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Deleted in liver cancer-1 (DLC-1): An emerging metastasis suppressor gene

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Abstract

While significant progress continues to be made in the early detection and therapeutic management of primary tumors, the incidence of metastatic disease remains as the major cause of mortality. Accordingly, the development of novel effective therapies that can ameliorate dissemination and secondary tumor growth are a clinical priority. The identification of genetic and functional alterations in cancer cells that affect factors implicated in the metastatic process is critical for designing preventive and therapeutic strategies. Evidence implicating the protein deleted in liver cancer 1 (DLC1), a Rho GTPase activator, in metastasis has accumulated to a point where DLC1 may be considered as a metastasis suppressor gene. This review will present evidence supporting an anti-metastatic role for DLC-1 in several human cancers and discuss the mechanisms contributing to its inhibitory effects. In addition, promising opportunities for therapeutic interventions based on DLC1 function and downstream pathways involved in the metastatic process are considered.

Keywords

metastasis; DLC-1; tumor cell signaling

INTRODUCTION

Dissemination of cells from primary tumors, and establishment of colonies in distant sites, i.e. metastasis, is the leading cause of cancer mortality and so understanding the molecular basis that enables this phenomenon is a predominant issue in cancer biology. The establishment of metastatic disease requires completion of a complex multistep process involving various cellular and genetic or epigenetic alterations. These changes can impact the functional balance between factors that either support or suppress the development of metastatic lesions [1].

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Given that most differentiated cells are not capable of initiating dissemination or surviving and thriving through transit and establishment at a secondary site, the idea that metastasis suppressor genes are active in normal cells has been posited. A metastasis suppressor gene would be one that codes for a factor that reduces the propensity or efficiency of a tumor cell to metastasize. In some cases, the same factor may also meet criteria for a tumor suppressor gene, but if not active or sufficient to prevent the establishment of a particular primary tumor, it may still limit the dissemination and establishment of secondary lesions.

In this review we focus on the anti-metastatic role of DLC1, a member of the deleted-in-liver cancer family of proteins which includes DLC1, DLC2 and DLC3. All three members share the Rho GTPase-activating (RhoGAP) domain, a StAR-related lipid-transfer (START) domain and sterile alpha motif (SAM) domain [2–4]. Since being cloned in 1998, the DLC1 gene has been extensively investigated. DLC1 expression has been noted to be frequently down regulated or inactivated in many human cancers, and shown to confer tumor suppressive effects in numerous cancer models [4–10].

The human DLC-1 gene encodes a 1,091 amino acid protein that is highly homologous to the rat p122-RhoGAP [2, 11]. Rho-GTPase-activating proteins (RhoGAP) are important regulators in the switching between the active GTP-bound state and the inactive GDP-bound state of Rho proteins. The Rho proteins are members of the Ras superfamily and are involved in a variety of cellular functions, including the regulation of cell proliferation and actin cytoskeleton organization [12, 13], and have been implicated in oncogenic transformation and cancer progression [14, 15]. RhoGAPs catalyze the conversion of the active GTP-bound Rho proteins to the inactive state, thereby attenuating their signal transduction activities. The RhoGAPs act as negative modulators of RhoA, RhoB, RhoC, and CDC42 [16–20]. Inactivation of DLC1, via changes in expression or activity, may represent the most frequent mechanism for aberrant activation of Rho GTPases in human oncogenesis.

A potentially crucial role for DLC1 is in the control of Rho signaling that regulates actin cytoskeleton and focal adhesions. Notably, some of the strongest indications that morphogenetic defects contribute directly to human cancer have emerged from studies on the DLC1 gene [21], however, Rho-GAP independent mechanisms have also been linked to tumor cell phenotype, showing that the function of DLC1 in malignancy is complex [16, 18, 17, 22, 23]. Murine models have often been employed in the study of DLC1 functions and interactions. In a recent, well-designed study it was clearly demonstrated that DLC1 inactivation in mouse embryo fibroblasts can lead to neoplastic transformation. In addition to inactivation of DLC1 and concomitant increased activity of Rho and Cdc42, neoplastic transformation depended on the activation of Cdk4/6 resulting from decreased expression of the Cdk4/6 inhibitors p15^{Ink4b} and p16^{Ink4a}. Several of the genes in these pathways are known to be altered in human cancers [24]. Experiments with human and mouse hepatoma cells investigating upstream factors have provided conclusive evidence that protein kinase A (PKA) can regulate the Rho-GAP activity and tumor suppressive capacity of DLC1 [10].

DLC1 has been proposed as a ‘gatekeeper gene’ or a ‘tumor suppressor gene’ (TSG) because the presence of DLC1 activity appears to have inhibitory effects on tumor cell

growth and survival. Inactivation of gatekeeper or TSG genes can contribute directly to cancer formation and progression [25], and DLC1 was shown to function in this role particularly well in studies using a mouse liver tumor model [4, 6]. Although DLC1 may not fully meet the criteria of a TSG in all contexts, there is general agreement that DLC1 is a pivotal regulator of many of the tumor cell behaviors thought to be essential for the successful completion of the metastatic cascade, i.e. cell motility, migration, invasion of local tissue, intra- and extravasation of the bloodstream and lymphatics. All of these features of malignant cancer cells rely upon the plasticity of cytoskeletal structures, for example the assembly/disassembly of focal adhesion complexes [26–31]. Some of the evidence implicating DLC1 as a metastasis suppressor gene (MSG) is discussed below.

ANTI METASTATIC EFFECT OF DLC1 IN VARIOUS COMMON CANCERS

Breast cancer

Transcriptomic analysis of an isogenic breast tumor xenograft model identified a multigene expression signature that correlated with metastatic phenotype. The signature associated with non-metastatic cells was highly enriched for GTPase signaling components [32], and the most differentially expressed of these components was DLC-1. Restoration of DLC-1 in metastatic cells (M4A4) to levels observed in an isogenic non-metastatic clone (NM2C5) resulted in the inhibition of migration and invasion *in vitro* and a significant reduction in the ability of these cells to form pulmonary metastases in athymic mice. The natural mechanism responsible for the down-regulation of DLC1 in the metastatic cells of this model was found to be hypermethylation of the DLC1 promoter. The metastatic M4A4 cells lacking DLC1 expression exhibit a high level of actin stress fibers and focal adhesion complexes compared to the non-metastatic NM2C5 cells. In additional experiments, it was shown that the nonmetastatic cells (NM2C5) cells of the breast xenograft model can successfully disseminate but have a dormant phenotype *in vivo*, i.e. solitary tumor cells reach and remain viable in the secondary organs for several months after inoculation [33]. Perhaps DLC1 and associated factors can mediate the dormancy of tumor cells, enabling metastases if a threshold of activity is perturbed by additional cellular or environmental events. The effect of DLC-1 on tumor dormancy remains to be addressed directly experimentally [27].

Similarly, but in a different clonal model of experimental organ-specific metastasis, DLC1 was found to be down regulated in breast tumor cell populations highly metastatic to bone [34]. The study did not manipulate DLC1 specifically, so its role was not well-defined in this model, but the fact that parental cells in both models form tumors but have differential metastatic propensity associated with DLC1 activity suggests that metastasis requires a set of functions beyond those underlying the emergence of the primary tumor. In another experimental model the transcription factor GATA3 was identified as being influential in the ability of an aggressive breast cell line to metastasize to the lung. Overexpression of GATA3 in this cell line significantly reduced the pulmonary metastatic burden in nude mice. Of interest was the finding that GATA3 up-regulated DLC1 in these metastasis-compromised cells. Accordingly, loss of expression of GATA3 has been associated with aggressive human breast tumor development and poor patient survival [35]. These data support a pivotal role in for DLC1 in regulating the efficiency of breast tumor metastases.

A recent analysis of a large cohort of breast cancer patients from China revealed that DLC1 mRNA expression was significantly higher in adjacent normal tissue, benign lesions and minimally invasive specimens than in highly invasive carcinomas with synchronous metastases. The study concluded that DLC-1 plays an important role in breast carcinoma progression, invasiveness and metastasis [36]. This is consistent with earlier results demonstrating that restoration of DLC1 expression in primary cell lines derived from metastatic breast adenocarcinomas lacking endogenous DLC1 caused cell growth inhibition *in vitro* and prevented the development of tumors in athymic nude mice [37]. On the genetic front, a multivariate analysis of allelic imbalance (AI) in invasive breast tumors with known clinical outcome showed that AI on the short arm of chromosome 8 at band p22, the site that harbors the DLC1 gene, is associated with breast cancer mortality, independently of other pathological factors [38]. Both genetic and environmental factors are important in the development of breast cancer, and diet is thought to play an important role. A low incidence of breast cancer in Asia has been attributed in part to a high intake of flavonoids. Interestingly, flavone treatment has been shown to restore DLC1 expression in aggressive and metastatic breast carcinoma cells that are DLC1-deficient due to promoter hypermethylation. The flavonoid induced increase in DLC1 resulted in induction of cell proliferation, cell cycle arrest, and apoptosis, and led to the deregulation of several proto-oncogene and TSGs [39]. Family member DLC2 is also proposed to exert oncosuppressive effects on tumor cells, and so the expression of both genes was examined by quantitative RT-PCR in a series of primary ductal carcinomas obtained from patients with regional lymph node metastases. A significantly lower expression of either DLC1 and DLC2, and usually both, was detected in nearly 100% of these tumors, suggesting that deficiency of DLC function facilitates breast carcinoma progression to metastasis [40].

Prostate cancer

The molecular basis for the malignant progression and dissemination of prostate cancer is poorly understood. A prostate cancer isogenic model, similar to those described in breast cancer, has been derived to investigate metastasis-associated factors. A cell line C4-2-B2, which metastasizes to bone, was isolated from LNCaP cells that are non-tumorigenic and non-metastatic [41]. Comparative analyses revealed that the difference in metastatic potential of the model was associated with changes in DLC1 expression. The amount of DLC1 mRNA in the non-metastatic LNCaP cells was about five times that in the metastatic counterpart. Adenovirus-mediated DLC1 transfer into C4-2-B2 inhibited cell proliferation and anchorage independent growth *in vitro* and induced apoptosis. It also induced cell cycle arrest, inhibited the activation of RhoA and the formation of actin stress fibers [19]. Furthermore, it has been demonstrated that silencing of DLC1 in nonmalignant prostate epithelial cells enhances angiogenesis. As tumor growth and metastasis are dependent on angiogenesis, DLC1-mediated pathways might be potential therapeutic targets for both events [42]. PC-3 is also a metastatic cell line and both PC-3 and C4-2-B2 have been used to examine DLC1 interactions with β -catenin and E-cadherin [43, 44]. The DLC1 binding motifs for both proteins have been identified and resulting complexes have been shown to localize within adherens junctions (AJs). The presence of DLC1- β -catenin complexes reduces Rho GTP levels at the plasma membrane and increases E-cadherin expression, resulting in the disruption of cytoskeleton organization and stabilization of AJs. This process

results in reduced invasiveness of both C4-2- B2 and PC-3 cells through modulation of the Rho pathway. The functional relevance of these interactions was assessed by knocking down α -catenin in DLC1-positive cells, an experiment that had multiple effects, underscoring a complex role for DLC1 in cellular architecture [43]. E-cadherin, a cell-cell adhesion molecule that regulates tumor cell invasion, is down regulated in many advanced human cancers. It has been demonstrated that the expression of DLC1 increases E-cadherin expression, which in turn, suppresses the invasive capacity of the two highly metastatic prostate cancer cell lines [44].

Liver cancer

Loss of heterogeneity (LOH) on the short arm chromosome 8 (8p) is among the most genetic common alteration in hepatocellular carcinoma (HCC), and is associated with liver dysplastic nodules and both primary and metastatic HCC (reviewed in [45]). In an early study, loss of 8p was detected in 8 metastatic HCC tumors but only in 3 corresponding primary tumors suggesting that the region containing the DLC1 gene might contribute to the development of HCC metastasis [46]. Subsequently, evidence-implicating DLC1 in metastasis of HCC has been obtained by examination of HCC cell lines and surgical samples from HCC patients. A striking correlation between DLC1 expression and the degree of invasiveness and metastatic propensity has been observed. DLC1 expression was invariably and significantly lower in highly invasive tumors relative to less invasive cases, and lower DLC1 expression is consistently observed in metastatic versus non-metastatic HCC cell lines [47]. Down-regulation of DLC1 was also detected in hepatitis C virus-positive HCC patients pending liver transplantation [48]. Experimentally, the effect of DLC1 in the dissemination of human HCC cells has been generated using two cell lines; Focus and 7703K, derived from patients with aggressive HCC, both lacking DLC1 expression. Restoration of DLC1 expression in these cell lines resulted in inhibition of *in vitro* cell proliferation and *in vivo* tumorigenicity to some extent [49]. Both cell lines are aggressive *in vivo* and cells disseminate to the liver and lung, but this was significantly suppressed by stable DLC1 transfection. The anti-metastatic effect of DLC1 was associated with activation of RhoA GTPase and alterations in cytoskeleton organization and cell adhesion [50]. The role of Rho GTPase signaling downstream of DLC1 on cell motility and invasiveness may be critical to the cascade of events leading to metastasis [28].

HCC models have been used to address the anti-metastatic role of DLC1 in several studies. A novel mechanism that negates the metastasis suppressive function of DLC1 in HCC was identified. Phosphorylation of a single serine residue of DLC1 by activated Akt abolished the tumorigenicity and metastasis of transformed hepatoblasts in a mouse model, without affecting DLC1 RhoGAP activity [51]. Conversely, it has been shown that phosphorylation of DLC1 at Ser549 by cyclic AMP-dependent PKA enhances DLC1 RhoGAP activity through structural dimerization, and thus promotes the activity of DLC1 leading to the inhibition of hepatoma cell growth and motility *in vitro* and metastasis *in vivo* [52]. HCC cells that are sensitive or resistant to zebularine, a potent DNA methylation inhibitor, have been derived for mechanistic study. The most aggressive of the cell lines, HuH7, metastasizes to the lung and brain and was shown to express negligible levels of DLC1 expression [53]. Treatment of xenografts of this cell line with zebularine resulted in a

reduction of the size and frequency of pulmonary metastases in athymic mice [54]. The multi-functional role of DLC1 was further illustrated using these HCC cells. Depletion of megakaryoblastic leukemia 1 and 2 (MKL1/2), known coactivators of the transcription factor serum response factor, was shown to abolish HuH7 xenograft growth and to induce senescence. But this effect was dependent on the loss of DLC1 and the consequential activation of RhoA. The MKL1/2 depletion also resulted in Ras activation, elevated p16 expression and hypophosphorylation of the retinoblastoma protein. Studies have suggested that DLC1 exerts its tumor cell suppressive effects by inducing cellular senescence via the p16/pRb pathway, and so depletion of MKL1/2 is mimicking the effects of DLC1 restoration [55].

Lung cancer

A considerable amount of data has accumulated that implicates DLC1 in lung cancer development and dissemination, and the monitoring of DLC1 has been proposed as a reliable prognostic marker for lung cancer outcome. The transduction of DLC1 into cells lacking endogenous expression abolished the development of non small carcinoma (NSCLC) xenografts in nude mice [56], and the functional interactions between DLC1 and other proteins was first investigated in NSCLC. DLC1 has been shown to interact with tensin 1, S100A10 and caveolin1 (CAV1-1), all factors known to have cooperative effects on tumor progression and metastasis [22, 57, 58]. The involvement of DLC1 in the fatal metastatic aspect of NSCLC was reported in a study designed to investigate the role of the microRNA-200 family in tumor metastasis. Re-expression of all members of the miR-200 family resulted in the down regulation of DLC1 mRNA and protein expression in human metastatic H1299 NSCLC cells. These results may provide a basis for new therapeutic approaches that indirectly target DLC1 function [59]. A study designed to identify the genes mediated by TIMP-2, a tissue inhibitor of metalloproteinase 2 in NSCLC cells revealed a link to DLC1. Beyond regulation of MMP activity, TIMP-2 promotes the transcription of a number of genes that can potentially inhibit tumorigenesis and metastasis. Tumor cell growth inhibition induced by TIMP-2 was accompanied by up regulation of DLC1, the secreted protein EFEMP1, and E-cadherin [60], mirroring the effects seen in metastatic prostate carcinoma cells described above [44].

In several independent studies the DLC1 gene and its products have been recognized as reliable prognostic biomarkers for lung cancer. A multiplexed methylation profile of TSGs in lung tumors identified DLC1 and SCGB3A1 as the most frequently methylated genes, and DLC1 methylation status was significantly associated with lung metastasis [61]. In a genome-wide mRNA expression analysis of 256 lung adenocarcinoma patients, a 12-gene signature, including DLC1, was derived for lung cancer prognosis and prediction of chemotherapy response. As in other studies, down regulation of DLC1 was associated with poor prognosis in NSCLC and its overexpression was linked with chemo-resistance to Erlotinib, Paxitrel and Cisplatin. Interestingly, low expression of DLC1 was related to certain degree to resistance to Etoposide and Carboplatin [62]. Further evidence underlining the prognostic value of DLC1 expression in lung cancer has been generated by examination of protein coding and non-coding gene expression in 291 lung adenocarcinoma tissues from Japan, the United States and Norway. Four genes, BRCA1, HIF1A, DLC1, and XPO1 were

found to be significantly associated with prognosis for early stage lung cancer patients across all geographic locations. It was proposed that altered expression of any of those genes would generate aggressive tumors that are either more likely to metastasize or to acquire resistance to chemotherapies [63].

Multiple myeloma

The events involved in progression of multiple myeloma (MM) mimic the metastatic process to some extent. Transduction of DLC1 into MM cell lines lacking DLC1 expression resulted in suppression of cell migration and invasion, events associated with a significant reduction of RhoA activity and in changes in the amount and distribution of actin content. Cell migration and tissue invasion are required for myeloma cell movement within the bone marrow and the formation of metastatic lesions in secondary sites [64]. In a comprehensive analysis of DNA methylation and gene expression of 193 MM samples, it has been shown that DNA methylation directly regulates a rather small number of genes in this disease. A positive methylation-expression correlation was identified for DLC1 and for p16, a tumor suppressor gene, and a negative correlation was observed for two other genes, IGF1R and IL17RB. These results confirm and strengthen the importance of DLC1 in pathogenesis and the dissemination of malignant cells [65].

Other major cancers

Potential DLC1 function in the metastatic process among other common forms of cancer that deserve further investigation include ovarian, renal, nasopharyngeal, pancreatic and urothelial cancer. Examination of 76 cases of advanced epithelial ovarian carcinoma revealed low expression of DLC1 and concomitant high expression of phosphorylated focal adhesion kinase pFAK. The combination of the status of these 2 factors had prognostic value in 60 cases [66]. Similarly, in a recent study examining 75 samples of ovarian carcinoma the level of DLC1 protein conversely correlated with the expression of plasminogen inhibitor 1 (PAI-1), a factor that is associated with the metastasis and invasion of ovarian carcinoma cells. Furthermore, patients with negative expression of DLC1 and positive expression of PAI-1 had the worst overall survival rates within the cohort. The study concluded that the combination of DLC1 and PAI-1 could serve as an independent prognostic factor for ovarian carcinoma [67].

The level of DLC1 mRNA and protein expression was measured in matched tumor and non-tumor tissue samples from 62 patients with clear cell renal cell carcinoma (RCC). A high level of DLC-1 mRNA and protein expression was detected in the majority of normal renal specimens. Furthermore, DLC-1 expression inversely correlated with advanced histological grade and stage consistent with evidence in other cancers, in primary tumors with synchronous metastases DLC1 expression was lower than in those without metastases [68]. Recent studies in urothelial carcinoma found that a reduction in DLC1 expression was associated with poor differentiation, high risk of metastasis and shorter survival, and DLC1 was considered as a promising marker of disease prognosis [69].

DLC1 FUNCTION IN CANCER

Proapoptotic activity

The induction of apoptosis when cells are under stress is a major attribute of MSGs. In two independent studies with prostate, liver and lung tumor cells, cellular and molecular alterations indicative of the induction of programmed cell death by DLC1 have been demonstrated. In prostate carcinoma cells, the restoration of DLC1 expression induced apoptosis, but only after chemical inhibition of antiapoptotic Bcl-2 protein expression [19]. Similarly, in HCC cells, the induction of apoptosis was associated with cleavage of caspase 3 and a reduced level of the antiapoptotic Bcl-2 [49]. In a non-small cell lung carcinoma (NSCLC) study, transduction of DLC1 induced marked changes in cell morphology. DLC1-mediated actin cytoskeleton-based morphological alterations and DLC1 protein nuclear translocation occur prior to inhibition of cell migration and before the induction of apoptosis. These observations led to the conclusion that DLC1 functions in the cytoplasm as an inhibitor of tumor cell proliferation and migration, but in the nucleus as an inducer of apoptosis [70].

DLC1 interaction with proteins involved in initiation and progression of cancer

The multi domain structure of DLC1 suggested that DLC1 might interact with proteins other than Rho GTPases. Through yeast two-hybrid screening, several DLC1 interacting proteins have been identified, and their interaction have been confirmed in human cells. Identification of such protein interactions is essential to deciphering the mechanisms that regulate the function of DLC1 and may provide novel therapeutic targets. A recent review article comprehensively covers the range of protein-protein interactions that modulate DLC1 function [10]. Here, we briefly discuss a few proteins that may have relevance in the metastatic process. Members of the tensin family of focal adhesion proteins were the first DLC1 binding partners identified. The impact of this interaction has been examined in HCC, NSCLC and breast carcinoma cells [71, 22, 5]. It has been demonstrated that the binding of tensin 3 to DLC1 via an actin-binding domain reduces epithelial growth factor receptor-driven migration and anchorage-independent growth of breast cancer cells. The same study proposed DLC1 as a valid therapeutic target and an important marker for metastatic cancer as well as a predictor of cancer outcome [72].

Several other interacting proteins including TSGs have been identified and found to negatively or positively modulate the activity of DLC1. For example, DLC1 interaction with phosphatase and tensin homolog (PTEN), a TSG frequently deleted or mutated in sporadic breast, prostate, endometrium and brain tumors, was examined and it was shown that simultaneous depletion of both genes enhanced cell migration of the non-invasive breast carcinoma cell line MCF7 [73]. DLC1 also interacts with p120Ras-GAP (RASA1). This interaction involves the DLC1 Rho-GAP catalytic domain and the SH3 domain of Ras-GAP and results in a dramatic reduction of DLC1 Rho-GAP activity *in vitro*. Consistent with this, it has been found that ectopic overexpression of Ras-GAP in a Ras-GAP-insensitive tumor line impaired the growth-suppressing activity of DLC1 and increased RhoA activity *in vivo* [23].

DLC1 interactions with two proteins that are known to be involved in tumor progression and metastasis, namely S100A10 (also known as p11) is of particular interest. S100A10 is a proinflammatory protein and a cell surface receptor for plasminogen that regulates pericellular proteolysis and promotes tumor cell invasion. The DLC1 interaction with S100A10 triggers a cascade of events required for the acquisition of suppressive effects in NSCLC cells. The initial and critical event was identified as the displacement of annexin-2 from the binding site of DLC1, thus making S100A10 accessible to ubiquitin-dependent degradation. This results in a decrease of steady-state level of S100A10 expression, a reduction of plasminogen activation, and consequently inhibition of *in vitro* cell migration, invasion, colony formation, and anchorage-independent growth [57]. A novel DLC1 interaction with Caveolin-1 (CAV-1), a TSG that is known to affect tumor progression and metastasis was also identified in NSCLC cell studies. Interaction of DLC1 with CAV-1 was found to be mediated by the DLC1 START domain. Through mutation of the DLC1 START domain, it was revealed that DLC1 requires CAV-1 to limit neoplastic growth. From the same study, examination of two public mRNA datasets identified low DLC1 as clinically associated with poor outcome [58].

THERAPEUTIC PROSPECTS BASED ON DLC1 FUNCTIONS, INTERACTIONS AND LEVEL OF EXPRESSION

The analyses of isogenic metastatic and non-metastatic breast, prostate and HCC clones, has invariably shown the down regulation of DLC1 in metastatic cells relative to non-metastatic counterparts. The absence, or relative decrease of DLC1 expression in primary tumors is a valid indicator for the development of metastasis and a poor prognosis. Thus, it seems reasonable to propose that the restoration of DLC1 could have therapeutic utility. Impacts could occur at the primary tumor, and/or through reducing the probability of metastatic disease establishment. Insights into the function and mechanisms of DLC1 as a MSG could facilitate the manipulation of DLC1-regulated complexes for therapeutic benefit. An important guidance for therapy of metastatic disease has been recently outlined and underscores the need for the application of drugs that prevent cancer dissemination shortly after diagnosis [74]. The relative inefficiency of tumor cells to form distant metastases is generally recognized, as only a fraction of the tumor cells overcome the barriers of the multi-step metastatic process. Several options for strategic therapeutic interventions are seemingly feasible in various forms of cancer. The selection of patients with solid tumors and hematological malignancies for any type of therapeutics should be based on an initial gene expression profile. Those patients with compromised DLC1 expression, and/or other factors known to interact and modulate DLC1 expression would be suitable for a targeted therapeutic approach. Perhaps the most amenable approach would be the pharmacological intervention of Rho GTPase signaling that is activated by the loss of DLC1 suppressor function.

A preclinical therapeutic evaluation of an antitumor agent closely associated with DLC1 function has been the newly developed inhibitor of protein geranylgeranyltransferase type I (GGT1). Because frequent loss of DLC1 expression in various cancers increases Rho activation and Rho proteins are geranylgeranylated in tumor cells it has been predicted that

GGT1 would suppress tumor growth [75]. This prediction was validated by testing the antitumor effect of P61A6, a GGT1 inhibitor, on the growth of PANC1 human pancreatic cancer cells deficient in DLC1 expression. The P61A6 treatment had a robust inhibitory effect on the development of xenotransplanted tumors in nude mice [75]. The inhibitory effect of this compound is not limited to pancreatic cancer, as most recently similar effects have been obtained in lung cancer [56]. The effect of P61A6 was examined in NSCLC cell lines H358, H23 and H1507. P61A6 inhibited cell proliferation in all three lines, but in H358 cells which are defective in DLC1 expression, P61A6 also inhibited anchorage-dependent and independent growth, and tumor development in nude mice. This suppressive effect was associated with RhoA activation, accumulation of G1 phase cells, and a decrease of cyclin D1/2 expression [76].

As described above, the activity of Rho GTPases is elevated in many human cancers and their metastases, and the oncosuppressive effect of DLC1 requires RhoGAP activity, which negatively regulates Rho GTPases, most commonly RhoA [14, 77, 20]. Inhibition of the RhoA pathway and Rho kinase, a downstream effector of Rho, are the most promising options for therapeutic interventions in this DLC1-regulated pathway [7, 6]. A number of effective small molecule Rho kinase (ROCK) inhibitors are already available and there will likely be more to come. Among the ROCK inhibitors, Y-27632 and its derivative Wf-536 exert anti-metastatic activity in liver cancer and melanoma in *in vivo* models [45]. A new potent and selective ROCK inhibitor, OXA-06 that is superior to Y compounds, might be a promising therapeutic compound in patients with NSCLC and other forms of cancer [77]. In addition, targeting prooncogenic pathways activated by DLC1 down-regulation could be therapeutically effective for the suppression of metastases in NSCLC and other forms of cancers [58].

Another promising therapeutic target emerged from the analysis of p120 Ras-GAP interactions with DLC1 [23]. Evidence showing that Ras-GAP promotes the growth of cells containing mutant Ras suggest that Ras-GAP might represent a valid therapeutic target. Approximately 30% of all tumors carry a mutant Ras and there is a particular need for improved therapeutic approaches to such tumors, which are often associated with treatment resistance and poor prognosis. An anti-Ras-GAP SH3 monoclonal antibody or an aptamer that specifically binds the Ras-GAP SH3 domain could be effective inhibitors of tumor cell proliferation [23]. Indeed, an approach based on a Ras-GAP strategy shows promise. TAT-RasGAP317–326, a peptide corresponding to the 317–326 sequence of p120 RasGAP coupled with a cell-permeable TAT-derived peptide, sensitizes the death response of various tumor cells to several anticancer treatments [78]. Recent investigations show that this peptide can also hampers cell migration and invasion through marked modification of the actin cytoskeleton and focal adhesion redistribution [79]. Interestingly, DLC1 was found to be essentially required for TAT-RasGAP317–326 to promote cell adherence and inhibit migration. Once again, it can be envisioned that combination strategies that couple therapeutics with manipulation of the pivotal DLC1 gene would be particularly powerful.

SCREENING FOR PHARMACOLOGICAL AGENTS THAT UP-REGULATE DLC-1 GENE EXPRESSION

Down-regulation or inactivation of the DLC-1 gene during tumor development appears to be primarily due to either deletions at the genomic level, or to aberrant methylation or histone deacetylation at the gene promoter. In an early study, the role of promoter methylation in down-regulation of DLC1 was established by screening several cell lines derived from HCC, colon and prostate tumors [80]. Subsequently, numerous analyses have shown that such promoter methylation is the major mechanism responsible for inactivation of the DLC-1 gene in various solid tumors and hematological malignancies (reviewed in [4]). For example, the analysis of 73 surgical samples of HCC showed that the level of DLC1 methylation in highly invasive HCC was significantly higher than in low invasion cases [81]. A recent methylation analysis of 68 patients with pancreatic ductal adenocarcinoma showed that DLC1 hypermethylation strongly correlated with lymph node metastasis [82]. Promoter methylation and LOH result in DLC1 inactivation in nasopharyngeal tumors [83], and promoter methylation is highly prevalent in lung tumors [61]. Accordingly, epigenetic therapeutic strategies based on the manipulation of DNA methyltransferases and histone deacetylases to restore DLC1 expression in cancer cells has been highlighted as a potential approach [7]. Given the considerable interest and progress in epigenetic therapy, a number of promising antineoplastic agents have been developed and used successfully in clinical trials. Perhaps the combined action of positive DLC1 manipulation and HDAC inhibitors could be exploited for a more effective cancer therapy. To evaluate the potential benefits of this approach, a preclinical evaluation was undertaken to determine the combined effects of DLC1-mediated transduction and exposure to suberoylanilide hydroxamic acid (SAHA), a powerful HDAC inhibitor, in 22Rv1 prostate cells. These cells do not express endogenous DLC1 due to histone deacetylation. DLC1 transduction of these cells reduced RhoA activity and the addition of SAHA enhanced this effect. SAHA alone significantly inhibited on RhoA activity. This combinatorial regimen almost completely inhibited xenograft tumor growth in nude mice, suggesting that this protocol has potential as a therapeutic option in metastatic prostate cancer [84]. Among a variety of DNA methylation inhibitors, zebularine that preferentially targets cancer cells, is considered one of the most effective compounds. As described above, xenotransplanted, zebularine-sensitive HCC cells with disabled DLC1 expression had a considerably lower ability to form metastases to the lung [54].

A recent study demonstrated that DLC1 is a target of repression by enhancer of zeste homolog 2 (EZH2), the catalytic subunit of the Polycomb Repressive Complex 2 that trimethylates histone H3 lysine 27 (H3K27me3) [85]. EZH2 is over-expressed in several human cancer types and is a potential target for therapy [86]. Treatment of cell lines derived from various forms of cancer with the EZH2 small molecular inhibitor, 3-Deazaneplanocin A (DZNep), restored DLC1 expression, showing that EZH2-mediated H3K27me3 epigenetic regulation of DLC1 contributes to the pro-metastatic role of EZH2 through the silencing of TSGs and MSGs [85]. Finally, despite the fact that DLC1 is one of the genes most frequently deregulated in a multitude of cancers, its potential as a target for cancer gene therapy has not been thoroughly addressed, but a recent study evaluated the feasibility

in lung and pancreatic cancer. If tumor cell targeting can be achieved reliably in the future, then DLC1 could be a valid candidate for gene therapy of primary or secondary lesions.

CONCLUSIONS

Accumulating evidence shows that DLC1 function has to be absent or compromised in order for tumor cells to efficiently establish distant, secondary lesions, prompting the use of metastasis suppressor gene terminology. Importantly, the suppressive function is observed across all cancers tested experimentally, and the association of absent or diminished expression level with metastatic disease is supported by numerous human tissue studies. The defined involvement of DLC1 in the metastatic process offers realistic opportunities for the development of effective therapeutic strategies aimed at reducing the incidence of metastatic disease, the most fatal aspect of cancer.

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