Tumor/cancer stem cell marker doublecortin-like kinase 1 in liver diseases

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Abstract

Liver diseases are the fourth leading cause of mortality among adults in the United States. Patients with chronic liver diseases such as viral hepatitis, fibrosis, and cirrhosis have significantly higher risks of developing hepatocellular carcinoma (HCC). With a dismal five-year survival rate of 11%, HCC is the third most common cause of cancer-related deaths worldwide. Regardless of the underlying cause, late presentation and a lack of effective therapy are the major impediments for successful treatment of HCC. Therefore, there is a considerable interest in developing new strategies for the prevention and treatment of chronic liver diseases at the early stages. Cancer stem cells (CSCs), a small cell subpopulation in a tumor, exhibit unlimited self-renewal and differentiation capacity. These cells are believed to play pivotal roles in the initiation, growth, metastasis, and drug-resistance of tumors. In this review, we will briefly discuss pivotal roles of the CSC marker doublecortin-like kinase 1 (DCLK1) in hepatic tumorigenesis. Recent evidence suggests that anti-DCLK1 strategies hold promising clinical potential for the treatment of cancers of the liver, pancreas, and colon.

Keywords: Hepatocellular carcinoma, doublecortin-like kinase 1, cancer stem cell, liver diseases, epithelial-to-mesenchymal transition, cancer treatment

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Introduction

Chronic liver diseases such as long-term viral infections with hepatitis B and C viruses (HBV, HCV), non-alcoholic steatohepatitis (NASH), and cirrhosis affect millions of people worldwide and thus pose significant global health challenges.^{1–3} Patients with chronic liver diseases are at an increased risk of developing hepatocellular carcinoma (HCC), which typically arises from the setting of cirrhosis. HCC is one of the most common cancers in the world with over half a million new cases globally each year and is the third most common cause of cancer-related deaths worldwide.^{3–7} Its prognosis is poor, with a dismal five-year survival rate of 11%. The risk of HCC increases strikingly in HCV carriers who also have obesity and diabetes. The majority of HCC cases are normally diagnosed at late stages, with only 30–40% of HCC cases properly diagnosed at an early stage.⁸ Patients with early stage HCC have a promising prognosis and a five-year survival rate around 70–75% due to effective curative treatment options such as surgical resection, local ablation, and liver transplantation.^{7–9} However, these options are not effective in a large

group of patients with HCC, and patients with advanced HCC have a median survival of less than one year.^{9,10} The US Food and Drug Administration-approved sorafenib, a small molecule multikinase inhibitor, was shown to increase survival only by two to three months in advanced HCC patients.¹¹ In addition, patients frequently develop resistance to sorafenib.¹² The molecular basis of HCC aggressiveness and chemoresistance is poorly understood. It has been suggested that circulating and/or tumor-resident cancer stem cells (CSCs) play pivotal roles in such events.¹³

CSCs in liver diseases

Tumor/cancer-initiating stem cells are a minor subpopulation of cells within a tumor that display unlimited self-renewing capabilities and the ability to differentiate into heterogeneous lineages that form the bulk of the tumor.¹⁴ CSCs exhibit intrinsic resistance to chemo/radiation therapies because these treatments fail to deplete the CSCs within the tumor. The surviving residual CSCs can then recapitulate the growth of the original tumor resulting in relapse of the cancer.¹⁵ As a result, there is considerable interest in

developing strategies to effectively target these cells. A growing body of work suggests that CSCs are implicated in hepatocarcinogenesis as a result of various types of liver injury such as hepatitis and metabolic dysregulation. The HCV core protein upregulates the pluripotency factors OCT4, NANOG, and Lin28A.¹⁶ The viral protein was also found to enhance NOTCH1, CD133, and c-Kit, a stem cell factor receptor involved in self-renewal and differentiation of several types of stem cells.¹⁶ In the setting of obesity, hepatic CSCs can be activated through enhanced expression of the leptin receptor OB-R and receive oncogenic signals from adipocytes, as marked by increased expression of OCT4 and SOX2.¹⁷ Thus, chronic hepatic injury-induced CSCs may contribute to the initiation, development, and maintenance of HCC.

Doublecortin-like kinase 1 in liver diseases

Doublecortin-like kinase 1 (DCLK1) is a member of the doublecortin gene family and contains a distinct

N-terminus microtubule-binding domain with two DCX motifs (Figure 1(a)). Structural and functional characterization of these tandem DCX motifs (designated as N-DCX or DCX1 and C-DCX or DCX2) suggests that DCX1 specifically binds to microtubules, whereas DCX2 interacts with tubulin dimers as well as microtubules.¹⁸ These functional features in DCX may enhance tubulin polymerization and/or crosslinking between microtubules. The C-terminus domain contains a serine-threonine kinase that is structurally similar to calmodulin-dependent protein kinase 1G (Figure 1(b)), but lacks a canonical calmodulin-binding site.^{19,20}

The DCLK1 gene contains two promoter sites. The upstream 5'(α)-promoter encodes a full-length DCLK-1 (~82kDa long forms DCLK1/2, 729/740 aa, respectively) and is regulated by β -catenin. The alternate β -promoter, located in Intron V, encodes a C-terminus kinase-containing region (termed as DCLK₁-S or DCLK3/4, ~45–50 kDa, 422 aa) of the full-length DCLK1 and is regulated by NF κ B.²² It is also possible that multiple alternatively spliced DCLK1

Figure 1 Organization of DCLK1 protein and its four isoforms. (a) The numbering of amino acid residues shown in the diagram are according to Uniprot (<http://www.uniprot.org>). The numbers with DCLK1 represent isoforms 1, 2, 3, and 4. (b) *In silico* modeling of the C-terminus half of DCLK1 (DCLK1 isoform 3 representing C-terminus half of the full-length isoform 1) using SWISS-MODEL (<http://swissmodel.expasy.org>).²¹ The structure was deduced using the template for human calmodulin-dependent protein kinase 1 G (CAMK1G at 1.70 Å, PDB ID: 2JAM). The conserved protein structure shows two distinct domains: the N-terminus is enriched in β -pleated sheets, whereas the C-terminus predominantly contains α -helices. ATP is a natural ligand docked at the active site of DCLK1 and makes direct contacts with the conserved E99 and V101 residues of the DCLK1-3, which corresponds to amino acids E406 and V408 in DCLK1-1/2 isoforms. (A color version of this figure is available in the online journal.)

mRNAs translate into DCLK1 variants or calpain cleavage of full-length DCLK1 may result in smaller forms.^{19,23} It is widely accepted that DCLK1 is expressed as at least four isoforms in different organs (designated here as DCLK1-1, DCLK1-2, DCLK1-3, and DCLK1-4, where the last digit represents the specific isoform [Figure 1(a)]). Although detailed studies on the structure–function relationship of these isoforms are not available, it is highly likely that these isoforms display functional differences and distinct subcellular localizations.²⁴

DCLK1 was initially characterized as a protein involved in brain development and neuronal migration.^{20,25} Recently, the microtubule-associated activity of DCLK1 has been shown to be involved in dendritic growth, remodeling, and cargo trafficking.^{26,27} CSCs in several gastrointestinal cancers including pancreatic, esophageal, colon, and liver cancer were found to overexpress DCLK1.^{28–31} A lineage tracing study in *Apc*^{Min/+} mice revealed that DCLK1 was selectively expressed by intestinal CSCs in response to injury.³² This and several other studies have provided strong evidence that DCLK1 can be regarded as a CSC marker in liver and gastrointestinal cancers.^{28–31,33,34}

DCLK1 in chronic hepatitis

The HCV virus requires intact microtubules for replication and viral trafficking within the hepatocyte since replication complexes directly interact with microtubules.^{35,36} Interestingly, hepatoma cells expressing an HCV subgenomic replicon displayed enhanced DCLK1 expression relative to control cells, and confocal microscopy revealed co-localization of DCLK1 with HCV complexes and microtubules.²⁸ In a JFH1 HCV infection model, cells exhibited marked DCLK expression following infection with the viral particles.²⁸ DCLK1 appears to promote HCV replication since siRNA-mediated knockdown of DCLK1 significantly diminishes HCV RNA and HCV NS5B polymerase levels.²⁸ Inhibition of HCV replication in hepatoma cells leads to downregulation of CSC-related proteins DCLK1, CD133, Lgr5, Lin28, AFP, and c-Myc expression.²⁸ Liver biopsies of patients infected with HBV and HCV (major HCC risk factors) also exhibit DCLK1 expression in multiple hepatic cell types.^{28,33}

Pro-inflammatory and oncogenic roles of DCLK1

DCLK1 overexpression is observed in the liver of patients with chronic inflammation, cirrhosis, and HCC. Liver tissues from patients with chronic HCV infection have been shown to express high levels of DCLK1 in epithelial and stromal cells, lymphocytes, and bile ducts, which correlates with the expression of the pro-inflammatory calprotectin subunit S100A9. In contrast, normal liver tissues are usually negative for both proteins except in Kupffer cells (hepatic macrophages), which typically show S100A9 expression.³³ DCLK1 overexpression has been shown to enhance S100A9 expression whereas its downregulation diminishes S100A9 levels to a considerable level. These observations set a precedent that DCLK1 potentially

regulates hepatic inflammatory pathways because S100A9 is an important subunit of pro-inflammatory calprotectin complexes.³³ The S100A8/A9 complex is primarily expressed in myeloid cells and is involved in neutrophil chemotaxis, adhesion, and microtubule reorganization during endothelial transmigration.^{37–39} Additionally, S100A8/A9 expression can be induced in other cell types such as endothelial cells, keratinocytes, and epithelial cells in response to inflammation and injury.⁴⁰ Aside from the primary pro-inflammatory functions, S100A8/A9 also exhibits pro-tumorigenic properties such as induction of nuclear factor kappa B (NF- κ B), MAPK signaling, and epithelial-to-mesenchymal transition (EMT).³⁸ Knockdown of DCLK1 in hepatoma cells diminishes S100A9 expression, which in turn is likely to downregulate inflammation via NF κ B pathway.³³ Given the inflammatory and oncogenic roles of S100A8/A9, there is interest in targeting S100A8/A9 for the treatment of cancer. The oral S100A9 inhibitor tasquinimod has shown partial efficacy against prostate cancer. However, the drug did not significantly prolong the survival of patients with castrate-resistant prostate cancer in a recent phase III clinical trial.⁴¹ Since DCLK1 appears to be an upstream inducer of S100A9, it can be speculated that DCLK1 activation may continually drive the expression of the pro-inflammatory S100A9 making it resistant to S100A9-targeted therapies.

A key histologic finding of chronic HCV infection is the presence of lymphoid infiltrates in the liver. Immunohistochemical analysis of liver biopsies from HCV-infected patients showed enhanced DCLK1 and S100A9 staining in CD20⁺ B-cell aggregates in areas of portal inflammation.³³ It is known that HCV is capable of infecting B-cells due to lymphotropism, and HCV infection is associated with plasma cell dyscrasias such as mixed cryoglobulinemia as well as lymphoproliferative disorders through unclear mechanisms.^{42,43} Thus, the detection of DCLK1 and the pro-inflammatory S100A9 in CD20⁺ B-cells may provide new insights into HCV-related hematologic disorders.

The evidence for DCLK1 as a regulator of oncogenesis in liver diseases came from recent studies using primary human hepatocytes and mouse models. Normal healthy liver tissue does not express detectable levels of DCLK1 protein. However, primary hepatocytes derived from normal human liver tissue acquire DCLK1⁺ phenotypes when cultured as spheroids in Matrigel culture. These spheroids form heterogeneous hepatic and non-hepatic lineages.³³ These findings reiterate the liver's remarkable level of cellular plasticity which may be driven by DCLK1 signaling. DCLK1 can also be experimentally induced in C57BL/6 mice livers following diethylnitrosamine (DEN)/carbon tetrachloride (CCl₄) treatment within a month.⁴⁴ In patients with cirrhosis and HCC, DCLK1 overexpression correlates with c-Myc oncogene and BRM/SMARCA2 (part of the SW1/SNF1 chromatin remodeling complexes) suggesting that DCLK1 has a role in cirrhosis and HCC via chromatin remodeling.³³ It was recently reported that increased levels of DCLK1 is detectable in the plasma of patients with cirrhosis and HCC, but not in normal patients.⁴⁵ The use of DCLK1 as a potential

biomarker for the detection and/or management of HCC should be further explored.

DCLK1 in other gastrointestinal diseases

The inflammatory and oncogenic roles of DCLK1 have been extensively studied in the pancreas, intestine, and colon in the settings of radiation-induced damage and chemical carcinogenesis.^{29,34,46} Genetic lineage tracing in transgenic mouse models has revealed that DCLK1-positive cell number is increased many folds in intestinal and colonic polyps of *APC^{+/-min}* mice with inflammation.³² It is now believed that a subpopulation of DCLK1⁺ cells is extremely long lived and functions as CSCs. Ablation of these cells results in the rapid collapse of established polyps while having no discernable effects on adjacent normal tissues.^{34,47} It has been suggested that patients with inflammatory bowel diseases (IBD), such as ulcerative colitis and Crohn's disease, have increased risks of developing colon cancer. IBD-associated colon cancers typically have acquired genetic alterations (e.g., TP53, Rb, and DNA repair genes) and epigenetic dysfunction as a result of the inflammatory insult.⁴⁸ Stem cells in the intestinal crypts play important roles in responding to intestinal inflammation, regenerating mucosal integrity following injury, and initiating tumorigenesis.⁴⁹ In a dextran sulfate sodium-inducible colitis mouse model, the deletion of intestinal DCLK1⁺ cells resulted in worsening of intestinal inflammation, as demonstrated by elevated pro-inflammatory cytokines and chemokines, as well as reduced epithelial proliferation following injury.⁵⁰ Analogous to colitis-induced colon cancer, pancreatitis also significantly increases the risk of pancreatic cancer. In a recent study using a cerulein-induced pancreatitis mouse model, DCLK1 was found to mark a subpopulation of pancreatic progenitor cells involved in the regenerative process following pancreatic injury and displayed tumor-initiating capabilities.⁵¹ These findings underscore the involvement of DCLK1 in regulation of inflammatory signaling pathways and post-injury cellular regeneration that may predispose cells to neoplasia. Future research should be aimed at delineating the predicted DCLK1-S100A9-NFκB axis in various chronic inflammatory diseases.³³

DCLK1's role in EMT and metastasis

A major obstacle in the management of HCC is the complication of metastasis. Since HCC is typically detected at late stages, many patients often initially present with signs of extrahepatic metastasis. In order to metastasize to distant sites, cancer cells downregulate cell-cell adhesion proteins and acquire pro-migratory phenotypes in a coordinated process called EMT. Cancer cells can repress the expression of E-cadherin by promoting transcription factors Zeb1/2, Twist, and Snail, which downregulate E-cadherin expression and promote cellular detachment.⁵² Upregulation of vimentin also causes changes in cytoskeletal elements that ultimately promote alterations in the cellular architecture and increases cellular motility.⁵³ Furthermore, increased expression and secretion of matrix metalloproteinases

(MMPs) results in the degradation of the extracellular matrix which allows tumor cells to invade through the basement membrane and into other tissues.⁵² Our transcriptome analysis of liver cancer tissues (LIHC) from The Cancer Genome Atlas (TCGA)^{54,55} revealed that several EMT-related proteins and transcription factors are co-expressed with DCLK1 (Figure 2). Specifically, DCLK1 expression positively correlates with the expression of ZEB2, TWIST1, vimentin, MMP-2, MMP-14, and VEGF-C (Figure 2). These findings and published data on pancreatic cancer⁵⁶ suggest that DCLK1 is involved in HCC metastasis and that targeting of DCLK1 may reduce aggressive progression of HCC.

Targeting DCLK1 in liver diseases and cancers

Direct acting anti-virals (DAAs) against HCV that causes chronic liver diseases in a majority of patients have been shown to be highly effective in curing infection in patients with different genotypes.⁵⁷⁻⁵⁹ The risk of HCC occurrence in patients with HCV-associated cirrhosis is not reduced despite successful treatment with DAAs.⁶⁰ In addition, liver diseases such as cirrhosis, alcohol-induced liver diseases, NASH, and those contributed by metabolic disorders (diabetes, obesity) are rising globally.^{7,61} Most chronic liver diseases and HCC are presented at late stages for which there are no effective treatments. Recent investigations suggest that DCLK1 is induced in hepatocytes during adverse conditions and has the potential to drive tumorigenesis including metastasis and angiogenesis (Figure 2). Concerted efforts by many investigators have provided ample evidence that DCLK1 is potentially an important target for the treatment of inflammatory and neoplastic diseases. Inhibition of DCLK1 expression and/or activities using siRNAs, shRNAs, cross-reactive kinase inhibitors, or natural products has revealed DCLK1's proliferative and neoplastic properties. These approaches have been tested in pre-clinical models and may have translational potential into clinical application.^{44,45,62-65} However, the development of small molecules with therapeutic potential for targeting DCLK1 remains a challenge.

Potential impacts of DCLK1-targeted therapy

DCLK1 has been directly targeted using siRNAs and shRNAs to demonstrate the consequences of DCLK1 down-regulation as a proof of concept. As discussed earlier, siRNA-mediated knockdown of DCLK1 in hepatocytes expressing the HCV subgenomic replicon inhibited HCV replication as demonstrated by a marked reduction of HCV RNA and viral NS5B polymerase levels. Thus, continued research is still needed to uncover if targeting DCLK1 will simultaneously diminish viral infection and neoplastic changes in patients with HCV-related cirrhosis and HCC.

We have provided evidence that it is possible to effectively target tumors of various organs in xenografts and cell culture models by downregulating DCLK1 using specific RNA interference. We have successfully used biodegradable poly(lactic-co-glycolic acid) nanoparticles to meet the challenges of siRNA delivery to the tumor sites. In xenograft

Figure 2 Positive correlation between the levels of DCLK1 mRNA expression and factors involved in EMT. The graphs were generated using TCGA expression data for liver cancer (LIHC, $n = 190$) and analyzed with cBioPortal.⁵⁵ Linear correlation for DCLK1 expression with the expression of MMP-2, MMP-14, TWIST1, Vimentin, ZEB2, and VEGF-C is shown. Data are presented with Pearson coefficient (r); p -values indicate statistical significance. (A color version of this figure is available in the online journal.)

models representing liver, colon, and pancreatic tumors, siRNAs against DCLK1 (siDCLK1) resulted in a significant reduction in tumor growth, upregulation of the tumor suppressor miRNA let-7 a, and downregulation of EMT, c-Myc, Notch-1, and VEGF.^{62,66,67} Thus, targeting DCLK1 holds promise in cancer treatment.

In liver cancer cells, overexpression and knockdown assays suggest that DCLK1 functions upstream of S100A9 and regulates expression of the S100A9 subunit of calprotectin. shDCLK1 silencing caused downregulation of the pro-inflammatory S100A9 and inhibited cancer cell migration.³³ Knockdown of DCLK1 via siRNA also caused significant reduction in the growth of liver cancer xenografts as well as inhibition of EMT markers. It would be interesting to investigate the impacts of combined anti-DCLK1 strategies with the S100A9 inhibitor tasquinimod for the treatment of inflammatory diseases and inflammation-induced neoplasia.

Kinase inhibitors for DCLK1

DCLK1-specific kinase inhibitors have not yet been developed. However, two kinase inhibitors originally developed for targeting other kinases have been found to display cross-reactivity with the DCLK1 kinase domain. XMD8-92, originally developed as an inhibitor of MAPK7/BMK1 ($K_d = 80$ nM), was found to cross-react with DCLK1/2 ($K_d = 190$ nM).⁶⁸ The inhibitor was shown to downregulate DCLK1 expression and caused growth inhibition of AsPC-1 pancreatic cells and mouse xenograft tumors.⁶³ Additionally, XMD8-92 causes a significant reduction in the levels of EMT markers (ZEB1, ZEB2, SNAIL, and SLUG) and pluripotency factors (KLF4, OCT4, and SOX2).⁶³ The viability of hepatoma

cell lines and HCV replicon-expressing cells are considerably diminished with XMD8-92 treatment above $2.5 \mu\text{M}$ (unpublished data). The inhibitor of leucine-rich repeat kinase 2 (LRRK2), LRRK2-IN-1, also cross-reacts with the DCLK1 kinase domain. The drug inhibits HCT116 (colon cancer) and AsPC-1 (pancreatic cancer) cells by reducing cell growth, inducing cell cycle arrest, and halting expression of c-Myc and EMT markers.⁶⁴

Impact of indirect interference with DCLK1 functions

DCLK1 associates with microtubules, catalyzes microtubule polymerization, and regulates its dynamic instability.^{20,27,69} Thus, inhibiting the microtubule-associated functions of DCLK1 may be effective at thwarting intracellular transport, cell growth, and cell division in tumors. Fluvastatin, a cholesterol-lowering drug that inhibits HMG-CoA reductase, causes downregulation of DCLK1, induction of microtubule bundling, and halting of the cell cycle in the G1 phase, whereas other drugs in the statin class did not.⁶² Fluvastatin also reduces the HCV RNA levels, reiterating DCLK1's role in HCV replication. The use of fluvastatin as an adjuvant to antiviral therapy has been explored in patients with HCV.^{70–73} Statins have also been reported to exert anti-inflammatory properties by inhibiting COX-2 and prostaglandin E1 production in hepatic myofibroblasts and anti-tumor activities such as ERK-mediated apoptosis.^{74,75} Since statins are generally well tolerated in patients, the use of fluvastatin in particular should be explored as potential adjuvant to HCC therapy.

Natural products and related small molecules may also be effective in blocking DCLK1's tumorigenic characteristics. Curcumin, a dietary polyphenol found in turmeric,

was shown to inhibit DCLK1 expression and growth of HCT-116 colon cancer cells.⁷⁶ Recently, we reported that the resveratrol analog (Z)-3,5,4'-trimethoxystilbene (Z-TMS) exhibits hepatoprotective effects against DEN/CCl₄-induced injury and concomitantly downregulates hepatic DCLK1 expression in mice. Mechanistically, Z-TMS causes bundling of DCLK1 with microtubules, arrests cell cycle progression at G2/M phase via reduction of CDK1, induction of p21^{cip1/waf1} expression, and inhibition of Akt (Ser⁴⁷³) phosphorylation.⁴⁴ Z-TMS-induced DCLK1 sequestration with microtubule bundles is likely to interfere with HCC growth because only CSCs, but not normal hepatocytes, overexpress the protein. In addition, Z-TMS appears to be a potent anti-proliferative agent against hepatoma cells and erlotinib-resistant lung adenocarcinoma cells (H1975) harboring the T790M EGFR mutation.⁴⁴

Conclusion

The existing paradigm that facultative stem/progenitor cells are called upon to produce mature hepatocytes during chronic injury has been challenged by recent reports.^{77–79} These studies suggest that adult hepatocytes exhibit significant plasticity during renewal and regeneration of adult liver following injury. Persistent HCV replication induces stem cell-like properties in the cells. These observations support the concept that tumor/cancer stem-like cells might be generated from mature hepatocytes following chronic injury. Published evidence suggests that DCLK1 induction and its overexpression following hepatic injury are likely to contribute to tumorigenesis including maintenance and dissemination of tumor cells in circulation. Emerging evidence suggests that anti-DCLK1 agents interfering with its expression, microtubule-DCLK1 dynamics, or kinase activities can downregulate CSC properties, tumor growth, and EMT. Although recent advances since its first report in 1998⁸⁰ have proven DCLK1 as a bona fide tumor target in liver, pancreas, colon, and intestine, specific small molecule inhibitors are needed to be developed.

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DECLARATION OF CONFLICTING INTERESTS

CW Houchen has ownership interest (including patents) and is a consultant/advisory board member for COARE Biotechnology Inc. No potential conflicts of interest were disclosed by the other authors.

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