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Emerging role of caldesmon in cancer: A potential biomarker for colorectal cancer and other cancers

Alnuaimi AR *et al*. CaD in colorectal cancer

Abstract

Colorectal cancer is a devastating disease, mainly because of metastasis. As a result, there is a need to better understand the molecular basis of invasion and metastasis and to identify new biomarkers and therapeutic targets to aid in managing these tumors. The actin cytoskeleton and actin-binding proteins are known to play an important role in the process of cancer metastasis because they control and execute essential steps in cell motility and contractility as well as cell division. Caldesmon (CaD) is an actin-binding protein encoded by the CALD1 gene as multiple transcripts that mainly encode two protein isoforms: High-molecular-weight CaD, expressed in smooth muscle, and low-molecular weight CaD (l-CaD), expressed in nonsmooth muscle cells. According to our comprehensive review of the literature, CaD, particularly l-CaD, plays a key role in the development, metastasis, and resistance to chemoradiotherapy in colorectal, breast, and urinary bladder cancers and gliomas, among other malignancies. CaD is involved in many aspects of the carcinogenic hallmarks, including epithelial mesenchymal transition *via* transforming growth factor-beta signaling, angiogenesis, resistance to hormonal therapy, and immune evasion. Recent data show that CaD is expressed in tumor cells as well as in stromal cells, such as cancer-associated fibroblasts, where it modulates the tumor microenvironment to favor the tumor. Interestingly, CaD undergoes selective tumor-specific splicing, and the resulting isoforms are generally not expressed in normal tissues, making these transcripts ideal targets for drug design. In this review, we will analyze these features of CaD with a focus on colorectal cancer and show how the currently available data qualify CaD as a potential candidate for targeted therapy in addition to its role in the diagnosis and prognosis of cancer.

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Core Tip: The actin-binding protein caldesmon (CaD) plays an important role in cancer development, metastasis, and resistance to chemotherapy. CaD has emerged as a significant player in carcinogenesis, as it features many cancer hallmarks, including epithelial mesenchymal transition, angiogenesis, and immune evasion. Interestingly, CaD undergoes selective tumor-specific splicing, and the resulting isoforms are generally not expressed in normal tissues. These data qualify CaD as an attractive candidate for targeted therapy in addition to its role in the diagnosis and prognosis of cancer.

INTRODUCTION

The global cancer burden has increased to approximately 19.3 million cases and 10 million cancer deaths in 2020[1]. Colorectal cancer is the second most common prevalent cancer, with 5253335 cases, and the third most common cancer worldwide, with 1931590 new cases, in 2020[1,2]. Almost half of the patients with colorectal cancer succumb to the disease[1,2]. Cancer morbidity and mortality are essentially due to the ability of cancer cells to invade, metastasize, and destroy normal tissues. Cancer cells, which undergo this complex process, have the ability to survive in the hostile microenvironment, a process mediated by the accumulation of multiple genetic and epigenetic mutations and the activation of a multitude of signaling pathways fueled, generally, by a state of genetic instability[3].

Cancers of epithelial origin (carcinomas), such as those of the colon, shed away their adhesion molecules and acquire mesenchymal markers that enable invasion and metastasis in a process known as epithelial to mesenchymal transition (EMT)[4-6]. The actin cytoskeleton is an important player in cell motility, division, and contractility among other cellular processes[7]. Multiple actin-binding proteins (ABPs) control these functions of the actin cytoskeleton[8]. ABPs form a growing family of more than 160

proteins that can bind actin monomers, polymers, or both[9]. ABPs can be divided into two broad categories, depending on their effect on actin filament dynamics[10]. The first category controls cytoskeletal responses to external stimuli by regulating G-actin/F-actin turnover. This category includes Arp2/3, ADF/cofilin, profilin, and gelsolin. The second category promotes the formation of higher-order structures, such as actin filament meshwork or bundles. This category includes tropomyosin, caldesmon (CaD), and filamin[10].

CaD is encoded by the CALD1 gene in multiple isoforms (Figure 1, and Supplementary Figure 1). High-molecular-weight CaD (h-CaD; 120-150 kDa) is restricted to smooth muscle cells of visceral and vascular origin, and it has been used in diagnostic histopathology as a specific marker for tumors of smooth muscle or myofibroblast origin. The low-molecular weight CaD (l-CaD; 70-80 kDa) isoforms are expressed in nonsmooth muscle cells[11-13]. CaD, particularly l-CaD, has emerged as a significant player during the development and progression of many types of cancers. For some cancers, such as urinary bladder cancer, glioma, and glioblastoma, the literature consistently suggests an oncogenic role of CaD (Table 1). However, the available data for some other cancers, such as stomach and breast cancer, show contrasting effects of l-CaD, as shown in Table 2. Therefore, we set out to clarify the role of CaD during carcinogenesis, with a focus on colorectal cancers. We will highlight the role of CaD in cancer development and progression, resistance to various therapeutic modalities, and immune evasion. We will also discuss the role of CaD in EMT, modulation of the tumor microenvironment, and tumor-specific splicing.

CAD AND THE ACTIN CYTOSKELETON

Cell motility, which is required for cancer cell invasion into surrounding tissue, intravasation, and metastasis, is driven by cycles of actin polymerization, cell adhesion, and acto-myosin contraction. The actomyosin system in smooth muscle cells is regulated by myosin-linked and actin-linked molecules. The myosin-linked mechanism is essentially based on myosin phosphorylation by Ca^{2+}/calmodulin-dependent myosin

light chain kinase and dephosphorylation by a type 1 myosin phosphatase, which is targeted to myosin by a regulatory subunit[14]. The actin-linked mechanisms are mediated *via* complex interactions among a growing family of ABPs; a more detailed discussion of these proteins and mechanisms can be found in specialized reviews[15-19].

CaD and tropomyosin are crucial components of the actin-linked mechanism that regulates the acto-myosin contractile system in smooth muscle. CaD was initially identified as an inhibitory factor for the actin-myosin interaction, in which CaD-induced inhibition can be released by Ca^{2+}/calmodulin. Subsequently, CaD was found to play an important role in cell motility by regulating the contractile system in both smooth muscle and nonmuscle cells[12]. CaD is conserved in almost all vertebrate cells and stabilizes actin filaments directly by binding along the sides of F-actin; it also enhances the binding of tropomyosin to actin[20].

H-CaD has been used as a diagnostic biomarker of smooth muscle and mesenchymal tumors[21-29], while nonmuscle l-CaD is broadly implicated in many aspects of cell motility, including cell migration[30], focal adhesion assembly[31], and podosome dynamics[32]. In cultured and transfected cells, overexpression of the actin-binding domain, or full length, of l-CaD promotes cell movement and facilitates the formation of cytoplasmic processes, while cell contractility is inhibited and the number of focal adhesions is decreased[31].

THE EMERGING ROLE OF CAD IN CARCINOGENESIS

CaD has emerged as an attractive molecule that potentially controls significant steps in tumor formation, cell division, invasion, metastasis, and response to therapy. Early work has shown that the expression and distribution of CaD are different in normal fibroblasts and their transformed counterparts[33]. In normal fibroblasts, myosin, CaD, and tropomyosin were distributed along the stress fibers as expected but were not seen at their termini known as 'focal adhesions/adhesion plaques'[33]. In contrast, these contractile proteins were concentrated within 'podosomes', which are cell-adhesive structures located within the protrusions of the ventral cell surface of transformed cells

and are associated with high motility. Podosomes have previously been shown to have short F-actin bundles[34,35], together with actin-associated regulatory proteins, such as fimbrin[36] and gelsolin[37]. In transformed cells, CaD appears to play a major role in podosome structure and function due to its localization mainly in the podosome core domain with short F-actin bundles, in contrast to myosin and tropomyosin. Thus, CaD was associated with high motility of the podosomes of transformed cells, while the stable adherence of focal adhesions of normal cells was suggested to be due to the lack of this system[33]. The significance of these findings stems from observations of the podosomes of transformed cells being most dynamic adhesive structures with high motility (short half-life), leading to metastasis and invasion, while the focal adhesions of normal cells were not capable of performing these functions[38].

The role of CaD, particularly the light isoform (l-CaD), in solid tumors has been analyzed in various study types, including clinical, bioinformatics, and functional/experimental studies. A comprehensive summary of this literature is supplied in Table 1. This summary does not include the classical use of CaD/h-CaD as a marker for smooth muscle and related tumors, which is not the focus of this review but can be found in other publications/reviews[21-29]. The majority of the publications suggest an oncogenic role of CaD, particularly l-CaD, in many cancer types, such as breast cancer[39], urinary bladder carcinoma[40,41], oral cavity squamous cell carcinoma[42], and colorectal cancer[43], including early onset[44], gastric cancer[45], and lung cancer[46], and it was associated with a poor prognosis in bladder cancer in an *in silico* analysis[47] (Table 1). Moreover, the serum level of l-CaD was found to be high in glioma patients; hence, it is suggested to be a potential serum marker for glioma[48]. Some of the aforementioned studies clearly indicated that the transcript studied or expressed was l-CaD, but others did not specify the transcript. Even in the last case, it is most likely that the transcript responsible for these actions is nonsmooth muscle is l-CaD because h-CaD expression is most likely to be restricted to smooth muscles and their tumors.

In contrast, a smaller number of publications have reported contradictory results (Table 2). Following an earlier report that CaD is a cell motility suppressor[49], tumor suppressor functions were shown *in vitro* using breast[50,51], colon[50], thyroid, and prostatic cancer cells[52], and CaD was suggested to be a metastasis suppressor in gastric cancer[53]. Overall, the overwhelming majority of the recent literature supports the idea that l-CaD exerts multiple oncogenic potentials by upregulating tumor cell motility, angiogenesis, and cell division, as well as modulating the tumor microenvironment. Furthermore, l-CaD overexpression was associated with resistance to immunotherapy and chemotherapy and poor overall survival in multiple cancer types (Table 1).

CAD, TRANSFORMING GROWTH FACTOR-BETA SIGNALING, AND EMT

Cancer cells activate EMT to move and migrate from the primary tumor to other parts of the body. EMT is an essential process of cellular plasticity for normal tissue and organ development, yet it is also involved in an array of oncogenic processes, including proliferation and invasion, angiogenesis, stemness, and resistance to chemoradiotherapy[73,74]. The process involves major changes in the phenotype of cancer cells within the primary tumor marked by loss of an epithelial phenotype and gain of a mesenchymal phenotype. EMT is the first of many steps leading to metastasis. Different factors are involved in activating EMT, such as environmental factors, signaling molecules, and transcription factors. EMT is tightly controlled in normal tissues by maintaining a balance between EMT transcription factors, while in cancer, the process is much more complicated. Once the primary cancer is formed, different triggers stimulate the movement of tumor cells for nourishment, exchange of nutrients and/or immune escape. These factors, such as hypoxia, oxidative stress, nutrient deprivation, and inflammation, activate a set of transcription factors, including transforming growth factor-beta (TGF-β), Wnt, SNAIL, TWIST and MAPK/ERK-ZEB1, among others[73-77]. All of these signaling pathways participate in crosstalk with each other and share interconnected regulatory components, which together with their targets form a

complex network[78]. Comprehensive transcriptomic analysis of a large cohort have shown that EMT is the most dominant program in colorectal cancer[79].

TGF-β signaling is a potent inducer and one of the best-characterized EMT pathways. Although TGF-β potently promotes tumor progression *via* mechanisms that include activation of the EMT program and the resulting invasion of carcinoma cells into surrounding nonneoplastic tissue, it may negatively control the initial stages of tumor formation through its antiproliferative effects. However, some tumor cells solve this problem by inactivating other components of the pathway, such as SMADs[80,81], rather than TGF-β itself. The expression levels of cytoskeletal-associated proteins, including the actin binding protein CaD, increase during TGF-β1-induced EMT[69]. CaD was shown to play a key role in TGF-β-driven EMT of normal murine mammary epithelial cells. Nalluri *et al*[60] found that induction of EMT by TGF-β1 is mediated by increased expression together with increased levels of phosphorylated CaD, which was associated with increased focal adhesion number and size and increased cell contractility. CALD1 appears to play a major role in colorectal cancer *via* EMT induction because its expression is significantly and specifically upregulated in the consensus molecular subtypes 4 subtype, which is characterized by TGF-β signaling activation together with other EMT phenotype indicators, such as invasion of the stroma by malignant cells and marked angiogenesis[82]. Moreover, Calon and coworkers showed that the poor prognosis of colorectal cancer is linked to TGF-β signaling in stromal cells that results in CALD1 overexpression[83].

CAD CONTRIBUTES TO TUMOR ANGIOGENESIS

The HeLa l-CaD I and II splice variant and protein isoforms were initially cloned from HeLa S3 in 1992[11]. L-CaD was found to be associated with actin filaments (stress fibers) and tropomyosin in quiescent cells, but l-CaD, tropomyosin and myosin were not seen at the focal adhesions end of these fibers[33]. Endothelial cells (ECs) and endothelial progenitor cells (EPCs) are quiescent under normal conditions. However, these cells are activated in tumors under hypoxia and other environmental stimuli to start to

proliferate and migrate in the process of angiogenesis. Upon activation of ECs/EPCs, changes in focal adhesions occur, and simultaneous remodeling of F-actin causes changes in cell shapes[84]. These events enable the navigation of EC tips during angiogenesis and the recruitment of circulating EPCs from bone marrow to the site of neoangiogenesis. The HeLa l-CaD-containing cell protrusions were found to be specific for tumor ECs/EPCs and have never been observed in normal ECs[68]. Consistent with this finding of podosomes in ECs[85], Zheng *et al*[68] found a variety of motility-related cell protrusions, such as filopodia, microspikes, lamellipodia, podosomes, membrane blebs and membrane ruffles, in the activated ECs/EPCs of various human tumors under a histologically preserved microenvironment. HeLa l-CaD appeared to be invariably expressed in the subregions of these cell protrusions. Furthermore, HeLa l-CaD-positive multinucleated ECs/EPCs were observed in the glioma samples, among other tumor samples. These cells appeared to be highly motile because they were ubiquitously distributed in the tumor tissue sections[68]. Multinucleation is considered to be a sign of aborted cytokinesis and is associated with the activation of aortic EC motility and podosome formation[85,86].

The expression of HeLa l-CaD was restricted to the tumor vasculature and was not found in normal blood vessels of cancers derived from various organs, including breast, lung, kidney, colon, stomach, ovary, uterus, prostate, thyroid, and liver[87]. HeLa l-CaD was preferentially expressed in the early stage of tumor neovascularization. The available data suggest that HeLa l-CaD can be considered a marker for angiogenic ECs during the early stages of tumor neovascularization[87]. Taken together, these findings suggest that HeLa l-CaD is implicated in the migration of ECs/EPCs in human neoplasms, where they contribute to tumor angiogenesis[68].

A recent study of the mechanisms underlying the effect of l-CaD on microvascular facilitation and architecture in glioma showed that l-CaD is associated with abnormal microvessels in anaplastic astrocytoma and glioblastoma (an aggressive grade IV astrocytoma)[66]. The mechanism of such action was suggested by biofunction prediction to occur by modulating tumor angiogenesis, as ECs and pericytes were more apparent

in the tumor microenvironment of high CALD1 expression samples. Histological and immunofluorescence examination of tumor tissue showed that CaD was associated with vessel architecture in astrocytoma and glioblastoma[66,68]. In stage III/IV mismatch-proficient colorectal cancer, CALD1 was upregulated and associated with angiogenesis, as detected by bioinformatics 'Weighted gene coexpression network analysis' (WGCNA)[55].

L-CAD IS A TUMOR-SPECIFIC SPLICE VARIANT

Alternative splicing is an attractive mechanism of mutation acquisition by cancer cells, as it has the potential to expand a limited number of genes into very complex proteomes and endow them with altered functions, localization, binding properties, and stability[71,88-90]. The CALD1 gene undergoes alternative splicing in cancer tissues, including colon, urinary bladder, and prostate tissues, and these variants are mostly tumor specific. Thorsen *et al*[54] found that the long CALD1 isoform, including an extended form of exons 5 and 6, was absent or reduced in bladder, colon, and metastatic prostate cancer. The dominant splice variant in these tumors is most likely to be transcript variant 2 encoded by WI-38 L-CADII[57]. Other cytoskeleton-associated proteins, such as Tropomyosin 1, ACTN1, and vinculin, were identified as significant candidates for alternative splicing in these tumors in the same study[54], supporting the role and importance of actin cytoskeleton modification in tumor progression[34,35,91]. It is known that splice variants can exert antagonistic functions in tumors, such as the well-known case of the B cell lymphoma (BCL)-X long isoform (BCL-X$_L$), which has an antiapoptotic function, and its short isoform BCL-X$_S$, which is proapoptotic[92]. Indeed, the identified cancer-specific splice variants of CALD1 are predicted to encode proteins with potentially altered functions[54]. Thus, the finding of CALD1 tumor-specific splice variants can explain the reported contrasting effects of the two isoforms, h-CaD and l-CaD, and could explain the oncogenic role of l-CaD in many types of cancers.

The splice variant identified by Thorsen *et al*[54] was confirmed to be tumor specific and associated with metastatic disease and poor overall survival in colorectal cancer[57].

Abnormal splicing was associated with upregulation of l-CaD in glioma tumor tissue samples and body fluids[48,67,93]. Cancer-specific splice variants may potentially be used as diagnostic, prognostic, and predictive biomarkers of various tumors. Moreover, the specificity of these isoforms to cancer cells compared with normal cells makes CaD an ideal selective therapeutic target in cancers[94].

CAD AND RESISTANCE TO THERAPY

CaD was implicated in resistance to multiple modalities of cancer therapy, including chemotherapy, radiotherapy, hormonal therapy and immunotherapy (Figure 2).

CaD and resistance to chemotherapy

It has long been shown that the F-actin associated with transformed cells is different from that of normal cells not only in morphology and function but also in its insensitivity to drugs[95]. The association between CaD and resistance to various forms of cancer therapy has been documented in many cancer types. Dai et al[64] showed that non-small-cell lung cancer (NSCLC) cells enter a state of dormancy upon exposure to 5-fluorouracil (5-FU) and subsequently acquire resistance to this therapy. The mechanism of this resistance involves the accumulation of p53, activation of the ubiquitin ligase anaphase-promoting complex and TGF-β/SMAD signaling that results in EMT, followed by mesenchymal-epithelial transition. Chemotherapy-induced EMT-transformed NSCLC cells showed higher expression of CaD associated with increased invasion potential; however, these EMT-transformed NSCLC cells were arrested in the cell cycle in G0-G1 and lost their ability to divide during this phase[64]. The role of CaD in resistance to 5-FU was documented in locally advanced rectal cancer patients[56].

CaD and antihormonal therapy

CaD was associated with resistance to the targeted antihormonal drug tamoxifen in estrogen receptor (ER)-positive recurrent breast cancer[59]. This study was based on a proteomic analysis to identify a predictive signature for tamoxifen therapy outcomes in

recurrent breast cancer. CALD1 and annexin-A1 (ANXA) were the most differentially expressed proteins and were confirmed by immunohistochemical staining of an independent set of tumors. CALD1 expression showed a significant association with a shorter time to progression, independent of other clinicopathological predictive factors. The majority of proteins that were correlated with ANXA1 were also correlated with CALD1, but a direct link between the two genes (CALD1 and ANXA1) and the mechanism underlying the association have yet to be clarified. CALD1, in particular, was associated with ER downregulation and nuclear factor-kappaB (NF-κB) signaling[59].

CaD and immunotherapy

CALD1 was among the top genes associated with both overall survival and disease-free survival in bladder cancer according to bioinformatics analysis. Tumors with low levels of CALD1 expression had a better prognosis than tumors with high CLAD1 expression[47]. This finding was confirmed in a recent study, and the mechanism was linked to immunomodulation *via* upregulation of programmed death ligand 1 (PD-L1) in bladder cancer[61]. PD-L1 has the potential to suppress the immune response in both physiological and pathological pathways by interacting with its corresponding receptor, PD-1[96,97]. PD-L1 expressed by tumor cells binds to PD-1 on the cytotoxic T-cell surface and thus attenuates immunosurveillance in the tumor microenvironment. Li *et al*[61] found that PD-L1 is associated with CALD1 in bladder cancer cells and that both are induced by interferon-gamma *in vitro*. CALD1 silencing significantly reduced cell viability in T24 bladder cancer cells *in vitro* and *in vivo* in nude mouse xenografts. The authors suggested that CALD1 promoted the expression of PD-L1 *via* the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway[61]. It is likely that the CALD1 effect on PD-L1 is active in other cancers, such as colon cancer, and can exert immunomodulation through this axis because PD-L1 expression is also upregulated *via* JAK/STAT3 after fibroblast growth factor receptor 2 stimulation in colorectal cancer[98].

A recent bioinformatics-based report showed that CALD1 was highly expressed in gastric cancer compared with adjacent normal tissue and that this high expression was associated with poor overall survival in these patients. There was a strong correlation between CALD1 expression and gene markers of M2 macrophages (CD163, VSIG4, membrane-spanning 4A) and Treg and T-cell exhaustion markers (FOXP3, CCR8, STATA5B, TGF-β1, T cell immunoglobulin and mucin domain 3) in gastric cancer. These findings suggest that CALD1 plays an important role in M2 polarization, T-cell exhaustion, and immune modulation in gastric cancer[58].

CAD AND COLORECTAL CANCER

The available data show that CaD plays an important role in the development, progression and response to therapy of colorectal cancer, as detailed below (Figure 3).

CaD contributes to colorectal cancer development

An early hint that CaD could play a role in colorectal cancer development came from the study in 2008 of alternative splicing in cancer by exon array analysis. Briefly, the identified tumor-specific CALD1 variant was missing an extended form of exons 5 and 6 and was predicted to encode proteins with potentially altered functions[54]. This finding implied an oncogenic role of l-CaD in colon cancer, as discussed in more detail above (see "l-Caldesmon is a tumor-specific splice variant").

Based on the proteomic finding of aberrant expression of CaD isoforms in colon cancer, Kim *et al*[43] set out to analyze the particular role of the short isoform l-CaD in colorectal cancer and liver metastasis. They observed a significantly higher expression level of l-CaD in primary colon cancer and liver metastasis than in the corresponding normal tissues. However, h-CaD did not differ among these groups. There was a tendency to have a poor response to chemoradiotherapy in patients with high expression of l-CaD in their tumors, which was confirmed *in vitro* by small interfering RNA (siRNA) silencing of l-CaD and monitoring the response to 5-FU treatment in colon cancer cell lines[43]. L-CaD was suggested to exert these effects by relieving the cell

cycle inhibition exerted by p21^{Cip1} (cyclin-dependent kinase inhibitor 1, or CDK-interacting protein 1) and blocking apoptosis. Furthermore, silencing l-CaD downregulated NF-κB[43], an important signaling pathway that can stimulate tumor cell proliferation, survival, and angiogenesis by controlling a wide network of genes and molecules, such as tumor necrosis factor-α, interleukin-6, BCL2, and vascular endothelial growth factor[99]. Silencing l-CaD also downregulated phosphorylated mammalian target of rapamycin[43], a pathway that regulates not only tumor cell proliferation but also the tumor immune response and metabolism[100]. Collectively, a study showed that high expression of l-CaD in colorectal cancer is associated with increased metastatic properties and a decreased response to therapy[43].

A recent study confirmed that l-CaD transcript 2 is the dominant transcript and is associated with metastatic disease and poor overall survival in colorectal cancer[57]. Interestingly, CALD1 was among the top upregulated genes implicated in the development of early-onset colorectal cancer based on a comprehensive bioinformatics analysis[44]. This finding may shed light on the pathogenesis of early-onset colorectal cancer, which is a heterogeneous category of colorectal cancers that is more common in Eastern than in Western countries[101,102]. The association of CALD1 together with other genes involved in cellular mobility and vascular smooth muscle contraction with early-onset colorectal cancer can explain the aggressive nature of this subset of tumors[44].

A recent study by Zheng et al[55] utilized a new bioinformatics tool, WGCNA, to clarify the basis of the poor response to immunotherapy in mismatch-proficient, stage III/IV colorectal cancer and showed that CALD1 was upregulated and associated with protumorigenic M2 macrophage infiltration. M2 macrophages are believed to be an important contributor to the failure of immunotherapy due to their anti-inflammatory, immunosuppressive, and proangiogenic characteristics[103]. CALD1 was negatively correlated with fractions of plasma cells, CD8 T cells, CD4 memory-activated natural killer cells, and dendritic cells[55]. High expression of CALD1 was significantly correlated with angiogenesis, TGF-β, and trans-endothelial migration. Taken together, these data are consistent with the published literature on the importance of the crosstalk

between angiogenesis and TGF-β in macrophage recruitment and M2 polarization[104,105], but the role of CALD1 in this scenario remains to be clarified. Cancer cell proliferation, invasion, and migration abilities were suppressed after reducing CALD1 expression *via* siRNA silencing *in vitro*[55].

Only one article suggested that ectopic expression of CaD in a panel of cell lines of various lineages, including the HCA7 colorectal cancer cell line, reduced the number of podosomes/invadopodia and suppressed cell invasion, but no further functional analysis or clinical correlation was presented. The vector used, pcDNA3.1(+)-HA-CaD, was supposed to contain l-CaD[50]. However, the cell line used, HCA7, is an atypical colorectal cancer cell line with an unusual cytogenetic profile and other characteristics[106,107]. Overall, the available literature suggests that l-CaD, particularly splice variant 2, is a colorectal cancer splice variant that exerts protumorigenic characteristics and is associated with angiogenesis, invasion, metastasis, immune evasion, and poor prognosis in colorectal cancer.

CaD as a prognostic biomarker of colorectal cancer

As discussed above, Kim *et al*[43] showed that colon cancer patients with high expression of l-CaD in their tumors had a poor response to chemoradiotherapy. L-CaD could exert these effects by inhibiting p21^{Cip1} and blocking apoptosis[43]. Lian *et al*[57] showed that l-CaD was associated with metastatic disease and poor overall survival in colorectal cancer. The WGCNA-based study of Zheng *et al*[55] showed that CALD1 was significantly associated with a worse prognosis in mismatch proficient, stage III/IV colorectal cancer. However, chemotherapy and tumor stage remained significantly correlated with overall survival. Both CALD1 and tumor stage were independent prognostic predictors in the GSE41258 validation dataset used in that study.

Calon *et al*[83] performed a comprehensive bioinformatics analysis to clarify the characteristics of the poor-prognosis subtypes of colorectal cancer in three common classification systems. Although these three classification systems were based on distinct global gene expression profiles in independent cohorts of colorectal cancer and

differed regarding the number of the identified tumor subtypes[108-110], they all concluded that poor patient outcome in colorectal cancer is associated with the expression of stem cell and mesenchymal genes[111]. Calon *et al*[83] found that among the poor-prognosis gene sets common to at least two of the three molecular classifications, 31% (including CALD1) stained solely the tumor stroma, and 62% stained both stromal and tumor cells in the Human Protein Atlas Dataset[112]. Intriguingly, CALD1 mRNA and protein expression were upregulated in cancer-associated fibroblasts (CAFs) and other stromal cell populations in contrast to epithelial tumor cells[83]. However, CaD was identified in pure colon cancer parenchymal tissue from cell lines (containing no stroma), and both l-CaD and h-CaD were observed by western blot or transcriptomics analysis of colorectal carcinoma cells in other studies[43,55,113]. Moreover, the functional consequences of l-CaD silencing were shown to impact the mobility, response to therapy and signaling pathways in colorectal carcinoma cells in these studies[43,55]. Interestingly, Calon *et al*[83] showed that the poor prognosis of colorectal cancer is linked to TGF-β signaling in stromal cells that results in CALD1 overexpression, providing evidence linking CALD1 to TGF-β signaling in the tumor stroma.

Jensen *et al*[113] reported that the CALD1 gene was upregulated in the transcriptome of more than one colorectal cancer cell line (HT29, LoVo) that acquired resistance to SN38 (a potent irinotecan metabolite). Moreover, proteomic analysis of locally advanced, nonmetastatic colorectal cancers treated with neoadjuvant chemoradiotherapy, including 5-FU, showed that CALD1 was among the top genes overexpressed in nonresponders[56]. In this study, the authors verified the mRNA expression of CALD1, as well as the presence of gene sequence variants, in the colorectal cancer cell line set of the 'Colorectal Cancer Atlas' available from http://colonatlas.org/index.html.

ROLE OF CAD IN OTHER CANCER TYPES

Gastric cancer

Bioinformatics analysis suggested that CALD1 is a novel target of the TEA domain family member 4 gene that mediates gastric cancer development by stimulating cell

proliferation and migration[45]. Another bioinformatics-based analysis showed that high expression of CALD1 is associated with poor overall survival and with immune infiltration in gastric cancer[58]. Conversely, Hou *et al*[53] showed that CaD expression was decreased in metastasis-derived gastric cancer cell lines as well as in resected biopsies of metastatic gastric cancer to lymph nodes compared with the primary tumors. Functional analysis showed that knockdown of CALD1 using siRNA in these cells resulted in an increase in cell migration and invasion. The first two studies[45,58], suggesting an oncogenic role of CaD in gastric cancer, were based upon bioinformatics analysis of a large series of gastric cancer, yet they did not supply a functional analysis of CALD1 action, while Hou *et al*' study focused on metastatic gastric cancer[53]. Thus, controversy remains, and further work is needed to clarify the role of CaD in gastric cancer.

Breast cancer
Two independent studies have shown an inverse relationship between ER and CaD. In the first study, silencing of ER in an ER-positive breast cancer cell line upregulated CALD1, concomitantly with the acquisition of more aggressive oncogenic features, including increased growth rates, loss of cell-to-cell adhesion and increased motility[39]. The second study was based on clinical breast cancer samples and aimed to identify predictive markers of tamoxifen resistance in recurrent breast cancer. ANXA1 and CALD1 were the most differentially expressed proteins, and they were associated with the downregulation of ER *via* activation of NF-κB signaling, which blocks apoptosis and causes cancer cells to become estrogen-independent[59]. Another study suggested that CaD can exert its carcinogenic effects in mouse mammary cells *via* EMT induction. The expression level and phosphorylation state of CaD increased as a function of time after induction of EMT by TGF-β1, and these changes in CaD correlated with an increased focal adhesion number and increased cell contractility[60].

In contrast, two publications showed the tumor suppressive functions of CaD. In the first, ectopic expression of l-CaD reduced the number of podosomes/invadopodia and

suppressed cell invasion in breast cancer cells[50]. The second showed that cGMP-dependent protein kinase I enhanced breast cancer cell motility and invasive capacity by phosphorylating CaD and that knockdown of endogenous CaD in MDA-MB-231 breast cancer cells exerted promigratory and proinvasive effects[51]. Thus, more work is needed to clarify the role of CaD in various molecular subtypes of breast cancer as well as in large cohorts of clinical samples.

Bladder cancer

The role of CaD in bladder cancer has been comprehensively studied, and the published literature consistently supports an oncogenic role of CaD in bladder cancer, as shown in Table 1. CaD is significantly overexpressed in bladder cancer tissue compared with normal urothelial tissue[40]. L-CaD is overexpressed in primary nonmuscle invasive bladder cancer and is significantly associated with tumor progression. Functional studies have shown that l-CaD mediates morphological changes associated with increased cell motility and invasive characteristics in bladder cancer cells and can inhibit apoptosis *in vitro* and *in vivo*[41,61]. CALD1 was significantly correlated with histological grade, stage, and lymphatic metastasis of bladder cancer in the Cancer Genome Atlas (TCGA) and Gene Expression Omnibus databases[61]. High CALD1 expression was associated with a poor prognosis[47], including poor overall survival[61].

CALD1 has been linked to JAK/STAT activation and PD-L1 overexpression[61]. The role of CALD1 in promoting bladder cancer progression by remodeling the tumor microenvironment was supported by the recent finding of CALD1 expression in CAFs as well as macrophages and T cells in the bladder tumor microenvironment[62]. Finally, noncoding RNA regulation of CALD1 was studied in bladder cancer and was found to occur *via* MIR100HG, which can promote the proliferation, migration and invasion of bladder cancer cells. MIR100HG inhibits the expression of miR-142-5p, which targets CALD1, thus relieving CALD1 from this inhibitory effect. Consequently, upregulated CALD1 results in the induction of aggressive features in bladder cancer cells[63].

Glioma

CALD1 expression was associated with a high pathological grade and poor clinical outcome in a bioinformatics analysis of glioma samples from the TCGA and Chinese Glioma Genome Atlas databases. "Biofunction prediction" suggested that CALD1 modulated tumor angiogenesis in these tumors[66]. Single-cell RNA sequencing (scRNA-seq), a technique that can define cellular states within both normal and disease tissues, including the immune phenotypes in the tumor microenvironment[114], showed that CALD1 was upregulated in neoplastic cells and was involved in the tumorigenic processes of gliomas. Dysfunctional l-CaD also led to a decline in cell mobility in glioblastoma cells[66]. L-CaD is abnormally spliced in glioma vasculature, and the resultant altered expression of the protein isoforms in ECs/EPCs plays a role in the neoangiogenesis of various human tumor types[67]. Finally, the serum level of l-CaD was elevated in glioma patients, and this elevation was significantly higher than the l-CaD serum levels in other brain tumor patients[48].

CONCLUSION

Traditionally, scientific interest in CaD has been focused on its application in diagnostic histopathology to diagnose smooth muscle and related tumors using h-CaD or "total CaD" antibodies. However, the nonsmooth muscle isoform l-CaD has recently attracted much interest for its variable actions during carcinogenesis. In contrast to the initial expectation, based upon its role in inhibiting actin-myosin interaction and smooth muscle contraction, a growing list of studies are showing pro-oncogenic roles in various cancers. Some controversy remains, as a few studies suggested that CaD can exert a tumor suppressor role that needs to be clarified, together with the detailed mechanism of action of CaD in cancer cells of various lineages. The availability of new technologies for the study of ABP biology and functions could assist in these tasks[115-117]. Our comprehensive analysis of the available publications to date showed that CaD, particularly l-CaD, plays an important role in the development, metastasis, and resistance to chemotherapy in colorectal cancers and other cancer types. Furthermore,

CaD is implicated in angiogenesis and immune evasion in specific types of cancers, such as those of the urinary bladder. It is highly likely that the role of CALD1 in immune modulation in bladder cancer could be a general mechanism that is applicable to colorectal cancer and many other tumors. Few publications have focused on the analysis of the localization of CaD in the stroma and the role it plays in various components of the tumor microenvironment, which is an important research priority. Interestingly, CaD undergoes selective tumor-specific splicing, and the resulting isoforms are not generally expressed in normal tissues. These data qualify CaD as a potential candidate for targeted therapy in addition to its role in diagnosis and prognosis.

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