REVIEW

Apoptosis regulation in adrenocortical carcinoma

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Abstract

Apoptosis evading is a hallmark of cancer. Tumor cells are characterized by having an impaired apoptosis signaling, a fact that deregulates the balance between cell death and survival, leading to tumor development, invasion and resistance to treatment. In general, patients with adrenocortical carcinomas (ACC) have an extremely bad prognosis, which is related to disease progression and significant resistance to treatments. In this report, we performed an integrative review about the disruption of apoptosis in ACC that may underlie the characteristic poor prognosis in these patients. Although the apoptosis has been scarcely studied in ACC, the majority of the deregulation phenomena already described are anti-apoptotic. Most importantly, in a near future, targeting apoptosis modulation in ACC patients may become a promising therapeutic.

Key Words

- adrenocortical tumors
- adrenocortical carcinomas
- apoptosis
- molecular deregulations

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Adrenocortical tumors

Adrenal cortex tumors (ACT) are common tumors with a reported prevalence above 4% in most populations (1). However, the majority of ACT are benign, non-functioning and incidentally discovered during imaging studies performed for unrelated clinical reasons (1, 2). On the other side, adrenocortical carcinomas (ACC) are rare but usually have an aggressive behavior and a poor prognosis (1, 3, 4, 5, 6). According to the 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 (4). This dismal clinical outcomes of ACC patients is related to the diagnosis at an advanced clinical stage and because there is no effective adjuvant or neoadjuvant therapy for late-stage diagnosed patients (7). Only few targeted therapies based on the advances in the knowledge of adrenal tumor pathophysiology were developed and up until now they generally failed in clinical settings. One of the main reasons associated to adrenal tumor cells resistance to chemotherapy is compromised apoptosis signaling, a fact that deregulates the balance between cell death and survival, thus facilitating tumor development, invasion and resistance to treatment (8, 9, 10, 11). In the following section, we will briefly review the regulation of apoptosis and then explore the abnormalities in apoptosis in ACC that have been reported to date.

Caspases alterations

There are two main apoptosis activation pathways: the extrinsic one that involves activation of cell death receptors and the intrinsic cascade. The latter is also known as the mitochondrial pathway, as it involves the permeation of the mitochondrial outer membrane followed by the release of apoptotic intervenients (12, 13). Both apoptosis pathways converge into caspase activation and cellular disintegration (12).

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Table 1 Classification of caspases according to the point of entry into the apoptosis cascade.

Initiator caspases		**Executioner caspases**	**Inflammatory caspases**
Extrinsic pathway	Intrinsic pathway		
Caspase 8	Caspase 2 ↑	Caspase 3 ↓	Caspase 1
Caspase 10	Caspase 9 ↓	Caspase 6	Caspase 4
		Caspase 7	Caspase 5
			Caspase 12

↓ represents the caspases that are underexpressed in adult ACC,
↑ represents the overexpression caspases.

Caspases are a family of cysteine proteases that play a crucial role at apoptosis (14, 15). Caspases can be classified as initiators or executioners, according to the point of entry into the apoptosis cascade (Table 1) (15). Initiator caspases such as caspase-8 and caspase-10 are characterized by containing a death effector domain (DED), involved in the extrinsic apoptosis pathway. Alternatively, the caspases involved in the intrinsic apoptosis pathway such as caspase-2 and caspase-9 contain a caspase recruitment domain (CARD). These caspases are able to cleave and activate executioner caspases: caspase-3, caspase-6 and caspase-7. A third group of caspases including caspase-1, caspase-4, caspase-5 and caspase-12 participate in innate immune responses rather than in the apoptotic cascade (16).

Initiator caspases are produced as inactive monomeric procaspases and are activated by dimerization via the designated 'induced proximity model' (16, 17). The executioner caspases are also produced as inactive procaspases and they require cleavage by the initiator caspases in order to be activated (16, 18). Executioner caspases, when activated are able to cleave and activate Rho-associated protein kinase (ROCK-1) and p21-activated kinase 2 (PAK-2), by removing the inhibitory domain. Both, ROCK-1 and PAK-2 induce myosin regulatory light-chain (MLC) phosphorylation that results in cell shrinkage and cell membrane blebbing (19, 20). In addition, executioner caspases cleave DNA fragmentation factors (DFF) in two fragments: the DFF40 and its inhibitor DFF45. This cleavage allows DFF40 translocation into the cell nucleus, which induces DNA double-strand breaks resulting in DNA fragmentation (Fig. 1) (21, 22).

In ACC occurring in adulthood, the expression of genes that encode initiator caspases (caspase-2 and caspase-9) involved in the intrinsic apoptosis pathway was found to be altered (Table 1) (23). The expression of *CASP9* was decreased in ACC while the *CASP2* was increased when compared with adrenocortical adenomas (ACAs) (23). The executioner *CASP3* expression was found to be decreased in ACC compared with ACAs as well as with normal adrenal glands. On the contrary, in ACT occurring in childhood, the caspases *CASP3*, *CASP8* and *CASP9* have been studied and their expression was not found to be different in malignant when compared to benign ACT (24). Concerning prognostic differences in children with ACC, a low *CASP3* expression was found

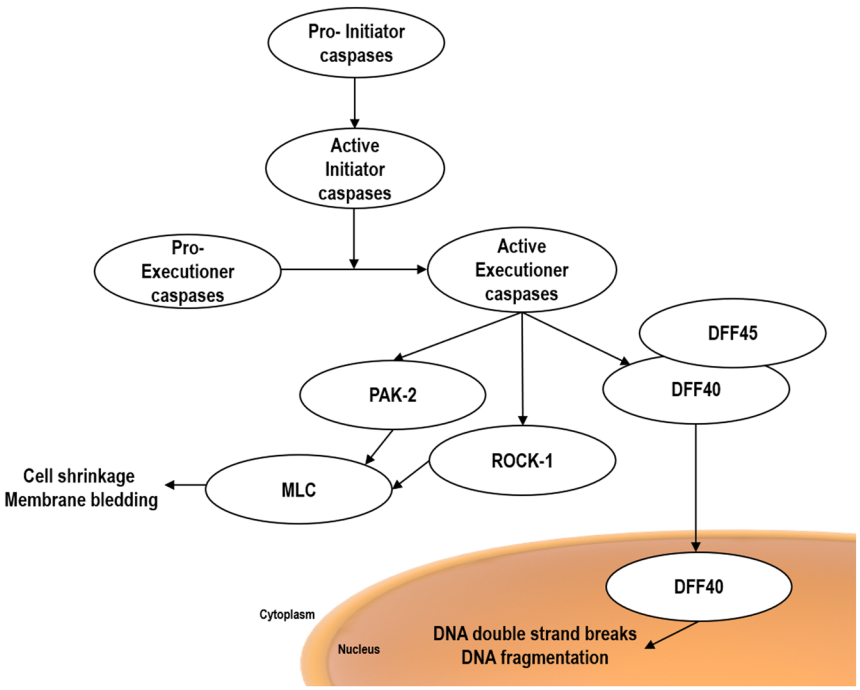

Figure 1
Schematic representation of apoptosis regulated by caspases. After activation of the initiator caspases, they activate executioner caspases by cleavage. Executioner caspases, when activated cleave and activate ROCK-1, PAK-2 and DFF40/45 leading to cell shrinkage, membrane blebbing and DNA fragmentation.

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to be associated with a lower 5-year event-free survival, while low levels of *CASP9* were associated with a higher 5-year event-free survival (24). Also, a significant negative correlation between *CASP3* expression and tumor size was found in adults with ACC.

Extrinsic apoptosis signaling pathway alterations

The apoptosis extrinsic pathway is triggered by extracellular ligands that activate the death receptors located in the plasma membrane. These receptors possess a cytosolic death domain (12, 25, 26).

The most well-known ligands and their respective death receptors are fatty acid synthetase ligand (FasL)/fatty acid synthetase receptor (Fas); TNF-related apoptosis-inducing ligand (TRAIL)/death receptor 4 and 5 (DR4 and DR5) and tumor necrosis factor α (TNF-α)/tumor necrosis factor receptor 1 (TNF1-R) (12, 25, 26).

FasL binding to Fas, triggers the aggregation of Fas trimers and the recruitment of the death domain-containing protein (FADD) that binds to Fas death domain (Fig. 2) (12, 27). Besides, FADD presents a DED domain that allows initiator caspases binding, such as pro-caspase 8, creating a death-inducing signaling complex (DISC) (12, 26). DISC formation then causes the cleavage of pro-caspase 8 into active caspase 8. The activation of initiator caspases can then cleave and activate executioner caspases (13, 16). Moreover, caspase 8 also cleaves and activates BH3 interacting domain death agonist (Bid) that releases mitochondrial cytochrome c, which can also activate caspase 3 (28).

The activation of DR4 and DR5 by TRAIL triggers a similar pathway as the one described for Fas-induced apoptosis with the recruitment FADD and pro-caspase 8 to form DISC (Fig. 2) (12, 13).

Fas expression was found to be reduced and FasL expression increased in ACC when compared with ACA and normal adrenal glands (29). Another group reported that *Fas* gene expression was absent in all analyzed ACC and present in ACA and normal adrenal glands (30). On the other side, soluble Fas antigen (sFas) plasma levels were also observed to be higher in patients with ACT than in healthy blood donors. sFas plasma levels were particularly higher in patients with aldosterone producing adenomas. In addition, sFas plasma levels were positively correlated with the ACC size (31, 32). In childhood ACT, *Fas* gene expression was lower in ACT when compared with normal adrenal glands, but no differences were observed between ACC and ACA (24).

TNFα binding to the extracellular domain of TNF1-R leads to the binding of the TNF receptor-associated death domain (TRADD) adaptor protein to the intracellular domain of TNF1-R, resulting in the recruitment of the receptor interacting protein (RIP), FADD and TNF-R-associated factor (TRAF). FADD recruitment then leads into the signaling apoptosis pathway, while RIP activates the nuclear factor of κB (NF-κB) through stimulation of the inhibitor of κB-kinase (IKK) and TRAF activates the JNK pathway stimulating the transcription factor, activating protein-1 (AP-1) (Fig. 2) (12, 25, 26).

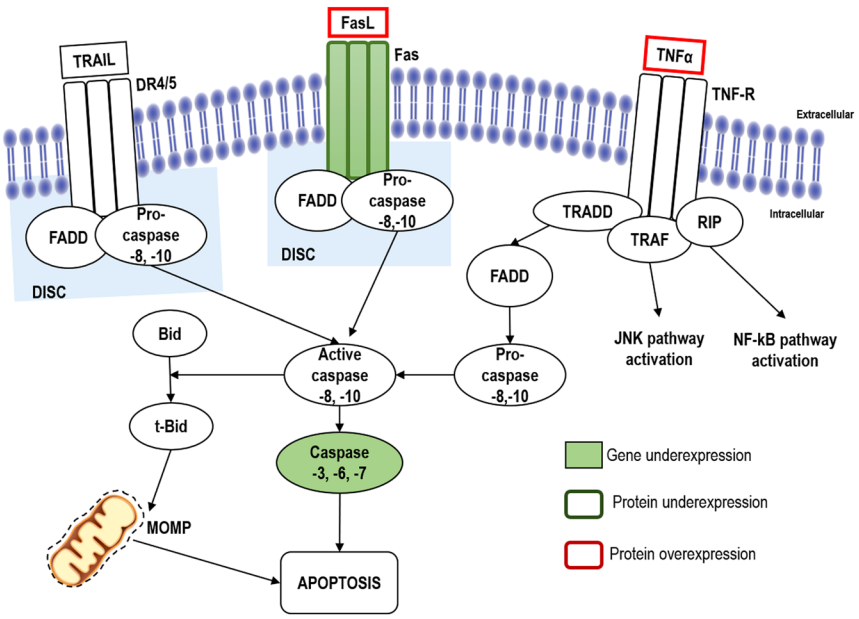

Figure 2
Schematic representation of extrinsic apoptotic pathway. Stimulation of death receptors of the TNF-R, Fas and DR4/5 by their respective ligands, results in receptor aggregation and recruitment of FADD and caspase-8 and caspase-10. These caspases become activated and cleaves the executioner caspases-3, caspase-6 and caspase-7, leading to apoptosis. Abnormalities in mRNA and in protein expression alterations already described in adrenocortical carcinomas are highlighted in solid and open squares, respectively.

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Although *TNF* gene expression was not found to be altered in ACC, the tumor necrosis factor alpha-induced protein 3 (*TNFAIP3*), a ubiquitin-modifying enzyme that negatively regulates TNF response, was found to be overexpressed in ACC when compared to the normal adrenal gland. Moreover, high *TNFAIP3* expression was significantly associated with poor overall survival of ACC patients (33). *TNFRSF19*, a gene that encodes a member of the TNF receptor superfamily, was also found to be overexpressed in ACC and it was associated with poor prognosis in another study (23).

TNFα serum levels were significantly higher in patients with ACT before adrenalectomy when compared to healthy subjects. TNFα serum levels were particularly higher in patients with ACC and aldosteronomas. In contrast, soluble TNF receptors (TNF1-R and TNF2-R) serum levels were similar in ACT patients and in healthy subjects. Following adrenalectomy, TNFα levels decreased in patients with ACC, non-functioning ACA and in patients with aldosteronomas. On the other hand, reduction of TNF1-R and TNF2-R serum levels was observed only in patients with unilateral aldosteronomas (34).

TNF expression was significantly lower in childhood ACC when compared with ACA and associated with a lower 5-year event-free survival (24). In these pediatric patients, a strong and moderate immunoreactivity for TNF-α protein expression was associated with a higher 5-year event-free and overall survival (24).

Tumor necrosis factor receptor-associated factor 4 (TRAF4), a mediator of the TNF-induced signaling pathway, is able to inhibit the Fas-induced apoptosis (35). *TRAF4* overexpression was associated with poor prognosis in patients with ACC (23).

Intrinsic apoptosis pathway signaling alterations

The intrinsic apoptosis pathway is triggered by intracellular stimuli that include DNA damage, absence of growth factors, oxidative stress and endoplasmic reticulum stress (13). These stimuli lead to mitochondrial outer membrane permeation (MOMP) resulting in the release of pro-apoptotic factors such as cytochrome c and other intermembrane space proteins such as Smac/DIABLO, HtrA2/Omi, apoptosis-inducing factor (AIF) and Endonuclease G (EndoG) (Fig. 3). Cytochrome c binds to the apoptotic protease-activating factor-1 (APAF-1) inducing its oligomerization, a conformational change that leads to apoptosome formation. The apoptosome then binds and activates pro-caspase 9 to caspase 9, which is then able to activate the executioner caspases 3, 6 and 7 (12, 36, 37). Smac/DIABLO and HtrA2/Omi potentiate caspase activation, since they are antagonists of the inhibitors of apoptosis proteins (IAPs). IAPs are able to inhibit activated executioner caspases stopping the caspase-dependent apoptosis (26, 38, 39). After being released from mitochondria, caspase-independent cell death effectors AIF and EndoG translocate to the nucleus and trigger nuclear condensation and DNA fragmentation (40, 41).

MOMP is regulated by the members of the Bcl-2 family (26, 42, 43). There are three types of Bcl-2 family proteins based in their apoptotic function and Bcl-2 homology domains (BH1, 2, 3 and 4) (42): (1) the anti-apoptotic proteins that contain 3 or 4 BH domains, like Bcl-2, Bcl-x_L and Bcl-w; (2) the pro-apoptotic proteins that contain 2 or 3 BH domains, like Bax, Bak and Bok (3) the BH3-only proteins that are also pro-apoptotic but have only the BH3 domain, like Bad, Bik, Bid, Noxa and Puma (26, 44, 45, 46, 47, 48, 49, 50, 51). Anti-apoptotic proteins are able to inhibit MOMP by sequestering BH3-only proteins and/or Bax/Bak (43, 52, 53). MOMP only occurs when the anti-apoptotic Bcl-2 family proteins are inhibited and Bax or Bak are activated by the activator BH3-only proteins thus inducing Bax/Bak oligomerization (43, 53).

BCL2 expression levels were found to be higher in ACC when compared with non-functioning ACA, while the *Bax* gene expression was found to be similar among all the different ACT analyzed by Ando *et al.* (54). This finding was not confirmed in subsequent studies where *BCL2* and *BCLXL* gene expression was similar among ACC, ACA and normal adrenal glands (30, 55). *Bax* gene expression was absent in ACC, implying a reduction in apoptosis, whereas it was always present in ACA and normal adrenal glands (55). In another study, the *BCL2L12* gene that encodes another anti-apoptotic Bcl-2 family protein was increased in the ACC, while the *Bok* gene that encodes a pro-apoptotic Bcl-2 family protein were described to be increased in ACC with poor prognosis (23).

In pediatric patients with ACT, *BCL2* gene expression was similar in ACC, ACA and normal adrenal glands. However, lower *BCL2* gene and protein expression were associated with poor prognosis in these pediatric patients with ACC (24). In contrast, another study in pediatric patients did not find an association between Bcl-2 protein expression and prognosis (56).

In adult patients, Bcl-2 protein expression was found to be similarly increased in ACC and non-functioning ACA when compared with normal adrenal glands in a

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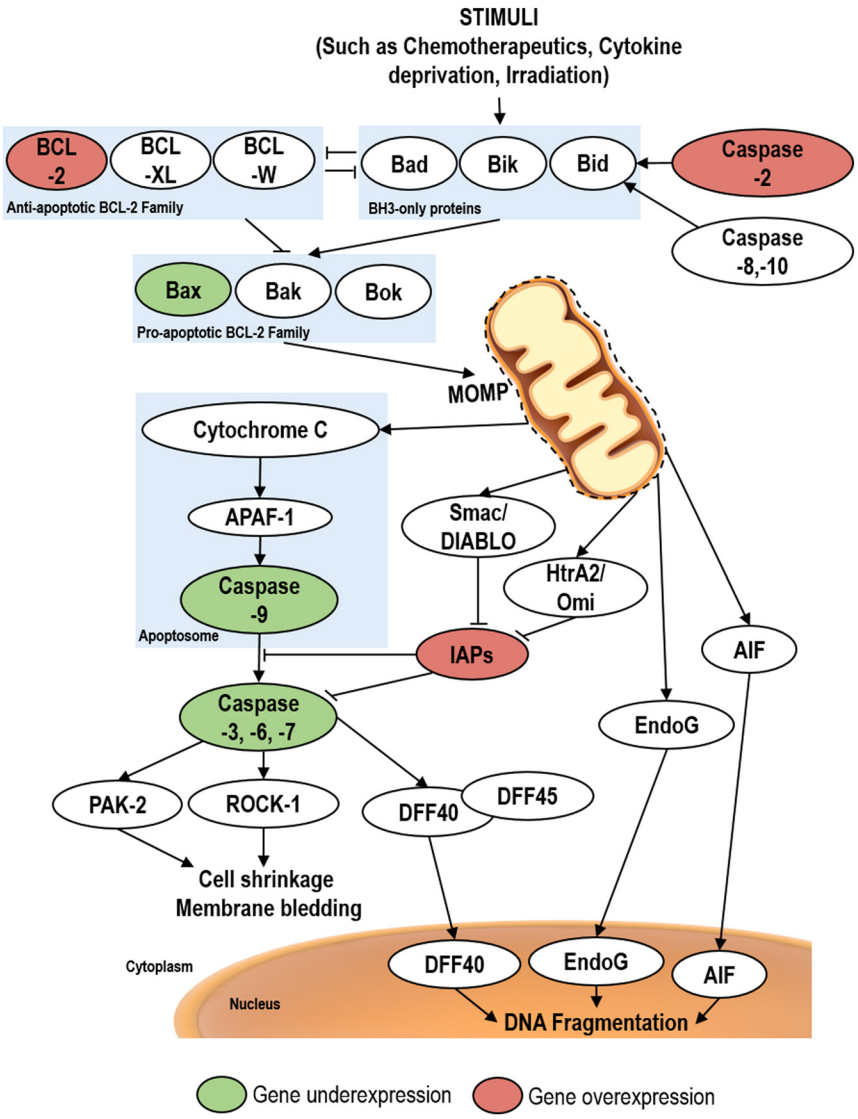

Figure 3
Schematic representation of intrinsic apoptotic pathway. Stress signals leads to the pro-apoptotic BCL-2 family proteins activation that induce the mitochondrial outer membrane permeation (MOMP). MOMP allows the release of the Cytochrome C, AIF, EndoG, HtrA2/Omi and Smac/DIABLO. Cytochrome C release leads to the formation of apoptosome complex that triggers caspase-3, -6, -7 activation. They cleave ROCK-1, PAK-2 and DFF40/45 leading to cell shrinkage, membrane blebbing and DNA fragmentation. HtrA2/Omi and Smac/DIABLO inhibit IAPs, avoiding the caspase inhibition by them. EndoG and AIF lead to DNA fragmentation. Abnormalities in the expression of genes involved in the intrinsic apoptotic pathway in adrenocortical carcinomas are highlighted in red or green circles.

single study (57). However, these results have not been confirmed in several other studies (58, 59, 60, 61).

The IAPs family have been highly studied in the context of the cancer due to their key role in the regulation of apoptosis mostly through the direct inhibition of executioner caspases (62, 63).

Altieri *et al*. studied three elements of the IAPs family: MLIAP/livin/BIRC7, survivin/BIRC5 and XIAP/BIRC4. They found that increased livin mRNA and protein levels were promising markers for malignancy diagnosis in ACT. *BIRC5* expression was found to be similar between ACC and ACA but higher than that in normal adrenal glands (64). Contrarily, other authors found that *BIRC5* expression is increased in the ACC group (23, 65). Survivin expression was found to have a negative impact on overall survival in patients with ACC (64); increased survivin was found in more aggressive tumors (23, 65). Although increased in ACC, no association was observed between livin expression and ACC aggressiveness features (64).

Cell cycle and apoptosis regulation

Cell cycle instabilities can lead to apoptosis. Some cell cycle genes are also involved in apoptosis regulation, of which *TP53*, *c-myc* and *pRB/E2F* are the most well-known examples (12, 66, 67).

p53 is involved in both intrinsic and extrinsic apoptotic pathways. However, its main role occurs in the intrinsic pathway (68, 69). After DNA damage, p53 and MDM2 are phosphorylated, which inhibits their interaction resulting in p53 accumulation in the nucleus

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and activation of transcription of various pro-apoptotic genes including *Bax*, *Noxa*, *Puma*, *BID*, *Fas*, *APAF-1*, *DR5*. In addition, p53 accumulation in the nucleus represses anti-apoptotic genes transcription, such as *BIRC5* that encodes survivin, a member of the IAP family (68, 69, 70, 71, 72, 73). Moreover, p53 can bind directly to the anti-apoptotic proteins, Bcl-2 and Bcl-xL, and also translocate to mitochondria inducing Bax/Bak oligomerization, thus promoting MOMP (74). It has also been described that P53 overexpression increases Fas levels at the cell surface, by promoting its trafficking from Golgi complex. Finally, p53 can also activate DR5 (75, 76).

TP53 mutations usually result in the loss of p53 tumor suppressive activity, mainly due to repression of p53 target genes transactivation (77). *TP53* mutations in ACT was demonstrated to be absent in the majority of ACA. In contrast, the prevalence of somatic *TP53* mutations in sporadic ACC can varies from 20 to 30% of the cases (78, 79, 80, 81). Besides that, the majority of ACC patients with mutated *TP53* were observed to have poor outcomes (78). Aberrant p53 protein expression was also found to be associated with decreased disease-free survival (80). However, in ACC p53 expression was found to be highly variable (5–52%). Thus, p53 expression cannot be considered a reliable molecular marker to identify ACC (58, 61, 82, 83, 84). Still, loss of heterozygosity (LOH) in chromosome 17q13 harboring *TP53* was demonstrated to be present in approximately 80% of ACC. Nevertheless, LOH at 17q13 is not always associated with *TP53* mutations suggesting that other genes in the same chromosomal region contribute to ACC biology (85, 86, 87).

TP53 gene germline pathogenic variants are typically associated with Li-Fraumeni or Li-Fraumeni-like syndrome, which is associated with an hereditary predisposition to neoplasms, in particular to pediatric ACC (88).

Contrarily to the majority of mutations associated with LFS that are in p53 DNA-binding domain, 80% of pediatric ACC have mutations in p53 oligomerization domain, in particular in the exon 10 of the short arm of chromosome 17 (p.R337H). p.R337H mutation appears to be particularly prevalent in Southeast and Southern Brazilian populations, where the incidence of pediatric ACT is estimated to be 10–15 times higher than worldwide (89, 90, 91). *In vivo* studies found that this mutation is associated to an increased DNA damage, a mildly decreased apoptosis and cell cycle arrest. These tumor-suppressive activity alterations have been proposed to be sufficient to induce tumorigenesis and to confer tumor growth advantages (92, 93). Otherwise, genomic profiles of ACC with p.R337H mutation were found to be similar to ACC with a different *TP53* mutation (94). Pediatric ACT with p.R337H mutation were found to have a significantly lower expression of apoptosis-related genes when compared to non-neoplastic adrenals (24).

The cross-talk between c-Myc and p53 is important for driving cell decision to undergo apoptosis in response to stress (95, 96). c-Myc can also drive apoptosis in a manner that does not require p53 (96, 97). c-Myc is able to suppress anti-apoptotic Bcl-2 family members and to induce the expression or activation of pro-apoptotic Bcl-2 proteins, such as Bax, Bak and Bim and transcriptionally activate *Bax* (95, 98, 99, 100). Besides that, c-Myc overexpression sensitizes cells to the apoptotic action of TNF and TRAIL death ligands (101). Furthermore, c-Myc also inhibits the activation of anti-apoptotic c-Jun kinases and NF-kB (95, 102). In ACC, c-Myc was found to be underexpressed compared to ACA and to the normal adrenal cortex (23, 103, 104, 105, 106).

Loss of pRB induces apoptosis and the mechanisms behind this phenomena are mainly related to the action of the E2F transcription factors, the well-known targets of pRB (107). The cytochrome c, AIF and SMAC are transcriptionally regulated by E2F1 (108, 109, 110). Death-inducing-protein (DIP) is located in the mitochondria and mediates E2F1-induced apoptosis, in a p53-independent pathway. In E2F1-activated cells, DIP suppression results in a decrease of apoptosis (111, 112). The loss of pRB was suggested to be a marker of poor prognosis as it appears to be a characteristic of the more aggressive ACC (113). A significant overexpression of *E2F* genes was found in ACC (104, 114). However, contrarily to expected, *DIP* was found to be underexpressed when compared with ACA (23).

miRNAs and apoptosis

miRNAs are non-coding RNAs that are able to silence their target genes at post-transcriptional level. Functional studies showed that abnormal miRNA expression is a key to cancer development by abnormally regulating several cellular processes, including apoptosis. Studies on ACC showed that deregulated miRNAs expression is able to negative modulate ACC apoptosis (115, 116).

miRNA-483-3p was found to be overexpressed in ACC compared with ACA (116, 117). This miRNA is able to protect the cancer cells from apoptosis through the negative modulation of the pro-apoptotic protein PUMA. A combination of the expression of the miR-483-3p and Smad4, a critical effector in the TGF-β signaling pathway, was demonstrated to have more powerful diagnostic

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accuracy than the classic pathology Weiss score system alone (117). Another study found that miR-483-3p is an excellent marker for the differential diagnosis between ACC and ACA. Higher miR-483-3p was found in ACC and there was no overlap between ACC and ACA (118).

Wu *et al.* found that miRNA-205 was significantly decreased in ACC compared with ACA. miRNA-205 was able to activate Bcl-2, in ACC tumor cells, leading to the activation of the intrinsic apoptosis pathway by activating caspase-9 and caspase-3 (119).

miR-195 is also downregulated in ACC compared to ACA (120). Low expression of miR-195 was significantly associated with poor overall survival (120). In the ACC cell line H295R, the restoration of the miR-195 expression led to increased expression of caspase-3, resulting in decreased cell viability. This finding in ACC confirmed the role of miR-195 on promoting apoptosis (116). In line with this finding, in colorectal tumors the miR-195 targeted Bcl-2 and induced apoptosis (121).

Apoptosis as a therapeutic target in ACC

Several *in vitro* and *in vivo* studies have attempted to improve the prognosis of patients with cancer by targeting apoptosis pathways either by targeting the overexpressed anti-apoptotic proteins or by stimulating pro-apoptotic molecules expression (9). These studies reported great potential of apoptosis-targeted therapies. Some of these have focused on reducing anti-apoptotic proteins such as the Bcl-2 family proteins, IAPs or c-FLIP (cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein) in order to allow the activation of apoptosis (62, 63, 122, 123). In ACC few studies were performed in order to evaluate the efficacy of those therapies (64, 65, 124, 125).

Gossypol is a polyphenolic compound extracted from cotton plants with the ability of binding to the anti-apoptotic proteins, Bcl-2 and Bcl-x_L inhibiting them (126). In 1991, a study found that this compound has multiple inhibitory effects on adrenal steroidogenesis (127). Some years later, a trial of this compound was performed in 18 patients with metastatic ACC and the authors found that three patients that were refractory to chemotherapeutic agents had partial responses and one patient had stable disease. However, the majority of the patients had tumor progression (124). The low therapeutic response of Gossypol was considered similar to the other medical therapies available. Later, some studies found that Gossypol presents two stereoisomers and that the (-) Gossypol stereoisomer is the one with highest affinity for Bcl-2 and Bcl-x_L (126, 128, 129). Since the drug used before was a mixture between the two stereoisomers, the use of the (-) Gossypol alone may have better effects compared to what was observed in the ACC patients. The compound (-) Gossypol, actually known as AT-101, has already been tested in clinical trials in patients with prostate cancer, leukemia and lung cancer (130, 131, 132). In ACC, only pre-clinical *in vitro* and *in vivo* studies were performed with that drug. Schteingart *et al.* studied the effects of (-) Gossypol in two ACC cell lines, H295 which is a cell line with low levels of Bcl-x_L and RL25, that presents high levels of Bcl-x_L. The authors demonstrated that the expression of Bcl-x_L influences the effects of the drug, since better results were found in the RL25 cell line with a complete suppression of the tumor growth (125).

Some studies also performed pre-clinical studies in order to evaluate the efficacy of the IAPs in inhibit ACC. Sbiera *et al.* knocked down survivin expression by siRNA transfection in an ACC cell line and it doubled the apoptosis rates comparing to the non-transfected cells treated with etoposide. The authors also studied if survivin inhibition could be a sensitizing factor to chemotherapeutic drugs. However, the treatment with addition of etoposide to the survivin siRNA-transfected ACC cells showed only a slight increase in the apoptosis rate (65).

Altieri *et al.* studied the efficacy of another IAP (livin) in ACC cell lines. Using livin transfected H295R cells, they observed the expected decrease in *Casp3* levels. However, no differences in the cell viability and proliferation were observed, suggesting that additional mechanisms are possibly involved in the already complex mechanism of the apoptotic cascade in ACC (64).

Evaluation of the apoptosis are highly used to verify the cancer efficiency of drugs but mostly in pre-clinical studies. In ACC cell lines, the efficacy of a given drug in increasing the apoptosis rate has been mainly studied using the Annexin V/Propidium Iodide assay, TUNEL assay and the final apoptotic molecules involved, such as the executioner caspases (133, 134). Unfortunately, it does not give us the full comprehensive mechanistic understanding of tumoral apoptosis inductor. Table 2 summarizes the available studies targeting ACC and their relationship with apoptosis.

Clinical implications of apoptosis alterations in ACC

The balance between cell death and survival becomes compromised when apoptosis signaling is deregulated, thus conferring advantages for tumorigenesis and

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Table 2 Drugs that positively increased the ACC apoptosis rate in *in vitro* and *in vivo* studies.

Anti-ACC drugs	Drug type	Method to evaluate apoptosis	Samples type	Ref.
AMG 900	Aurora kinase inhibitor	Annexin V-FITC apoptosis detection assay	ACC cell line NCI-H295	(141)
ATR-101 (PD132301-02)	Adrenalytic activity inhibitor	TUNEL assay	NCI-H295R xenographs	(142)
		Annexin V-FITC apoptosis detection assay	ACC cell line NCI-H295R	
Cholesterol free sHDL nanoparticles in combination with cisplatin, etoposide or mitotane	efflux cholesterol inducer	Annexin V-FITC apoptosis detection assay	ACC cell lines: SW-13 and NCI-H295R	(143)
Docosahexenoic acid	n-3 polyunsaturated fatty acid/mTOR complex 1/2 inhibitor	Annexin V-FITC Apoptosis Detection assay	ACC cell lines: SW-13 and NCI-H295R	(144)
		TUNEL assay	SW-13 xenographs	
DZNep	EZH2 (histone modifier) inhibitor	*BCL2*, *BCL-XL* and *BIRC5* expression/Caspase 3 activity assay/TUNEL assay	ACC cell line NCI-H295R	(145)
Erlotinib and NVP-AEW541 combination	EGFR and IGF1R inhibitor	Annexin V-FITC apoptosis detection assay	ACC cell lines: SW-13 and NCI-H295R	(134)
Fingolimod (FTY720)	Sphingosine kinase 1 antagonist	Annexin V-FITC apoptosis detection assay	ACC cell lines: SW-13 and NCI-H295R	(146)
G-1	Non-steroidal G protein-coupled estrogen receptor agonist	Annexin V-FITC Apoptosis Detection assay/Caspase 9 and 3/7 activity assay/TUNEL assay	ACC cell line NCI-H295R	(147)
Metformin	Biguanide cationic agent	Annexin V Apoptosis Detection assay/Bcl-xl, Bcl-2, Bcl-w and Cleaved Caspase 3 expression	ACC cell line NCI-H295R	(148)
Mitotane (drug used in ACC patients)	Steroidogenesis inhibitor and cytostatic antineoplastic	Annexin V Apoptosis Detection assay/*Bax, Bak* and *Bcl-2* expression	ACC cell line NCI-H295	(149, 150)
		Caspase 3/7 activity assay	ACC cell line NCI-H295R	(151)
Niclosamide	Anti-helminthic agent	Caspase 3/7 activity assay	ACC cell lines: BD140A, SW-13 and NCI-H295R	(152)
Rapamycin	mTOR complex 1 inhibitor	Cleaved Caspase 3 expression	Adrenocortical tumors from AdTAg mice	(153)
Rottlerin	Anti-helminthic or fertilization antagonist	TUNEL assay	SW-13 xenographs	(133)
		Annexin V-FITC Apoptosis Detection assay	ACC cell lines: SW-13 and NCI-H295R	

ACC, adrenocortical carcinomas; AMG, urora kinases inhibitor; Bak, B-cell lymphoma 2 homologous antagonist/killer; Bax, B-cell lymphoma 2-associated X protein; BCL-2, B-cell lymphoma 2; BCL-W, B-cell lymphoma-like 2; BCL-XL, B-cell lymphoma-extra-large; BIRC5, Baculoviral IAP repeat containing 5; DZNep, Deazaneplanocin A; EGFR, epidermal growth factor receptor; EZH2, enhancer of zeste homolog 2; FITC, fluorescein isothiocyanate; IGFR, insulin growth factor receptor; mTOR, mammalian target of rapamycin; NCI, National Cancer Institute; sHDL, synthetic high-density lipoprotein; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

tumor growth (8, 9, 10, 11). The detailed knowledge on the status of apoptosis regulation in ACC can result in pivotal clues that could be translated into the clinical practice, by modifying current therapeutic interventions. Although few alterations in apoptosis regulation were reported in ACC, some of these were shown to be promising.

miR-483-3p, a negative modulator of pro-apoptotic proteins, is overexpressed in ACC depicting a poor prognosis. So miR-483-3p was appointed not only to be an important mechanistic alteration for ACC tumorigenesis and tumor progression, but also demonstrated its diagnostic utility by depicting a more powerful diagnostic accuracy to assess ACC tumor grade than the Weiss score that is currently used in routine clinical practice (116, 117, 118).

TP53 was described as one of ACC driver genes in several studies, including the most recent pan-genomic studies (81, 135). LOH in the chromosome were *TP53* is harbored and *TP53* mutations are the most frequent alterations observed in ACC, leading to abnormal cell cycle progression and apoptosis inhibition (78, 79, 80, 81, 85, 86, 87). Targeted therapies with the goal of recovering or reactivating p53 function in *TP53* mutants were

already developed and are currently being tested for the treatment of several tumors, other than ACC (e.g. PRIMA-1^{MET} and MDM2 antagonists) (136). The high prevalence of alterations related to *TP53* in ACC makes these tumors good candidates for *TP53* modulators therapies.

Survivin, a member of the apoptosis inhibitor protein family, was found to have a negative impact in ACC patients' survival and was pointed as a promising ACC drug target (23, 64, 65). Although the development of effective survivin inhibitors was hampered by a few reported challenges, several potent survivin inhibitors were already tested in clinical trials for some cancers (137, 138), other than ACC.

Finally, some authors demonstrated that anti-apoptotic molecules expression, such as members of the Bcl-2 family, are able to influence the effectiveness of some drugs, providing an additional rationale for the outcomes variability observed in clinical trials (139). So, when possible, profiling ACC for anti-apoptosis genes expression could be recommended in order to improve the predictability of outcomes achieved and even enable a more personalized treatment approach for ACC patients. (Reviewer #1, Comment #1)

Conclusions

Apoptosis evading is one of well-known process leading to cancer cells proliferation and expansion. It is also a very important hallmark of cancer (140). Tumor cells are not only characterized by genetic alterations leading to increased proliferation but also by a compromised apoptosis signaling, a fact that adds to the imbalance between cell death and survival, leading to tumor development, invasion and resistance to treatment (8, 9, 10, 11). Decreased apoptosis in ACC associated with their aggressiveness and the resistance to treatment was observed in ACC patients. In ACC, pro-apoptotic factors are generally inhibited and anti-apoptotic ones are generally increased. However, although promising, the attempts to revert these alterations in ACC cell lines and in xenograft models have led to insufficient results to date. More studies are needed in order to integrate the key players involved in the apoptosis in ACC and to translate this information into the clinical practice. Unraveling all defects in apoptosis in ACC may have a significant importance either as therapeutic targets or molecular markers for ACC diagnosis or prognosis. So far, none of these expectations has been accomplished.

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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