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Apoptosis-inducing proteins with reduced expression in breast cancer: A review article

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ABSTRACT

Breast cancer is considered one of the most abundant malignancies with high morbidity and mortality. Traditional cancer treatments possess various weaknesses, including a lack of specificity and numerous side effects. Novel cancer therapies aim to overcome the shortcomings of traditional therapies and offer more efficient and safe treatments. Designing and identifying novel proteins that can practically and specifically inhibit tumors by inducing apoptosis can interest researchers in this field. This review discusses recent studies on the use of four promising proteins, KAI1, Apoptin, BIF-1, and DFF-40, for breast cancer treatment. This study also focuses on alterations in the expression of the androgenic proteins and novel strategies for better penetration and delivery of these proteins. Lastly, prospects for protein-based targeted cancer therapy and future studies in this field are highlighted.

1. Introduction

Breast cancer is the second most common type of cancer globally, only after lung cancer among the 34 recognized types [1]. In 2020, there were more than 2.3 million new cases of breast cancer and also 685,000 related deaths. It is estimated that the incidence of breast cancer will increase by reaching approximately 3 million cases annually in 2040 [2]. This malignancy is classified into several subgroups based on histology and type [3]. These variations are referred to as cancerous cell growth patterns. About 75–80 % of breast cancers are "invasive carcinoma of no special type (NST), unlike the other subgroup called as "special types" which are different from in terms of tumoral cells morphology and the presence of tubular or glandular structures [4].

Breast neoplasm is one of the most common malignancies among women [5] with high mortality rate as well as poorer prognosis even more than lung cancer in this gender [6].

The balance between cell proliferation and apoptosis in the normal breast cells is disrupted in cancer cells, the anti-apoptotic signaling pathway is activated that can lead to uncontrolled cell proliferation, treatment resistance, and cancer cell recurrence [7–9]. In breast cancer cells, several factors, including growth factors, damaging of DNA, UV, and reactive oxygen radicals (ROS) can induce unbridled cell proliferation through mitochondria, nuclear factor κB, and MAPK-mediated pathways to disrupt the balance of pro-apoptotic and anti-apoptotic effects. Therefore, targeting the apoptosis pathways is a unique strategy to identify drug candidates derived from natural products for breast cancer treatment [10].

Apoptosis resistance mechanisms increase the cancer cell's survival and decreases their sensitivity to therapeutics [11]. Apoptotic pathways with different regulation in normal and tumor cells is a good approach to develop cancer-specific strategies. The balance between pro- and anti-apoptotic factors generally determines the cancer cell behavior; in tumor cells, activation of molecules in the upstream of apoptotic signaling pathways or inhibition of anti-apoptotic factors can induce apoptosis [12]. The presence of high levels of anti-apoptotic factors, such as inhibitors of apoptosis proteins (IAPs) in cancer cells, but not normal cells may account for this insensitivity to apoptosis induction in these population of cells. On the other hand, apoptosis-inducing factors in normal cells may be down-regulated in cancer cells. Changes in the expression of these apoptosis-inducing molecules can contribute to escape of these cells from apoptosis and long survival and worse prognosis of cancer [13]. One of the known proteins that plays a role in apoptosis induction and is even known as a tumor suppressor is P53, which is mutated in various cancers and loses its function, which causes

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tumor cells to the escape from apoptosis [14,15]. In breast cancer, mutations in the p53 gene are linked to more aggressive disease and poorer overall survival. p53 plays a crucial role in regulating cell survival by inhibiting the PI3K/AKT survival pathway in epithelial tumors. This inhibition is essential for p53-mediated apoptosis in malignant cells [16].

In one study, several human breast cancer cell lines were analyzed for the BCl-2 expression as an anti-apoptotic protein and tumor suppressor gene p53. This study found an inverse correlation between the expressions of two proteins. This means that the increased expression of BCl-2 is associated with a decrease in the expression of P53 protein with tumor-suppressing activity [17]. In another study, decreased expression of the apoptotic protein BIF-1 (Bax interacting factor 1) was found in breast cancer [18]. Reducing the expression of DNA fragmentation factor 40 (DFF-40) as an apoptotic factor is another example of this category [19]. There are several examples of apoptotic proteins with reduced expression in breast cancer. The current narrative review aims to take a comprehensive look at the list of apoptotic proteins that are reduced in their expression in breast cancer and can be used as urgent drug candidates for cancer treatment and as a tumor marker with diminished expression in breast cancer.

2. KAI1 (CD82)

Recent studies have unveiled the multifaceted role of KAI1 (CD82) in breast cancer patients, offering valuable insights into its potential as a therapeutic target and diagnostic marker [20]. A schema of structure and various functions of this protein are summarized in Fig. 1. Several studies have shed light on the intricate relationship between KAI1 expression and the progression of breast cancer, revealing its role as a metastasis suppressor gene [21]. The down-regulation or loss of KAI1 expression is particularly pronounced in advanced stages of breast cancer, which is often associated with a poor prognosis. Several studies have highlighted the substantial down-regulation of KAI1 expression in breast cancer, with a particular emphasis on its diminished presence in estrogen receptor (ER)-positive breast cancer, confirmed by Christgen et al.'s in 2008. In the mentioned study, a 7.5-fold reduction in KAI1 mRNA levels in ER-positive tumors compared to their ER-negative counterparts was shown. Furthermore, an intriguing correlation between KAI1 and ESR1, unraveling the intricate hormonal control of KAI1 expression. Crucially, the regulatory mechanism governing KAI1's expression isn't confined solely to transcriptional control; it also intersects with therapeutic interventions. For instance, the study ventured into the effects of ER antagonists on KAI1 expression by exposing ER-positive breast cancer cell lines, such as MCF-7 and T-47D, to fulvestrant as an ER antagonist. This revealed the ability of fulvestrant, to up-regulate KAI1 expression and concurrently inhibit cell proliferation and migration in ER positive breast cancer cells. These findings hold substantial promise, suggesting a potential avenue for endocrine therapies targeting KAI1 as a therapeutic strategy in the context of ER-positive breast cancer.

Kussaibi et al. also showed in 2019 that a significant proportion of breast cancer samples exhibited loss of KAI1 expression, and this loss was associated with adverse prognostic indicators, including loss of expression of ER and PR, as well as the over-expression of HER2. Moreover, this study centered its focus on investigating "alternative splicing" as a potential mechanism contributing to KAI1's loss of function in breast cancer patients at a tertiary hospital in Saudi Arabia. They collected 90 breast cancer tissue samples, 38 metastatic lymph node samples, and 70 normal breast tissue samples for comparison. Among the 90 breast cancer samples, a substantial 67 % (60/90) displayed a loss of KAI1 expression. In the remaining 33 % (30/90) with detectable KAI1 expression using the TS82B antibody, only 11 % (10/30), indicating the presence of wild type KAI1 (complete protein). In another 22 % (20/30) of the cancer samples, no detectable KAI1 expression was found, suggesting the presence of truncated-KAI1. This study's significant revelation lay in the detection of alternative splicing of KAI1 in several breast cancer samples, leading to the formation of a truncated form of the protein. Furthermore, no significant correlations were identified with factors such as cancer grade, cancer type, patient age, or P53 expression. However, this truncated-KAI1 expression was notably associated with an advanced cancer stage, particularly featuring lymph node metastasis, indicating a poor prognosis [20]. These findings underscore the importance of alternative splicing as a contributing mechanism to KAI1's loss of function in breast cancer and emphasize its significance in predicting disease progression.

Also, in 2018, Miller et al. investigated the effects of KAI1 variants (wild type and splice variant) on breast cancer cells. The study elucidated their roles in integrin expression, cell adhesion, wound healing, focal adhesion kinase (FAK) activation, proliferation, and epidermal growth factor receptor (EGF-R) up-regulation. The study's findings unveiled distinct effects of KAI1-WT and KAI1-SP on breast cancer cells. In MDA-MB-231 cells, both KAI1 variants reduced the expression levels of integrin αvß3, with KAI1-WT demonstrating a more significant reduction (up to 50 %) compared to KAI1- SP (up to 30 %). Conversely, in MDA-MB-435 cells, both KAI1 variants increased αvß3 levels, with KAI1-

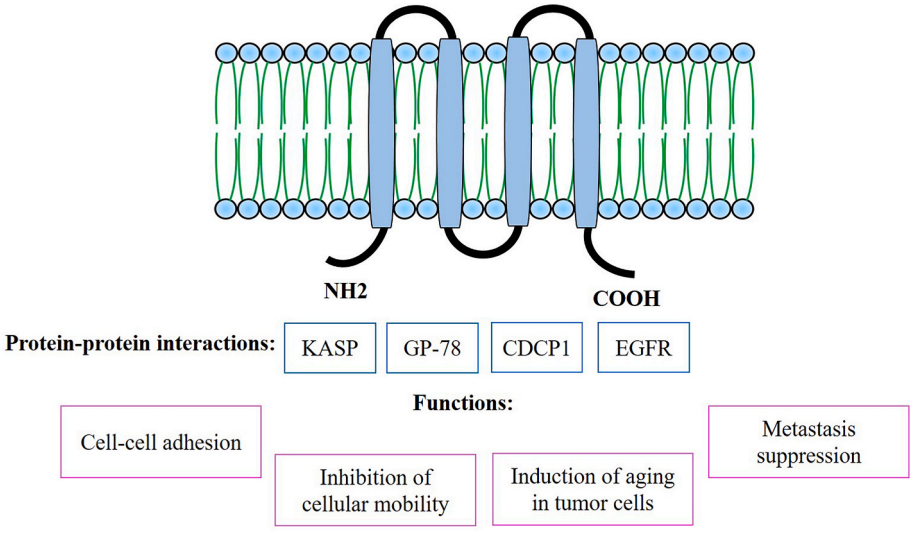

Fig. 1. KAI1 structure and function. This transmembrane protein interacts with various proteins leads to the strong cell-cell adhesion and inhibition of metastasis and mobility of cells. KASP: KAI1/CD82-associated surface protein, EFDR: epidermal growth factor receptor, CDCP1: CUB-domain-containing protein 1.

WT causing up to a 2.5-fold rise and KAI1-SP leading to a remarkable 6.5-fold increase. In MDA-MB-231 cells, KAI1-WT and KAI1-SP enhanced cell adhesion, with KAI1-SP showing a more profound effect. Particularly, KAI1-SP significantly increased adhesion, up to 8.3-fold on vitronectin (VN). Similar patterns were observed in MDA-MB-435 cells, with KAI1-SP transfectants exhibiting the highest adhesion onto VN, up to 2.7-fold. Moreover, the researchers observed that KAI1-SP promoted faster wound healing and compared to KAI1-WT in MDA-MB-231 cells. FAK activation was significantly elevated by KAI1-SP, whereas KAI1-WT had no notable impact, particularly in MDA-MB-231 cells. The study also assessed proliferative activity, revealing that both KAI1-WT and KAI1-SP enhanced cell proliferation, with KAI1-SP exhibiting a more pronounced effect.

Interestingly, KAI1-SP not only increased cell growth but also up-regulated EGF-R [22]. So, KAI1-SP demonstrated a more significant impact on various aspects, including wound healing and cell proliferation, accentuating its potential to promote cancer progression and metastasis.

In another study conducted by Latha et al., in 2019, researchers collected 100 biopsy samples from breast cancer cases in Delhi, India. They performed a comprehensive analysis of KAI1 expression at both the transcriptional and translational levels. KAI1 expression was found to be reduced in 58.3 % of cases and was entirely negative in 20 % of cases. Notably, KAI1 expression was remarkably decreased in breast cancer both at the gene and the protein levels compared to benign breast disease. Among the cases, 64.7 % of those with lymph node involvement and 50 % of those without lymph node involvement displayed reduced KAI1 expression. The difference between these two groups of lymph nodes has observed 1.7-fold down-regulation of KAI-1 mRNA expression in node-positive breast cancer cases. KAI1 expression was notably lower in cases with lymph node involvement and larger tumor sizes in comparison to those without lymph node involvement and smaller tumor sizes. This difference was statistically significant, with a 1.9-fold down-regulation in the mRNA level of KAI1 in cases. It's noteworthy, however, that similar to other studies, KAI1 expression did not exhibit significant associations with various other prognostic variables, including patient age, tumor grade, menopausal status, and receptor status (ER, PR, and Her2). These findings highlighted strong associations between KAI1 expression levels and axillary lymph node status as well as advanced tumor staging, emphasizing the potential of KAI1 expression as a predictive marker for lymph node metastasis and tumor staging in breast cancer patients [23].

KAI1's role in regulating angiogenesis, on the other hand, has gained significant attention. Primarily expressed in pericytes (PCs), KAI1 counters angiogenic factors, opening new avenues for treatments targeting angiogenesis in breast cancer. For example, a study conducted by Lee et al., in 2021 delves into the anti-angiogenic properties of KAI1 in endothelial cells (ECs) and PCs. The study employed KAI1 knockout mice and conducted both *in vitro* and *in vivo* investigations. The findings of the study revealed that KAI1 is primarily expressed in PCs, and its localization in lipid rafts of PCs relies on palmitoylation. Palmitoylation, a crucial mechanism governing the functions of tetraspanin family members, including CD9, CD81, KAI1 (CD82), and CD151, was found to be significantly higher in PCs than in ECs. Transcriptome analysis of KAI1 knockout PCs revealed a reduced expression of "negative angiogenic regulators" and "blood vessel remodeling genes." Specifically, the levels of Sulf1 and Leukemia inhibitory factor (LIF) were significantly decreased in Kai1−/− PCs. Further investigation into the KAI1-LIF axis signaling pathway in PCs unveiled that Src inhibition could reverse the effects of KAI1 overexpression on LIF expression. Src was identified as a pivotal component in this pathway, with the downstream mechanism involving the activation of p53 and Pbx 1 binding elements in the LIF gene-regulating element. Importantly, it was confirmed that p53 serves as a transcription factor specifically activating the LIF gene-regulating element. KAI1 exerts its antiangiogenic effects through two distinct mechanisms. Firstly, it activates LIF expression via the Src/p53 axis, leading to the downregulation of angiogenic genes. Secondly, KAI1 directly binds to VEGF-A (Vascular Endothelial Growth Factor-A) and PDGF-BB (Platelet-Derived Growth Factor-BB), thereby inhibiting VEGF and PDGF signaling. KAI1 negatively regulates angiogenesis via the LIF signaling pathway and by direct interaction with angiogenic cytokines like VEGF-A and PDGF-BB. The treatment with recombinant human KAI1 (rhKAI1) has been shown to reduce angiogenesis in three-dimensional hybrid spheres comprising cancer and blood vessel cells. Furthermore, KAI1 has demonstrated anti-angiogenic effects in *vivo* breast cancer and retinal neovascularization models, underscoring its potential as a therapeutic target to inhibit angiogenesis [24].

A study conducted by Han et al., in 2015, delved into the expression of CD82 (KAI1) and its clinical significance in Cancer Stem Cells (CSCs). The research involved 325 IDC (Invasive Ductal Carcinomas) Chinese patients for the expression of CD82. The study found that in IDC tissues, positive expression rates were 42.2 % (137/325) for CD82 protein. A positive relationship was observed between the expression of CD133 and CD44 and the MVD score, as well as the tumor grade, lymph node metastasis, and tumor-node-metastasis (TNM) stages. Interestingly, CD82 exhibited a negative association with these factors. However, the positive expression of CD133, CD44, CD82, and the MVD score had no significant relationship with factors such as patient age, tumor diameter, or tumor site. The survival time of the CD82-positive expression group was significantly longer than that of the negative expression group. Conversely, a negative association was observed between CD82 expression and CD133 expression, CD44 expression, the MVD score, and Her-2 expression [25].

Taken together, recent studies have revealed the versatile role of KAI1 in breast cancer, highlighting its potential as a therapeutic target and diagnostic marker. These studies emphasize its impact on cancer progression and metastasis, especially in ER-positive breast cancer, where it's often down-regulated. KAI1 variants, like KAI1-SP, have shown significant effects on various aspects of cancer cell behavior, such as adhesion and proliferation. Additionally, alternative splicing of KAI1 has been associated with advanced cancer stages. KAI1's anti-angiogenic properties, particularly in PCs, have opened new avenues for angiogenesis-focused treatments. Lastly, its relationship with markers like CD133, CD44, and CD82 provides valuable insights into cancer stem cells and prognostic factors in breast cancer. Table 1 summarized several studies related to the KAI1 protein in breast cancer.

3. Apoptin

Apoptin is a 14 KDa proline-rich protein from the chicken anemia virus (CAV) with 121 amino acid residues that possess a selective apoptosis-inducing effect on human cancer cells through a p53-independent pathway. While in normal cells, proteasomes degrade it and cause it to lose its cytotoxicity, tumor cells phosphorylate Apoptin on threonine-108, which might be the reason for its tumor-specific activity [26]. These effects give Apoptin a remarkable tumor-selective activity in cancer treatment [26–29]. Multiple studies have evaluated the selective effect of Apoptin in different cancers, including hepatoma, osteosarcoma, melanoma, cholangiocarcinoma, colon carcinoma, lung cancer, breast cancer, prostate cancer, cervix cancer, and gastric cancer [30].

In a study by Ezami et al. Apoptin was expressed in *Escherichia coli* BL21 DE3 using recombinant DNA technology. Apoptin's effect on MCF-7, A549, and Vero cell lines was measured by MTT assay. Interestingly, lower concentrations of Apoptin showed the highest decrease in the proliferation of the Vero cell line. This opposite effect of the concentration of Apoptin on the lethality of cancerous and Vero cell lines indicates the selective effect of this protein on normal and tumor cells. This study concluded that recombinant Apoptin can destroy tumor cells with a selective effect on normal and cancerous cells [29]. With all the benefits mentioned for Apoptin, two major issues exist regarding its use as an antitumor agent. Firstly, no membrane receptors on human cells can

Table 1
Summarizing the multifaceted role of KAI1 (CD82) in breast cancer.

Study	Key Findings	Significance	Year
Christgen et al.	– 7.5-fold reduction in KAI1 mRNA in ER-positive tumors compared to ER-negative. - Fulvestrant upregulated KAI1 and inhibited proliferation/migration in ER-positive cells.	- Correlation between KAI1 and ESR1. - Potential therapeutic strategy for ER-positive breast cancer.	2008
Han et al.	- CD82 expression negatively correlated with tumor grade, lymph node metastasis, and TNM stages. - Positive CD82 expression associated with longer survival.	- Highlights CD82's prognostic value in invasive ductal carcinoma.	2015
Miller et al.	- KAI1 variants reduced/increased integrin levels based on cell type.- KAI1-SP promoted faster wound healing, adhesion, and proliferation.- Upregulation of EGF-R by KAI1-SP.	- KAI1-SP enhances cancer progression and metastasis. - Variants impact adhesion, proliferation, and signaling.	2018
Latha et al.	– 58.3 % cases with reduced KAI1 expression. – 1.7-fold downregulation in node-positive cases.- Significant association with lymph node metastasis and advanced staging.	- KAI1 as a predictive marker for lymph node metastasis and tumor staging.	2019
Kussaibi et al.	– 67 % of 90 breast cancer samples showed loss of KAI1 expression. - Alternative splicing led to truncated-KAI1 associated with advanced cancer stages. - No correlations with grade, type, or P53 expression.	- Highlights alternative splicing as a mechanism for KAI1 loss. - Predicts disease progression and metastasis.	2019
Lee et al.	- KAI1 negatively regulates angiogenesis by activating LIF and inhibiting VEGF/PDGF signaling. - Anti-angiogenic effects observed *in vitro* and *in vivo*.	- KAI1 as a therapeutic target for angiogenesis inhibition in breast cancer.	2021

integrate with apoptin, leading to poor protein uptake in the cancerous and healthy cells. Secondly, this protein is hard to dissolve and preserve in large quantities outside the body. To overcome these two problems, Hou et al. used a recombinant form of Apoptin (folic acid-modified protein with GST: glutathione S transferase). Targeting folate receptors on the cancer cell membrane by folic acid causes the whole protein to enter the cell easily. Also, the folate-GST-Apoptin complex can be produced and preserved in the industry, with good solubility in PBS. This study assessed the proliferation of two breast cancer cell lines (MCF-7 and MDA-MB-231) treated with rApoptin for 24–72 h. The IC50 of rApoptin on these two cell lines was reported 3.2 and 5.7 μg/ml for 24 h. Viability of both cell lines was decreased dose and time-dependently. rApoptin caused a 50 to 70 percent reduction in the number of colonies compared with the control group in both cell lines. Furthermore, the apoptosis rate was increased dose-dependently in the rApoptin group compared with the control group. Western blotting showed that *p*-Akt and *p*-Nur77, considered two of the most critical molecules in the Apoptin signaling pathway, had a significant up-regulation in MCF-7 and MDA-MB-231 cells. Furthermore, rApoptin showed a significantly smaller tumor size than the control group, and necrosis was more evident in the rApoptin group compared with the control. However, tumor weight was not significantly altered. Western blotting showed elevated levels of Bax, Cyt *c*, *p*-Akt, and *p*-Nur77, but the expression of Bcl-2 was significantly reduced *in vivo* in the rApoptin group.

Finally, this study concluded that rApoptin can enter MCF-7 and MDA-MB-231 cell lines and effectively induce apoptosis in tumor cells via the apoptosis-inducing mechanism. Thus, it can be considered a favorable agent in breast cancer treatment [30].

In the other project, Shoae-Hassani et al. conducted a research in which they studied the apoptotic effect of apoptin expressing recombinant λ phage nanobioparticle (NBP) on five breast cancer cell lines (BT-474, MDA-MB-361, SKBR-3, UACC-812, and ZR-75) and non-cancerous bone marrow stem cell (BMSC) *in vitro*. They also studied the effect of this recombinant phage on mice models with BT-474 tumor induced *in vivo*. The MTT assay showed that cell growth of BT-474 and SKBR-3 was inhibited in a time-dependent manner. The MDA-MB-361 cell line was the most inhibited by only a 10 % survival rate. Flow cytometry showed apoptosis was induced in BT-474, SKBR-3, and ZR-75 cells. For the *in vivo* experiment, the NBPs did not show any specific homing site and were detectable in the mice's kidneys, livers, brains, and hearts, but phages were more accumulated in the tumors. NBPs inhibited cancerous cells 96 h after injection. No sign of apoptosis was seen in the histochemistry assay of the brain and heart tissues of NBP-treated mice. The tumor volumes of IM and IV groups were 4 and 2.8 times less than control groups. The survival rate of tumor-bearing mice without NBP treatment over three months was zero percent (all mice died in three months), while the survival rate of NBP-treated groups over three months was 60 % [31].

Wu et al. investigated the possible use of albumin as a vector for apoptin to treat breast cancer via a construct as HSA-PEI-pcDNA-Apoptin. After treatment, the viability of the treated group was reduced by 14.3 %, while the other two groups did not show a significant difference in viability according to the MTT assay. Flow cytometry showed that HSA-PEI-pcDNA-Apoptin significantly induced apoptosis in the MCF-7 cell line. For *in vivo* investigation, HSA-PEIpcDNA-Apoptin was injected into the tail vein of the MCF-7-bearing mice once daily for 25 days. The tumor weight and volume significantly decreased in the HSAPEI-pcDNA-Apoptin group. Western blotting showed apoptin was expressed in normal cells (liver, lung, and kidney) and tumor cells of the HSA-PEI-pcDNA-Apoptin group. The expression of apoptin in the tumor tissue was higher than in the normal tissues that showed apoptin expression. HE-stained lung and liver tissues in the HSA-PEI-pcDNA-Apoptin group exhibited no pathological changes, indicating that apoptin has not damaged the normal tissues. Thus, the *in vivo* experiment showed that HSA-PEIpcDNA-Apoptin can effectively and specifically inhibit breast cancer in the nude mice bearing the MCF-7 cancer cell line [32].

The other study, in this case, conducted by Tang which constructed self-assembled mRNA nanospheres (mRNA-NSs) that deliver apoptin mRNA to cancer cells. MTT assay showed pure mRNA-NSs have very low toxicity on 4T1 and human lung fibroblast cells, but TransIT-X2 can significantly increase their toxicity. The survival rate and cloning ability of 4T1 cells decreased significantly when treated with mRNA-NSs@Dox. Western blotting showed mRNA-NSs@Dox induced expression of apoptin after 48 h, while apoptin was not detectable in the free dox group. Expression of Bax and cleaved caspase-3 were also significantly increased in the mRNANSs@Dox group. For *in vivo* investigation, mRNA-NSs@Dox, mRNA-NSs, free dox, or saline was injected in the tail vein of 4T1 breast cancer mice models every three days. The mRNA-NSs@Dox group had the lowest tumor growth rate compared to other groups, including the free Dox group. mRNA-NSs@Dox tumor volume was 87 and 67 percent reduced compared to the control and free dox group. Immunofluorescence staining showed KI67 (which indicates the degree of malignancy) was significantly reduced in mRNA-NSs@Dox Group [28].

Wang et al. constructed a recombinant adenovirus (Ad-Apoptin-hTERTpE1a) that caused the specific expression of apoptin in breast cancer cells and investigated its synergism with paclitaxel on cancer lines (MCF-7 and MDAMB-231) and non-malignant breast epithelial cells (MCF-10A). Based on the data, 50 MOI Ad-VT + 4 nmol paclitaxel

was shown to have a good synergic effect and was selected for *in vitro* inhibition tests on MCF-7 and MDA-MB-231 cell lines. Ad-VT was cytotoxic against MCF-7 and MDA-MB-231 cells but did not induce toxicity against MCF-10A. Paclitaxel, on the other hand, was cytotoxic against MCF-10A. Paclitaxel cytotoxicity against MCF-10A was reduced when combined with Ad-VT. Ad-VT inhibition of the cancer cells could reach as high as 40 % without adverse effects on normal cells, while alone paclitaxel showed toxicity towards normal cells. The combination of Ad-VT and paclitaxel reduced toxicity and caused a cancer cell inhibition of higher than 65 %, which was higher than Ad-VT or paclitaxel alone. Ad-VT or combined with paclitaxel induces apoptosis in MCF-7 and MDA-MB-231 cells, but the proportion of apoptotic cells in Ad-VT and paclitaxel was higher. Furthermore, the apoptosis induced by paclitaxel group did not significantly differ from the control group, while Ad-VT and AdVT + paclitaxel groups showed a significantly higher apoptosis level (~55 and ~75 %, respectively). The caspase activity assay showed increased caspase-3, 6, and 7 levels in both cell lines treated with Ad-VT. This effect was not seen in the paclitaxel groups. Ad-VT + paclitaxel groups showed higher caspase- 3, 6, and 7 levels than Ad-VT groups. The MMP of Ad-VT was significantly higher than that of the control and paclitaxel groups. Combined treatment showed this effect more significantly. These results showed that Ad-VT might induce apoptosis through the endogenous apoptosis pathway. Furthermore, Ad-VT inhibits cell migration and invasion in MCF-7 and MDA-MB-231 cell lines. Combined therapy showed this effect more significantly, while paclitaxel did not inhibit cell migration. The control group showed the highest luminance intensity during and at the end of six weeks of treatment of mice harboring tumor cells. The tumor volume measurement also reported that combined treated groups had more inhibitory effects than Ad-VT or paclitaxel alone groups. The average tumor inhibition rate of 10^9 PFU/100 μl + 20 mg/kg paclitaxel reached 73.1 % at the highest. The average survival time of each group was reported as follows: control~31.8 days, 20 mg/kg~33.2 days, 10 mg/kg~33.6 days, Ad-VT~37.4 days, 10^9 PFU/100 μl Ad-VT + 20 mg/kg paclitaxel ~40.2 days, and 10^9 PFU/100 μl Ad-VT + 10 mg/kg paclitaxel~39.6 days. The survival rate of Ad-VT with 20 mg/kg paclitaxel and Ad-VT with 10 mg/kg paclitaxel was 80 % and 70 %, respectively. As a final conclusion, this study concluded that the combination of Ad-VT with paclitaxel can lead to synergic effects in the inhibition of breast cancer without damage to normal cells [26].

The other study in which apoptin was used as an apoptotic protein is its fusion to Azurin derivate P28; Azurin is a 14 kDa peptide found in *Pseudomonas aeruginosa*. This peptide selectively enters the cancer cells through a caveolin-mediated pathway and the formation of a complex with P53 protein. Although this protein considerably enters cancer cells specifically, it does not show much inhibitory activity against tumor cells [26]. Noei et al. designed and produced a novel chimeric P28-apoptin protein that uses P28 as cell homing peptid and apoptin as apoptotic agent. The chimeric protein was expressed by *E. coli* BL21 (DE3). MCF7, MDA-MB.231 and HEK293 were treated for 48 h. MTT assay indicated that chimeric protein induced toxicity towards MCF7 and MDA-MB-231 in a concentration-dependent manner (IC50 = 38.55 μg/ml and 43.11). Toxicity in the HEK293 cells was reported to be mild. The chimeric protein significantly decreased the survival of the MCF7 compared with the apoptin. MDA-MB-231 cells showed reduced survival with chimeric protein compared with the apoptin [33].

Hazrati et al. conducted a study to further investigate the same chimeric protein's antitumor effects. The 4T1, MDA-MB-468, Vero, and HEK293 cells were treated with this fusion protein for 48 h. The IC50 values for these cell lines were 1.41 μM, 1.38 μM, 6.13 μM, and 264.49 μM. Furthermore, treatment with chimeric protein increased the rate of late apoptotic cells compared to the control. The protein uptake was significantly higher in 4T1 cells than Vero cells, and FITC-labeled p28-apoptin significantly binds more to the tumor tissue than normal human breast tissue. The p28-apoptin showed lower hemolysis in comparison with PBS. When 4T1-bearing mice were treated with 250 μg of p28-apoptin, showed a reduction in tumor growth. However, the tumors continued to grow in size. After treatment with 500 μg of the chimeric protein, the tumors were significantly smaller than those treated with 250 μg. The control group showed metastases in lung tissue, while lung metastasis was not observed in the p28-apoptin-treated group. Significantly fewer mitotic cells and reduced angiogenesis were observed in the p28-apoptin-treated group compared to the control group. Altogether, these two studies demonstrated that fusing p28 as a cell-penetrating peptide with cytotoxic proteins such as apoptin can markedly increase the efficacy of these proteins as antitumor agents [26].

4. BIF-1

In the first study by Runkle et al., in 2012, a novel tumor-suppressive protein named Bif-1 was reported in triple-negative metastatic breast cancer and identified Bif-1 (also known as SH3GLB1 and Endophilin B1) as a potential therapeutic target for intervention.

Fig. 2 illustrates the mechanism of BIF-1 in mitochondrial-dependent apoptosis by its interaction with the BAX apoptotic protein.

Knockdown or suppression of Bif-1 delays EGFR intracellular trafficking and lysosomal degradation, leading to sustained activation of Erk1/2—a signaling pathway associated with cancer progression—-while also disrupting Rab7 recruitment to early endosomes, reducing Rab7 activation, and enhancing tumor cell migration, thereby underscoring the critical role of Bif-1 in regulating EGFR endocytosis and metastatic potential.

Substantial suppression of Bif-1 expression led to delayed EGFR degradation and sustained receptor activation upon EGF stimulation. Notably, this effect was accompanied by sustained activation of Erk1/2, a downstream mediator of EGF signaling.

These findings were demonstrated in MDA-MB-231, HeLa, PLC-PRF-5, and HCT116. Furthermore, while Bif-1 suppression did not affect EGFR internalization, it markedly reduced EGFR co-localization with lysosomal markers, implicating Bif-1 in facilitating EGFR trafficking to lysosomes for degradation. On the other hand, the impact of Bif-1 on the activation of Rab 5 and Rab7, pivotal regulators of endocytic trafficking was also investigated. These results emphasize the essential function of Bif-1 in regulating EGFR endocytosis through its impact on endosome maturation, pointing to its potential as a therapeutic target for cancer treatments that aim to disrupt EGFR signaling pathways. The inhibition of Bif-1 caused shifts in the intracellular distribution of acidic vesicles and the pH balance. A reduction in fluorescence intensity in Bif- 1-knockdown cells was shown, indicating decreased acidity within vesicles compared to wild-type counterparts.

In this study, utilizing MDA-MB 231 and LM2 cell models, the impact of Bif-1 suppression on cytoskeletal reorganization and chemotactic cell migration in response to growth factors was elucidated. Bif-1 knockdown cells displayed increased membrane ruffling, microspikes, and filopodia projections upon stimulation with EGF or FBS, indicative of an augmented migratory phenotype. Importantly, Bif-1-deficient cells maintained these migratory characteristics for an extended period compared to control cells, suggesting sustained migratory potential. Bif-1 suppression markedly increased cell migration in response to FBS and EGF gradients. Notably, treatment with the EGFR tyrosine kinase inhibitor, gefitinib, effectively attenuated EGF-induced cell migration in both control and Bif-1 knockdown cells, underscoring the specificity of Bif-1-mediated effects on migration via EGFR signaling pathways. Overall, these findings underscore the essential role of Bif-1 in restricting breast cancer cell invasion and migration [24].

In the second study by Mohammadi et al., in 2020, researchers examined the correlation between Bif-1 gene expression levels and hormone receptor statuses, including ER, PR, and HER2, in breast cancer patients, also comparing these findings to patients lacking these hormone receptors. The study analyzed the expression of the Bif-1 gene in normal breast tissue samples (n = 50) and breast cancer tumors (n = 50) utilizing RT-PCR to measure expression levels.

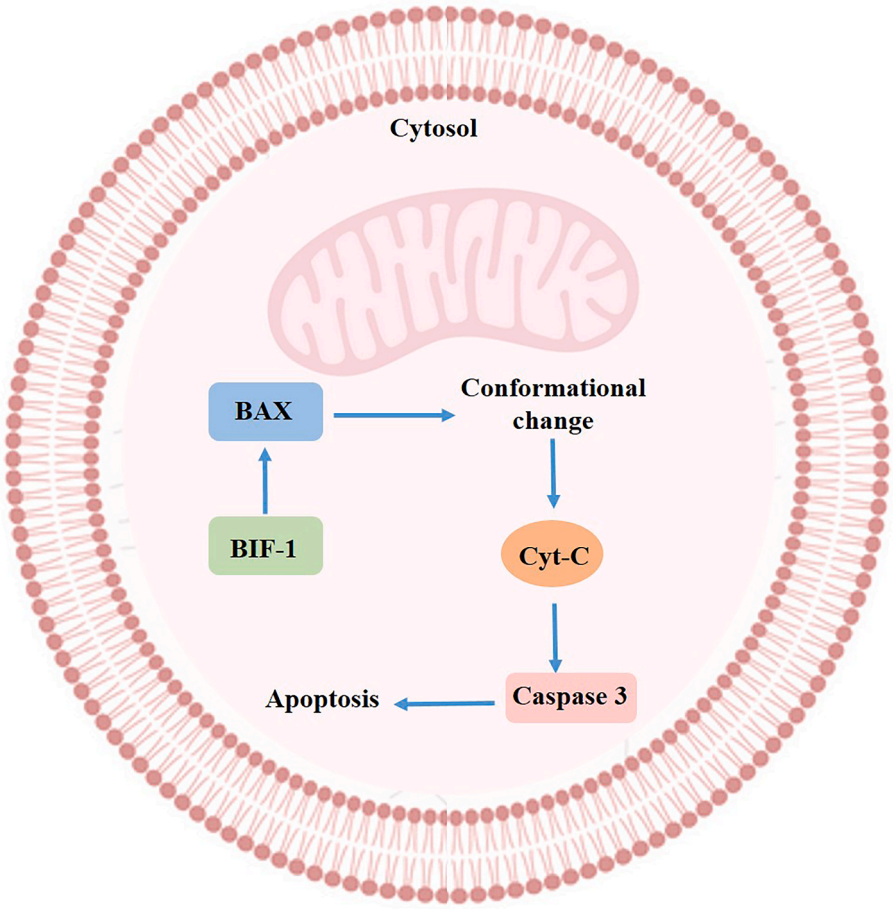

Fig. 2. Schema of BIF-1 function in apoptosis induction. BIF-1 leads to the BAX conformational change and its activation induce the intrinsic apoptosis pathway. BIF-1: BAX interacting factor-1, Cyt-C: Cytochrome-C.

Expression levels were categorized and indicated as 68 % down-expression, 20 % normal, and only 12 % overexpression. So, this significant down-regulation of Bif-1 mRNA in tissues with breast cancer compared to normal controls supports the gene's role as a tumor suppressor. This finding suggests a potential role for Bif-1 in breast cancer progression, as evidenced by its varying expression in tumor versus normal tissues. As for protein level, the Bif-1 protein abundance in breast cancer tissues was lower than in adjacent normal tissues. Additionally, this study evaluated Bif-1 gene expression in various patient groups including lymph node involvement, stage of disease, various breast cancer types, tumor size, and the status of hormone receptors. Bif-1 expression showed no significant correlation with most clinicopathological characteristics.

Specifically, no statistically significant relationship was found between Bif-1 gene expression and lymph node involvement, stage of disease, various breast cancer types, or tumor size. However, Bif-1 expression was higher in patients with at least one hormone receptor, whereas it was reduced in those with triple-negative breast cancer. Nonetheless, no significant correlation was observed between the expression of Bif-1 and the individual hormone receptors including ER, PR, and HER2.

In conclusion, the study highlighted Bif-1 gene expression as a potential biomarker for treatment response in breast cancer. With its decreased expression in breast cancer patients compared to normal tissues, Bif-1 alterations could serve as prognostic indicators. However, the researcher suggested further research with larger sample sizes and various geographical areas for a deeper understanding of Bif-1's role in breast cancer and its potential as a therapeutic target [25].

5. DFF40

DNA fragmentation factor 40 (DFF40) is a DNase responsible for DNA fragmentation in the final stages of apoptosis [34]. In normal cells, DFF45 inhibits the activity of DFF40, but in apoptotic cells, activated caspase 3 breaks down the DFF45, activating DFF40 [35]. Fig. 3 presents the mechanism of DFF40 and its activation by caspase 3, followed by induction of DNA fragmentation. Alterations and flaws in the expression of DFF40 have been reported in several human cancers [36]. Thus, this protein can be a reasonable target for novel cancer treatment methods.

Bagheri et al. demonstrated research investigating the synergic cytotoxicity of DFF40 gene-based therapy and sulfonamides (acetazolamide, sulfabenzamide, sulfathiazole, and sulfacetamide) on T-47D breast cancer cell lines. A plasmid containing DFF40 coding sequence was used for the transfection of cells. DFF40 transfected groups showed 80-folds DFF40 mRNA expression compared to the transfected cells with null plasmid. MTT assay showed that the only significant decrease in viability of the DFF40 over-expressing groups was observed in acetazolamide and sulfabenzamide-treated groups. A comparison of the different cell cycle stages showed that cell cycle arrest was not the leading cause of cytotoxicity in DFF40 over-expressing groups. Sulfabenzamide and sulfathiazole caused partial arrest in the G2/M and S + G2/M stages in both DFF40 and vector-only groups. Overexpression of

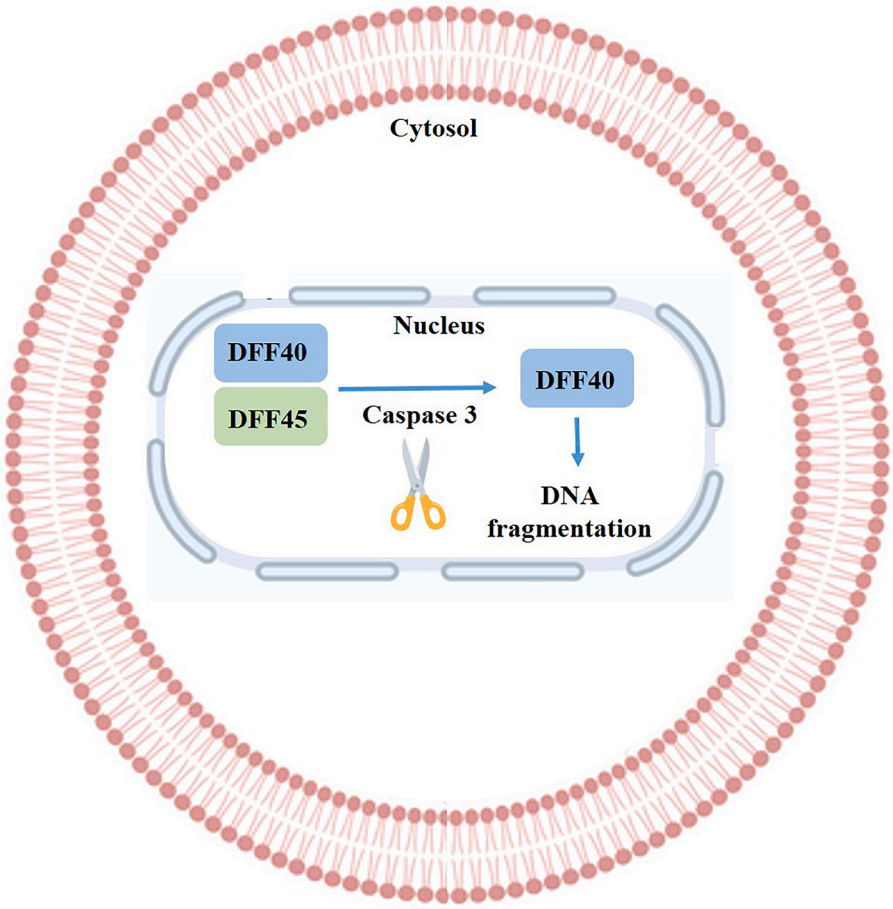

Fig. 3. Schema of DFF40 in the final stage of apoptosis. DFF40: DNA fragmentation factor-40.

DDF40 combined with acetazolamide resulted in internucleosomal DNA fragmentation, a sign of the late stages of apoptosis. This effect was not observed in other groups. Overall, this study demonstrated that over-expression of DFF40 increases the apoptotic effect of some sulfonamides like acetazolamide [35].

In a similar study, the same team investigated the effect of DFF40 overexpression on the T-47D cell line's response to doxorubicin. pIRES2 (vector only) or pIRES2-DFF40 transfected cells were incubated with doxorubicin for 24, 48, and 72 h. MTT assay showed that the IC50 of doxorubicin in the pIRES2 group was the same as the registered IC50 of doxorubicin alone. On the other hand, the MTT assay showed that the cytotoxicity of doxorubicin was increased by nearly 50 % in the pIRES2-DFF40 group after 48 and 72 h of incubation. The expression of DFF40 and DFF450 were not altered in the doxorubicin-treated pIRES2 group, but the expression of caspase-3 was increased. DFF40 overexpression did not affect DFF45 and caspase-3 expression alone, but when accompanied by doxorubicin, it showed elevated levels of DFF40, DFF45, and caspase-3. No change in cell cycle distribution was observed in pIRES2 or pIRES2-DFF40 groups with or without doxorubicin treatment. About 24 % of doxorubicin-treated pIRES2 group cells were apoptotic. In comparison, this number increased to 50 % in the doxorubicin-treated pIRES2-DFF40 group, indicating that over-expression of DFF40 increased the percentage of apoptotic cells. DNA fragmentation was observed time-dependently in doxorubicin-treated pIRES2-DFF40 after 48 h. This effect was not detectable in the doxorubicin-treated pIRES2 group after 24 and 48 h. Finally, overexpression of DFF40 did not significantly affect cell migration in T47D cells [36].

In another study, the Gonadotropin-releasing hormone receptor (GnRHR), which is not commonly expressed in normal human cells but is overexpressed in tumor cells, was established as an attractive target for selective cancer therapy.

Barazesh et al. evaluated the effect of DFF40 in fusion to GnRH on three breast cancer cell lines (MCF7, MDA-MB231, and SKBR3). MTT assay showed that 24 h of incubation with GnRH-DFF40 concentration-dependently decreased the survival rate of all three cell lines compared to DFF40 and negative control.

Western blot test showed that fusion protein was internalized and accumulated in the MDA-MB-231 cells (overexpressing GnRHR) up to 24 h after treatment, and then the total fusion protein content decreased. MDA-MB231 cells incubated with GnRH-DFF40 showed DNA laddering. However, this effect was not observed in MDA-MB231 and HepG2 with no or deficient GnRH receptor expression treated with DFF40 or PBS. Nuclease activity was not concentration-dependent and did not vary in IC50 and 1/2IC50 concentrations. GnRH-DFF40 and DFF40 had DNase activity against naked plasmids in a cell-free system. All three cell lines treated with GnRH-DFF40 showed a significant increase in sub G1 population and partial S + G2/M arrest. The percentage of apoptotic cells in MCF7, which was most affected, after treatment at IC50 concentrations, were as follows: GnRH-DFF40 (54.65 %), DFF40 (7.46 %), and PBS (0.36 %).

The GnRH-DFF40 treatment group exhibited a reduction in MDA-MB-231 cells movement up to 56.2 % and 49.9 % compared to the PBS and DFF40 groups, respectively. GnRH-DFF40 decreased 76.43 % and 61.3 % of cell migration activity in MDA-MB231 cells compared to PBS

and DFF40 [34].

In the other study, internalizing RGD (iRGD with sequence as CRGDK/RGPD/EC) was attached to DFF40 via recombinant DNA technology and assessed for its cytotoxic and apoptotic effects on two breast cancer cell lines with different αvβ3 expressions (MDA-MB231 as αvβ3 positive and MCF-7 as αvβ3 negative cell line).

MTT assay showed that both recombinant proteins (DFF40 and DFF40-iRGD) had cytotoxic effects in a time and concentration-dependent manner. In the MDA-MB231 cell line, DFF40-iRGD was significantly more cytotoxic than DFF40 in similar concentrations. Cytotoxicity of both proteins was increased time-dependent, but after 72 h, the difference in cytotoxicity was less than 24 and 48 h.

In the MCF-7 cell line, after 24 h of treatment, there was no significant difference in cytotoxicity between the two proteins. Treatment with 0.5 μg/ml concentration of DFF40-iRGD caused signs of apoptosis in 76 % of cells and a survival rate of 24 %.

In summary, the designed DFF40-iRGD showed selective cytotoxicity and promising results regarding its apoptosis-inducing and inhibitory effects on tumor cells [37].

6. Conclusion

This paper reviewed four proteins that have received attention recently because of their influential role in inducing apoptosis for breast cancer treatment. Three of the four apoptosis-inducing proteins surveyed are endogenous proteins involved in apoptosis and anti-apoptosis homeostasis in normal cells and tissues. Reduction in their expression level in cancer cells causes an imbalance in their activity. Therefore, in addition to the fact that each of these apoptotic proteins can compensate for the effect of reducing their expression in cancer cells and induce apoptosis via exogenous replacing by recombinant DNA technology production, it can be said that these proteins can be used as tumor markers in identifying cancer; at least judge the prognosis of the disease based on their expression level. Of course, the important point in this context is that these apoptosis-inducing proteins generally have decreased expression levels in cancer, while tumor markers have increased expression levels.

On the other hand, they are not specific to a unique type of cancer. Nevertheless, checking the expression level of these proteins can encourage us to use their replacement therapy for the apoptotic death of cancer cells in the future. The point that can be obtained from this review article is that in using each of these apoptotic agents, there is a need to use a targeting moiety to specifically apply these agents only on cancer cells to reduce adverse effects in normal cells. Another solution is using therapeutic gene strategies for targeted entry into cancer cells or exclusive expression in cancer cells through tumor-specific promoters. However, as shown in this review article, most of the interventions carried out have been limited to the use of apoptosis-inducing proteins at the level of *in vitro* and *in vivo* animal studies, and clinical trials have not been started in any cases.

Therefore, it is still not possible to correctly judge the success of using these agents for cancer treatment. This narrative review can only provide a general summary of apoptotic proteins with reduced expression in breast cancer, based on which therapeutic strategies are designed. However, the effective use of each of these strategies is conditional on determining their efficacy and safety during the clinical trial phases.

CRediT authorship contribution statement

Mohammad Mehdi Khaleghi: Writing – original draft, Data curation. **Faezeh Rouhi:** Writing – original draft, Data curation. **Kourosh Eslami:** Writing – review & editing, Writing – original draft, Data curation. **Fatemeh Shafiee:** Writing – review & editing, Data curation.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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