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Cell cycle regulation in adrenocortical carcinoma

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Abstract

Adrenocortical carcinomas (ACCs) are rather rare endocrine tumors that often have a poor prognosis. The reduced survival rate associated with these tumors is due to their aggressive biological behavior, combined with the scarcity of effective treatment options that are currently available. The recent identification of the genomic alterations present in ACC have provided further molecular mechanisms to develop consistent strategies for the diagnosis, prevention of progression and treatment of advanced ACCs. Taken together, molecular and genomic advances could be leading the way to develop personalized medicine in ACCs similarly to similar developments in lung or breast cancers. In this review, we focused our attention to systematically compile and summarize the alterations in the cell cycle regulation that were described so far in ACC as they are known to play a crucial role in cell differentiation and growth. We have divided the analysis according to the major transition phases of the cell cycle, G1 to S and G2 to M. We have analyzed the most extensively studied checkpoints: the p53/Rb1 pathway, CDC2/cyclin B and topoisomerases (TOPs). We reached the conclusion that the most important alterations having a potential application in clinical practice are the ones related to p53/Rb1 and TOP 2. We also present a brief description of on-going clinical trials based on molecular alterations in ACC. The drugs have targeted the insulin-like growth factor receptor 1, TOP 2, polo-like kinase1, cyclin-dependent kinase inhibitors, p53 reactivation and CDC25.

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Introduction

Adrenocortical carcinomas (ACCs) are rather rare tumors, and this constituted a major limitation to conduct wide and comprehensive molecular characterization studies. In spite of this, a relatively large number of studies identified a wide array of molecular alterations, but usually in a relatively limited number of ACC samples.

Molecular studies that aimed to characterize the cell cycle deregulations found in adrenocortical tumors (ACTs) are the subject of this systematic review. This comprehensive review aims to clarify some apparently

contradictory results and rather complex molecular interactions. The data presented in this review intends to be a useful working resource for researchers interested in ACC to identify new diagnostic, prognostic and therapeutic tools to improve outcomes for ACC patients.

Adrenocortical carcinomas

ACCs have an annual incidence of 1–2 cases per million persons worldwide (1, 2, 3, 4, 5). ACCs can be functional,

producing hormonal and metabolic syndromes that can occasionally accelerate their identification or are discovered incidentally during imaging for unrelated clinical reasons (4, 6). In some patients with very large tumors, the symptoms of abdominal discomfort or pain due to the mass effect will lead to adrenal imaging and diagnosis. The detection of non-functioning ACTs (both benign and malignant) increased significantly over the last years, due the widespread use of CT, MRI and abdominal ultrasonography (6, 7).

The potentially dark prognosis of ACC is very different from adrenocortical adenomas (ACAs), which stresses the importance of differential diagnosis between the two entities (8, 9). However, the evaluation of the potential malignant evolution of ACTs is not always easy to assess. So far, the size and morphological characteristics of the lesion on the imaging studies is the initial strongest predictor of the adrenocortical malignant potential; according to the European Society of Endocrinology Clinical Practice Guideline (10), unilateral ACTs larger than 6cm should be treated surgically. Those between 4 and 6cm should undergo additional imaging investigation to better define malignant potential. In addition to size, a threshold value of 10 Hounsfield units (HU) of unenhanced CT scan was shown to have a high specificity and sensibility for the differential diagnosis of adrenocortical malignant lesions (10, 11). After tumor removal, the pathological diagnosis includes evaluation of tumor macroscopic characteristics, again including size, but also the presence of an intact capsule, areas of necrosis and hemorrhage, adjacent organ invasion and lymph node metastasis. At the microscopic level, the modified Weiss scoring system helps in the assessment of malignancy potential whenever metastasis has not been identified (12, 13). A few molecular and immunohistochemistry markers are also helpful to define ACC diagnosis (11, 13, 14). The major alterations found in the ACCs are the overexpression of IGF-2 and the constitutive activation of the Wnt/ β -catenin pathway. Although the former one was identified as a useful marker for ACC by several authors, it was not yet validated to be used in clinical practice. Ki-67 proliferation index has also been found to be an important marker for the prediction of recurrence (15), and it is used to guide the management of ACC patients.

The disease stage and margin-free resection are among the major prognostic factors in ACC (16, 17). The stage classification proposed by the European Network for the Study of Adrenal Tumors (ENSAT) in 2009 is the most accurate to define ACC prognosis (5, 17). This staging system, defines stage I and stage II as localized tumors

with a size of ≤ 5 or > 5 cm respectively. Stage III ACCs present infiltration in surrounding tissue or regional lymph, whereas stage IV is characterized by the presence of distant metastasis. Using ENSAT classification, the 5-year disease-specific survival rate is approximately 82% for stage I, 61% for stage II, 50% for stage III and 13% for stage IV (17). The high proportion of ACC is diagnosed in an advanced stage leading to a poor prognosis (4). The secretion of abnormal levels of steroids by ACCs also confers substantial morbidity (18).

The behavior of ACCs differs between patients. Almost 50% of stage I and II adrenal carcinomas will not be cured by surgery even when complete margin-free resection is achieved, while others with similar pathological characteristics are cured by surgery and patients remain disease free. Recent studies indicate that prognosis is related to significant variability of tumors at the molecular level (19).

Pan-genomic characterization of ACCs

Assié *et al.* and Zheng *et al.* recently performed comprehensive genomic characterizations of ACCs with the major goal of identifying stratification profiles of ACC patients with distinct clinical outcomes (20, 21). The studies include data on gene expression, exome sequencing, miRNA expression, copy number alterations, SNPs, loss of heterozygosity (LOH), mutations, DNA methylation and even protein expression (20, 21). Much of this information is not yet translated into complete understanding of molecular pathways, but they have confirmed some previous findings and identified potentially very important novel contributing pathogenic alterations.

Using exome sequencing and SNP array analysis, the studies identified recurrent alterations in several genes implicated in the Wnt pathway, cell-cycle regulation, chromatin remodeling and chromosome maintenance, as ACC drivers.

Indeed, the major findings of the integrated pan-genomic studies were the identification of distinct ACC molecular subgroups strongly associated with patient's outcome. The subgroup of aggressive ACCs presented higher expression of cell-cycle-related genes and accumulation of more mutations in the ACC drivers.

Based on the methylation on CpG islands in gene promoter regions, the aggressive ACC group was further divided into two other subgroups: a group of patients with intermediate outcome and a group with the

poorer outcome, which was characterized by having hypermethylation in those regions (20, 21).

Cell cycle

Since alterations in the cell cycle regulators were pointed-out as very important ACC drivers by the already mentioned pan-genomic studies but also by several smaller studies, we decided to gather the current knowledge on cell cycle dysregulation in ACCs in this review.

The cell cycle is a complex process that results in cell division. It includes four distinct phases going from the cell growth, DNA replication, partition and distribution of the duplicated chromosomes and cell division (Fig. 1) (22). This process is regulated by complex intracellular and extracellular signaling cascades that coordinate the different phases of the cell cycle to ensure a successful cell division (22, 23). Deregulation of the cell cycle is one of the most frequent events in tumor development. Therefore, elucidation of the underlying mechanisms of its alterations in tumorigenesis could lead to novel therapeutic targets (24, 25).

Figure 1

Schematic representation of the mammalian cell cycle. Cell cycle is mainly regulated by cyclin-dependent kinase (CDK): CDK2, CDK4, CDK6 and Cdc2; cyclins (cyclin A, B, D and E) and CDK inhibitors (p15^{Ink4B}, p16^{Ink4A}, p18^{Ink4C}, p19^{Ink4D}, p21^{Cip1}, p27^{Kip1}, p57^{Kip2}). In red are represented the overexpressed cell cycle regulators and in green the underexpressed regulators in ACCs. A full colour version of this figure is available at <https://doi.org/10.1530/EJE-17-0976>.

Cell cycle regulation is generally grouped in three waves that correspond to the transition points of the cell cycle: G1 to S; G2 to M and M to G1 (Fig. 1) (26). The majority of the cell cycle regulation alterations in ACCs were found to be present in the G1-to-S and G2-to-M transitions (27).

Alterations in the regulation of G1-to-S phase found in ACCs

Pan-genomic studies identified the p53-Rb1 pathway as the second most altered pathway found in the ACCs (20, 21), while the first one the Wnt B-catenin pathway is not related to the cell cycle. The p53-Rb1 pathway was already known as the major checkpoint of the G1 phase of the cell cycle. Briefly, in response to DNA damage, ataxia telangiectasia mutated (ATM) or ataxia telangiectasia and Rad3-related protein (ATR) are activated and phosphorylate p53 through Chk1/2. Phosphorylated p53 dissociates from the murine double minute 2 (Mdm2) and is thereby stabilized. Active p53 upregulates the endogenous p21 mRNA and protein levels (28). Overexpression of p21 blocks the phosphorylation of the retinoblastoma protein (pRb) by cyclin E/cyclin-dependent kinase (CDK)2, preventing the E2F-mediated gene expression induction required for cells to enter S phase (29).

Inactivating mutations or homozygous deletion of *TP53* (16%), *CDKN2A* (11%) and *RB1* (7%) and high levels of amplification of *CDK4* (2%) and *MDM2* (2%) were found to occur in 40 cases of the 122 ACCs analyzed by Assié *et al.* (21). These alterations were mainly found in the ACCs with poorer prognosis. Zheng *et al.* (20) also reported deactivating alterations (mutations and deletions) in *TP53* (21%), *CDKN2A* (16%) and *RB1* (7%) and activating alteration in *CDK4* (7%), *MDM2* (7%) and *CCNE1* (6%), the gene that encodes the cyclin E. Altogether, the p53/Rb1 pathway was altered in 44.9% of the analyzed ACCs by Zheng *et al.* (20). Since the majority of the *TP53* mutations observed affect the DNA-binding domain or the oligomerization domain, these alterations prevent the role of p53 as a tumor suppressor, leading to an important dysfunctionality of the G1 checkpoint. This point, together with a deregulation of the major molecules implicated in the G1 phase: cyclin E, CDK4 and Rb1, will lead to an uncontrolled ACC cell proliferation.

Alterations in p53 pathway in ACCs

The study of the p53 and their regulators in ACC started many years ago, after the discovery that the

Li-Fraumeni syndrome confers susceptibility to ACCs and is characterized by the presence of *TP53* germline mutations in approximately 71% of families with that syndrome (30, 31). Although germline *TP53* mutations were later found to be rare in adult patients with ACC, somatic *TP53* mutations were documented to be more frequent (32, 33, 34, 35, 36). In a large cohort of adult Caucasian patients with ACC, a 3.9% prevalence of *TP53* germline mutation was found (35); in contrast, 13% of adult patients (aged <40 years), carried a *TP53* germline mutation (35). Germline mutations in *TP53* were observed in 50–80% of children with sporadic ACC (37, 38, 39). Many studies analyzed the presence of somatic *TP53* mutation in ACCs and verified that its prevalence varies from 20 to 30% in sporadic ACCs (32, 34, 40). The majority of patients with ACC and mutated *TP53* had a poor outcome (40).

Mutations of *TP53* usually result in loss of p53 protein function. Several studies evaluated p53 expression in ACTs, and it was demonstrated to be absent in the majority of ACAs (41, 42). Among ACCs, p53 expression has been found to be highly variable ranging between 5 and 52%, denoting the inadequacy of this marker to identify malignancy in ACT (41, 42, 43, 44, 45). Even in childhood ACCs, p53 expression demonstrated no prognostic significance (46).

p53 and MDM2 (mouse double minute 2) form an auto regulatory negative feedback loop allowing the maintenance of low cellular p53 levels in the absence of stress (47). p53 stimulates the expression of MDM2 and MDM2 inhibits p53 activity by promoting its degradation, blocking its transcriptional activity and promoting its nuclear export (47, 48). As already described, DNA damage promotes phosphorylation of p53 and MDM2, mediated by ATR and ATM and avoid MDM2-p53 interaction and thus stabilizing p53 (Fig. 2) (47). Protein p19^{ARF} also stabilizes p53 by inhibiting the nuclear export of MDM2 by tethering MDM2 in the nucleolus (49).

The amplification of the *MDM2* gene was observed in a minority of the human ACCs, as reported by several studies, including the pan-genomic studies (20, 21, 50, 51, 52, 53). Using the immunohistochemistry technique, MDM2 protein expression showed no differences between ACAs and ACCs (42, 45). The underexpression of *ATR* gene was also observed in ACCs and was associated with poor survival (27, 53). Mutations in *ATM* gene were also described in ACCs by some authors (50, 51).

Figure 2

Schematic representation of p53 regulation. p53 transcriptional activity is inhibited by MDM2 that promote p53 ubiquitination. DNA damage leads to p53 phosphorylation via ATM/ATR, preventing its association with MDM2. Besides that, p19^{ARF} also inhibits MDM2 preventing p53 ubiquitination. Stabilized p53 goes to the nucleus where it is able to transcriptionally upregulate genes involved in apoptosis and cell cycle arrest. Gene and protein alteration already described in the ACCs are indicated. A full colour version of this figure is available at <https://doi.org/10.1530/EJE-17-0976>.

CDK, cyclins and CDKi status in ACCs

Cell cycle progression from G1 to S phase is driven by the CDK family of serine/threonine kinases (CDK-2, -4 and -6), their regulatory partners, the cyclins (D and E) and their inhibitors, CDK inhibitors (CDKi) (p15, p16, p21, p27 and p57) (54, 55, 56).

In the pan-genomic studies (20, 21) and in other smaller studies, one of the most frequent DNA copy number changes in ACCs are gains in chromosome (chr)12, combined with the amplification of *CDK4* (located at chr12q14.1) and *CDK2* (located at chr12q13) (53, 57, 58, 59, 60). Reinforcing its importance, gains in chr12q13.2 have been associated with ACCs poor survival

rates (53). Overexpression of *CDK2* and *CDK4* has also been reported in ACC (61). At the protein levels, *CDK4* and *CDK2* overexpression was detected in the human ACC cell line (H295R) and in the majority of the ACCs (62). Schmitt *et al.* verified that all the ACCs studied presented *CDK4*-positive staining, but the same occurred in the majority of ACAs; however, ACAs had a very weak positivity when compared to ACCs (63).

Cyclin E is the regulatory subunit of the cyclin E-*CDK2* complex, and together they control the progression through G1 phase (23, 64, 65). Cyclin E dysregulation is often observed in several tumor cells; it is thought to be involved in the tumorigenesis process and to be an important prognosis marker for some tumors (64, 66, 67). There are two subtypes of cyclin E, the cyclin E1 and the cyclin E2, encoded by the genes: *CCNE1* at chr19q12, and *CCNE2* at chr8q22.1, respectively (65). *CCNE1* and *CCNE2* overexpression and amplification has been reported in ACCs by several authors, including in the recent pan-genomic studies (20, 61, 68, 69, 70, 71). Overexpression of the cyclin E protein was observed in ACCs and found to be significantly associated with the histologic grade and with shorter disease-free survival (62, 67, 71).

CDKi are negative regulators of cell cycle progression and potentially act as tumor suppressors, through the inhibition of the cyclin/CDK complexes. The CDKi, p57 is encoded by the *CDKN1C* gene located at chr11p15.5, where *IGF2* is also located (5, 72). Genetic rearrangements at the chr11p15.5 are common in ACCs, leading to the well-known phenomenon of *IGF2* overexpression (5, 73, 74, 75). The majority of ACCs and virilizing ACAs present low p57 and H19 expression and high *IGF2* expression (73). In contrast to normal adrenal glands and most ACA where the p57 mRNA is highly expressed, suggesting that p57 has a physiological role in normal adrenal cortex growth, the combination of low p57 and H19 expression and high *IGF-2* expression seems to be involved in ACT malignancy (73). Low expression of *CDKN1C* gene and p57 protein has been found in adult and childhood ACC. This was not due to mutations since no *CDKN1C* mutations were detected, suggesting that other mechanisms, such as abnormalities of imprinting or methylation, could be responsible for its low expression (8, 34, 62, 69, 76). Downregulation of p57 in ACCs was found to be associated with *CDK2* increased activity (62).

CDKN2A can encode, by alternative splicing, for p16, a *CDK4/6* inhibitor, and p14, the p53 stabilizer, whereas *CDKN2B* encodes another *CDK4/6* inhibitor, the p15 (50, 77). Alterations in both genes, namely the deletion

of *CDKN2A* and *CDKN2B*, were observed in 14.3% and 10.7% of the analyzed ACCs respectively. Allelic losses on chr9p21, where *CDKN2A* and *CDKN2B*, are harbored were described (20, 78).

p21 and p27 are the CDKis involved in the regulation of cyclin E-*CDK2* and cyclin D-*CDK4/6* complexes in the G1-S transition (Fig. 1) (79, 80). p21 is encoded by *CDKN1A* gene and its expression is regulated by p53, while p27 is encoded by *CDKN1B* gene, a gene that is rarely mutated in the context of cancer (79, 80, 81). The p21 protein expression was observed in both benign and malignant ACT, although a significantly higher proportion ACCs presented positive expression (42). Babinska *et al.* also found an increased expression of p21 in ACCs when compared with ACAs and a significant correlation between its expression and the occurrence of metastasis (82). Still, other studies did not find the significant differences between benign and malignant ACT, and there is evidence that its expression is inconsistent regardless of other molecular abnormalities, similarly to the p53 expression (34, 45). Nakazumi *et al.* observed p27 expression to be decreased in the ACCs when compared with ACAs (83). However, other authors have found opposite results, with increased p27 expression in ACCs when compared to ACAs (42, 45).

RB-E2F complex status in ACCs

In G1 phase, in order to allow the transition to S phase, CDK-cyclin complexes are responsible for the inactivation of the pRb through phosphorylation (84). Activated pRb binds to the transactivation domain of E2F to form a pRB-E2F complex that changes the chromatin structure at the E2F-responsive promoter by recruiting histone deacetylase to the pRB-E2F complex (Fig. 3) (84, 85). Then, this complex binds to the promoter of some genes such as DNA polymerase subunits, cyclin A and cyclin E, which are required for S phase entry, leading to the transcription repression of those genes, and cell cycle arrest in phase G1 (84, 86).

Upregulation of genes with binding sites for E2F seems to be a common event in many tumor types and has also been observed in ACCs (71, 87). Gains in the chr20, namely chr20q and chr20q11, where *E2F1* is harbored have been reported as a common event in ACCs (57, 58, 60). However, other studies, including the pan-genomic studies, have not reported gains in chr20 as a common occurrence in ACCs (20, 21, 88, 89).

Inactivating mutations, deletions and allelic losses of *RB* gene were observed in several tumors and seem to

Figure 3

Schematic representation of Rb regulation.

Hyperphosphorylation of Rb by the CDK/cyclin complexes prevents the binding of RB to the transcription factor E2F, allowing the transcription of genes required for S phase entry. Gene and protein alteration already described in the ACCs are indicated. A full colour version of this figure is available at <https://doi.org/10.1530/EJE-17-0976>.

be associated with an increase in cancer susceptibility (84, 90). Ragazzon *et al.* found that the loss of pRB was exclusively found in the subgroup of aggressive tumors, suggesting that the *RB1* has an important role in the last events of the ACC and could be used as a prognostic marker (91). Among the ACCs with pRB loss, the majority presented an allelic loss at the *RB1* locus (91). de Fraipont *et al.* confirmed that mRNA expression of *RB1* was reduced in the malignant ACTs (92). Gupta *et al.* also reported differences in the pRB1 staining between benign and malignant ACTs, whereas among the ACTs studied by Vargas *et al.*, both benign and malignant ACTs, were positive for pRB1, with no differences between these two groups (93, 94).

c-Myc is a pivotal regulator of the cell cycle being able to activate and repress pathways affecting G1-to-S phase progression in mammalian cells. c-Myc overexpression leads to the loss of CDK inhibition resulting in the inactivation of pRb through phosphorylation, release of E2F and cell cycle progression to S phase. Furthermore, c-Myc induces the activity of E2F, directly and

subsequently activates transcriptional of DNA synthetic enzymes (54, 95).

Amplification of protooncogene *c-myc* is frequently observed in a wide variety of human neoplasms through a variety of mechanisms (95, 96). In contrast to the observation in the majority of other tumors, several studies revealed that *c-myc* is underexpressed in ACCs compared to ACAs and to the normal adrenal cortex (69, 70, 71, 97, 98). The location of c-myc protein has also been correlated with ACT malignancy, since ACCs express c-myc both in the cell cytoplasm and nuclei while ACAs only express it in the cell nuclei (99). Since overexpression of c-myc induces cell proliferation and ACCs are rapidly proliferating tumors, c-myc was expected to be overexpressed in ACCs; however, the opposite was found to occur in this type of cancer (95, 97).

G2-to-M phase transition in ACCs

In ACCs, alterations in the G2-to-M transition are less frequently reported than changes in the G1 to S. In addition, these changes were mostly reported in isolated studies and rarely in an integrated approach.

The most important role of the G2 phase is to ensure that the chromosomes have been accurately replicated without mistakes or damages, in order to allow the cycle to progress to mitosis (100).

G2-to-M transition is mainly regulated by the CDK, Cdc2, also known as CDK1 (101, 102). Cdc2 is able to form a complex with either cyclin B or cyclin A (Fig. 1) (101, 103).

Cdc2 is activated by a combination of some required steps: (1) the phosphorylation of Thr¹⁶¹ by the Cdk-activating kinase (CAK) in order to open the catalytic region of Cdc2; (2) the nuclear translocation of Cdc2/cyclin B1 complex by the polo-like kinase 1 (Plk1) phosphorylation of Ser¹⁴⁷ on Cdc2 and (3) the dephosphorylation of Thr¹⁴ and Tyr¹⁵ by the division 25 (Cdc25) phosphatase family, Cdc25A, Cdc25B and Cdc25C (104, 105, 106, 107).

Activation of Cdc25C is achieved by phosphorylation of Ser¹⁹⁸ by Plk1, leading to nuclear export of the Cdc25C (107). After that, Cdc25C is hyperphosphorylated by the complex Cdc2–cyclinB1, leading to a positive feedback loop, increasing the Cdc2–cyclinB1 activity (Fig. 4A) (104).

Cdc25 is a dual-specificity phosphatase that not only activates the complexes cyclin B-Cdc2, but also the cyclin A-CDK2 and cyclin E-CDK2 at key cell cycle transitions: the isoform Cdc25A regulates the G1/S transition, whereas

Figure 4

Schematic representation of CDC2/cyclin B regulation. In unstressed cells, Plk1 phosphorylates Cdc25C, Wee1 and Myt1, leading to Cdc2/cyclin B activation and entry in the mitosis (A). In the presence of DNA damage, Chk1/2 are phosphorylated via ATM/ATR and are able to induce Cdc25C nuclear exclusion and to activate Wee1, leading to the inactivation of Cdc2/cyclin B and cell cycle arrest (B). Gene and protein alteration already described in the ACCs are indicated. A full colour version of this figure is available at <https://doi.org/10.1530/EJE-17-0976>.

the isoforms Cdc25B and Cdc25C act at G2/M, controlling the entry into mitosis (108, 109). Inactivation of Cdc25C is reached through phosphorylation of Ser²¹⁶, creating a binding site for the 14-3-3 protein. Then the Cdc25C goes to the cytoplasm preventing Cdc25C and Cdc2 interaction (Fig. 4B) (104, 110). This phosphorylation is mediated by Chk1, Chk2, C-TAK1 and Plk3 and also through the complexes cyclin B-cdc2 or cyclin A-cdc2 formation (104).

Deregulation of Cdc25 isoforms expression and activity, leading to unrestrained proliferation, has been found in some tumors (108, 109). In ACCs, CDC25 isoforms gene overexpression have been described (27, 69, 70), in parallel with the gain-of-chromosome region ch5q31.2, where CDC25C is harbored (111, 112). The overexpression of CDC2 was also reported in ACCs, by several studies, with a higher expression in the more aggressive cases (68, 69, 70, 71, 113). Overexpression of Plk1, the kinase responsible for Cdc2/cyclin B1 complex nuclear translocation and Cdc25c phosphorylation, has

been associated with tumor development and considered a possible prognostic marker for some tumors (114, 115, 116, 117, 118, 119). Furthermore, Plk1 inhibitors are in preliminary tests for tumor treatment (120, 121, 122). Overexpression of *PLK1* has been found in ACCs when compared with the non-functioning ACAs, cortisol-producing ACAs and with the normal adrenal glands (69). Plk1 inhibitors, such as the small-molecule BI-2536, have been tested in ACC cell lines, SW-13 and H295R, and found to significantly reduce the tumor cell growth, suggesting that Plk1 inhibitors deserve to be further investigated as a potential therapeutic approach in ACCs (123, 124).

Inactivation of the Cdc2–cyclin B complexes, responsible for mitosis initiation is a pre-requisite to exit mitosis (Fig. 1) (101, 102). Cyclin B presents various isoforms: Cyclin B1 that is encoded by *CCNB1*, cyclin B2 that is encoded by *CCNB2* and cyclin B3, that is the less well-characterized cyclin B, encoded by *CCNB3* (125, 126). Overexpression of *CCNB1* or the correspondent chromosome gains were described in ACCs comparing to ACAs and normal adrenal glands (27, 69, 70, 113, 127). Cyclin B1 protein expression was also found to be increased in ACCs but in spite of a high specificity (100%), it had a low sensitivity (43%) for predicting malignancy. In consequence, cyclin B1 is only useful for confirming malignancy but is not helpful for its exclusion when it is negative (113).

CCNB2 overexpression was also found in ACCs, particularly in the more aggressive forms. However, the correspondent chromosomal alterations were not observed (27, 69, 70, 127).

The Myelin Transcription Factor 1 (Myt1) and Wee1 are proteins able to inhibit the Cdc2, through inhibitory phosphorylation at Thr¹⁴ and Tyr¹⁵ (Fig. 4) (100, 109, 128, 129). Once activated, cyclin B–Cdc2 complexes phosphorylate Wee1 and Myt1 to promote their inactivation, allowing an even larger amplification of Cdc2 activation (106). Wee1 is predominantly a nuclear protein that has been found to associate with centrosomes, whereas Myt1 is present in the cytoplasm bound to membrane structures (128, 130). *WEE1* gene overexpression has been found in ACCs when compared with ACAs and normal adrenal glands (68), but there was no *MYT1* gene overexpression in ACCs (69).

Topoisomerases (TOPs) are enzymes that are involved in the biological processes that require strand unwinding, such as replication, transcription and maintenance of genome stability, as they are able to introduce transient breaks in DNA (131, 132). The TOP1 introduces single-strand breaks in the DNA, and TOP2 introduces double-strand

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breaks. Type II TOPs are required to segregate replicated chromosomes (131, 133). TOP2A, is a TOP2 isoform, present only in the S, G2 and M phases of the cell cycle of proliferating tissues (131, 133). Several authors confirmed TOP2A overexpression in ACCs (61, 68, 69, 70, 71, 127), which was associated with higher ACC aggressiveness (70). TOP2A protein overexpression was associated with significantly poorer overall and disease-free survival (93, 134, 135, 136). Iino *et al.* have also suggested that TOP2A could be an even better proliferation marker than Ki-67, since some cells expressing TOP2A failed to express Ki-67 (134). Chromosomal gains at chr17, where TOP2A is harbored, were also found in ACAs, suggesting that it may be an early event in the tumorigenesis of ACT (60).

Clinical implications for the diagnosis and treatment of ACCs

Considerable advances in the understanding of adrenocortical neoplasia molecular pathology have occurred over the recent years. In spite of this, ACC continues to have a poor prognosis, as they are frequently diagnosed in advanced disease stages and harbor molecular alterations responsible for an aggressive biological behavior. Thus, much efforts are required to identify the main drivers in ACC pathogenesis in order to design the appropriate treatments that are still missing. Nonetheless, recent molecular studies allowed identification of two subsets of ACC tumors with distinctive cell proliferation rates and clinical prognosis (20, 21, 70). Tumors with worst prognosis depict a high histological grade and present a greater frequency of gene mutations, DNA methylation and a specific miRNA expression profile involving cell cycle and proliferation; more differentiated tumors tend to harbor metabolism instead of cell cycle-related molecular alterations and have improved clinical outcomes, as evidenced by higher survival rates and lower ENSAT stage (20). There is, however, some overlap between the two tumor categories highlighting the need to pursue the distinctive mechanisms.

The morphological characteristics and the initial molecular studies in ACC supported the hypothesis that proliferation was the most important feature leading to tumor development and dissemination. Genomic analysis has allowed to identify particular gene subsets that are overexpressed while others are under-expressed, whereas more innovative investigations focusing on epigenetic analysis and microRNAs have highlighted additional determinants of tumor biological behavior. Concerning

the cell cycle alterations, overexpression of the cell-cycle-related genes can distinguish the ACC with good and poor prognosis, the last group being the one with an increased expression; in contrast, the adenomas rarely presented those types of alterations and thus those features are possible predictors of malignancy and prognosis (137, 138).

One of the most frequent alterations found in the ACC is LOH in chr11p15, where CDKN1C and IGF2 are localized. IGF2 expression has been demonstrated to be a useful marker for predicting malignancy after ACT surgical removal (139, 140). The influence of IGF2 overexpression in the ACC tumorigenesis is well established and reasonably understood. In contrast, CDKN1C under expression is frequently found in ACCs, but its role is not yet fully understood (73, 139). Besides its relevance as a diagnostic tool, these alterations have also raised the expectation of being possible treatment targets. Indeed, the use of IGF1-R inhibitors for ACC was tested in several clinical trials but so far failed to improve significantly patients' progression-free survival and overall survival (141, 142, 143). Subsequently, as IGF1-R is a well-known mTOR pathway regulator, the combined use of simultaneous IGF1R and mTOR inhibitors to reduce ACC growth was tested. However, the only observable effect was disease stabilization for at least 6 months in 42% of the patients (144).

TOP2A, whose protein expression is associated with poor overall and disease-free survival (93, 134, 135, 136) is particularly interesting. In ACC, TOP2A seems to be a better proliferation marker even when compared to Ki-67, as some TOP2A-expressing cells fail to express Ki-67 (134). Roca *et al.* evaluated the TOP2A gene and protein expression in a large series of 98 ACC patients and showed that a high TOP2A expression in ACC tumor is significantly associated with higher response to the combined chemotherapy EDP plus mitotane (EDP-M) and with a longer time to tumor progression. These results suggest that TOP2A could also have a potential clinical relevance as a predictor for the EDP-M treatment response (145). TOP2A inhibition has already been tested in *in vitro* and in clinical trials with some promising results (135). *In vitro* anti-cancer activity of 14 different agents targeting TOP2A was evaluated. Among these agents the one that demonstrated to have the highest anticancer activity was aclarubicin, and this compound will probably be tested in future clinical trials for the treatment of locally advanced and metastatic ACC (135). Moreover, clinical trials including etoposide and doxorubicin, TOP2 enzyme inhibitors, in combination

with cisplatin (EDP) used in advanced ACCs resulted in a better progression-free survival when compared to the mitotane and streptozotocin combination (NCT00094497) (5, 146, 147).

PLK1 regulates multiple steps of cell division and DNA stability/repair. PLK1 expression levels are positively correlated with a poor survival in ACC patients, suggesting PLK1 as a good prognostic marker that could also be a good candidate for targeted therapy (124, 148). TKM-080301 is a lipid nanoparticle formulation of a siRNA targeting PLK1. Eight ACC patients (NCT01262235) received two cycles of the treatment and four patients showed a good response in stabilizing disease including a 13% reduction of tumor size in one patient, thus suggesting that the role of PLK1 inhibition for ACC treatment should be further investigated (149).

The fact that the completed clinical trials produced insufficient outcomes highlights that there is still much to be learned about the molecular pathology of these tumors. Thus, in addition to previous and ongoing clinical trials, other pharmacological targets based on the recent molecular findings related to the cell cycle alterations found in ACC are likely to start being tested in the near future. These include CDK inhibitors, drugs targeting p53 reactivation and CDC25 inhibitors.

The CDK/cyclin complexes have a critical role in the regulation of cell cycle transition that if disrupted can ultimately lead to uncontrolled proliferation. Thus, these are certainly molecular attractive targets for cancer treatment (25). The overexpression of CDK4/6/2 and 1, as well as the overexpression of cyclins involved in the G1-S transition (CCNE1/2) and in the G2-M (CCNB1/2) were recurrently observed in ACC (53, 57, 58, 59, 60, 68, 69, 70, 71, 113). CDK inhibitors are being tested for several advanced solid tumors, but there are no registered clinical trials aiming to assess their efficacy in ACCs. Besides, despite the results of the initial clinical trials using CDK inhibitors being disappointing, the recent use of highly selective CDK inhibitors, specifically targeting CDK4 and CDK6, combined with patient stratification, showed a more substantial and promising clinical activity (25). Indeed, a small molecule that is a selective CDK4/6 inhibitor (SHR6390) is in clinical trial for advanced solid tumors, currently in a recruiting phase and thus could represent an opportunity to include patients with ACCs (NCT02684266).

TP53 was recognized as one of ACC driver genes by the pan-genomic studies (20, 21). Inactivating mutations were found to be present in 21.3% of the ACCs (20, 21). So, the recovery of p53 'tumor suppressor gene' function,

Table 1 G1/S phase regulators altered in adrenocortical carcinoma (ACC) compared with adrenocortical adenoma (ACA).

	ACC	ACA	Ref.
CDK4	↑ 64.7% cases were strongly positive	↓ 13.6% cases were strongly positive	(63) ^a
Cyclin E	↑ 2-fold	↓ 2-fold	(71) ^a
	↑ Labeling index median of 15%	↓ Labeling index median of 0%	(67) ^a
<i>CCNE1</i>	↑ 10.4-fold	↓ 10.4-fold	(68) ^b
	↑ 3.56-fold	↓ 3.56-fold	(70) ^b
Cyclin D1	↑ 1.27% of stained area	↓ 0.1% of stained area	(45) ^a
	= 5.4% of positive cases	= 0% of positive cases	(42) ^a
p53	↑ 59% of positive cases	↓ 0% of positive cases	(41) ^a
	↑ 52.4% of positive cases	↓ 11.8% of positive cases	(153) ^a
	= 5.4% of positive cases	= 0% of positive cases	(42) ^a
	= 7.39% of stained area	= 2.99% of stained area	(45) ^a
<i>CDKN1C</i>	↓ 26.3% of positive cases	↑ 100.0% of positive cases	(62) ^c
p21	↑ 69.4% of positive cases	↓ 36.4% of positive cases	(42) ^a
	= 1.59% of stained area	= 1.25% of stained area	(45) ^a
p27	↓ Labeling index of 48.9%	↑ Labeling index of 59.4%	(83) ^a
	↑ 94.4% of positive cases	↓ 68.8% of positive cases	(42) ^a
	↑ 9.37% of stained area	↓ 3.89% of stained area	(45) ^a
<i>CDKN3</i>	↑ 6.84-fold	↓ 6.84-fold	(68) ^b
	↑ 5.28-fold	↓ 5.28-fold	(70) ^b
	↑ Higher than 5-fold	↓ Lower than 5-fold	(71) ^b
	↑ 2.69-fold	↓ 2.69-fold	(113) ^b
pRB1	= 100% of positive cases	= 100% of positive cases	(94) ^a
	↓ 13.3% of cases with abundant staining	↑ 73.3% of cases with abundant staining	(93) ^a

^aImmunohistochemistry analysis; ^bgenome microarray analysis; ^cNorthern Blot analysis.

↑, higher; ↓, lower; =, similar.

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using MDM2 antagonist (such as RO5503781) and the reactivation of mutant *TP53*, using PRIMA-1^{MET}, already tested for other malignancies (150), also have the potential to be used in ACCs with *TP53* inactivated.

Altered expression of CDC25 gene isoforms, repeatedly described in ACCs, could represent a potential treatment target for these tumors (27, 69, 70). However, despite the fact that several natural and synthetic molecules with distinct structural features targeting CDC25 with good pre-clinical results have been identified (151, 152), no registered clinical trials using CDC25 inhibitors for ACC are ongoing.

Finally, in addition to the specific molecules that were identified as potential targets for ACC treatment, there are a few other molecules that despite not having yet been considered for their therapeutic potential, will eventually prove very valuable as new diagnostic and prognostic molecular tools in the near future (Tables 1, 2 and 3).

Table 2 G2/M phase regulators altered in adrenocortical carcinoma (ACC) compared with adrenocortical adenoma (ACA).

	ACC	ACA	Ref.
CDC2	↑ 7.70-fold	↓ 7.70-fold	(68) ^b
	↑ Higher than 5-fold	↓ Lower than 5-fold	(71) ^b
	↑	↓	(69) ^b
	↑ 3.76-fold	↓ 3.76-fold	(113) ^b
	↑ 5.85-fold	↓ 5.85-fold	(70) ^b
Cyclin B1	↑ 43% of positive cases	↓ 0% of positive cases	(113) ^a
CCNB1	↑ Higher than 8-fold	↓ Lower than 8-fold	(127) ^b
	↑ 7.05-fold	↓ 7.05-fold	(70) ^b
	↑	↓	(69) ^b
CCNB2	↑ 2.88-fold	↓ 2.88-fold	(113) ^b
	↑ 8.88-fold	↓ 8.88-fold	(68) ^b
	↑ Higher than 8-fold	↓ Lower than 8-fold	(127) ^b
	↑ 5.61-fold	↓ 5.61-fold	(70) ^b
	↑ Higher than 14-fold	↓ Lower than 14-fold	(69) ^b
CDC25C	↑ 2.22-fold	↓ 2.22-fold	(70) ^b
	↑	↓	(69) ^b
TOP2A	↑ Labeling index mean of 6.13	↓ Labeling index mean of 0.72	(134) ^a
	↑ Labeling index mean of 37.5	↓ Labeling index mean of 1.4	(93) ^a
	↑	↓	(135) ^a
TOP2A	↑	↓	(136) ^a
	↑ 6.86-fold	↓ 6.86-fold	(68) ^b
	↑ Higher than 5-fold	↓ Lower than 5-fold	(71) ^b
	↑ 3.54-fold	↓ 3.54-fold	(70) ^b
	↑ Higher than 9-fold	↓ Lower than 9-fold	(69) ^b
	↑	↓	(135) ^c

^aImmunohistochemistry analysis; ^bgenome microarray analysis; ^creal-time quantitative RT-PCR.

↑, higher; ↓, lower; =, similar.

Table 3 Relevant cell cycle regulator to define clinical prognosis.

Possible prognostic markers	Study
pRB1 loss	(91)
<i>RB1</i> mutations	(20, 21)
<i>chr17p13</i> LOH	(20, 21, 34, 41)
<i>CCNE1</i> amplification	(28)
Cyclin E overexpression	(62)
<i>CDK4</i> amplification	(20, 21)
<i>CDKN2A</i> deletions	(20, 21)
<i>CCNB2</i> overexpression	(27, 69, 70, 127)
<i>TOP2A</i> overexpression	(93, 134, 135, 136)

Results from the pan-genomic studies Zheng *et al.* and Assié *et al.* are highlighted (20, 21).

Conclusion

Our aim was to deliver an integrative review of what has been reported regarding cell cycle disruptions in ACC, so that this comprehensive and integrated knowledge could then be used to optimize disease diagnosis and treatment.

The rarity of the ACC represents an important limitation to accomplish a complete molecular characterization of cell cycle alterations. Despite this limitation, collaborative studies focusing in a wide array of molecular pathways have been performed. The results of these studies are summarized in this review, highlighting the complexity of the molecular alterations that may contribute for an aggressive biological behavior in ACC.

The molecular mechanisms underlying the traditional pathological features have already proved to be useful molecular markers for differential diagnosis and tumor stratification and prognosis. In addition, targeting the mechanisms leading to uncontrolled cell proliferation may have a much required potential for ACC treatment. From a clinical perspective, these have already led to the implementation of targeted treatments aiming to be used alone or in combination with the classical adrenolytic drug mitotane. However, we must state that some genomic alterations were analyzed in a non-functional perspective and so up until now their functional impact is most of the times, unknown.

In conclusion, the main goal for the future should be translating what was learned, and summarized in this review, together with the comprehensive information resulting from the pan-genomic studies into better tools to identify and evaluate ACC, as the final objective to develop specific medical therapy personalized for each patient to improve their disease-free intervals and survival.

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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