Emerging role of BAD and DAD1 as potential targets and biomarkers in cancer (Review)

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Abstract. As key regulators of apoptosis, BAD and defender against apoptotic cell death 1 (DAD1) are associated with cancer initiation and progression. Multiple studies have demonstrated that BAD and DAD1 serve critical roles in several types of cancer and perform various functions, such as participating in cellular apoptosis, invasion and chemosensitivity, as well as their role in diagnostic/prognostic judgement, etc. Investigating the detailed mechanisms of the cancerous effects of the two proteins will contribute to enriching the options for targeted therapy, and may improve clinical treatment of cancer. The present review summarizes research advances regarding the associations of BAD and DAD1 with cancer, and a hypothesis on the feasible relationship and interaction mechanism between the two proteins is proposed. Furthermore, the present review highlights the potential of the two proteins as therapeutic targets and valuable diagnostic and prognostic biomarkers.

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Abbreviations: DAD1, defender against apoptotic cell death 1; OST, oligosaccharyltransferase; ER, endoplasmic reticulum; Mcl-1, myeoid cell leukemin-1; PKA, cAMP dependent protein kinase; p-BAD, phosphorylated BAD; PAK1, P21-activated kinase 1; MPNs, myeloproliferative neoplasms; JAK, Janus kinase; PP2C, protein phosphatase 2C; CSCs, cancer stem cells; OVCA, ovarian cancer; HCC, hepatocellular carcinoma; EMT, epithelial-mesenchymal-transition; SCLC, small cell lung carcinoma; TNBC, triple-negative breast cancer; EC, endometrial cancer; AML, acute myeloid leukemia; Bim, Bcl-2 interacting mediator of cell death protein; EBV, Epstein-Barr virus; AMPK, protein kinase AMP-activated catalytic subunit α1; TSBN7, temperature-sensitive nephrogenic cell line; DmDAD1, *Drosophila melanogaster* DAD1; Perk, protein kinase R-like endoplasmic reticulum kinase; Atf4, activating transcription factor 4; CfDAD1, *Chlamys farreri* DAD1; SUMO, small ubiquitin-related modifier; NET, neuroendocrine tumor; SPTP, solid pseudopapillary tumor of the pancreas; PSA, prostate-specific antigen; RPN1, ribophorin I; TRE, 12-O-tetradecanoylphorbol-13-acetate response element; CRE, cAMP response element; AP1, activator protein 1; CREB, CRE element binding, DNA-binding transcriptional regulator

Key words: BAD, DAD1, apoptosis, cancer, biomarker

Contents

1. Introduction

In recent years, the achievements of targeted therapy for cancer treatment have been self-evident (1,2). The difference between targeted therapy and conventional chemotherapy is that the cytotoxicity of normal cells is greatly reduced in targeted therapy due to its specific targeting (3,4). With the development of molecular biology and the gradual unfolding of mechanisms employed by tumor-associated factors, molecular targeted therapy will become the principal direction of antitumor treatment. However, the heterogeneity of drug resistance in tumor cells and the limited number of alternative targets are also clinical bottlenecks, which must be addressed (5). Therefore, it is urgent to identify and elucidate the abnormally activated or silenced signaling pathways in cancer cells, which are useful in exploring valuable therapeutic targets.

Tumorigenesis partly results from dysregulation of apoptosis, leading to apoptosis evasion of cells, which then become cancerous (6,7). There are three pathways to regulate apoptosis (death receptor cell death pathway, endoplasmic reticulum (ER) cell death pathway and mitochondrial cell death

pathway), among which the mitochondrial cell death pathway is mainly regulated by the Bcl-2 family proteins (8). BAD, a member of the Bcl-2 family, acts as the main pro-apoptotic protein that regulates the cellular survival-apoptosis balance, and its phosphorylation may contribute to cancer progression (9). In addition, defender against apoptotic cell death 1 (DAD1), a subunit of oligosaccharyltransferase (OST) acting on N-glycosylation residing in the ER (10), is a negative regulator of programmed cell death associated with the ER cell death pathway (11). Increasing experimental evidence has indicated deep engagement of the two proteins in tumorigenesis, particularly in cellular apoptosis, invasion, chemosensitivity and diagnostic/prognostic judgment. Therefore, their key roles in signaling transduction pathways and their close association with cellular behavior may provide insights and novel alternative molecular agents for targeted therapy of cancer.

2. Physiological characteristics and cancerous activity of BAD

Bcl-2 family and overview of BAD. The Bcl-2 family, a group of cooperative proteins, exerts a great influence on the regulation of apoptosis via the mitochondrial cell death pathway (12,13). Bcl-2 homology domains (BH1-4) have been determined to be a collective characteristic of Bcl-2 family members, and two of the member proteins can form homo- or heterodimers as an essential functional unit to promote or suppress apoptosis (14). The effects of the Bcl-2 family are antagonistic, which means that some of the members, including BAD, Bax and BH3 interacting domain death agonist, serve a pro-apoptotic role in cellular modulation, while others, including Bcl-2, myeoid cell leukemin-1 (Mcl-1) and Bcl-xL, appear to suppress apoptosis (12). The mechanism underlying the regulation of the mitochondrial cell death pathway has been demonstrated to be the action of the Bcl-2 family proteins, which determine the permeability of transition pore embedded in the mitochondrial membrane (15,16). Upstream apoptosis signals make the non-selective pore an irreversible access point between the cytoplasm and mitochondrial matrix mediated by the Bcl-2 family of proteins (15,16). Mitochondrial matrix-deprived cytochrome c combines with apoptotic protease activating factor-1 and caspase-9 proenzyme to form apoptosomes in the cytoplasm, which then activate caspase-9, triggering a cascade reaction of apoptotic proteases to subsequently induce apoptosis (15,16).

BAD was first cloned from a mouse cDNA library, and the homologous human gene was cloned later (17). BAD comprises 168 amino acids, of which Ser112, Ser136 and Ser155 are the three known regulatory residues, which can be sequentially phosphorylated by several kinase proteins (17). Among them, ribosomal protein S6 kinase and cAMP dependent protein kinase (PKA) mediate the phosphorylation of Ser112, Akt mediates the phosphorylation of Ser136 (18), and PKA preferentially mediates the phosphorylation of Ser155, which is located in the center of the BH3 domain (19-21). Normal phosphorylation at the three residues helps to maintain cytoplasmic sequestration of BAD, and thus, apoptosis is attenuated (22). Phosphorylation of BAD at Ser26 by the IκB kinase complex inhibits the pro-apoptotic activity of BAD (23). A novel synthetic compound, *N*-cyclopentyl-3-((4-(2,3-dichlorophenyl) piperazin-1-yl) (2-hydroxyphenyl) methyl) benzamide, a specific inhibitor of BAD phosphorylation at Ser99, could suppress the vitality of cancer cells *in vivo* and *in vitro* (24). Normally, phosphorylated BAD (p-BAD) combines with the amphipathic groove of chaperone 14-3-3 (25). BAD is different from most Bcl-2 family members, as it has no C-terminal transmembrane domain that anchors the outer mitochondrial membrane and nuclear envelope (17). Therefore, BAD is sequestered in the cytoplasm, and apoptosis is inhibited (22). In the presence of survival signals, dephosphorylated BAD, which is generated by phosphatases, disassociates from 14-3-3 and begins to displace Bax in the Bcl-2/Bax or Bcl-xL/Bax heterodimer to form a Bcl-2/BAD or Bcl-xL/BAD heterodimer via the BH3 homologous domain in a concentration-dependent manner (17). Additionally, free Bax homodimerization is increased, and apoptosis is initiated by the Bax homodimer integrated into the outer mitochondrial membrane (17).

To simplify, subcellular relocation to the mitochondria of BAD that triggers apoptosis is tied to its phosphorylation status at the three amino acid residues. The switch between p-BAD and dephosphorylated BAD determines its role in the pathway, i.e., pro-apoptosis or pro-survival, wherein 14-3-3, several kinases and phosphatases are key regulators (26). In addition to apoptosis-related roles, BAD also has multiple non-apoptotic functions, such as regulation of the cell cycle (27-29), autophagy (30), immune engagement (31,32), glucose metabolism (15,33), and control of localized translation (34), all of which are closely associated with its cellular effects in cancer. Among all these pathways converging due to BAD, the phosphorylation status coordinates the multiple functions of BAD, and its BH3 domain is utilized. p-BAD manages cytoplasmic sequestration to prevent apoptosis, while other metabolic pathways, such as suppression of gluconeogenesis and activation of oxidative metabolism of glucose in the mitochondria of liver cells (33,35), are activated to promote survival according to different ligands matched with BH3. Therefore, the central status of BAD in several apoptosis-related and other metabolism-related signaling pathways may result in it being an appealing target in cancer.

Expression and function of BAD in cancer. BAD is usually expressed in the colon, stomach, prostate, kidney, brain and adipose tissues (36). The tumor-associated effects of abnormal levels of p-BAD are reflected in several aspects as described subsequently.

Cell proliferation, survival and apoptosis. Several types of cancer cells have been detected to exhibit higher levels of p-BAD than corresponding immortalized normal cells *in vivo* and *in vitro*, and cell apoptosis or survival is regulated by the BAD phosphorylation status (17,37). The effects of p-BAD on cell proliferation, survival and apoptosis are summarized in Table I.

Sastry *et al* (38) determined that there are two signaling pathways that phosphorylate BAD to protect prostate cancer cells from apoptosis under the stimulus of epidermal growth factor. One signaling pathway induces phosphorylation at Ser112 via Ras/MEK. Another signaling pathway induces phosphorylation at Ser136 via Ras-related C3 botulinum toxin

Table I. Interaction of (p-)BAD with relevant genes and effect on cell proliferation, survival and apoptosis.

First author/s, year	Tissue/cell type	Interaction of (p-)BAD with relevant genes	Effect	(Refs.)
Sastry *et al*, 2006	Prostate cancer	Ras/MEK and Rac/PKA1 signaling pathways mediate the phosphorylation of BAD at Ser112 and Ser136, respectively.	Prevents apoptosis	(38)
Smith *et al*, 2009		Silencing of BAD by shRNA.	Suppresses cell proliferation	(46)
Kulik, 2019		Upregulation of p-BAD by the activation of the ADRB2/PKA signaling pathway.	Inhibits apoptosis	(48)
She *et al*, 2005	PTEN-deficient tumor cell lines	Phosphorylation defect of BAD at Ser112 and Ser136 via EGFR/MEK/MAPK and PI3K/Akt signaling pathways, respectively.	Promotes apoptosis	(39)
Polzien *et al*, 2011	B-Raf-mutated cancer cell lines	Raf kinase phosphorylates BAD at Ser134.	Promotes proliferation	(40)
Winter *et al*, 2014	JAK-depleted myeloproliferative neoplasms	JAK2 phosphorylates BAD.	Promotes survival	(44)
Stickles *et al*, 2015	Colorectal adenocarcinoma, breast cancer, endometrial adenocarcinoma and ovarian cancer	PP2C deletion induces higher levels of p-BAD at Ser155.	Promotes growth	(47)
Mann *et al*, 2019	Breast cancer	Phosphorylation of BAD at Ser118 increases Ser99 phosphorylation, 14-3-3 binding and Akt activation. BAD stimulates mitochondrial complex I activity.	Promotes growth and survival Facilitates growth and sensitizes cells to apoptosis in response to complex I blockade	(49)
Lu *et al*, 2019	Ovarian cancer	Artificial fusion p53-BAD locates to the mitochondria.	Promotes apoptosis	(50)

p-BAD, phosphorylated BAD.

substrate (Rac)/P21-activated kinase 1 (PAK1) (38). However, She *et al* (39) reported that phosphorylation of Ser112 and Ser136 could also be mediated by the EGFR/MEK/MAPK and phosphatidylinositol 3-kinase/Akt signaling pathways, respectively. Furthermore, survival signaling-induced kinases, such as PAK1 and Raf, promote the proliferation of cancer cells in the presence of wild-type BAD (39). Polzien *et al* (40) determined that Raf kinases could phosphorylate BAD at Ser134 to promote cell proliferation in B-Raf-V600E-containing tumor cells. Furthermore, replacement of Ser134 with alanine leads to phosphorylation, suggesting that BAD phosphorylation at Ser134 is essential for sufficient cell proliferation (27). Myeloproliferative neoplasms (MPNs) may result in activating mutations of the Janus kinase (JAK) gene (41-43). A previous study has reported that the phosphorylation of BAD induced by JAK2 promotes cell survival in JAK-depleted MPN cells (44). Additionally, in cells sensitive to JAK inhibitor, treatment with JAK inhibitor results in dephosphorylation of BAD and affects its combination with Bcl-xL, initiating apoptosis (44).

Huang *et al* (45) revealed that overexpression of BAD inhibits the proliferation of tumor cells *in vitro* and reduces tumor volume *in vivo* by promoting cell apoptosis and suppressing cell proliferation. Smith *et al* (46) observed contrasting results compared with Huang *et al* (45) in prostate cancer cells, wherein increased BAD expression could promote cell proliferation and silencing of BAD by short hairpin RNA suppressed cell proliferation. Stickles *et al* (47) demonstrated that protein phosphatase 2C (PP2C) deletion leads to higher levels of p-BAD at Ser155, which is beneficial for cell proliferation *in vitro*. Sastry *et al* (9) determined that the phosphorylation of BAD is indispensable for the survival of cancer stem cells (CSCs). Deficient expression of p-BAD induces apoptosis of CSCs, which could be reversed by the BH3 mimetic ABT-737, revealing that only the BH3 homologous domain is essential in BAD (9). Furthermore, the downregulation of BAD weakens the frequency and renewal capacity of CSCs (9). Kulik (48) reported that upregulated levels of p-BAD, together with Mcl-1 mediated by the activation of the adrenoceptor β2 (ADRB2)/PKA signaling

pathway, are responsible for increased inhibition of apoptosis of prostate cancer cells. Furthermore, Mann *et al* (49) verified the two distinct mechanisms underlying the BAD-regulated increase in cell proliferation in breast cancer cells. Specifically, phosphorylation of BAD at Ser118 increases Ser99 phosphorylation, 14-3-3 binding and Akt activation, which promotes cell proliferation and survival. On the other hand, BAD stimulates mitochondrial oxygen consumption in a novel manner downstream of substrate entry into the mitochondria (49). BAD stimulates complex I activity that facilitates cell proliferation and sensitizes cells to apoptosis in response to complex I blockade, which may result in large but non-aggressive breast cancer (49). These results suggest that BAD-induced apoptosis may not only depend on mitochondrial membrane reactions between Bcl-2 family proteins, but it may also be associated with oxidative metabolism.

Lu *et al* (50) designed a novel chimeric gene fusion p53-BAD to overcome the dominant negative inhibition of wild-type p53 and multiple genetic aberrations in ovarian cancer (OVCA). By introducing Ser122A and Ser136A mutations to prevent phosphorylation at the two residues, p53-BAD constructs could always be located in the mitochondria. Furthermore, they observed that p53-BAD constructs exhibited higher pro-apoptotic activity, which was direct and rapid via the mitochondrial cell death pathway (50). This pro-apoptotic effect was consistent in several OVCA cell lines, regardless of the endogenous p53 status (50).

It is worth mentioning that Datta *et al* (32) explored the physiological significance of BAD phosphorylation for cell survival *in vivo*. They generated BAD^{3SA} mutant mice, in which the three phosphoregulatory residues were shifted to alanine; thus, endogenous BAD was not responsive to survival signaling. They demonstrated that growth factor-mediated BAD phosphorylation is indispensable to prevent cells from undergoing apoptotic stimuli. Notably, they validated that the levels of BAD phosphorylation via growth factors could raise the threshold at which mitochondria release cytochrome c in response to apoptotic stimuli. In summary, the levels of BAD phosphorylation may be a sensor that determines the extent to which cells undergo apoptosis, and it is also one of the mechanisms employed by survival factors to block apoptosis (32).

Invasion and distant metastasis. A previous study revealed that the expression levels of BAD in hepatocellular carcinoma (HCC) are associated with vascular invasion (51). Cekanova *et al* (52) reported that the levels of BAD and p-BAD in clinical breast cancer tissues are lower than those in normal breast tissues. The expression levels of several proteins associated with invasiveness (c-Jun, Akt and signal transducer and activator of transcription proteins), epithelial-mesenchymal-transition (EMT; transcription factor Sp1 and β-catenin) and metastasis (vascular endothelial growth factor) are decreased by BAD in BAD-overexpressing breast cancer cells (52). The novel anti-invasion and EMT inhibition functions of BAD are distinct from its traditional role in prompting the mitochondrial cell death pathway (52). Furthermore, 33 out of 60 clinical salivary gland adenoid cystic carcinoma cases exhibited high expression levels of BAD, and the expression levels of BAD were associated with distant metastasis (53).

Clinical characteristics. Hu *et al* (51) reported that the expression levels of BAD are decreased in clinical HCC tissues compared with non-tumorous adjacent tissues. The expression levels of BAD are negatively associated with several clinical characteristics, including α-fetoprotein levels, clinical stage and tumor size. Furthermore, subsequent multivariate analyses revealed that BAD can act as an independent indicator of overall HCC survival. This study demonstrated that BAD may act as a potential biomarker for poor prognosis in clinical HCC (51). Furthermore, Yu *et al* (54) reported similar results in small cell lung carcinoma (SCLC), and notably decreased expression levels of BAD were detected in clinical SCLC specimens compared with in neighboring non-tumorous tissues. The downregulated levels of BAD were significantly associated with overall survival, disease-free survival and several clinical characteristics (e.g., tumor recurrence, tumor size and clinical stage) of patients with SCLC (54). Multivariate analyses further indicated that BAD can act as an independent indicator of overall survival in SCLC (54). Another study on triple-negative breast cancer (TNBC) constructed a BAD pathway gene expression signature score system derived from principal component analysis to evaluate the overall expression and activation of the BAD pathway, and the results demonstrated that BAD pathway expression was associated with triple-negative status and overall survival (55).

Chemosensitivity. Chon *et al* (56) revealed that BAD phosphorylation has an important cisplatin sensitivity in endometrial cancer (EC) cells. Since BAD can be phosphorylated by PP2C, they observed that higher levels of p-BAD resulted in lower chemosensitivity to cisplatin in PKA small interfering RNA (siRNA) EC cells. However, p-BAD presented higher chemosensitivity to cisplatin in EC cells when PKA dephosphorylation was knocked down (56).

Interestingly, Hayakawa *et al* (57) reported that treatment of both cisplatin-sensitive and cisplatin-resistant OVCA cell lines with cisplatin could result in the phosphorylation of BAD at both Ser122 and Ser136, which was later determined to be mediated by the ERK and Akt cascades, respectively. Furthermore, they determined that inhibition of either of the two cascades could render OVCA cells more sensitive to cisplatin (57). Marchion *et al* (58) observed that the parallel effect of p-BAD increased with cisplatin resistance both in OVCA cells and in primary patients. Apart from the evidence presented, there are several other kinases or phosphatases derived from the BAD apoptosis pathway that are associated with the evolution of cisplatin resistance by exerting influence on p-BAD status. For example, Bansal *et al* (59) selected CDK1 and PP2C to validate OVCA sensitivity to cisplatin. Lower expression levels of PP2C and higher expression levels of CDK1 increased cisplatin resistance. In addition, they revealed that downregulation of CDK1 by siRNA infection increased cisplatin sensitivity (59). Taken together, these results demonstrated that inhibition of p-BAD enhanced chemosensitivity in OVCA chemotherapy (57-59).

Yu *et al* (60) reported that Bcl-2(-)BAD(+) breast cancer cells exhibited higher chemosensitivity to four types of anticancer

drugs (epirubicin, 5-fluorouracil, navelbine and cisplatin) than other breast cancer cells [Bcl-2(+)BAD(-) or Bcl-2(+)BAD(+)]. Therefore, the joint detection of Bcl-2 and BAD expression may help in chemotherapy drug selection. Boac *et al* (55) demonstrated that patients with TNBC express higher levels of p-BAD isoforms than patients with breast cancer that is not triple negative, and the levels of p-BAD-Ser136 are different, while the differences in p-BAD-Ser112 and p-BAD-Ser155 levels are not significant. Furthermore, the study demonstrated that targeted inhibition of kinases known to phosphorylate BAD results in increased sensitivity to nonspecific chemotherapeutic agents, such as cisplatin, *in vitro* (55). In a later report, BAD enhanced docetaxel sensitivity by facilitating longer mitotic arrest and activating cell death in mitosis *in vivo* and *in vitro* (61). Notably, death in mitosis has been observed to be an abnormal type of apoptosis, one that was dependent on Bcl-2 interaction and caspase activation; in fact, it was necroptosis (61). This type of BAD-enhanced docetaxel-mediated necroptotic cell death is dependent on reactive oxygen species, which indicates the chemosensitivity amplification effect of BAD in breast cancer (61).

In acute myeloid leukemia (AML), Yu *et al* (62) developed a system to quantify the chemosensitivity of dormant AML cells. The results revealed that two BAD mimetics, ABT-199 and ABT-737, were both able to effectively target dormant primary leukemia cells and decrease the dormant fraction of leucineaminopeptidase cells to 84 and 80%, respectively, revealing their good efficacy against cells protected by dormancy. Yiau *et al* (63) compared the alterations in the expression levels of CD34 and BAD in blood samples collected from patients with AML before and at day 3 after induction therapy. They observed that the average percentages of CD34 and p-BAD were higher in chemoresistant than chemosensitive samples, indicating potential CD34 signaling-associated chemotherapy resistance via p-BAD in AML (63).

Zhou *et al* (64) explored the relationship between low glucose levels and hypoxia-induced autophagy and chemoresistance in HCC cells. The study revealed that autophagy induced by low glucose and hypoxia in central solid tumors could reduce the protein expression levels of BAD and Bcl-2 interacting mediator of cell death (Bim), and elevated chemoresistance of HCC cells. Furthermore, they observed that chemotherapy-induced apoptosis could be reduced or promoted by RNA interference or upregulation, respectively. These results revealed that the downregulation of BAD and Bim is involved in the chemoresistance of HCC (64).

Constitutive engagement with other dominant molecules. Kim *et al* (65) reported that Epstein-Barr virus (EBV)-derived microRNA, microRNA-BART20-5p, inhibits BAD-mediated caspase-3-dependent apoptosis by targeting BAD in gastric carcinoma. The study demonstrated that BAD could act as a potential target of BAD in EBV-associated gastric carcinogenesis (65). Tang *et al* (66) revealed that downstream molecules, such as caspase-3, are influenced by BAD, together with cytokines affected by the NF-κB signaling pathway. Remodeling is performed when Akt is knocked out in primary liver cancer cells, which induces an altered inflammatory response and apoptosis. Additionally, they revealed that carnosic acid nanoparticles could activate the NF-κB signaling pathway and that the overexpression of caspase-3 can moderate inflammation, as well as promote apoptosis in Akt-knockout liver cancer cells (66). Zhao *et al* (67) observed that downregulation of prostate cancer associated transcript 1 in esophageal cancer cells results in upregulated BAD expression, inhibits cell proliferation, decreases migration and invasion, and enhances apoptosis. Liu *et al* (68) reported that inhibition of protein kinase AMP-activated catalytic subunit α1 (AMPK) with dorsomorphin (a specific AMPK inhibitor) in AMPK-driven hematological cancer types upregulates the expression levels of BAD to induce apoptosis.

Mansouri and Percival (69) observed that the anticancer effect of cranberry extract may result in a decrease in Akt induced in HL-60 cells, which leads to an increase in dephosphorylated BAD and subsequent activation of the intrinsic apoptosis pathway. Endo *et al* (70) demonstrated that the pro-apoptotic effect of curcumin is partly associated with BAD transfer from the cytoplasm to the mitochondrial membrane to trigger the mitochondrial cell death pathway by inhibiting the expression of 14-3-3 in the cytoplasm. An Akt-dependent manner was determined to be utilized to promote the dephosphorylation of BAD by curcumin (70). Furthermore, Gao *et al* (71) revealed that the JNK-p21/BAD signaling pathway may be involved in the process of cell proliferation inhibition and cisplatin resistance mediated by downregulation of cell death inducing DFFA like effector A protein in esophageal cancer.

3. Overview of DAD1 and its cancerous role

Biological characteristics and function of DAD1. DAD1 was originally cloned from a temperature-sensitive nephrogenic cell line (TSBN7) (11). Since a somatic mutation of DAD1 in a temperature-sensitive cell line was responsible for the induction of apoptosis when shifted to a non-permissive temperature, it was proposed that DAD1 may inhibit apoptosis and it was named based on this function (11). Subsequently, a number of studies have demonstrated another role of DAD1, acting as a subunit of OST, which participates in aspartic acid-mediated N-linked glycosylation (72,73). Human DAD1 gene mapping at chromosome 14q11-q12, encoding 113 amino acids (74), is widely expressed in thyroid, adrenal, kidney and lung cells (75). The molecular structure of DAD1 is conserved and stable during biological evolution, which indicates that it has an important function in cellular modulation (76).

Research on the function of DAD1 mainly focuses on two aspects: DAD1 as a crucial component of OST in catalyzing N-linked glycosylation and DAD1 as a pivotal negative regulator in programmed cell death. N-linked glycosylation is a type of co-translational or post-translational modification of proteins. The newly synthesized N-glycosylated chains are added to the asparagine residues of the peptide chains by the OST complex. However, to the best of our knowledge, the mechanism by which DAD1 regulates apoptosis remains unclear. One of the apoptotic mechanisms proposed is the DAD1 loss-induced N-linked glycosylation block (77). Researchers have demonstrated that deletion of DAD1 in hamster TSBN7 cells induces apoptosis (11). However, cycloheximide (a protein synthesis inhibitor) inhibits this process, while Bcl-2, a conventional anti-apoptotic

Table II. Aberrant expressive alterations of DAD1 gene in diverse cancer cells.

First author/s, year	Cancer type	Protein or mRNA alterations of DAD1 gene	(Refs.)
Tanaka *et al*, 2001	Hepatocellular carcinoma	mRNA upregulated	(89)
Bandres *et al*, 2004	Colorectal carcinoma	Protein upregulated	(90)
Kulke *et al*, 2008	Small bowel carcinoid tumor	Protein upregulated	(91)
Zhu *et al*, 2014	Solid pseudopapillary tumor of pancreas	Protein downregulated	(94)
Schnormeier *et al*, 2020	Chronic lymphocytic leukemia	Protein upregulated	(95)
Ayala *et al*, 2004; True *et al*, 2006; Bhasin, 2015	Prostate cancer	Protein upregulated	(96,97,100)
Wang *et al*, 2016	Invasive bladder cancer	mRNA downregulated	(98)
Yoon *et al*, 2010	Cisplatin-resistant ovarian cancer	Protein and mRNA upregulated	(99)

DAD1, defender against apoptotic cell death 1.

molecule, does not (11). Notably, apoptosis induced by DAD1 deletion could not be rescued by Bcl-2, suggesting that DAD1 may serve a pivotal role in the ER pathway rather than the mitochondrial and death receptor pathways. Brewster *et al* (78) and Hong *et al* (79) have reported that DAD1 is necessary for development beyond the blastocyst stage, and its deletion promotes apoptosis in mouse embryos. However, in terms of T cell development and activation, DAD1 enhances T cell proliferation instead of preventing apoptosis *in vivo* (80).

Gene cloning and/or functional exploration of heterogenetic DAD1 has been performed and validated in other species, including *Chlamydomonas* (81), Hessian fly *Mayetiola destructor* (82) and bay scallop *Argopecten irradians* (83). Furthermore, enhanced expression levels of DAD1 have been suggested to be accountable for unanticipated stimulus in case of cell injury or apoptosis (83). Zhang *et al* (76) demonstrated that DAD1 in *Drosophila melanogaster* (DmDAD1) contributes to tissue enrichment, and upregulation of DmDAD1 facilitates N-linked glycosylation. Furthermore, feasible mechanisms have been proposed in terms of the deletion of DmDAD1, resulting in subsequent apoptosis (76). Loss of DmDAD1 leads to blocked N-linked glycosylation and the accumulation of unfolded or misfolded peptide chains, and enhancement of ER stress. Defects in DmDAD1, which employs the JNK pathway downstream to implement apoptosis, activate the protein kinase R-like endoplasmic reticulum kinase (Perk)/activating transcription factor 4 (Atf4) signaling pathway (76). On the other hand, compensatory proliferation of neighboring cells is driven by the Perk/Atf4 signaling pathway to sustain tissue homeostasis (76). Furthermore, Wang *et al* (84) cloned the homologous gene of DAD1 in *Chlamys farreri* (CfDAD1) and observed that suppression of CfDAD1 with specific dsRNA injection results in increased cell apoptosis. In addition, high mRNA expression levels of CfDAD1 are detected in the hepatopancreas and gill, which are regarded as immune battlefields, indicating its key role in the innate immunity of scallops (84). Another study revealed the interaction between DAD1 and Mcl-1, an anti-apoptotic member of the Bcl-2 family, and apoptosis triggered by DAD1 depletion could be inhibited by Mcl-1, indicating the feasible interaction between the two apoptosis pathways (85). Notably, the anti-apoptotic effect of DAD1 has also been determined in humans *in vivo*. The expression levels of DAD1 are increased in neutrophils from patients with sepsis after multiple traumas (86). Furthermore, increased expression levels of the DAD1 gene have been observed in thymocytes of enhancer Eα's downstream CTCF binding sites $(EACBE)^{-/-}$ mice (87). The increased DAD1 expression in EACBE-deleted $CD4^+CD8^+$ double-positive thymocytes can be explained by the increased interaction between enhancer Eα and DAD1 (87). EACBE is essential for the sub-topologically associating domains boundary, which separates the Tcra-Tcrd locus and the downstream region including the DAD1 gene (87). DAD1 has also been reported to be an adipokine candidate in adipose tissue (88).

Cancerous role of DAD1. Since DAD1 is a negative regulator of apoptosis, the anti-apoptotic function of DAD1 may pose potential advantages for tumor cells to allow them to infinitely proliferate, and research on this aspect highlights the role of DAD1 in cancer therapy. The aberrant expressive alterations of the DAD1 gene in different cancer types are summarized in Table II.

Aberrant expression in cancer. Tanak *et al* (89) identified that DAD1 mRNA, and antisecretory factor-1, gp96 and CDC34, are highly expressed in HCC cells compared with adjacent non-tumorous liver tissues or normal liver tissues. Bandres *et al* (90) reported that DAD1 expression is upregulated in colorectal carcinoma with lymph node metastasis compared with that without lymph node metastasis, indicating the potential positive lymph node involvement. Kulke *et al* (91) reported that the expression levels of DAD1 in small bowel carcinoid tumor cells are higher than those in normal mucosa or the surrounding stroma. In addition, Wilson (92) conducted a meta-analysis to explore the genes involved in small ubiquitin-related modifier (SUMO) signaling pathways. In a meta-analysis, 10 out of 15 analyzed studies reported that DAD1 is co-expressed with SUMO1, which was the highest co-expression finding with SUMO1 (92). This result indicates that DAD1 might act as a member of the SUMO signaling pathway in cancer. Ter-Minassian *et al* (93)

Table III. Genetic alterations associated with BAD and DAD1 signaling pathways.

A, BAD

First author/s, year	Cellular behavior	Gene alteration and outcome	(Refs.)
Sastry *et al*, 2006	Proliferation, survival and apoptosis	Phosphorylation of BAD on Ser112 and Ser136 via Ras/MEK and Rac/PKA1 signaling pathways attenuates apoptosis.	(38)
She *et al*, 2005		Phosphorylation defect of BAD on Ser112 and Ser136 via EGFR/MEK/MAPK and PI3K/Akt signaling pathways promotes apoptosis.	(39)
Polzien *et al*, 2011		Phosphorylation of BAD on Ser134 by Raf kinases promotes. proliferation	(40)
Winter *et al*, 2014		Phosphorylation of BAD induced by JAK2 promotes survival.	(44)
Stickles *et al*, 2015		High levels of p-BAD induced by PP2C deletion promote cell proliferation.	(47)
Kulik, 2019		Upregulated p-BAD co-expressed with Mcl-1 leads to increased apoptosis inhibition.	(48)
Mann *et al*, 2019		Phosphorylation of BAD at Ser118 increases 14-3-3 binding and Akt activation to promote growth.	(49)
Cekanova *et al*, 2015	Invasion	Overexpressed BAD decreases gene expression to inhibit invasion (cyclin D1, MMP 10, c-Jun, Akt and STATs), metastasis (Snail) and EMT (Sp1, β-catenin, GSK-3β).	(52)
Chon *et al*, 2012	Chemosensitivity	PP2C siRNA leads to higher levels of p-BAD and cisplatin resistance.	(56)
Hayakawa *et al*, 2000		Phosphorylation defect of BAD at Ser112 and Ser136 mediated by the ERK and Akt cascade, respectively, sensitizes ovarian cancer cells to cisplatin.	(57)
Marchion *et al*, 2011		Cisplatin resistance is associated with BAD signaling pathway genes, including Bax, Bcl-xL and PP2C/PPM1A.	(58)
Bansal *et al*, 2012		CDK1 (BAD signaling pathway gene) siRNA increases cisplatin sensitivity.	(59)
Yiau *et al*, 2019		CD34 and p-BAD are increased in chemoresistant samples.	(63)

B, DAD1

First author/s, year	Cellular behavior	Gene alteration and outcome	(Refs.)
Zhang *et al*, 2016	Proliferation, survival and apoptosis	DAD1 defect-induced endoplasmic reticulum stress triggers Perk-Atf4 signaling pathway to induce apoptosis. Perk-Atf4 signaling pathway indirectly induces compensatory cell proliferation.	(76)
Bhasin, 2015		Extracellular DAD1 interacting with Fas results in apoptosis.	(100)
Ayala *et al*, 2004	Invasion	Increased expression levels of DAD1, PIM2 and NF-κB is associated with enhanced perineural invasion.	(96)
Wang *et al*, 2016		Long non-coding RNA NONHSAG045391 is co-expressed with DAD1 to enhance invasion.	(98)
Yoon *et al*, 2010	Chemosensitivity	Overexpression of DAD1 is found in cisplatin-resistant ovarian cancer cells.	(99)

siRNA, small interfering RNA.

examined genetic associations with sporadic neuroendocrine tumor (NET) risk between patients with sporadic NET and healthy controls using a custom array containing 1,536 SNPs in 355 candidate genes. Ter-Minassian *et al* (93) demonstrated that DAD1 contained two of the SNPs found to be associated with NET risk, including in another independent duplication set, revealing that the DAD1-associated apoptosis pathway may participate in neuroendocrine tumorigenesis. Zhu *et al* (94) conducted a pioneering study on the proteomics of solid pseudopapillary tumor of the pancreas (SPTP), in which isobaric

Table IV. PROMO online prediction of transcription factor binding sites in the defender against apoptotic cell death 1 promoter region.

Transcription factor name	Start position	End position	Dissimilarity	String	RE equally	RE query
CREB [T00163]	1341	1349	3.614755	ACAACGTCA	0.10681	0.10409
AP1 [T00029]	1543	1551	14.681715	TGACTTGTT	0.27466	0.28161

AP1, activator protein 1; CREB, CRE element binding, DNA-binding transcriptional regulator; RE, random expectation.

tags for relative and absolute quantitation technology integrated in liquid chromatography-tandem mass spectrometry analysis were utilized to determine differentially expressed proteins in SPTP samples compared with normal pancreatic tissues. Bioinformatics analysis resulted in 1,171 qualified proteins. Immunohistochemistry was performed to confirm the differential expression of six representative proteins and revealed the downregulation of DAD1 in SPTP specimens (94). This suggests that DAD1, together with other abnormally expressed proteins, may be a potential biomarker of SPTP in clinical therapy. High expression levels and high variability of DAD1 have also been determined in chronic lymphocytic luekemia, one of the non-solid tumors (95).

Invasion. Ayala *et al* (96) observed that the expression levels of NF-κB, and its downstream agents DAD1 and pim-2 proto-oncogene, were increased in perineural prostate cancer cells. Concurrent to the positive association between DAD1 expression and Gleason score in prostate adenocarcinoma, higher levels of DAD1 expression are associated with cancerous epithelium and perineural invasion (97). Wang *et al* (98) revealed that DAD1 is one of 21 differentially expressed genes in bladder cancer. Downregulation of the lncRNA NONHSAG045391 co-expressed with DAD1 has been observed in invasive bladder cancer, suggesting that NONHSAG045391 may contribute to enhanced invasiveness by targeting DAD1 (98). However, to the best of our knowledge, the concrete mechanism remains obscure.

Cisplatin resistance. Cisplatin treatment of a cell line derived from a clinical patient with cisplatin-resistant OVCA facilitated DAD1 expression at both the transcription and protein levels, indicating that cisplatin resistance might partly result from the upregulation of DAD1 (99).

Novel performance. The role of DAD1 in prostate cancer has been previously analyzed (100). First, increased DAD1 expression was detected in samples derived from clinical patients with prostate cancer compared with that in normal adjacent tissues. Furthermore, the study revealed that different TNM grades and Gleason grades were associated with prominent differences in DAD1 expression levels, which gradually increased with the progression of prostate cancer, underlying its diagnostic or prognostic role as a biomarker (100). Receiver operating characteristic curve analysis revealed that serum DAD1 exhibited improved specificity and sensitivity compared with prostate-specific antigen (PSA) in distinguishing low Gleason and high Gleason prostate cancer (100). Additionally, Bhasin (100) determined that ribophorin I (RPN1), another subunit of OST, is essential for DAD1 retention in the ER. DAD1 could be exocytosed with the downregulation of RPN1; thus, intervention with DAD1 antibody was implemented to check if DAD1 exocytosis was necessary. As a result, the DAD1 antibody exhibited markedly increased cytotoxicity compared with the control antibody in cancer cells and suppressed cancer cell survival (100). Furthermore, Bhasin (100) pointed out that this type of apoptosis was the result of extracellular DAD1 interacting with Fas protein. This research highlighted the potential of DAD1 in targeted therapy of cancer.

4. Feasible relation and interaction mechanism between BAD and DAD1

Table III summarizes the genetic alterations involved in pathways with BAD and DAD1 participation and their effects on cellular behavior.

The apoptotic function of BAD has been greatly explored since its discovery, and the present review proposes a novel role of BAD. AI-Bazz *et al* (101) reported that BAD expression appeared to be nuclear in addition to its cytoplasmic location according to immunostaining in primary breast cancer. In addition, using proliferating breast cancer cell lines, Fernando *et al* (102) observed that endogenous BAD exists in both the cytoplasm and nucleus, whereas the levels of p-BAD in the nucleus are lower than those in the cytoplasm. Overexpression of BAD could augment the levels of p-BAD in the nucleus and inhibit the expression of cyclin D1 on the basis of phosphorylation at Ser75 and Ser99 and in combination with c-Jun (102). Using a chromatin immunoprecipitation assay, they further demonstrated that BAD could bind to the 12-*O*-tetradecanoylphorbol-13-acetate response element (TRE) and cAMP response element (CRE) in the promoter region of the natural cyclin D1 gene and possibly attenuate the transcriptional activity of c-Jun to suppress cyclin D1 expression (102). Activator protein 1 (AP1), a putative transcription factor, is a heterodimer of c-Jun and c-Fos that was also observed to be abolished by overexpression of BAD (102). These findings indicate that BAD may act as a DNA promoter binding protein and exert a transcription factor-like effect to downregulate the expression levels of targeted genes (102). Interestingly, the sequences of the DAD1 promoter region (~2.0 kb) were searched to predict the binding sites of transcription factors using PROMO (version 8.3; http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). The final results are listed and shown in Table IV and Fig. 1. It was identified that there were two putative transcription factors, CRE element

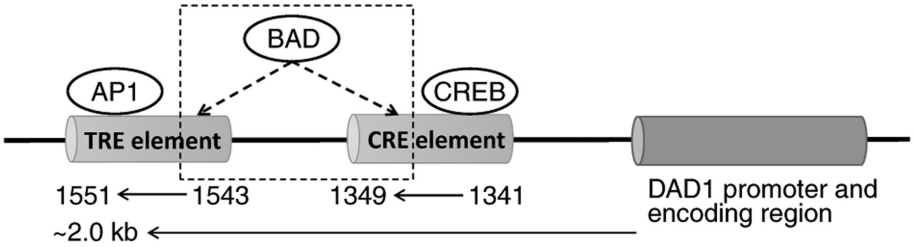

Figure 1. Potential transcription factor binding sites in DAD1 promoter region. Two putative transcription factors, CREB and AP1, are predicted to bind with CRE and TRE, respectively. The possible binding relationship between BAD and CRE/TRE is highlighted with a dashed outline. AP1, activator protein 1; CRE, cAMP response element; CREB, CRE element binding, DNA-binding transcriptional regulator; DAD1, defender against apoptotic cell death 1; TRE, 12-O-tetradecanoylphorbol-13-acetate response element.

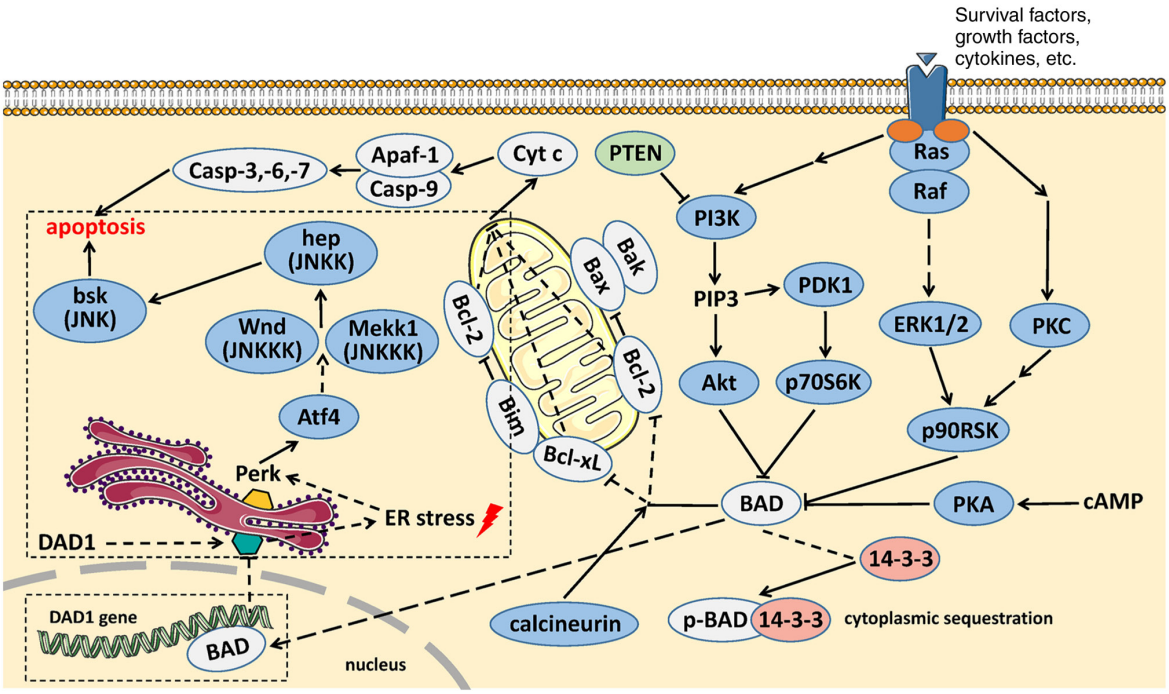

Figure 2. Feasible interaction between BAD and DAD1 and sequential apoptosis. Our hypothesis is highlighted with a dashed outline. By exerting a transcription factor-like function, the combination between BAD and the promoter region of the DAD1 gene results in DAD1 knockdown. Loss of DAD1 function triggers ER stress and activates the Perk-Atf4 signaling pathway, which sequentially activates the JNK signaling pathway via MEKK1, together with Wnd, and eventually initiates apoptosis (76). The apoptosis signaling pathways in Fig. 2 were created with reference to signaling pathway figures on the Cell Signaling Technology, Inc. website (104-106). ER stress, endoplasmic reticulum stress.

binding, DNA-binding transcriptional regulator (CREB) and AP1, which are able to bind CRE and TRE, respectively, in the DAD1 promoter region. In other words, BAD, as reported by Fernando *et al* (102), can bind with CRE and TRE in the cyclin D1 promoter region and may also bind with the CRE and TRE elements in the DAD1 promoter region. A previous study (103) revealed that overexpression of BAD in esophageal cancer cells could inhibit DAD1 expression, which could be restored when BAD expression is downregulated. Taken together, it was hypothesized that there is a negative regulatory relationship between BAD and DAD1 [Fig. 2; (104-106)]. BAD can act as a transcription factor by binding to the promoter of DAD1 to inhibit its expression. Our hypothesis suggests that BAD can act as a transcription factor-like protein to negatively regulate the expression of targeted genes and explains the relationship between BAD and DAD1 in apoptosis regulation and the crosstalk between two apoptotic signaling pathways: The mitochondrial cell death pathway and the ER cell death pathway. Further studies should be performed to support this hypothesis.

5. BAD and DAD1 as potential targets and biomarkers in cancer

Based on the studies presented, it can be clearly inferred that BAD and DAD1 serve an indispensable role in certain types of cancer development and progression, resulting from their key regulatory functions in apoptosis pathways and a number of other abilities affecting tumorigenesis (27-33,76,83,107). Therefore, it is possible to identify the two proteins as emerging useful targets and biomarkers in carcinogenesis and tumor therapies. Aberrant expression of both proteins in cancer cells is not always the same since their expression is influenced by cancer type and several unknown factors.

However, this suggests that their ectopic expression indicates the disorder of apoptosis signaling pathways. Therefore, drugs that target BAD and DAD1 can be utilized to restore or suppress the activity of apoptosis signaling pathways. Furthermore, as summarized in the present review, the expression levels of BAD and DAD1 are associated with apoptosis (11,17,38-49,76-79), invasion enhancement and metastasis (51-53,96-98), and chemoresistance (55-64,99). Therefore, it is possible to predict the potential of apoptosis, invasion and chemoresistance by detecting the expression levels of BAD and DAD1 in pathological samples. Apart from their collective application value, there are several insights presented for the two proteins. As mentioned in the cell proliferation, survival and apoptosis subsection of the present review, the phosphorylation status of BAD determines the incline of the apoptosis-survival balance (17,27,37-44). Therefore, examination of the phosphorylation status of BAD may contribute to the efficacy evaluation in response to treatment. On the other hand, kinases and phosphatases associated with BAD phosphorylation status can also be exploited as potential targets due to their plausible interactions with p-BAD. Furthermore, as summarized in the clinical characteristics subsection of the present review, the expression levels of BAD are associated with a number of clinical pathological characteristics of cancer, such as overall survival, clinical stage and tumor size, and thus, BAD also acts as an independent biomarker of prognosis (51-55). In terms of DAD1, the favorable proliferation inhibition by treatment with DAD1 antibody against prostate cancer cells *in vitro* has inventively demonstrated that DAD1 could act as a potential target in tumor therapy, together with its improved sensitivity and specificity as a diagnostic/prognostic biomarker compared with PSA (100).

6. Conclusion

The present review summarizes insights on the functional roles of BAD and DAD1, particularly in cancer, and contributions to apoptosis, invasion and chemosensitivity are emphasized. However, the underlying molecular mechanisms involved require further exploration. It is gradually becoming clear that the two proteins are mainly involved in tumorigenic signaling regulation, whether as a constitutive molecule picked up by other dominant molecules or as central target of signaling pathways, affecting cellular behavior independently. Finally, a hypothesis was proposed to reveal the feasible interaction mechanism between BAD and DAD1. It was highlighted that decreased DAD1 expression results from BAD binding to the DAD1 gene promoter region, exerting a novel transcription factor-like function. BAD and DAD1, two emerging molecules acting as targets and biomarkers in tumorigenesis, their specific functional mechanisms and their exploitation value should be given more importance when considering the broader clinical therapeutic applications.

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Authors' contributions

YL was the major contributor in writing of the manuscript, as well as in the preparation of the tables and figures. YW and HH searched and integrated all the supporting references, helped to refine the figures and tables and edited the manuscript. NY helped to improve the language and logic of the text in the present study. YC was responsible for the study design. Data authentication is not applicable. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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