié *et al.* 2014a, Juhlin *et al.* 2015, Zheng *et al.* 2016). Telomeric repeat-binding factor 2, *TERF2*, has also been identified as being focally amplified in 7% of cases (Zheng *et al.* 2016).

cAMP/PKA signalling pathway

In the most recent pan-genomic cohort, 8% of cases had inactivating mutations in the protein kinase cAMP-dependent regulatory type I alpha gene (*PRKAR1A*) (Zheng *et al.* 2016). Germline-inactivating mutations in this gene are associated with benign primary pigmented nodular adrenocortical disease and Carney Complex (Kirschner *et al.* 2000). This is an autosomal dominant condition characterised by endocrine neoplasia, such as adrenocortical adenoma, increased spotted skin pigmentation, cardiac myxomas and schwannomas (Carney *et al.* 1985). These conditions are usually associated with benign adrenal disease; however, malignant transformation has been reported (Anselmo *et al.* 2012). Further to this, an activating mutation in the catalytic subunit of the cAMP-dependent protein kinase A (PKA) (*PRKACA*) has been discovered in cortisol-producing adenomas (Beuschlein *et al.* 2014). Together this evidence is suggestive of a role of alteration in cAMP/PKA signalling

in the formation of adrenocortical tumours and in the development of ACC.

Methylome analysis

In addition to driver gene identification, two of these studies have also provided an in-depth analysis of the DNA methylation, miRNA analysis and chromosomal alterations in ACC (Assié *et al.* 2014a, Zheng *et al.* 2016). This information has validated previous work and helped further in defining prognostic groups. CpG islands are sequences of DNA rich in CG bases and are located in the promoter sequence of genes. Methylation status of these islands regulates gene expression. Global hypomethylation results in genomic instability and is observed in cancer (Kulis & Esteller 2010). However, hypermethylation of CpG islands in genomic areas encoding tumour suppressor genes is also recognised in malignancy (Kalari & Pfeifer 2010). In ACC, it has been reported that intergenic regions are hypomethylated compared to ACA (Rechache *et al.* 2012). In addition, there are over 200 hypermethylated promoter regions compared to ACA, including known tumour suppressor genes (Fonseca *et al.* 2012). The pan-genomic analyses showed that CpG island methylated phenotype (CIMP) was also associated with the previously identified disease clusters in ACC, C1A and C1B (Assié *et al.* 2014a, Zheng *et al.* 2016). High CIMP, referring to hypermethylated profiles, were found to be exclusively in the more aggressive ACC cluster C1A, with lower levels of methylation showing a better prognostic value even within this cluster (Assié *et al.* 2014a). Zheng and colleagues have taken this further to classify new clusters of clusters (CoC I–III) based on CIMP. Tumours with low CIMP fall into CoC I and are almost analogous with the previous cluster C1B. The old C1A has been further split into CoC II with intermediate CIMP and CoC III with high CIMP, which carries the worst prognosis (Zheng *et al.* 2016). Since this study another group has shown that DNA hypermethylation, measured using methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) is an independent marker of prognostication in ACC for overall and disease-free survival (Jouinot *et al.* 2016).

miRNAome analysis

The microRNA (miRNA) expression profile of tumours can also be used to differentiate ACC from ACA. MicroRNAs are small non-coding RNAs that act to regulate post-transcriptional gene expression. Interpreting the

functional significance of individual miRNA profiles can be difficult as each miRNA may act upon more than one mRNA transcript. The first large study looking into miRNA in adrenal cancer identified that ACC had significantly lower levels of expression of miR-195 and miR-335 than ACA (Soon *et al.* 2009). miR-7 is also significantly raised in normal tissue compared to ACC and ACA and is significantly more reduced in ACA vs ACC. This study also identified that low levels of miR-195 and higher levels of miR-483-5p were predictive of a more aggressive disease course within in ACC (Soon *et al.* 2009). miR-483 is located in intron 2 of the *IGF2* locus and has subsequently been shown to be the most recurrent marker of malignancy in both tumour samples (Patterson *et al.* 2011) and as circulating miRNA in serum (Chabre *et al.* 2013). The European-based pan-genomic study reaffirmed this finding when comparing their original ACC cohort with three normal adrenal samples (Assié *et al.* 2014a). Using consensus clustering, they identified three stable miRNA tumour clusters (Mi1–3). Mi3 was predictive of C1A and Mi1 predictive of C1B. Zheng and colleagues generated six different miRNA clusters, which did not appear to show associations with the CoC classification (Zheng *et al.* 2016).

Chromosomal alterations analysis

Another apparent marker of ACC compared to ACA is the quantitative chromosomal alterations that are present (Assié *et al.* 2014b). The pan-genomic studies all highlighted common profiles of copy number alterations (CNAs) (Assié *et al.* 2014a, Juhlin *et al.* 2015, Zheng *et al.* 2016). Two of the studies have concordant analyses from their cohorts that a noisy pattern of CNA (compared to quiet or chromosomal) was associated with a shorter event-free survival (Assié *et al.* 2014a, Zheng *et al.* 2016). Whole genome doubling was frequently associated with loss of heterozygosity in tumours and was also associated with a more aggressive phenotype (Zheng *et al.* 2016). A recent study has highlighted the potential diagnostic and prognostic benefit of assessing chromosomal alterations by correlating mutations and copy number variations to ACC histotype. A lower mutation burden was identified in oncocytic tumours and the highest in the conventional and myxoid ACCs (Vatrano *et al.* 2018).

Conclusion

In this review, we have detailed the pathobiology and genetic alterations in adrenocortical carcinoma through

integrating current understanding of homeostasis and self-renewal in the normal adrenal cortex with molecular profiling of tumours from recent genetic analyses. Pathways involved in self-renewal and development of the gland, namely Wnt/ β -catenin and cAMP/PKA, are recognised to be altered in large cohorts of ACC tumour samples. Activation of Wnt/ β -catenin signalling is present in 39–66% of cases studied and this would seem to play an important role in the overall pathogenesis of ACC (Assié *et al.* 2014a, Juhlin *et al.* 2015, Zheng *et al.* 2016). Given that Wnt/ β -catenin signalling has recently been shown to be inhibited by cAMP/PKA pathway activation, an event shown to occur in ACC though *PRKARIA*-inactivating mutations, the contribution of Wnt/ β -catenin dysregulation in ACC may be even higher (Drelon *et al.* 2016a). It is striking that all three of the pan-genomic analyses have identified activating *CTNNB1* and inactivating mutations in *ZNRF3* to be mutually exclusive events adding further weight to the role of the net effect of canonical Wnt signalling activation in this disease (Assié *et al.* 2014a, Juhlin *et al.* 2015, Zheng *et al.* 2016).

Activation of IGF2 signalling is the most frequently recognised molecular annotation in ACC, occurring in up to 85% of cases studied and proving, in adults, to be a good predictor of malignancy when comparing ACA and ACC (de Fraipont *et al.* 2005, Heaton *et al.* 2012, Zheng *et al.* 2016). It has proven difficult to pinpoint clearly a pathogenetic role for increased IGF2 expression in ACC as mice models have shown that overexpression of IGF2 alone can cause adrenal hyperplasia but not neoplastic transformation (Weber *et al.* 1999, Drelon *et al.* 2012). However, in the presence of constitutively active Wnt/ β -catenin signalling in these models, increased IGF2 expression does lead to the formation of a small number of ACCs. These studies are suggestive that more is at play, either in the form of other somatic mutations or epigenetic alterations (Drelon *et al.* 2012, Heaton *et al.* 2012). In addition, initial clinical trials using compounds that inhibit IGF2 signalling have failed to show significant tumour regression in adequate number of patients (Haluska *et al.* 2010, Lerario *et al.* 2014). A further trial with an oral IGF-1R inhibitor, linsitinib, showed no improvement in overall or disease-free survival in locally advanced and metastatic disease compared with placebo (Fassnacht *et al.* 2015).

IGF2 signalling in the adrenal has characteristics of a process involved in embryonic organ development rather than in gland self-renewal. These are namely, its paternal expression and its decrease in expression shortly after birth in normal human adrenals (Rainey *et al.* 2001). It is

possible that in addition to its putative cumulative effect in tumourigenesis, IGF2 expression signifies a more global molecular reversion to a developmental, stem/progenitor phenotype and that other as yet undefined paternally expressed genes encoding similar pathways may have a more active role in cancer formation.

Other pathways identified to be altered in ACC include p53 apoptosis/Rb1 cell cycle and alteration in chromatin modelling and maintenance. These changes are recognised in other malignancies also, and it is not yet clear how corruption of these pathways fit into the pathogenesis of ACC. The fact that two cohorts of molecularly profiled ACC showed activating β -catenin mutations and inactivating p53 mutations to be mutually exclusive events (Ragazzon *et al.* 2010, Juhlin *et al.* 2015) is suggestive that these may be separate pathways in the formation of this malignancy; however, larger studies have shown small numbers of cases with alterations in both (Assié *et al.* 2014a, Zheng *et al.* 2016). This is indicative of the heterogeneity of this condition, where recurrent driver mutations are not shared in 40% of cases (Assié *et al.* 2014a).

It is clear that ACC is a widely diverse condition in phenotype, molecular profile and pathogenesis. Recognition of the molecular alterations in these tumours along with better understanding of the pathways of physiological homeostasis in the normal adrenal cortex have improved our understanding of this condition enormously. The recent pan-genomic analyses have shown clear correlations between molecular profiles and prognosis and have intricately expanded the clustering of disease (Assié *et al.* 2014a, Zheng *et al.* 2016). However, we are yet to see the use of this wealth of molecular information affect therapeutic strategies in the clinical setting.

The knowledge of tumour molecular profiling has also aided in progression in a new area of diagnostics in cancer, the liquid biopsy. This refers to tumour-related material that is detectable in the bloodstream and includes circulating tumour cells, miRNA, circulating tumour DNA and exosomes. It offers the potential of molecular classification and thereby hopefully prognostication of adrenocortical tumours prior to surgery with a simple blood test. Recently, a study has shown that circulating tumour DNA can be detected in a subset of patients with ACC and that the DNA seemed to follow tumour dynamics including mutations in known driver genes in ACC (Garinet *et al.* 2018). However, in some patients, no circulating tumour DNA was detectable even in those with a large tumour burden, highlighting the challenges

in the development of this technique. Plasma analysis for microRNAs has shown that miR-483-5p, whose upregulation is recognised in tumours, is also upregulated in serum and plasma in ACC and levels were predictive of more advanced disease stages (Chabre *et al.* 2013, Salvianti *et al.* 2017). Both plasma ctDNA and miRNA have limitations in the amount of information derivable from prognostication purposes. This is not a problem for circulating tumour cells, which have the advantage of the possibility of genetic molecular profiling. These are starting to be used to profile other more common malignancies, and a proof-of-concept study has shown they are detectable in ACC compared with ACA (Pinzani *et al.* 2013); however, larger international collaborations with thorough molecular profiling are required to assess their actual prognostic value in ACC. Development of these techniques will provide a thorough molecular characterisation of ACC at the point of diagnosis.

It seems likely that the next tangible clinical effector in management of adrenocortical carcinoma will come in the form of improved disease staging and prognostication, initially histologically through advances in immunohistochemistry but also through molecular characterisation of disease post-operatively and eventually pre-operatively through liquid biopsy.

A better understanding of the mechanisms underlying self-renewal and stem cell hierarchy in human normal adrenal cortices, together with the identification of cell populations likely to be co-opted by oncogenic mutations, will facilitate further elucidation and definition of the molecular pathways involved in the pathogenesis of ACC. The combination of these advances will lead to the development of novel effective and personalised strategies to eradicate molecularly annotated ACCs.

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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