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14-3-3 ζ as a prognostic marker and therapeutic target for cancer

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Abstract

Importance of the field—The ubiquitously expressed 14-3-3 ζ protein is involved in numerous important cellular pathways involved in cancer. Recent research suggests 14-3-3 ζ may play a central role regulating multiple pathways responsible for cancer initiation and progression. This review will provide an overview of 14-3-3 proteins and address the role of 14-3-3 ζ overexpression in cancer.

Areas covered in this review—The review covers the basic role of 14-3-3 in regulation of multiple pathways with a focus on 14-3-3 ζ as a clinically relevant biomarker for cancer recurrence.

What the reader will gain—14-3-3 ζ overexpression has been found in multiple cancers; however, the clinical implications were unclear. Recently, 14-3-3 ζ has been identified as a biomarker for poor prognosis and chemoresistance in multiple tumor types, indicating a potential clinical application for using 14-3-3 ζ in selecting treatment options and predicting cancer patients' outcome.

Take home message—14-3-3 ζ is a potential prognostic marker of cancer recurrence and predictive marker for therapeutic resistance. The overexpression of 14-3-3 ζ in multiple cancers suggests that it may be a common target to intervene tumor progression; therefore, more efforts are needed for the development of 14-3-3 inhibitors.

Keywords

14-3-3 zeta; apoptosis; cancer; chemoresistance; prognostic marker

1. Introduction

Cancer is a group of diseases caused by a multi-step process involving the malfunction of genes that regulate cell proliferation, cell division, cell death, and tissue microenvironment. The malfunction of these genes leads to a series of acquired capabilities that allow uncontrolled growth and invasion of abnormal cells to other organs. Many types of cancer exist; yet, all malignant growths manifest a set of essential alterations that are shared among different tumor types. These “hallmarks of cancer” are defined as: self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis¹. Identifying alterations

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associated with multiple cancers and central to cancer progression is imperative to improve diagnosis and treatment.

The 14-3-3 family of proteins is known to interact with a multitude of targets that participate in essential cellular processes such as signal transduction, apoptosis, cell cycle and cell migration^{2, 3}. They have also been found to be overexpressed in multiple types of cancers². These findings indicate that 14-3-3 proteins may play a central role in regulating various biological pathways that contribute to cancer progression. Of the seven 14-3-3 isoforms, several recent studies have demonstrated that 14-3-3 ζ overexpression has a direct role in cellular transformation and is associated with poor prognosis in cancer patients. Therefore, in this review, we focus on the role of 14-3-3 ζ in cancer and expand the discussion on the clinical significance of 14-3-3 ζ . We bring a new paradigm in 14-3-3 research: the potential of 14-3-3 ζ as a therapeutic target in human cancers.

2. 14-3-3 Overview

14-3-3 proteins are a family of evolutionally highly conserved acidic proteins expressed in all eukaryotic organisms⁴. Moore and Perez first discovered 14-3-3 in 1967 when they fractionated soluble proteins from brain tissue. They named this abundant brain protein, 14-3-3, based on its particular migration pattern on two dimensional DEAE-cellulose chromatography and starch gel electrophoresis⁵. At least two or more 14-3-3 isoforms have been observed in all eukaryotic organisms. In mammals, there are seven distinct isoforms: β , γ , ϵ , ζ , η , σ , and τ that are encoded by seven different genes. 14-3-3 proteins lack endogenous enzymatic activity; however, they function through binding to phosphorylated-serine/threonine motifs on their target proteins. Following binding, target proteins can be regulated by 14-3-3 through a number of mechanisms including: changing the conformation of the protein, affecting protein activity or stability, facilitating protein complex formation, or altering protein subcellular localization.

Crystal structures have revealed that all 14-3-3 isoforms are strikingly similar in structure forming a flattened horseshoe structure⁶. 14-3-3 proteins exist as dimers, and each monomer in the dimer is composed of nine anti-parallel alpha helices with the dimer interface at the N-terminus. The concave inner surface of the horseshoe contains highly conserved residues while the outer surface is composed of variable residues. The inner domain of each monomer forms a groove, which is highly conserved amongst different species and mediates binding of 14-3-3 to its target proteins⁶. The primary amino acid sequences of the N terminus are quite variable while the crystal structure indicates the residues that create the dimer interface are quite conserved. It has been previously shown that 14-3-3 isoforms can form homodimers as well as heterodimers⁷. Therefore, the variability of the N terminus may be a mechanism for regulating the combinations of possible homo- and heterodimers between different 14-3-3 isoforms. Dimer formation is a functional characteristic of 14-3-3 proteins^{8, 9}. For example, it has been demonstrated that both monomeric and dimeric 14-3-3 molecules can bind to Raf-1; however, only the dimeric form supports Raf-1 kinase activity¹⁰. Thus, 14-3-3 dimerization is required for ligand regulation and may represent a regulatory mechanism controlling 14-3-3 binding.

The primary mechanism controlling 14-3-3 interaction with target proteins is serine/threonine phosphorylation of the interacting target protein⁴. Two prototype 14-3-3 binding motifs, RSXpSXP and RXY/FXXpSXP, where pS represents phosphoserine and X any amino acid, were derived from target binding proteins and extensive phospho-peptide library screening^{11, 12}. Many 14-3-3 interacting proteins contain one or more of these motifs; however, a broad range of substitutions, such as phospho-threonine, can be found in other proteins. Further, several non-phosphorylated proteins have been identified that interact with

14-3-3^{13, 14}. In addition to phosphorylation of 14-3-3 binding proteins, other mechanisms that directly regulate 14-3-3 have been shown to modulate 14-3-3 interaction with target proteins. Phosphorylation of the threonine 232 residue in 14-3-3 ζ and τ can disrupt 14-3-3 binding by altering the conformation of the C-terminal tail¹⁵. 14-3-3 dimers are normally stable and do not readily exchange; however, phosphorylation of the serine 58 residue on 14-3-3 ζ can disrupt binding by altering 14-3-3 dimerization^{7, 16}. Similar residues in other 14-3-3 isoforms can be phosphorylated that inhibit 14-3-3 binding and dimerization^{17, 18}. Sub-cellular localization of 14-3-3 may be another mechanism that directs or limits interacting proteins, especially in development and differentiation¹⁹. Furthermore, 14-3-3 ζ can be post-transcriptionally regulated by microRNA miR-375, leading to down regulation of both 14-3-3 ζ mRNA and protein, effectively altering the available pool of 14-3-3 in cells²⁰. Since 14-3-3 proteins can form homodimers or heterodimers, deregulation of mechanisms controlling 14-3-3 dimerization or expression may alter the balance of 14-3-3 dimers. This unbalance may disrupt cellular homeostasis leading to activation of 14-3-3 targeted pathways that contribute to the initiation of cancer.

3. 14-3-3 targets in cancer

14-3-3 was initially characterized as an activator of tyrosine and tryptophan hydroxylases and regulator of protein kinase C (PKC) activity^{21, 22}. With the advent of proteomics, a plethora of publications have reported that 14-3-3 proteins can interact with several hundreds of binding partners^{23, 24}. Thus, 14-3-3 proteins have been found to interact with target proteins involved in regulating multiple cellular processes, such as cell cycle control, protein trafficking, anti-apoptosis, metabolism, signal transduction, inflammation and cell adhesion/motility^{25, 26} (Figure 1). For examples, 14-3-3 proteins play a pivotal role during cell cycle progression²⁷ by binding and sequestering CDC25C in the cytoplasm thereby separating it from its nuclear substrate Cdc2 and allowing for entry into mitosis²⁵. 14-3-3 binding to the cardiac isoform of 6-phosphofructo-2-kinase/ fructose-2,6-bisphosphatase (PFK-2) implicates 14-3-3 in regulating glycolysis and metabolism²⁸. 14-3-3 proteins participate in the coordination of cell adhesion and motility by regulating the migration of keratinocytes through interactions with $\alpha 6 \beta 4$ and $\alpha 3 \beta 1$ integrins²⁹. 14-3-3 is also involved in the establishment and maintenance of mammalian epithelial polarity, as recently demonstrated in MDCK cells where 14-3-3 binding was shown to promote the dissociation of Par-1b from tight junctions after its phosphorylation by aPKC³⁰.

Although 14-3-3 proteins are highly homologous and have some overlapping binding partners, there is evidence that 14-3-3 isoforms may have distinct binding targets and isoform specific functions^{31–33}. Additionally, the tendency for isoforms to heterodimerize may also regulate the selectivity of potential binding partners and specificity of 14-3-3 interacting targets^{7, 33}. To date, 14-3-3s has been divided into two subgroups, with 14-3-3 σ defined as a tumor suppressor, while other 14-3-3 isoforms may have oncogenic roles. The down-regulation of 14-3-3 σ has been associated with a multitude of human epithelial cancers, including hepatocellular carcinoma³⁴, lung cancer³⁵ and breast cancer³⁶. 14-3-3 σ is considered a tumor suppressor mainly due to its unique role in positively regulating p53 and sustaining the G₂/M checkpoint in epithelial cells following DNA damage^{31, 37}. However, recent studies suggest 14-3-3 σ may also contribute to cancer development³⁸. Other 14-3-3 isoforms appear to be involved in pathways that contribute to transformation related properties. For example, some 14-3-3 isoforms have been identified to be involved in chemoresistance either in cell lines or patients tumors^{39, 40}. Single nucleotide polymorphisms in the 14-3-3 τ gene from soft tissue sarcoma (STS) patients were associated with an early onset of disease and an increased risk of tumor-related death in STS patients⁴⁰. Additionally, knockdown of 14-3-3 β with antisense induced apoptosis in cancer cell lines; whereas, 14-3-3 β overexpression contributed to transformation^{41, 42}. Likewise, a

number of 14-3-3 ζ target proteins are proto-oncogene and oncogene products that have a causative role in the development of human cancers, indicating that 14-3-3 ζ may directly contribute to cancer initiation and progression (Table 1) ^{43–47}.

Overexpression of 14-3-3 ζ in human cancers may contribute to transformation by inhibiting apoptosis, activating signaling pathways that promote growth, and/or sequestering tumor suppressor proteins. 14-3-3 ζ was found to play a major role in anti-apoptosis by directly binding with Akt-phosphorylated Bad, a pro-apoptotic protein, at one or more serine sites, thereby blocking the inhibition of Bcl-2 by Bad ⁴⁸. 14-3-3 ζ can also bind to the Forkhead transcription factor after its phosphorylation by Akt. 14-3-3 ζ negatively regulates Forkhead's function by sequestering it in the cytoplasm, preventing the transcription of the pro-apoptotic proteins Fas and Bax ⁴⁹. Similarly, 14-3-3 ζ enhances Akt-phosphorylated β -catenin-dependent transcription by stabilizing the β -catenin protein in the cytoplasm ⁵⁰. β -catenin co-localizes with activated Akt in intestinal stem cells (ISCs) suggesting 14-3-3 may have a role in stem cell regulation ⁵¹. 14-3-3 proteins can also bind to the insulin-like growth factor receptor (IGF1R) that plays an important role in regulating normal cell survival as well as promoting tumorigenesis. The major site for 14-3-3 ζ and β interaction with IGF1R occurs within a serine rich region that is critical for IGF1R mediated cell transformation ⁵². Mutation of the 14-3-3 binding site in IGF1R blocked colony formation in a soft agar assay and reduced the tumorigenicity in mice, suggesting 14-3-3 ζ and/or β may contribute to IGF1R mediated transformation ⁵³. The tuberous sclerosis (TSC) protein 1 and 2 are involved in tumor suppression by inhibiting ribosomal subunit S6 kinase (S6K) signaling pathways. TSC2 is phosphorylated by Akt and 14-3-3 ζ and β were found to interact with TSC2 in an Akt dependent manner ⁵⁴. 14-3-3 binding to TSC negatively regulates its function and overexpression of 14-3-3 ζ and β compromised TSC inhibition of S6K leading to S6K activation ⁵⁵. These studies indicate that 14-3-3 ζ may play a central role in the development and progression of human cancers.

4. 14-3-3 ζ and Cancer

14-3-3 ζ has been implicated in the initiation and progression of cancer and has been shown to be overexpressed in multiple cancer types (Table 2). Several studies have suggested that 14-3-3 ζ expression contributes to the transformation phenotype. Overexpression of 14-3-3 ζ in breast cancer cell lines enhanced anchorage-independent growth and inhibited stress-induced apoptosis, whereas down-regulation of 14-3-3 ζ reduced anchorage-independent growth, sensitized cells to stress-induced apoptosis, and reduced the onset and growth of tumor xenografts in vivo ⁵⁶. Also in breast cancer, 14-3-3 ζ was found to be elevated in metastatic cells compared to normal ductal epithelium ⁵⁷. In lung cancer, knockdown of 14-3-3 ζ sensitized cells to stress-induced apoptosis and enhanced cell adhesion and cell-cell contacts; two activities that are frequently being suppressed in cancer development ⁵⁸. Similarly, knockdown of 14-3-3 ζ was sufficient to restore the sensitivity of lung cancer cells to anoikis and impair their anchorage-independent growth ⁵⁹. In the context of cancer, overexpression of 14-3-3 ζ may lead to preferential activation of 14-3-3 ζ -targeted pathways and may be a central node regulating multiple pathways involved in cancer development (Figure 1). Additionally, some studies have suggested the possibility that overexpression of other canonical 14-3-3 isoforms may also contribute to tumorigenicity. It is known that 14-3-3 ζ proteins can form homodimers or form heterodimers with other isoforms. In the context of cancer, overexpression of 14-3-3 ζ may interfere with the balance of various dimer compositions within the cell. This would lead to substrate bias for different 14-3-3 home- and heterodimers, which may explain why increasing 14-3-3 ζ , even in the presence of other 14-3-3 proteins, leads to enhanced transformation.

A critical component for improved detection and treatment strategies for cancer is the identification of molecular and biochemical pathways that contribute to the transition from normal epithelium to the first definable stage of cancer. Overexpression of 14-3-3 ζ occurs in pre-malignant hyperplastic stages of oral and esophageal cancers⁶⁰⁻⁶². However the significance of 14-3-3 ζ overexpression in these cancers remains unknown. It was hypothesized that 14-3-3 ζ binding to NF κ B may be a link between chronic inflammation and cancer⁶⁰. Similarly in breast cancer, 14-3-3 ζ has been found to have elevated expression in atypical ductal hyperplasia and ductal carcinoma *in situ* (DCIS)^{63, 64}. For breast cancer, DCIS represents an early stage cancerous lesion in which malignant cells accumulate within the lumen of mammary ducts with no evidence of invasion through the basement membrane into the surrounding stroma⁶⁵. Our recent studies have identified 14-3-3 ζ as a possible key component in DCIS initiation and invasion into the surrounding tissue. 14-3-3 ζ overexpression in mammary epithelial cells (MEC) was shown to severely disrupt the acini architecture of MEC in 3-dimensional (3D) culture resulting in apoptosis resistance and luminal filling⁶³. 14-3-3 ζ overexpression in MEC conferred resistance to anoikis and induced hyperactivation of the phosphoinositide 3-kinase/Akt pathway which led to phosphorylation and translocation of the MDM2 E3 ligase and subsequently increased p53 degradation. Ectopic expression of p53 restored luminal apoptosis in 14-3-3 ζ overexpressing MECs. Furthermore, 14-3-3 ζ overexpression was found to be a “second hit” in a subset of ErbB2-overexpressing DCIS lesions facilitating the transition from non-invasive DCIS into life-threatening invasive breast cancer⁶⁶. Co-overexpression of 14-3-3 ζ and ErbB2 in breast cancers from patients was significantly correlated with distant metastasis. At the cellular level, overexpression of ErbB2 and 14-3-3 ζ in MECs increased cell migration and decreased cell adhesion, respectively. Increased expression of 14-3-3 ζ reduced cell adhesion by binding to and stabilizing expression of the TGF- β receptor I, which activated the TGF- β /Smads pathway. Consequently, activation of the TGF- β /Smads pathway up regulated SIP1, a master transcriptional regulator of epithelial-mesenchymal transition (EMT), leading to E-cadherin loss and epithelial to mesenchymal transition (EMT). In patient DCIS samples, increased expression of 14-3-3 ζ was clearly associated with increased expression of TGF receptor and EMT markers. Importantly, overexpression of both HER2 and 14-3-3 ζ in breast tumors was associated with poor prognosis and higher rates of metastatic recurrence in cancer patients. These studies indicate that 14-3-3 ζ may contribute to the development of early stage cancers and promote the transition to invasive cancers.

5. 14-3-3 ζ as a marker for cancer recurrence and chemoresistance

5.1. 14-3-3 ζ as a cancer prognostic marker

The discovery of new markers of disease recurrence and distant metastasis could help to identify patients for more aggressive treatment earlier in the course of cancer development. Additionally, identification of markers of therapeutic resistance would allow clinicians to choose treatment regimes and spare patients side effects from treatments that may not be effective. 14-3-3 ζ overexpression in multiple cancers and their interactions with multiple cellular pathways has implicated the important role of 14-3-3 ζ in cancer progression; however, the clinical significance of 14-3-3 ζ in human cancers has just begun to emerge.

The evolving 14-3-3 research indicates that 14-3-3 ζ may be a prognosis marker to predict cancer recurrence and treatment resistance. In head-and-neck/oral squamous cell carcinomas (HNSCCs), patients with 14-3-3 ζ positive tumors had a shorter disease-free survival than those with 14-3-3 ζ -negative tumors; however, the median time was not significantly different⁶⁷. Interestingly, HNSCC patients having overexpression of both 14-3-3 σ and 14-3-3 ζ had a significantly decreased median disease-free survival compared to patients showing no overexpression of these two proteins. Similarly, 14-3-3 ζ overexpression in non-

small cell lung carcinoma (NSCLC) was significantly associated with reduced survival and disease recurrence in patients ⁶⁸. This study also found high 14-3-3 ζ expression was significantly correlated with histological grade and clinical stage in NSCLC. Most importantly, among the clinical and histological parameters tested, overexpression of 14-3-3 ζ was the only independent predictor for disease recurrence and reduced overall survival in the cohort of NSCLC patients. We recently reported that 14-3-3 ζ overexpression in advanced stage breast cancer was significantly associated with disease recurrence and poor survival in breast cancer patients ⁵⁶. We found that among the clinical and histological parameters tested, 14-3-3 ζ was associated with ErbB2 expression and late stage tumors. In addition to the well-known prognostic markers in breast cancer, i.e., lymph node status and ErbB2 expression, 14-3-3 ζ was found to be an independent marker of disease recurrence. Furthermore, 14-3-3 ζ overexpression, ErbB2 overexpression, and positive lymph node status identified a sub-group of patients with a significantly increased risk of distant metastasis. In another study investigating the contribution of 14-3-3 ζ to ErbB2-mediated transformation, breast tumors co-overexpressing both ErbB2 and 14-3-3 ζ had significantly increased disease recurrence associated with increased risk of metastasis ⁶⁶. Together, these findings indicate that detecting 14-3-3 ζ protein expression levels in cancers may serve as a clinical marker for cancer recurrence.

5.2. 14-3-3 ζ gene amplification in cancer

A common feature of oncogenic transformation is DNA amplification, which can occur when gene copies of specific regions of the genome are increased due to redundant replication. Amplified genomic DNA can contain many copies of a gene and increased gene expression from these amplified regions may confer a selective advantage for cell growth. Amplification and overexpression of cellular oncogenes can be good predictors of clinical outcome and potential therapeutic targets. The 14-3-3 ζ gene (YWHAZ) is located on chromosome 8q22.3, an area frequently amplified in breast and other cancers ^{69, 70}. Several studies in different cancer types suggest that increased gene copy numbers of YWHAZ may be associated with increased protein expression of 14-3-3 ζ . Comparing array CGH data with expression data obtained by high density expression profiling in urothelial carcinomas ⁷¹, it was found that the region spanning chromosome 8q22, which included YWHAZ, was amplified in late stage urothelial tumors. Of the ten genes in this region, two genes, YWHAZ and POLR2K, were found to have a strong correlation between high copy numbers and high gene expression. In another study using a tissue microarray of head and neck squamous cell carcinomas (HNSCC), increased gene copy numbers of YWHAZ, associated with chromosome 8 polysomy, occurred in 30–40% of the cases ⁷². Increased 14-3-3 ζ expression was detected in 77% of the HNSCC samples from the tissue microarray with a subset of cases having both increased gene copy numbers of YWHAZ and increased 14-3-3 ζ protein expression. Similarly, amplification and chromosome 8 polysomy contributes to increased YWHAZ gene copy numbers in breast cancer ⁵⁶. Increased YWHAZ copy number determined by FISH was associated with strong 14-3-3 ζ protein expression detected by IHC staining in breast tumors. Another recent study in breast cancer found that amplification of the chromosomal 8q22 region contained 12 genes, including YWHAZ, associated with metastatic recurrence ⁷³. Amplification of 8q22 was correlated with increased expression of the 12 genes and was found to be a strong independent prognostic factor for breast cancer recurrence. These studies suggest amplification may be a common mechanism leading to 14-3-3 ζ overexpression in multiple cancers, although mechanisms independent of increased gene copy number, such as modulation of 14-3-3 ζ gene transcription, protein translation, or RNA and protein stability may also contribute to increased 14-3-3 ζ expression. Therefore, detection of 14-3-3 ζ gene amplification may also be developed as a clinical marker for cancer recurrence.

5.3. 14-3-3 ζ as a marker for cancer therapeutic resistance

Therapies using cytotoxic drugs or radiation form the basis for effective treatments of multiple cancers. However, the responses to chemotherapeutic agents can vary and drug resistance can often lead to treatment failures. One obstacle for the successful treatment of cancers by chemotherapeutic agents is the intrinsic or acquired resistance to drug treatments. Increased expression of 14-3-3 ζ in cancer and its role as an anti-apoptotic protein have been linked to resistance to anticancer therapies (Table 3).

In breast cancer, the expression of 14-3-3 ζ may be increased by tamoxifen treatment leading to tamoxifen resistance⁷⁴. Tamoxifen treatment increased expression of 14-3-3 ζ via the estrogen receptor (ER) in breast cancer cell lines; however, increased expression of 14-3-3 ζ was not regulated by estrogen. High expression of two tamoxifen stimulated genes, YWHAZ/14-3-3 ζ and LOC441453, were found to correlate significantly with disease recurrence following tamoxifen treatment in women with ER-positive breast cancers, suggesting 14-3-3 ζ overexpression may contribute to tamoxifen resistance. Additionally, amplification of 8q22 was associated with the increased expression of 12 genes in this region and chemo resistance in breast cancer⁷³. 8q22 amplification led to overexpression of 8q22 genes in tumor tissue, which was associated with poor prognosis in untreated cases and increased disease recurrence despite adjuvant chemotherapy. An siRNA screen of the 12 genes revealed that down regulation of YWHAZ and LAPTM4B increased sensitivity to chemotherapeutic drugs in breast cancer cell lines. The increased sensitivity was specifically to anthracyclines but not cisplatin or paclitaxel. Increased expression of YWHAZ or LAPTM4B in breast tumors was associated with poor outcome after adjuvant chemotherapy. Furthermore, increased expression of YWHAZ and/or LAPTM4B in tumors from breast cancer patients could predict a lower clinical response to epirubicin but was not associated with response to cisplatin or docetaxel. In diffuse large B cell lymphoma (DLBCL), 14-3-3 ζ was identified to be overexpressed in cell lines which were resistant to the standard anthracycline-based chemotherapeutic combination consisting of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP)⁷⁵. Knockdown of 14-3-3 ζ expression in DLBCL resistant cell lines restored the sensitivity of resistant cell lines to apoptosis induced by CHOP. Increased expression of 14-3-3 ζ was also detected in patients with DLBCL, and 14-3-3 ζ may lead to CHOP resistance in these patients. Similarly, elevated expression of 14-3-3 ζ was observed in a prostate cancer cell line resistant to 9NC6, a camptothecin derivative⁷⁶. Other 14-3-3 isoforms have also been identified to be involved in chemoresistance either in cell lines or in patients^{39, 40}.

These data support the use of FISH analysis of YWHAZ in combination with 14-3-3 ζ protein expression as clinically relevant markers to predict prognosis and response to anti-cancer treatment. 14-3-3 ζ status in patients' tumors may guide their treatment options. The significant association of 14-3-3 ζ overexpression with disease recurrence and chemoresistance also makes 14-3-3 ζ an attractive candidate for targeted therapy in multiple cancers.

6. 14-3-3 ζ as a target for anti-cancer therapy

Since 14-3-3 ζ overexpression can lead to apoptosis resistance, cancer recurrence and chemoresistance, it is imperative to design methods to effectively block the function of 14-3-3 ζ in human cancers. 14-3-3 ζ is not limited to cancer and may also represent a potential target for other diseases⁷⁷⁻⁷⁹. However, identification of 14-3-3 targeting molecules has been an underdeveloped area of research. Presently, only small interfering RNAs, antisense, and peptide inhibitors are used for targeting 14-3-3 ζ under experimental conditions.

6.1. Small Interfering RNA (siRNA)

Double stranded small interfering RNAs (siRNA) of 21 nucleotides can be used to inhibit specific genes without inhibiting global protein synthesis⁸⁰. The mechanism of siRNA involves the degradation of the targeted mRNA and therefore reduced protein abundance. This makes siRNA a powerful tool to study specific gene functions; however, the off-target effects of siRNA are a limiting factor for this technology⁸¹.

In lung cancer, 14-3-3 ζ knockdown by siRNA increased the sensitivity to cisplatin both *in vitro* and *in vivo*⁶⁸. We have shown that 14-3-3 ζ siRNA treatment of cancer cell lines sensitized cells to stress-induced apoptosis and effectively reduced the onset and growth of tumor xenografts⁵⁶. Several other studies in multiple cancer types have shown that knock down of 14-3-3 ζ via antisense or siRNA is effective at reducing tumor growth and sensitizing cancer cells to apoptosis inducing agents^{75, 82, 83}. These studies suggest using siRNA or antisense RNA to knockdown 14-3-3 ζ expression in tumors may be a potential therapeutic strategy; however, the clinical feasibility of using siRNA or anti-sense as therapies is still a challenge that needs to be met⁸⁴.

6.2. Peptide inhibitors: R18/Difopein

R18 is a small non-phosphorylated peptide identified from a phage library that non-selectively binds all 14-3-3 isoforms with high affinity and is a potent inhibitor of 14-3-3 ligand interaction⁸⁵. To facilitate delivery of R18 into cells and enhance its efficacy, two R18 coding sequences separated by a sequence coding for a short peptide linker were cloned into a mammalian expression vector. The translated product consisting of two R18 peptides linked together, called difopein (dimeric fourteen-three-three peptide inhibitor), was found to induce cell death alone, and enhance cell death by cisplatin^{86, 87}. In BCR-ABL transformed cells the R18 dimer effectively induced apoptosis, in part through liberation and reactivation of FOXO3a but not by disrupting 14-3-3/BCR-ABL association or BCR-ABL tyrosine kinase activity⁸⁸. Furthermore, cells expressing imatinib-resistant BCR-ABL mutants were more susceptible to apoptosis by R18 alone or in combination with signaling pathway inhibitors. Similarly, glioma cell lines showed increased apoptosis *in vitro* following knockdown of 14-3-3 via siRNA or inhibition via difopein⁸⁹. Interestingly, difopein effectively hindered proliferation and triggered apoptosis of tumor cells implanted into nude mice. While R18/difopein is an effective inhibitor of 14-3-3 target binding, it is a global inhibitor of 14-3-3 and is not specific to any isoform, such as 14-3-3 ζ . Isoform-specific inhibitors are not currently available, and research on isoform-specific inhibitors is an underdeveloped area in the 14-3-3 field.

These studies indicate that 14-3-3 ζ is a potential target in multiple cancers. Although current 14-3-3 ζ -targeting approaches are limited to knockdown experiments and peptide inhibitors, efforts are being focused to develop assays to discover 14-3-3 interacting molecules that may interfere with the key 14-3-3 interactions involved in disease^{90, 91}. It is foreseeable that other creative approaches (chemical inhibitors, shRNAs, etc.) will be developed in the future to specifically inhibit 14-3-3 ζ -mediated transformation. The discoveries of 14-3-3 ζ antagonists will not only lead to therapeutic interventions against diseases involving 14-3-3 ζ , it will expand our understanding of the biological functions of 14-3-3 ζ .

7. Conclusions

Recent studies have demonstrated that 14-3-3 ζ overexpression is significantly associated with disease recurrence and resistance to chemotherapeutic agents in cancer patients. Overexpression of 14-3-3 ζ has been associated with anchorage independent growth and a survival advantage under stress conditions. In contrast, knockdown of 14-3-3 ζ reduced

tumor growth and sensitized cells to chemotherapeutic agents. One of the major challenges in cancer treatment is to identify patients at high risk who would benefit from potentially unnecessary and toxic systemic therapy. 14-3-3 ζ has been identified as a clinically relevant prognostic marker for multiple cancers and may allow for identification of patients whose tumors are resistant to standard chemotherapies to receive more aggressive treatments. In addition, 14-3-3 ζ regulates known oncogenes and cooperates with HER2 to increase cancer invasion. These findings indicate that overexpression of 14-3-3 ζ may cooperate with known oncogenes leading to more aggressive cancers. Furthermore, multiple studies have found that 14-3-3 ζ gene amplification was associated with poor prognosis and resistance to common treatment regimens of cancer. Gene amplification may be a common mechanism leading to 14-3-3 ζ overexpression in multiple cancers. Although studies of larger cohorts are needed to validate these findings before moving to a clinical setting, these novel findings indicate 14-3-3 ζ as an important molecular marker for disease recurrence in cancer patients.

Even though therapeutic targeting of 14-3-3 is an underdeveloped area of research, several studies have validated 14-3-3 ζ as a target in cancer therapy. These data suggest that 14-3-3 ζ has the potential to serve as a therapeutic target for chemo-sensitization and effective tumor inhibition. Additionally, 14-3-3 ζ is known to modulate multiple downstream molecules of several survival signaling pathways. Therefore, targeting 14-3-3 ζ or the downstream pathways regulated by 14-3-3 ζ may sensitize cells to apoptosis and serve as effective anti-cancer strategies in patients whose tumors overexpress 14-3-3 ζ .

8. Expert Opinion

8.1. Definitive Role in Cancer

Many studies have shown that 14-3-3 ζ regulates central pathways critical for maintenance of the transformed phenotype and 14-3-3 ζ overexpression may activate or enhance numerous pathways that contribute to cancer development and progression. A missing component in the confirmation of the role of 14-3-3 ζ in cancer initiation and progression is the development of genetically engineered 14-3-3 ζ mouse models. Currently, no transgenic mouse models for 14-3-3 ζ exist in the literature. However, an initial unpublished study mentioned in a review by Tzivion et al indicated that a transgenic mouse model expressing the 14-3-3 ζ isoform developed various types of tumors in up to 15% of mice by 7–15 months². In addition, the tumor profile of mice infected with a Polyomavirus middle T antigen (MT) mutant defective in 14-3-3 binding showed a striking deficiency in the induction of salivary gland tumors but not other tumor types⁹². Our recent study also suggests that 14-3-3 ζ can cooperate with HER2 to induce early stage breast cancer to become invasive⁶⁶. These data suggest that 14-3-3 ζ plays a key role in the cooperative induction of tumors mediated by oncogenes. The development and characterization of 14-3-3 ζ transgenic and knockout mouse models will not only provide important information on the role of 14-3-3 ζ in normal development and cancer progression, but also facilitate the discovery of 14-3-3 ζ targeted therapies.

8.2. Powerful Cancer Prognosis Marker

Oncoprotein overexpression and oncogene amplification are often used as makers for predicting clinical outcome in cancer patients. Recent studies have demonstrated that increased expression of 14-3-3 ζ is associated with cancer recurrence and increased incidence of distant metastasis. In addition, 14-3-3 ζ overexpression and gene amplification have been associated with the development of resistance to multiple therapies. These studies have provided the basis for developing 14-3-3 ζ into a useful tumor marker. The utility of 14-3-3 ζ overexpression is not limited to a particular cancer type; 14-3-3 ζ can potentially be utilized as a global tumor marker and therapeutic target for multiple cancer types. However, in order

for 14-3-3 ζ to gain widespread clinical application, further clinical validation and standardization will need to be accomplished. Current studies have utilized small patient cohorts to establish the importance of 14-3-3 ζ in association with clinical outcomes. To further validate 14-3-3 ζ as a tumor marker, larger cohorts need to be investigated in both retrospective and prospective studies. A most important issue for 14-3-3 ζ marker development is the standardization of detection and staining methods. Recent studies implicating 14-3-3 ζ overexpression in cancers have used multiple methods of detection, including proteomic detection methods. A readily available method of detection and a standardized antibody for 14-3-3 ζ staining would strengthen results across studies. Our laboratory has successfully used standard immunohistochemistry (IHC) methods for paraffin embedded tissues with the 14-3-3 ζ clone C-16 from Santa Cruz. Validation of this antibody in our studies has shown this antibody to be specific for 14-3-3 ζ in both western blotting and IHC staining⁵⁶. In addition, a standardized method for scoring 14-3-3 ζ expression in tissues is needed to further compare results across studies.

14-3-3 ζ has been demonstrated to be a promising biomarker to identify high-risk patients for more aggressive and alternative therapy at earlier stages of cancer progression. Since 14-3-3 ζ is overexpressed in diverse tumor types, many patients will benefit from the validation of 14-3-3 ζ as a prognosis and chemoresistance marker. The development of clinical assays to detect 14-3-3 ζ expression in multiple tumor types will provide invaluable information for disease progression and treatment course in cancer patients.

8.3. Promising Therapeutic target

The overexpression of 14-3-3 ζ in multiple types of cancers and its significant association with poor prognosis indicate an immediate need to target 14-3-3 ζ . 14-3-3 ζ impacts multiple pathways and may act as a central hub for controlling numerous targets implemented in cancer progression (Fig. 1). In this “Spoke and Hub” hypothesis, a disruption at the hub would create interference in many cellular pathways. Previous *in vitro* studies utilizing siRNA and the 14-3-3 peptide inhibitor, difopein, have indicated 14-3-3 ζ as a potential target for cancer therapy. However, difopein targets all isoforms and has only been used in experimental studies. Targeting 14-3-3 ζ with siRNA has been an effective and isoform specific method for sensitizing cancer cells to apoptosis in experimental studies; however, the use of siRNA in the clinic is still under investigation and is hampered by inefficient delivery methods^{84, 93}. The invention of new 14-3-3 ζ -targeting agents/methods is an underdeveloped area of study.

Targeting 14-3-3 isoforms represents a major challenge since 14-3-3 does not have catalytic activities. Since 14-3-3 has been shown to regulate many targets, it is likely to have an important role in many normal cellular functions. Therefore, global inhibition of 14-3-3 functions might have unknown consequences in normal cells. Ideally, molecules that target 14-3-3 would be isoform specific and disrupt their dimerization or binding to specific targets. The protein structures of all 14-3-3 isoforms have been resolved and should facilitate the development of 14-3-3-targeting agents; however, this is a field in the early stages of development⁹⁰.

Alternatively, instead of targeting 14-3-3 ζ , therapies could be directed towards targets known to be regulated by 14-3-3 ζ . For example, many Akt downstream targets associated with cell survival are 14-3-3 binding partners. We have shown that 14-3-3 ζ overexpression in mammary epithelial cells can activate Akt⁶³. Furthermore, we have found that 14-3-3 ζ can activate PI3K, the upstream regulator of Akt (unpublished observation). This suggests that targeting the PI3K/Akt pathway in 14-3-3 ζ overexpressing tumors may be an effective therapy. In addition, we have recently found that 14-3-3 ζ can stabilize and activate the TGF receptor I, leading to activation of downstream signaling and EMT. The TGF pathway also

represents a potential target for treating 14-3-3 ζ overexpressing tumors⁹⁴. Targeting pathways activated by 14-3-3 ζ would accelerate the discovery of therapies that may be effective in patients with 14-3-3 ζ overexpressing tumors. This is an area of research which needs more focus and may bring the greatest benefit to patients with 14-3-3 ζ overexpressing tumors.

Article Highlights

- 14-3-3s, a highly conserved family of phospho-serine binding proteins, bind and regulate numerous targets involved in regulating multiple cellular pathways.
- Overexpression of 14-3-3 ζ is associated with diverse cancer types and regulates pathways that promote cancer initiation and progression.
- 14-3-3 ζ overexpression and gene amplification are correlated with poor prognosis and chemoresistance in cancer patients.
- 14-3-3 ζ is a potential therapeutic target in multiple cancers.
- Development of 14-3-3 ζ targeting agents is an under developed area of study.

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Figure 1.

14-3-3 regulates multiple pathways involved in cancer.

14-3-3 isoforms are homo- and hetero-dimeric, phospho-serine binding proteins. The 14-3-3 family proteins have been found to interact with and regulate several hundreds of proteins involved in multiple cellular pathways summarized in the figure. These pathways not only function to maintain normal cellular homeostasis, but, when deregulated, contribute to cancer initiation and progression. In this context, 14-3-3 may represent an ideal target for cancer therapy since it acts as a central hub regulating many pathways involved in cellular transformation common to various cancers.

Table 1Representative transformation-associated 14-3-3 ζ binding targets

14-3-3 ζ binding target	Reference
c-Raf-1	43
Polyomavirus middle T antigen	44
BCR/abl	45, 46
hTERT	47
IGF-IR	52, 53
β -catenin	50, 51

Table 2

Cancer types associated with 14-3-3 overexpression

Tumor Type	14-3-3 Isoform	Poor Prognosis	Reference
Breast	ζ	Yes	56 [†] , 57, 63, 64, 66 [†] , 73 [†] , 74 [†]
Lung	ζ, multiple	Yes	68 [†] , 95
Pancreas	ζ		96
Colon	ζ		97
Esophageal	ζ		61, 98
Stomach	ζ		99
Oral	ζ		100
Head and Neck	ζ	Yes	62, 67 [†] , 72, 101
Urothelial	ζ		71
Renal	ε		102
Brain, astrocytoma	β, η, multiple		103, 104
Brain, meningioma	multiple		105
Chronic myeloid leukemia	ζ		106
Diffuse large B cell lymphoma	ζ		75
Papillomavirus-induced carcinomas	ζ		107

[†] Studies associated with 14-3-3ζ overexpression and poor prognosis in cancer patients

Table 3Cancer treatments with reduced efficacy in patients with 14-3-3 ζ -overexpressing tumors

Treatment Type	Reference
tamoxifen	74
doxorubicin	73
epirubicin	73