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DCLK1, a putative novel stem cell marker in human cholangiocarcinoma

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Abstract

Background & Aims: Cholangiocarcinoma (CCA) is a very aggressive cancer showing high cancer stem cells (CSCs) presence. Doublecortin-like kinase1 (DCLK1) has been demonstrated as a CSC marker in different gastroenterological solid tumours. Our aim was to evaluate *in vitro* the expression and the biological function of DCLK1 in intrahepatic CCA (iCCA) and perihilar CCA (pCCA).

Approach & Results: Specimens surgically resected of human CCA were enzymatically digested, submitted to immunosorting for specific CSC markers (LGR5, CD90, EpCAM, CD133, CD13) and primary cell cultures were prepared. DCLK1 expression was analysed in CCA cell cultures by real-time quantitative polymerase chain reaction (RT-qPCR), Western Blot and immunofluorescence. Functional studies have been performed by evaluating the effects of selective DCLK1 inhibitor (LRRK2-IN-1) on cell proliferation (MTS-Assay, cell population doubling time), apoptosis and colony formation capacity. DCLK1 was investigated *in situ* by immunohistochemistry and RT-qPCR. DCLK1 serum concentration was analysed by enzyme-linked immunosorbent assay (ELISA). We describe DCLK1 in CCA with an increased gene and protein DCLK1 expression in $pCCA^{LGR5+}$ and in $iCCA^{CD133+}$ cells compared to unsorted cells. LRRK2-IN-1 showed an anti-proliferative effect in dose-dependent manner. LRRK2-IN-1 markedly impaired cell proliferation, induced apoptosis, decreased colony formation capacity and colony size in both iCCA and pCCA compared to untreated cells. *In situ* analysis confirm that DCLK1 is present only in tumours, but not

in healthy tissue. Interestingly, DCLK1 was detected in the human serum samples of iCCA (high), pCCA (high), HCC (low) and cirrhotic (low) patients, but it was almost undetectable in healthy controls.

Conclusion: DCLK1 characterizes a specific CSC-subpopulation of $iCCA^{CD133+}$ and $pCCA^{LGR5+}$ and its inhibition exerts anti-neoplastic effects in primary CCA cell cultures. Human DCLK1 serum might represent a serum biomarker for the early CCA diagnosis.

Keywords

Primary liver cancer, Invasiveness, Cancer Stem Cells, Biomarker, DCAMKL1

List of Abbreviation

BilIN: biliary intraepithelial neoplasm; CCA: cholangiocarcinoma; CSC: cancer stem cell; dCCA: distal CCA; DCLK1: doublecortin-like kinase 1; ELISA: enzyme-linked immunosorbent assay; EMT: epithelial-mesenchymal transition; GMP: good manufacturing practice; HepD: healthy hepatic bile ducts at hilum region;

HCC: hepatocellular carcinoma; iBEC: intrahepatic biliary epithelial cell; IC_{50}: half maximal inhibitory concentration; iCCA: intrahepatic CCA; pBEC: perihilar biliary epithelial cell; pCCA: perihilar CCA; PD: population doubling; PSC: primary sclerosing cholangitis; RT-qPCR: real-time quantitative polymerase chain reaction; WB: Western Blot

Cholangiocarcinoma (CCA) is a heterogeneous group of neoplasms of the bile ducts(1). CCA is a tumour that has an increasing incidence throughout the world, showing high mortality due to its aggressiveness, late diagnosis, metastatization and immunoregulation capacity(1, 2). The CCA is topographically classified into intrahepatic CCA (iCCA), perihilar CCA (pCCA) and distal CCA (dCCA)(3).

It has been demonstrated that there are pathological and molecular differences between iCCA and pCCA/dCCA(3-8).

Our research team has previously shown that CCA is rich of cancer stem cells (CSCs)(9), which are associated with an high rate of recurrence and chemotherapy resistance. Recently, several research groups have focused their attention on CSCs. CSCs exhibit many stem cell-like characteristics and CSCs recapitulate the origin of tumour heterogeneity after metastasis(10). CSCs have been subjected to extensive analysis and as therapeutic targets(10).

Doublecortin-like Kinase 1 (DCLK1) is a protein associated with microtubules in cytoplasm which catalyses their polymerization(11). DCLK1 has been found in CSCs of gastrointestinal tract tumours such as colon(12), pancreas(13) and hepatocarcinoma(11). Despite these discoveries, the role of DCLK1 in the tumours and its interaction with other protein is largely unknown.

Nakanishi *et al.*(14) exploiting the lineage tracking method, showed that DCLK1 can marks tumour stem cells in the intestine. In addition, Westphalen *et al.*(15) described the relationship between $DCLK1^+$ cells and the beginning of colon cancer. Also, Whorton *et al.*(16) showed that DCLK1 regulates pluripotency and angiogenesis in pancreatic cancer via a microRNA-dependent mechanism.

According to recent papers by Bailey *et al.*(17) pancreatic neoplasms expressing DCLK1 contain morphologically and functionally distinct subpopulations such a CSCs. Ito *et al.*(18) identified DCLK1 as a protein that is predominantly expressed in invasive and metastatic human pancreatic CSCs.

Currently, the presence of DCLK1 in CCA and its possible functional role in this tumour is unknown. In this study we investigated the DCLK1 expression in pCCA- and iCCA-subpopulations ($EpCAM^+$, $LGR5^+$, $CD90^+$, $CD13^+$, $CD133^+$ tumour cell subset).

Materials and Methods

Human CCA primary cell cultures and Tissue Sourcing

Samples of CCA and peritumoral noncancerous liver were obtained from patients presenting a single lesion, which were submitted to curative surgical resection at the ‘‘Paride Stefanini’’ Department of General Surgery and Organ Transplantation, Sapienza University of Rome, Rome, Italy; or at the Surgery, Hepatobiliary Unit, Catholic University of the Sacred Heart School of Medicine, Rome, Italy; or at the Hepato-Biliary Surgery, Regina Elena National Cancer Institute, Rome, Italy. Primary cell cultures were prepared by mechanical and enzymatic dissociation of specimens of human iCCA and pCCA samples. Primary cell cultures were prepared by mechanical and enzymatic dissociation of specimens of human iCCA and pCCA samples.

As described in previous papers(9, 19), pCCA and iCCA were selected and cultured in H69 medium and used for experiments over 30 passages from isolation.

Physiologic human primary cell cultures and Tissue Sourcing

Human biliary tree tissues from hilum region were used as physiologic control (HepD) of pCCA tissues, while, peripheral intrahepatic bile ducts from liver parenchyma was used as physiologic control (Healthy liver parenchyma) of iCCA tissues. EpCAM positive biliary epithelial cells isolated from liver parenchyma (iBECs) and EpCAM positive biliary epithelial cells isolated from hilium region, perihilar (p)BECs, were used as physiological controls of iCCA and pCCA, respectively. Perihilar biliary tree tissues and pBECs were obtained from organ donors from the "Paride Stefanini" Department of General Surgery and Organ Transplantation, Sapienza University of Rome, Rome, Italy. Informed consent to use tissues for research purposes was acquired from our transplant program. Peripheral intrahepatic bile ducts and iBECs were isolated from human foetuses (16–22-week gestational age) which were obtained by elective pregnancy termination from the Department of Gynaecology (Sapienza, University of Rome, Italy). Informed consent was acquired from the mother before abortion. pBECs and iBEC primary cells were cultures in Kubota's Medium (KM) as described previously by our laboratory (20-22).

Ethical approval

All experiments described above were approved by the Ethics Committee of the Policlinico Umberto I, Sapienza University Hospital, and all the procedures was in accordance with Good Manufacturing Practice (cGMP). No donor organs were obtained from executed prisoners or other institutionalised individuals.

Immortalized cell cultures

HT29 Cell Line human is a commercial cell line isolated from Caucasian colon adenocarcinoma grade II. It was used as internal control for each experiment and cultured in H69 medium. Moreover, H69 cell line, a cholangiocytes cell line is a commercial immortal non-malignant cell line isolated from BEC, were cultured in H69 medium.

Other Materials and Methods are described in the supplementary.

Results

Expression *in vitro*

Subpopulations of CSCs were immune-sorted from pCCA or iCCA and investigated by real-time quantitative polymerase chain reaction (RT-qPCR) and Western Blot (WB) to analyse the presence of DCLK1.

In the *p*CCA, the DCLK1 expression was evaluated by RT-qPCR. The highest DCLK1 gene expression was found in $pCCA^{LGR5+}$-subpopulation ($1.15*10^{-5}\pm3.23*10^{-6}$; N=6; $p<0.01$), this mRNA levels were significantly higher than DCLK1 mRNA levels in unsorted pCCA cells ($1.35*10^{-7}\pm1.76*10^{-8}$)(Fig.1A). Moreover, other subpopulations showed significant enhancer DCLK1 gene expression such as

$pCCA^{CD90+}$($4.99*10^{-6}$±$9.08*10^{-7}$; N=6; p<0.01), $pCCA^{EpCAM+}$($4.87*10^{-7}$±$7.09*10^{-8}$; N=6; p<0.001) and $pCCA^{CD13+}$($3.98*10^{-6}$±$9.23*10^{-7}$; N=6; p<0.01) respect to unsorted pCCA cells (Fig.1A). Instead, the $pCCA^{CD133+}$-subpopulation did not show significant different DCLK1 mRNA levels compared to unsorted pCCA (N=6). Similar results were observed in the protein analysis by WB. In all populations, it was observed a 82kDa band corresponding to the most studied isoform from previous studies(23). Fig.1A show the expression of DCLK1 protein in pCCA-subpopulation compare to unsorted pCCA cells. The $pCCA^{LGR5+}$(1.41±0.2; N=6; p<0.01), $pCCA^{CD90+}$(0.68±0.07; N=6; p<0.05), $pCCA^{133+}$(0.74±0.07; N=6; p<0.01), $pCCA^{EpCAM+}$(1.15±0.19; N=6; p<0.01;) $pCCA^{CD13+}$(1.3±0.03; N=6; p<0.01) expressed higher protein levels compare to unsorted control (0.45±0.14; N=6). In pCCA the higher genetic and protein expression of DCLK1 result in $LGR5^{+}$-subpopulation.

Notably, pBECs used as physiologic control of pCCA, did not express DCLK1 by RT-qPCR and WB analysis (data no shown).

In the iCCA, the DCLK1 expression in all sorted-subpopulation [$LGR5^{+}$($1.69*10^{-5}$±$2.22*10^{-6}$; N=6; p<0.01), $CD90^{+}$($2.95*10^{-5}$±$6.03*10^{-6}$; N=6; p<0.001), $CD133^{+}$($4.44*10^{-5}$±$4.61*10^{-6}$; N=6; p<0.001), $EpCAM^{+}$($6.24*10^{-8}$±$1.25*10^{-8}$; N=6; p<0.05), or $CD13^{+}$($2.51*10^{-5}$±$5.06*10^{-6}$; N=6; p<0.01)] was significantly higher than unsorted iCCA cells ($3.93*10^{-08}$±$5.46*10^{-09}$; N=6)(Fig.1B). In accordance with genic expression, DCLK1 protein expression was significantly higher in the sorted iCCA-subpopulations like $LGR5^{+}$(0.64±0.18; N=6; p<0.01), $CD90^{+}$(1.14±0.13; N=6; p<0.01) $CD133^{+}$(1.77±0.22; N=6; p<0.01), $EpCAM^{+}$(0.93±0.05; N=6; p<0.01) and $CD13^{+}$(1.74±0.19; N=6; p<0.01) compared to unsorted iCCA (0.2±0.05; N=6)(Fig.1B). In iCCA the higher genetic and protein expression of DCLK1 result in $CD133^{+}$- and $CD13^{+}$-subpopulation. Interestingly, iBECs used as physiologic control of iCCA, did not express DCLK1 by RT-qPCR and WB analysis (data no shown).

In HT29 cells, used as positive control, the genic expression of DCLK1 was increased in $LGR5^{+}$($1.14*10^{-5}$±$4.03*10^{-6}$; N=6; p<0.001), $CD90^{+}$($1.45*10^{-6}$±$2.29*10^{-7}$; N=6; p<0.01) and $CD13^{+}$ cells ($1.45*10^{-6}$±$3.09*10^{-7}$; N=6; p<0.01) compared to unsorted HT29 cells ($4.02*10^{-7}$±$2.74*10^{-7}$; N=6)(Fig.1C). Moreover, $CD133^{+}$ ($3.49*10^{-7}$±$6.41*10^{-8}$; N=6; p>0.05) and $EpCAM^{+}$($9.13*10^{-7}$±$1.66*10^{-7}$; N=6; p>0.05)-subpopulation did not show significative difference respect to unsorted HT29 cells (Fig.1C).

The DCLK1 protein expression was higher in $HT29^{LGR5+}$(1.39±0.21; N=6; p<0.01), $HT29^{EpCAM+}$(1.33±0.15; N=6; p<0.01) and $HT29^{13+}$(1.11±0.19; N=6; p<0.05)-subpopulation compare to unsorted HT29 population (0.87±0.17; N=6)(Fig.1C). Instead, $HT29^{90+}$ and $HT29^{133+}$ tumour cell subset did not show significative difference respect to unsorted HT29 cells.

Localization of DCLK1 in primary CCA cell cultures

Afterwards, we investigated the localization of DCLK1 protein in unsorted population and in $pCCA^{LGR5+}$, $iCCA^{CD133+}$, or $HT29^{LGR5+}$ sorted tumour cells, that have showed the highest DCLK1 mRNA and protein expression.

DCLK1 was localized in pCCA and iCCA primary cell line by IF (Fig.2). DCLK1 was observed in cytoplasmatic localization in all unsorted and sorted analysed population (Fig.2) as previously showed in

other tumours(12, 24). DCLK1 was not detected in iBECs and in pBECs (Fig.2) confirming gene expression and WB results which previously we have exposed.

HT29 unsorted cells showed a localization of cytoplasmic DCLK1 as already described in the literature(12).

Effects of DCLK1 inhibition on viability primary human cell cultures

Based on previous data we performed experiments to investigate the effects of DCLK1 inhibition on primary human unsorted and sorted cell cultures.

By MTS assay we evaluated the half maximal inhibitory concentration (IC_{50}) and viability of primary cell cultures after treatment with DCLK1 inhibitor (LRRK2-IN-1) for 72hrs.

The cell viability decreased in a dose dependent manner in all populations treated with LRRK2-IN-1 compared to untreated cells. Viability reduction was observed in $pCCA^{LGR5+}$ cells (IC_{50}=4.51μM; N=4; $p<0.01$) inhibitory concentrations compared to unsorted pCCA cells (IC_{50}=9.61μM; N=4)(Fig.3A), as well as the $CD133^{+}$ population (IC_{50}=9.36μM; N=4; $p<0.01$) versus the whole unsorted iCCA population (IC_{50}=14.72μM; N=4)(Fig.3B).

The $HT29^{LGR5+}$-subpopulation showed a higher IC_{50} (5.48μM; N=4) respect to unsorted HT29 (3.02μM; N=4)(Fig.3C). Furthermore, iBECs and pBECs viability were not influenced by LRRK2-IN-1 treatment (Fig.S1).

Moreover, supernatant from CCA cultures was collected and used to culture H69 non-malignant cholangiocyte cell line. MTS assay showed that all conditioned medium increases H69 proliferation compares to CTRL. H69 grown in conditioned medium, from CCA cell culture with LRRK2-IN-1 for 72hrs at the above found concentrations of IC_{50} for each CCA-subpopulation, showed reduced cell proliferation compared to H69 cultured with CCA conditioned medium without LRRK2-IN-1 (Fig.S8).

Effects of DCLK1 inhibition on CCA cell apoptosis

To investigate whether DCLK-1 inhibition by LRRK2-IN-1 can induce an effect on cell apoptosis, Annessin-V-FITC/Propidium-Iodide double labelling flow cytometry was carried out.

We treated the pCCA and iCCA cells with LRRK2-IN-1 for 72hrs at the above found concentrations of IC_{50} for each subpopulation. We observed a significant increase of apoptotic process in unsorted (treated minus untreated unsorted pCCA cells: 26.30%±7.48%; N=6; $p<0.05$) or in $pCCA^{LGR5+}$ cells (treated minus untreated $pCCA^{LGR5+}$ cells: 31.34%±4.37%; N=6; $p<0.01$) treated with LRRK2-IN-1 compared to the respective untreated control cells (Fig.3D).

Moreover, LRRK2-IN-1 increased apoptosis in unsorted iCCA when compared to untreated and unsorted iCCA (treated minus untreated unsorted iCCA cells: 21.47%±1.57%; N=6; $p<0.01$)(Fig.3E). The extent of apoptosis was massive in the $iCCA^{133+}$-subpopulation treated with LRRK2-IN-1 respect to untreated $iCCA^{133+}$ cells (treated minus untreated $iCCA^{133+}$ cells: 46.1%±5.44%; N=6; $p<0.01$)(Fig.3E). The apoptotic effect of LRRK2-IN-1 was confirmed in HT29 unsorted cells treated with LRRK2-IN-1 when compared to untreated cells (treated minus untreated HT29 unsorted cells: 24.87%±1.78%; N=6; $p<0.01$)(Fig.3F). No statistically significant increase was observed in apoptosis between $HT29^{LGR5+}$ cells and $HT29^{LGR5+}$ LRRK2-IN-1 treated cells.

Effects of DCLK1 inhibition on CCA cell proliferation.

In order to analyse the population doubling (PD) index we treated the pCCA and iCCA cells with LRRK2-IN-1 for 72hrs at the concentrations of IC_{50} for each subpopulation.

The PD index of the unsorted pCCA cells treated with LRRK2-IN-1 was significantly decrease (1.99±0.32; N=6; $p<0.01$) compared to untreated pCCA cells (3.74±0.07; N=6)(Fig.4A). Similarly, $pCCA^{LGR5+}$ cells treated with LRRK2-IN-1 underwent to a reduction of proliferation rate (0.99±0.54; N=6; $p<0.01$) when compared to $pCCA^{LGR5+}$ untreated cells that showed a stable growth overtime (4.15±0.08; N=6)(Fig.4A).

Unsorted iCCA treated with LRRK2-IN-1 cells showed an important population index decrease (0.67±0.43; N=6; $p<0.01$) compared to untreated cells that exhibited a stable growth overtime (4.09±0.07; N=6)(Fig.4A). Moreover, the PD index in $iCCA^{CD133+}$ cells treated with LRRK2-IN-1 (0.86±0.28; N=6; $p<0.01$) was significantly decrease compared to untreated $iCCA^{CD133+}$ cells (3.80±0.10; N=6)(Fig.4B).

In HT29 cells, treated with inhibitor, PD rate was reduced (HT29 unsorted: 3.74±0.20; and $HT29^{LGR5+}$: 3.22±0.32; N=6; $p<0.01$) compared to untreated cells (unsorted: 4.78±0.15; and $HT29^{LGR5+}$: 5.34±0.07; N=6)(Fig.4C).

Effects of DCLK1 inhibition on CCA colony formation

Colony formation capacity were analysed by measuring the colony number after 10 days of treatment with LRRK2-IN-1 or without (controls) at the concentrations of IC_{50} for each subpopulation.

pCCA, iCCA primary cell cultures and HT29 cell lines treated with LRRK2-IN-1 showed a significant reduction of colony formation capacity compared to controls cells ($p<0.05$)(Fig.4D). Moreover, we observed a trend toward a decrease of the size of colonies, measured by colony dimension index in pCCA, iCCA and HT29 cultures, treated with LRRK2-IN-1 compared to untreated controls respectively ($p<0.05$)(Fig.4E).

***In situ* expression of DCLK1**

To assess DCLK1 expression, we performed immunohistochemistry on normal and CCA samples. Interestingly, both intrahepatic (i.e. interlobular bile ducts) and extrahepatic bile ducts (hepatic ducts at the hilum) were mostly negative (less than 5% of epithelial cells) for DCLK1 in normal organs (Fig.S2). In the hepatic duct, peribiliary glands were also present and were mostly DCLK1 negative. In CCA samples, DCLK1 was expressed by tumour glands both in pCCA (semiquantitative (SQ) score: 2.50±0.55; $p<0.05$ vs. normal ducts) and iCCA (SQ score: 2.33±0.82; $p<0.05$ vs. normal ducts), with no significant differences between the two tumour subtypes (Fig.5A and 5B).

We then used double immunofluorescence to evaluate whether CSC-subpopulations co-expressed DCLK1 in the same cell (Fig.5C and 5D). In pCCA, DCLK1 was expressed by 54.8±12.7% of $EpCAM^+$ cells, by 25±6.7% of $CD133^+$ cells and by 80.2±5.7% of Lgr5+ cells. In iCCA, DCLK1 was expressed by 50.4±4.7% of EpCAM+ cells, by 74.8±4.9% of CD133+ cells and by 25.6±6.3% of $Lgr5^+$ cells.

These interesting results were confirmed by DCLK1 gene expression analysis from total tissue extracts by RT-qPCR(Fig.6A). The graph shown in Fig.6B shows a higher DCKL1 gene expression in pCCA ($9.23*10^{-3}$±$9.04*10^{-5}$; N=6; $p<0.001$) or in iCCA ($1.25*10^{-2}$±$2.56*10^{-3}$; N=6; $p<0.01$) when compared to healthy

hepatic bile ducts at hilum region (HepD)($2.26*10^{-8}\pm9.65*10^{-9}$, N=6) and healthy liver parenchyma ($7.39*10^{-5}\pm1.68*10^{-5}$, N=6), respectively (Fig.6B).

DCLK1 as putative diagnostic serum biomarker

Furthermore, we evaluated the DCLK1 concentration into human serum samples of patients with pCCA or iCCA, in patients with HCC, cirrhosis, PSC and in healthy subjects (Table S1). Higher DCLK1 levels were detected both in pCCA (4.35±1.76ng/ml; N=10; $p<0.01$ vs. cirrhotic, HCC and PSC patients) and iCCA patients (3.21±1.98ng/ml; N=10; $p<0.01$ vs. cirrhotic and PSC patients; $p<0.05$ HCC patients) when compared to cirrhotic (0.90±0.39ng/ml; N=10), HCC (1.20±0.39ng/m; N=10) and PSC patients (0.96±0.23ng/ml; N=10). The analysis of the variance confirmed that DCLK1 concentration into human serum of patients affected by iCCA and/or pCCA was sensibly higher compare to all the other groups (iCCA or pCCA vs others $p<0.05$; CCA vs others $p<0.01$). In the healthy group DCLK1 in the serum is undetectable (Fig.6B).

Correlation between DCLK1 and inflammation in CCA

To assess possible correlation between DCLK1 and tissue inflammation, we counted the number of $CD68^+$ macrophages within CCA samples and evaluated IL6 expression in $DCLK1^+$ cells (Fig.S3A). However, we did not find any significant correlation between macrophage number and DCLK1 expression, nor did we observe a significant overlap (co-expression) between $IL6^+$ and $DCLK1^+$ CCA populations (Fig.S3B). Furthermore, we analysed several tumours (CA19-9) and inflammation (IL6 and TNFα) biomarkers using enzyme-linked immunosorbent assay (ELISA) and observed a marked increase in all serum biomarkers analysed, similar to DCLK1 in serum samples (Fig.S4).

Discussion

For the first time we have demonstrated the DCLK1 overexpression in the CSC-CCA populations, in particular in $pCCA^{LGR5+}$ and $iCCA^{CD133+}$ and DCLK1 functional role in tumour proliferation and viability.

The results which lead to this conclusion are: 1) The expression of genetic and proteic DCLK1 changed based on expression of different pluripotent/multipotent markers analysed in different pCCA or iCCA cell - subpopulations (LGR5, CD90, CD133, EpCAM and CD13). 2) The highest DCLK1 genic and proteic expression was observed in the $pCCA^{LGR5+}$- and $iCCA^{CD133+}$-subpopulations by RT-qPCR and WB respectively and this expression was significantly highly when compared with the whole tumour cell population. 3) DCLK1 is located in the cytoplasm of CSC-CCA, without any relevant differences between sorted and whole populations, while in healthy tissues (pBECs and iBECs) were not detectable. 4) The specific inhibition of DCLK1 by LRRK2-IN-1 in CCA cells demonstrated the anti-proliferative effect of LRRK2-IN-1 was dose-dependent, inducing directly proportional dose-response through MTS assay and this inhibition was more relevant in pCCA than iCCA cells. 5) DCLK1 inhibition by LRRK2-IN-1 markedly impaired the cell proliferation and increased the PD time, induced apoptosis, decreased colony formation capacity and colony size in both pCCA and iCCA treated with LRRK2-IN-1 when compared to untreated

CCA cells. 6) Tumoral cells within iCCA and pCCA tissue samples expressed DCLK1 at IHC analysis. 7) Using ELISA, DCLK1 was only found in the serum of CCA, cirrhotic, HCC patients and, importantly, was never detected in healthy control serum samples.

Currently, there are no technique for early diagnosis of CCA, moreover no specific CCA-CSC markers are known(1). Furthermore, many research groups are studying several strategies to block CSCs activity, the cells responsible for tumour growth, recidivism and chemoresistance(1, 9, 25). Currently the drug therapy via radio-chemo-therapy is primarily for palliative purposes because it does not target CSCs(19, 26). In fact, surgical resection is the only effective therapy but it is low applicable given the numerous late diagnoses(1). Many research groups are trying to identify specific CSC markers in order to formulate a targeted therapy which able to block the CSCs(9, 24, 25). A new protein which can be expressed at low levels in gastrointestinal non-cancer stem cells and increased in gastrointestinal CSCs is DCLK1(24, 27). In the previous studies on colorectal and pancreatic cancers, DCLK1 has been identified as a co-marker in CSCs and its inhibition lead to tumour regression or disappearance(28). In this work, we demonstrate how the two subtypes of CCA, the iCCA and pCCA, present different levels of DCLK1 expression in cells sorted for stem (LGR5, CD90, EpCAM and CD13) and progenitor markers (CD133). This data confirmed both gene and protein expression of DCLK1 alter between iCCA and pCCA because of different cells of origin which are present in distinct stem cell niches located along the adult human biliary tree. As indicated in other metaplastic tumours and pathological conditions(11, 16, 18, 24).

The expression and the localization of DCLK1 has never been described in the CCA. DCLK1 in the CCA showed a cytoplasmic localization, as already described in colorectal cancer and pancreatic cancer where DCLK1 was found associated with microtubules(18, 29-31). However, no difference in cytoplasmic localization was found between the analysed subpopulations and the total population.

In many studies, it has observed that inhibition of DCLK1 by specific inhibitor (LRRK2-IN-1)(28) or by small interference RNA (siRNA)(24, 32, 33) leads to a reduction of colorectal and pancreatic tumour mass *in vivo*, and carcinogenesis and viability *in vitro*. Our data demonstrated that in the subpopulations with the higher DCLK1 expression, in both gene and protein levels, DCLK1 inhibition leads to significant decrease of cell viability and proliferation compared to whole populations. In fact, we observed high inhibitor efficiency in specific CSC-CCA-subpopulation when compared to whole CCA cells. Furthermore, the sensitivity to treatment in $pCCA^{LGR5+}$ cells was increased. In the previous studies, the DCLK1 pathway is hypothesized in the HCC(11) with the direct role on c-Myc and an indirect effect on NF-kB through S100A9 factor enhancing the tumour proliferation and cell mobility. We hypothesize that the same pathway is also active in CCA. This hypothesis is strengthened by the dose-response data obtained by MTS, in which the high concentration of inhibitor had more effect on cell viability, until the vitality is reduced about below 80%. In addition, the inhibition of DCLK1 leads to an increase of apoptosis in CSC-CCA-subpopulations. This indicates an involvement of DCLK1 protein in the resistance to apoptosis in the CSC-CCA, confirming this evidence, it has recently been seen that DCLK1 increases chemoresistance of colorectal cancer cells through the anti-apoptosis pathway(34). DCLK1 inhibition induced a marked decrease in cell proliferation and a reduced expression of NF-kB, a gene under Notch control (Fig.S7). Interestingly, Chandrakesan *et*

al. demonstrated that NOTCH expression decreased after DCLK1 knockdown in colon cancer cells(12). Therapeutic effects of DCLK1 inhibition by LRRK2-IN-1 in animal models should be subject of future research.

Our data showed a strong reduction in EMT gene markers expression and an impaired in cell migration after DCLK1 inhibition (Fig.S5 and S6). Consistent with these results, several research teams have demonstrated an important role of DCLK1 in the progression of different gastroenterological tumours(35-38).

Additionally, we assessed the correlation between DCLK1 and inflammation markers in tissue and serum. However, at this phase we do not have enough data to support a functional role of DCLK1 in tumour inflammation and thus further investigations are needed to better address this topic.

Finally, a potentially relevant result is that DCLK1 could be detected in the human serum samples of CCA patients but, surprisingly, it was almost undetectable in healthy controls. Interestingly, in liver cirrhosis, a recognized iCCA risk factor and in PSC, a recognized pCCA risk factor, we found very low DCLK1 serum levels. These results confirm findings observed in other tumour studies, in which the precancer condition showed lower DCLK1 levels than the tumour. In fact, Whorton *et al.*(16) and Sureban *et al.*(39) demonstrated in their studies that in the malignant condition the concentration of DCLK1 in the patient serum, in oesophageal adenocarcinoma and HCC respectively, was higher than pre-cancer conditions (Barrett's oesophagus and liver cirrhosis, respectively). Many research teams have associated DCLK1, analysed in different tumours, with metastasis, tumour invasion, and prognosis(18, 38, 40, 41). Moreover, Nakanishi *et al.*(14) demonstrated that the ablation of DCLK1 in tumour stem cells resulted in regression of intestinal polyps within mouse models. DCLK1 has been proposed as a marker of quiescent stem cells in both the pancreas and gastrointestinal tract(42). DCLK1 has displayed a high methylation frequency in CCA tissues(43). Intraepithelial neoplasms have been reported in the biliary tract as biliary intraepithelial neoplasms (BilINs), microscopic lesions unable to be identified macroscopically(44). DCLK1 marked a morphologically distinct and functionally unique population of pancreatic cancer-initiating cells in mouse models of pancreatic intraepithelial neoplasia, the pancreatic counterpart of BilIN(17). Finally, DCLK1 has been investigated in PSC through DNA methylation analysis of biliary brush samples as compared with CCA(45). Our results regarding DCLK1 expression in CCA serum samples and its low levels in at-risk conditions, e.g. liver cirrhosis and PSC, should be confirmed in larger investigations assessing the clinical use of DCLK1 as a serum biomarker in CCA.

Conclusion

In conclusion, DCLK1 expression characterizes specific CSC-subpopulations of $iCCA^{CD133+}$ and $pCCA^{LGR5+}$ and DCLK1 inhibition exerts anti-neoplastic in primary CSC-CCA cell cultures. Moreover, DCLK1 was detected in the serum of CCA patients while it was not observed in healthy people serum. Finally, it will be important to study the DCLK1 signalling pathway, because blocking its function could be effective therapeutic target in the future, against CCA-CSCs.

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Author Contributions Statement

L.N.: first author, corresponding author, conception and design of the study, generation, collection, assembly, analysis and interpretation of the data, main drafting and revision of the manuscript, approval of the final version of the manuscript.

S.D.M.: first author, conception and design of the study, generation, collection, assembly, analysis and interpretation of the data, main drafting and revision of the manuscript, approval of the final version of the manuscript.

G.C., I.Z., P.M.G.: analysis and interpretation of the data, drafting, revision and approval the final version of the manuscript.

V.C., V.A., D.C., O.V., S.S.: involved in the data collection, generation and analysis, approval the final version of the manuscript.

F.M., P.B.B.: were responsible for the collection of biliary trees from organ donors.

A.G., M.M., D.B., P.B.P.: were responsible for the collection of foetal biliary trees from therapeutic abort.

V.D.P., A.O., A.M.D., G.G., F.G.: were responsible for the collection of CCA surgical resection.

S.M., P.I., were responsible for the collection of PSC serum samples

G.C., E.G., D.A.: conception and design of the study, drafting and revision of the manuscript, approval of the final version of the manuscript.

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Conflicts of interest

All the authors have nothing to disclose.

Figure Legend

Figure 1. DCLK1 expression in the subpopulation of pCCA- and iCCA-CSCs primary cell culture.

Relative DCLK1 mRNA level expression analysis by RT-qPCR (left) and DCLK1 relative protein expression analysis by Western Blot (right) of pCCA (A), iCCA (B) and HT29 (C) whole and subpopulation.

Data expressed as mean±SD of N=6 experiments; *p<0.05 vs unsorted cells, §p<0.01 vs unsorted cells, ¤p<0.001 vs unsorted cells.

Figure 2. DCLK1 localization in subpopulation of pCCA- and iCCA-CSCs primary cell culture.

A) Cytoplasmic localization of DCLK1 (red) in $LGR5^+$ sorted and unsorted pCCA, nuclei are stained with DAPI (blue). No difference between $pCCA^{LGR5+}$ and unsorted pCCA cells were observed. No $DCLK1^+$ observed in unsorted healthy pBD cells. B) Cytoplasmic localization of DCLK1 (red) in $iCCA^{CD133+}$ and in unsorted iCCA, nuclei are stained with DAPI (blue). No difference between $iCCA^{CD133+}$ and in unsorted iCCA cells were observed. $DCLK1^+$ cells were not observed in unsorted healthy iBD cells. C) Cytoplasmic localization of DCLK1 (red) in unsorted HT29 (positive control), nuclei are stained with DAPI (blue).

Figure 3. DCLK1 inhibition decrease cell viability and enhancer apoptosis of subpopulation of pCCA- and iCCA-CSCs primary cell culture.

Viability and apoptosis analysis by MTS assay and flow cytofluorimetric assay respectively after treatment with LRRK2-IN-1, a specific DCLK1 inhibitor.

A) Curve dose-response of pCCA (up) and pCCA^{LGR5+}(down) cells with 0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20μM of LRRK2-IN-1 concentrations. Data expressed as mean±SD of N=6 experiments. The IC_{50} was indicated as a bold black point.

B) Curve dose-response of iCCA (up) and iCCA^{CD133+}(down) cells with 0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20μM LRRK2-IN-1 concentrations. Data expressed as mean±SD of N=6 experiments. The IC_{50} was indicated as a bold black point.

C) Curve dose-response of HT29 (up) and HT29 $^{LGR5+}$(down) cells with 0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20μM LRRK2-IN-1 concentrations. Data expressed as mean±SD of N=6 experiments. The IC_{50} was indicated as a bold black point.

D) Percentage of apoptotic unsorted pCCA and pCCA^{LGR5+} after treatment with LRRK2-IN-1 at the concentration of own IC_{50} or without treatment (controls). The dot plots below display the representative experiments. Data expressed as mean±SD of N=6 experiments; *p<0.05.

E) Percentage of apoptotic unsorted iCCA and iCCA^{CD133+} after treatment with LRRK2-IN-1 at the concentration of own IC_{50} or without treatment (controls). The dot plots below display the representative experiments. Data expressed as mean±SD of N=6 experiments; *p<0.05.

F) Percentage of apoptotic unsorted HT29 and HT29^{LGR5+} after treatment with LRRK2-IN-1 at the concentration of own IC_{50} or without treatment (controls). The dot plots below display the representative experiments. Data expressed as mean±SD of N=6 experiments; *p<0.05.

Figure 4. DCLK1 inhibition exerts an effect on proliferation and colony formation capacity of subpopulation of pCCA- and iCCA-CSCs primary cell culture.

Proliferation rate of A) unsorted pCCA and pCCA^{LGR5+} B) unsorted iCCA and iCCA^{CD133+} C) unsorted HT29 and HT29^{LGR5+} were analysed after 3 days of treatment with LRRK2-IN-1 at the concentration of own IC_{50} or without treatment (control) by PD. The primary cell culture treated with LRRK2-IN-1 showed a significant decrease of the PD compare to untreated cells. The resulting was expressed as mean±SD of N=6 experiments; §p<0.01 vs untreated control. D) Number of colonies of unsorted pCCA, pCCA^{LGR5+}, unsorted iCCA, iCCA^{CD133+}, unsorted HT29 and HT29^{LGR5+} after 10 days of treatment with LRRK2-IN-1 at the concentration of own IC_{50} or without treatment (controls). The primary cell culture treated with LRRK2-IN-1 showed a significant decrease in the number of colonies compared to untreated cells. Data expressed as mean±SD of N=6 experiments; §p<0.01. E) Colony size expressed as Colony Dimension Index of unsorted pCCA, pCCA^{LGR5+}, unsorted iCCA, iCCA^{CD133+}, unsorted HT29 and HT29^{LGR5+} after 10 days of treatment with LRRK2-IN-1 at the concentration of own IC_{50} or without treatment (controls). The primary cell culture treated with LRRK2-IN-1 show a significant decrease in the Colony Dimension Index

compared to untreated cells ($^{\S}p<0.01$ vs untreated control). Data expressed as mean±SD of N=6 experiments; $^{\S}p<0.01$.

Figure 5. *In situ* expression of DCLK1 and cancer stem cell markers in cholangiocarcinoma (CCA) samples

A) Haematoxylin and eosin (H&E) staining (upper panels) and immunohistochemistry for DCLK1 (lower panels) in perihilar CCA (pCCA) and intrahepatic CCA (iCCA). H&E staining shows histological heterogeneity between the two tumour subtypes. Immunohistochemistry for DCLK1 shows that both tumour subtypes express DCLK1. Original magnification: 20x. Areas in the circles are magnified below.

B) The graph reports mean and standard deviation (SD) of the semiquantitative (SQ) score for DCLK1 expression in pCCA and iCCA compared to the expression in cholangiocytes lining the hepatic ducts (HepD) and interlobular bile ducts (IBD), respectively ($p<0.05$ vs. normal samples). See also Fig.S2.

C) Double immunofluorescence for Lgr5/DCLK1 (upper panels) in pCCA, and for EpCAM/DCLK1 (middle panels) and CD133/DCLK1 (lower panels) in iCCA. Immunofluorescence staining shows how DCLK1+ cells (in red) represent a subpopulation of CCA cells expressing cancer stem cell (CSC) markers (i.e. Lgr5, EpCAM, and CD133, in green). Separate channels are also provided. Nuclei are displayed in blue. Original magnification: 20x.

D) The heat map shows the percentage of CSC+ cells co-expressing DCLK1 in pCCA and iCCA.

Figure 6. Putative role of DCLK1 as a diagnostic biomarker

A) DCLK1-relative mRNA levels in total extracts of human tumours are statistically higher compared to healthy human tissue from perihilar biliary ducts (healthy pBDs) and small biliary ducts (healthy sBDs), which were the healthy controls of pCCA and iCCA respectively. Data is expressed as mean±SD of N=6 experiments; $^{¤}p<0.001$ vs. healthy pBDs, $^{\S}p<0.01$ vs. healthy sBDs.

B) DCLK1 serum concentrations in pCCA, iCCA, cirrhotic, HCC, and PSC patients analysed by ELISA. DCLK1 was not detected in healthy controls and its average concentration increased in the serum of CCA patients ($p<0.01$ vs. healthy samples). Data expressed as mean±SD of N=5 experiments.

Reference

1. Banales JM, Cardinale V, Carpino G, Marzioni M, Andersen JB, Invernizzi P, Lind GE, et al. Expert consensus document: Cholangiocarcinoma: current knowledge and future perspectives consensus statement from the European Network for the Study of Cholangiocarcinoma (ENS-CCA). Nat Rev Gastroenterol Hepatol 2016;13:261-280.

2. Carnevale G, Carpino G, Cardinale V, Pisciotta A, Riccio M, Bertoni L, Gibellini L, et al. Activation of Fas/FasL pathway and the role of c-FLIP in primary culture of human cholangiocarcinoma cells. Sci Rep 2017;7:14419.

3. Komuta M, Govaere O, Vandecaveye V, Akiba J, Van Steenbergen W, Verslype C, Laleman W, et al. Histological diversity in cholangiocellular carcinoma reflects the different cholangiocyte phenotypes. Hepatology 2012;55:1876-1888.

4. Patel T. Cholangiocarcinoma--controversies and challenges. Nat Rev Gastroenterol Hepatol 2011;8:189-200.

5. Bragazzi MC, Ridola L, Safarikia S, Matteo SD, Costantini D, Nevi L, Cardinale V. New insights into cholangiocarcinoma: multiple stems and related cell lineages of origin. Ann Gastroenterol 2018;31:42-55.

6. Aishima S, Oda Y. Pathogenesis and classification of intrahepatic cholangiocarcinoma: different characters of perihilar large duct type versus peripheral small duct type. J Hepatobiliary Pancreat Sci 2015;22:94-100.

7. Nakanuma Y, Sato Y, Harada K, Sasaki M, Xu J, Ikeda H. Pathological classification of intrahepatic cholangiocarcinoma based on a new concept. World J Hepatol 2010;2:419-427.

8. Liau JY, Tsai JH, Yuan RH, Chang CN, Lee HJ, Jeng YM. Morphological subclassification of intrahepatic cholangiocarcinoma: etiological, clinicopathological, and molecular features. Mod Pathol 2014;27:1163-1173.

9. Cardinale V, Renzi A, Carpino G, Torrice A, Bragazzi MC, Giuliante F, DeRose AM, et al. Profiles of cancer stem cell subpopulations in cholangiocarcinomas. Am J Pathol 2015;185:1724-1739.

10. Yamashita T, Wang XW. Cancer stem cells in the development of liver cancer. J Clin Invest 2013;123:1911-1918.

11. Ali N, Chandrakesan P, Nguyen CB, Husain S, Gillaspy AF, Huycke M, Berry WL, et al. Inflammatory and oncogenic roles of a tumor stem cell marker doublecortin-like kinase (DCLK1) in virus-induced chronic liver diseases. Oncotarget 2015;6:20327-20344.

12. Chandrakesan P, Yao J, Qu D, May R, Weygant N, Ge Y, Ali N, et al. Dclk1, a tumor stem cell marker, regulates pro-survival signaling and self-renewal of intestinal tumor cells. Mol Cancer 2017;16:30.

13. Westphalen CB, Takemoto Y, Tanaka T, Macchini M, Jiang Z, Renz BW, Chen X, et al. Dclk1 Defines Quiescent Pancreatic Progenitors that Promote Injury-Induced Regeneration and Tumorigenesis. Cell Stem Cell 2016;18:441-455.

14. Nakanishi Y, Seno H, Fukuoka A, Ueo T, Yamaga Y, Maruno T, Nakanishi N, et al. Dclk1 distinguishes between tumor and normal stem cells in the intestine. Nat Genet 2013;45:98-103.

15. Westphalen CB, Asfaha S, Hayakawa Y, Takemoto Y, Lukin DJ, Nuber AH, Brandtner A, et al. Long-lived intestinal tuft cells serve as colon cancer-initiating cells. J Clin Invest 2014;124:1283-1295.

16. Whorton J, Sureban SM, May R, Qu D, Lightfoot SA, Madhoun M, Johnson M, et al. DCLK1 is detectable in plasma of patients with Barrett's esophagus and esophageal adenocarcinoma. Dig Dis Sci 2015;60:509-513.

17. Bailey JM, Alsina J, Rasheed ZA, McAllister FM, Fu YY, Plentz R, Zhang H, et al. DCLK1 marks a morphologically distinct subpopulation of cells with stem cell properties in preinvasive pancreatic cancer. Gastroenterology 2014;146:245-256.

18. Ito H, Tanaka S, Akiyama Y, Shimada S, Adikrisna R, Matsumura S, Aihara A, et al. Dominant Expression of DCLK1 in Human Pancreatic Cancer Stem Cells Accelerates Tumor Invasion and Metastasis. PLoS One 2016;11:e0146564.

19. Di Matteo S, Nevi L, Costantini D, Overi D, Carpino G, Safarikia S, Giulitti F, et al. The FXR agonist obeticholic acid inhibits the cancerogenic potential of human cholangiocarcinoma. PLoS One 2019;14:e0210077.

20. Nevi L, Carpino G, Costantini D, Cardinale V, Riccioni O, Di Matteo S, Melandro F, et al. Hyaluronan coating improves liver engraftment of transplanted human biliary tree stem/progenitor cells. Stem Cell Res Ther 2017;8:68.

21. Nevi L, Cardinale V, Carpino G, Costantini D, Di Matteo S, Cantafora A, Melandro F, et al. Cryopreservation protocol for human biliary tree stem/progenitors, hepatic and pancreatic precursors. Sci Rep 2017;7:6080.

22. Cardinale V, Puca R, Carpino G, Scafetta G, Renzi A, De Canio M, Sicilia F, et al. Adult Human Biliary Tree Stem Cells Differentiate to beta-Pancreatic Islet Cells by Treatment with a Recombinant Human Pdx1 Peptide. PLoS One 2015;10:e0134677.

23. Kuribara M, Jenks BG, Dijkmans TF, de Gouw D, Ouwens DT, Roubos EW, Vreugdenhil E, et al. ERK-regulated double cortin-like kinase (DCLK)-short phosphorylation and nuclear translocation stimulate POMC gene expression in endocrine melanotrope cells. Endocrinology 2011;152:2321-2329.

24. Gagliardi G, Bellows CF. DCLK1 expression in gastrointestinal stem cells and neoplasia. journal of Cancer Therapeutics and Research 2012;1.

25. Kennedy L, Hargrove L, Demieville J, Francis N, Seils R, Villamaria S, Francis H. Recent Advances in Understanding Cholangiocarcinoma. F1000Res 2017;6:1818.

26. Fraveto A, Cardinale V, Bragazzi MC, Giuliante F, De Rose AM, Grazi GL, Napoletano C, et al. Sensitivity of Human Intrahepatic Cholangiocarcinoma Subtypes to Chemotherapeutics and Molecular Targeted Agents: A Study on Primary Cell Cultures. PLoS One 2015;10:e0142124.

27. Mirzaei A, Madjd Z, Kadijani AA, Tavakoli-Yaraki M, Modarresi MH, Verdi J, Akbari A, et al. Evaluation of circulating cellular DCLK1 protein, as the most promising colorectal cancer stem cell marker, using immunoassay based methods. Cancer Biomark 2016;17:301-311.

28. Weygant N, Qu D, Berry WL, May R, Chandrakesan P, Owen DB, Sureban SM, et al. Small molecule kinase inhibitor LRRK2-IN-1 demonstrates potent activity against colorectal and pancreatic cancer through inhibition of doublecortin-like kinase 1. Mol Cancer 2014;13:103.

29. Westphalen CB, Quante M, Wang TC. Functional implication of Dclk1 and Dclk1-expressing cells in cancer. Small GTPases 2017;8:164-171.

30. Dai T, Hu Y, Lv F, Ozawa T, Sun X, Huang J, Han X, et al. Analysis of the clinical significance of DCLK1(+) colorectal cancer using novel monoclonal antibodies against DCLK1. Onco Targets Ther 2018;11:5047-5057.

31. Weygant N, Qu D, May R, Tierney RM, Berry WL, Zhao L, Agarwal S, et al. DCLK1 is a broadly dysregulated target against epithelial-mesenchymal transition, focal adhesion, and stemness in clear cell renal carcinoma. Oncotarget 2015;6:2193-2205.

32. Sureban SM, May R, Qu D, Weygant N, Chandrakesan P, Ali N, Lightfoot SA, et al. DCLK1 regulates pluripotency and angiogenic factors via microRNA-dependent mechanisms in pancreatic cancer. PLoS One 2013;8:e73940.

33. Wang W, Zhang H, Wang L, Zhang S, Tang M. miR-613 inhibits the growth and invasiveness of human hepatocellular carcinoma via targeting DCLK1. Biochem Biophys Res Commun 2016;473:987-992.

34. Li L, Jones K, Mei H. Doublecotin-Like Kinase 1 Increases Chemoresistance of Colorectal Cancer Cells through the Anti-Apoptosis Pathway. J Stem Cell Res Ther 2019;9.

35. Liu ZQ, He WF, Wu YJ, Zhao SL, Wang L, Ouyang YY, Tang SY. LncRNA SNHG1 promotes EMT process in gastric cancer cells through regulation of the miR-15b/DCLK1/Notch1 axis. BMC Gastroenterol 2020;20:156.

36. Yan R, Li J, Zhou Y, Yao L, Sun R, Xu Y, Ge Y, et al. Inhibition of DCLK1 down-regulates PD-L1 expression through Hippo pathway in human pancreatic cancer. Life Sci 2020;241:117150.

37. Makino S, Takahashi H, Okuzaki D, Miyoshi N, Haraguchi N, Hata T, Matsuda C, et al. DCLK1 integrates induction of TRIB3, EMT, drug resistance and poor prognosis in colorectal cancer. Carcinogenesis 2020;41:303-312.

38. Liu W, Wang S, Sun Q, Yang Z, Liu M, Tang H. DCLK1 promotes epithelial-mesenchymal transition via the PI3K/Akt/NF-kappaB pathway in colorectal cancer. Int J Cancer 2018;142:2068-2079.

39. Sureban SM, Madhoun MF, May R, Qu D, Ali N, Fazili J, Weygant N, et al. Plasma DCLK1 is a marker of hepatocellular carcinoma (HCC): Targeting DCLK1 prevents HCC tumor xenograft growth via a microRNA-dependent mechanism. Oncotarget 2015;6:37200-37215.

40. Gao T, Wang M, Xu L, Wen T, Liu J, An G. DCLK1 is up-regulated and associated with metastasis and prognosis in colorectal cancer. J Cancer Res Clin Oncol 2016;142:2131-2140.

41. Harada Y, Kazama S, Morikawa T, Emoto S, Murono K, Kaneko M, Sasaki K, et al. Prognostic impact of doublecortin-like kinase 1 expression in locally advanced rectal cancer treated with preoperative chemoradiotherapy. APMIS 2018;126:486-493.

42. Delgiorno KE, Hall JC, Takeuchi KK, Pan FC, Halbrook CJ, Washington MK, Olive KP, et al. Identification and manipulation of biliary metaplasia in pancreatic tumors. Gastroenterology 2014;146:233-244 e235.

43. Andresen K, Boberg KM, Vedeld HM, Honne H, Hektoen M, Wadsworth CA, Clausen OP, et al. Novel target genes and a valid biomarker panel identified for cholangiocarcinoma. Epigenetics 2012;7:1249-1257.

44. Zaccari P, Cardinale V, Severi C, Pedica F, Carpino G, Gaudio E, Doglioni C, et al. Common features between neoplastic and preneoplastic lesions of the biliary tract and the pancreas. World J Gastroenterol 2019;25:4343-4359.

45. Andresen K, Boberg KM, Vedeld HM, Honne H, Jebsen P, Hektoen M, Wadsworth CA, et al. Four DNA methylation biomarkers in biliary brush samples accurately identify the presence of cholangiocarcinoma. Hepatology 2015;61:1651-1659.

Figure. 1

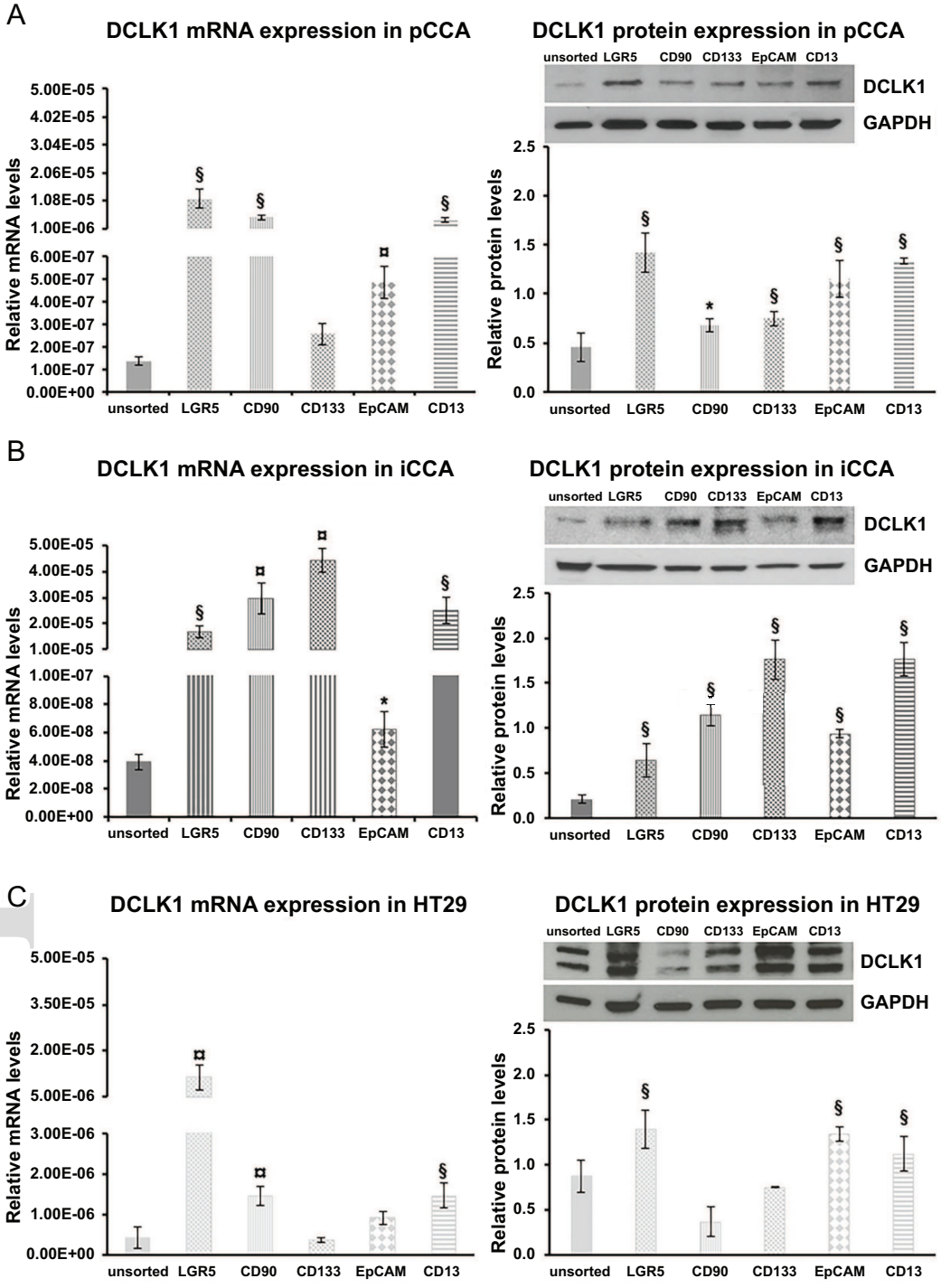
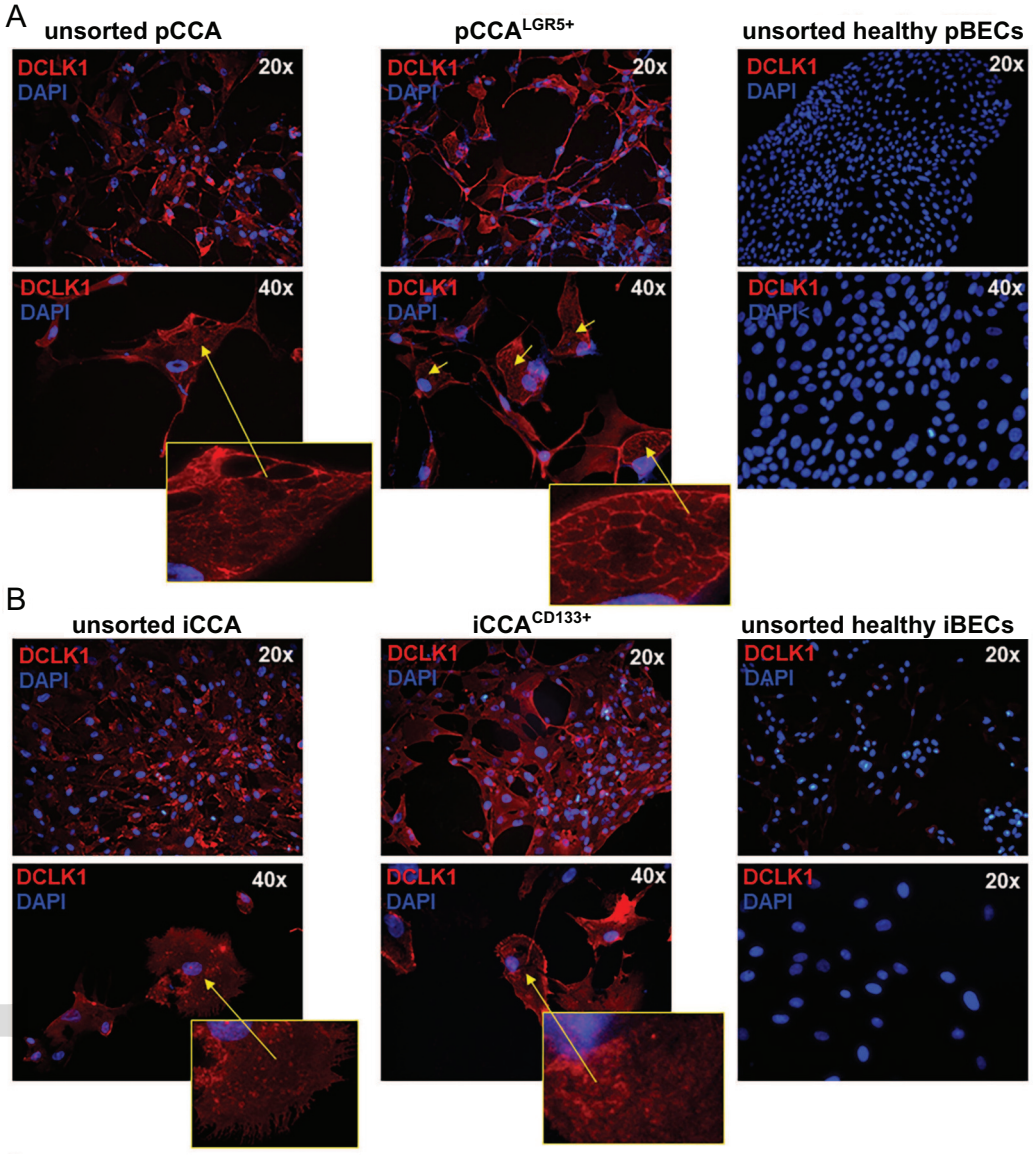


Figure. 2



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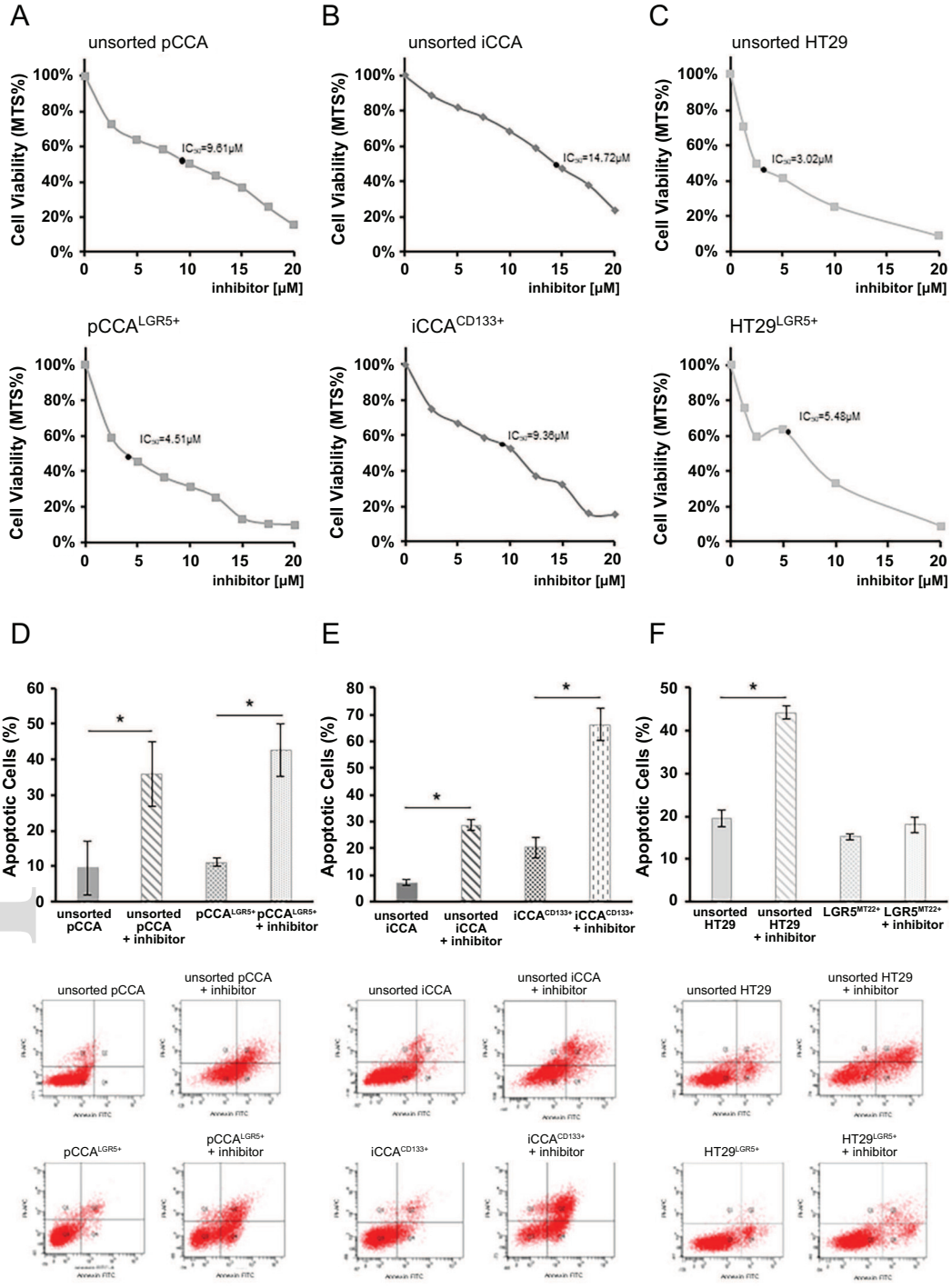

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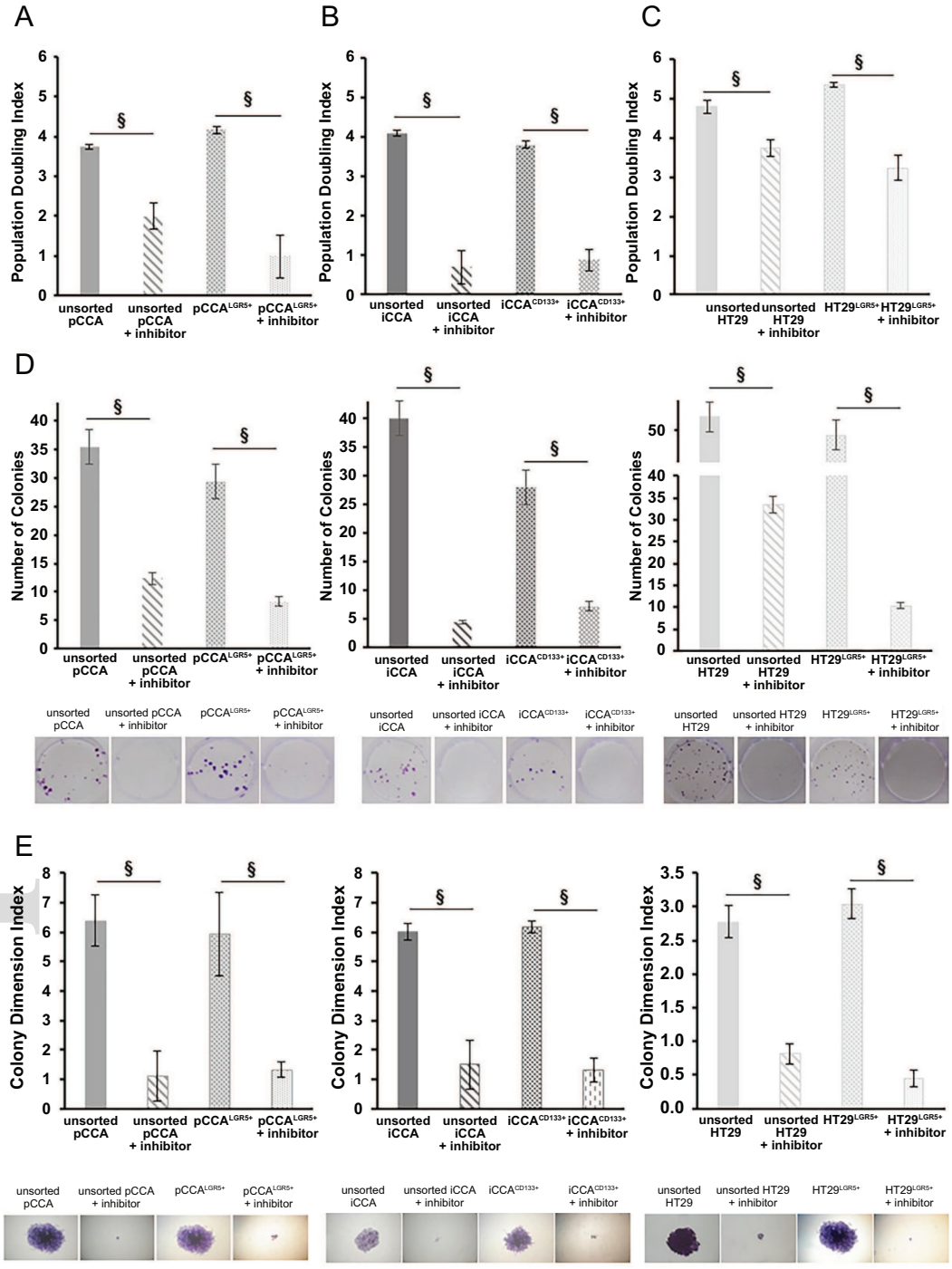

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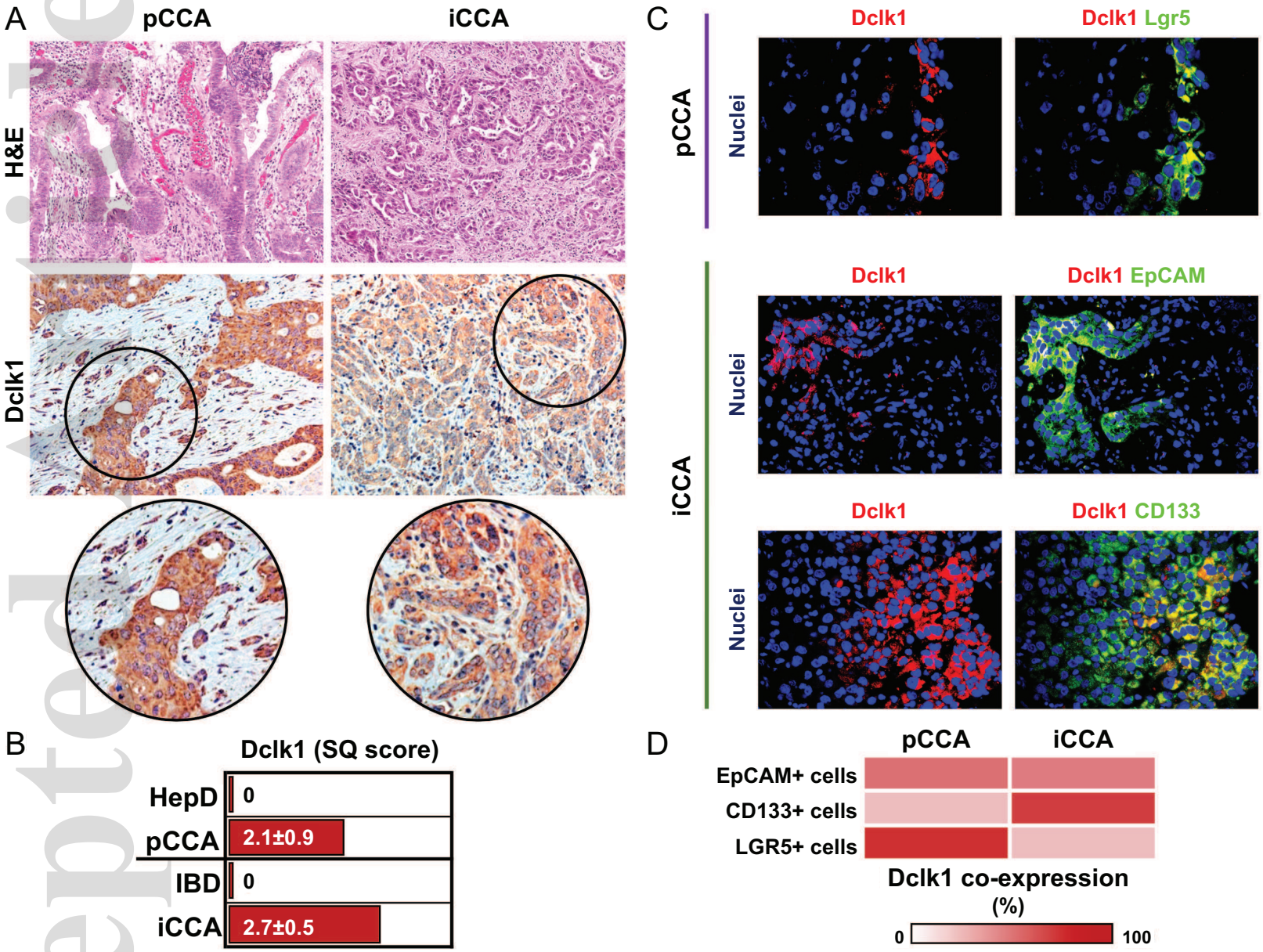

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Figure. 6

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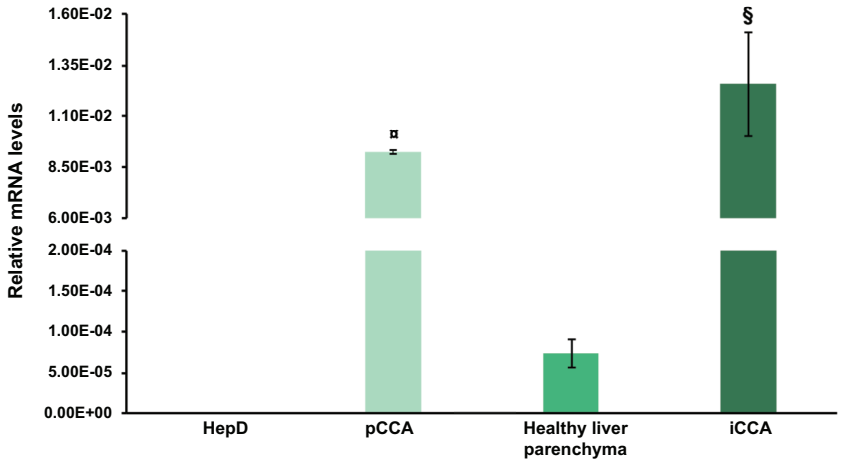

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