DCLK1, a Putative Stem Cell Marker in Human Cholangiocarcinoma

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

APPROACH AND RESULTS: Specimens surgically resected of human CCA were enzymatically digested, submitted to immunosorting for specific CSC markers (LGR5 [leucine-rich repeat-containing G protein-coupled receptor], CD [clusters of differentiation] 90, EpCAM [epithelial cell adhesion molecule], CD133, and CD13), and primary cell cultures were prepared. DCLK1 expression was analyzed in CCA cell cultures by real-time quantitative PCR, western blot, and immunofluorescence. Functional studies have been performed by evaluating the effects of selective DCLK1 inhibitor (LRRK2-IN-1) on cell proliferation (MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] assay, cell population doubling time), apoptosis, and colony formation capacity. DCLK1 was investigated *in situ* by immunohistochemistry and real-time quantitative PCR. DCLK1 serum concentration was analyzed by enzyme-linked immunosorbent assay. We describe DCLK1 in CCA with an increased gene and protein DCLK1 expression in pCCA^{LGR5+} and in iCCA^{CD133+} cells compared with unsorted cells. LRRK2-IN-1 showed an anti-proliferative effect in a dose-dependent manner. LRRK2-IN-1 markedly impaired cell proliferation, induced apoptosis, and decreased colony formation capacity and colony size in both iCCA and pCCA compared with the untreated cells. *In situ* analysis confirmed that DCLK1 is present only in tumors, and not in healthy tissue. Interestingly, DCLK1 was detected in the human serum samples of patients with iCCA (high), pCCA (high), HCC (low), and cirrhosis (low), but it was almost undetectable in healthy controls.

CONCLUSIONS: 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. (HEPATOLOGY 2021;73:144-159).

Cholangiocarcinoma (CCA) is a heterogeneous group of neoplasms of the bile ducts.[1] CCA is a tumor that has an increasing incidence

throughout the world, showing high mortality due to its aggressiveness, late diagnosis, metastasization, 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 a 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 tumor 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 catalyzes their polymerization.[11] DCLK1 has been found in CSCs of gastrointestinal tract tumors such as colon,[12] pancreas,[13] and hepatocarcinoma.[11] Despite these discoveries, the role of DCLK1 in the tumors and its interaction with other proteins is largely unknown.

Nakanishi et al.[14] exploited the lineage tracking method, showing that DCLK1 can mark tumor stem cells in the intestine. In addition, Westphalen et al.[15] described the relationship between DCLK1+ cells and the beginning of colon cancer. Whorton et al.[16]

also showed that DCLK1 regulates pluripotency and angiogenesis in pancreatic cancer through a microRNA-dependent mechanism.

According to recent papers by Bailey et al.,[17] pancreatic neoplasms expressing DCLK1 contain morphologically and functionally distinct subpopulations such as 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 tumor is unknown. In this study we investigated the DCLK1 expression in pCCA and iCCA subpopulations (EpCAM+ [epithelial cell adhesion molecule], LGR5+ [leucine-rich repeat-containing G protein-coupled receptor 5], CD [clusters of differentiation] 90+, CD13+, and CD133+ tumor 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,

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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 Hepatobiliary and Surgery Unit, 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 the hilum region were used as the physiologic control (HepD) of pCCA tissues, whereas peripheral intrahepatic bile ducts from liver parenchyma were used as the 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 the hilum region, perihilar (pBECs), were used as the 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 fetuses (16-22-week gestational age), which were obtained by elective pregnancy termination from the Department of Gynecology (Sapienza, University of Rome, Italy). Informed consent was acquired from the mother before abortion. The pBECs and iBEC primary cells were cultured in Kubota's Medium as described previously by our laboratory.[20-22]

ETHICAL APPROVAL

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

IMMORTALIZED CELL CULTURES

The HT29 human cell line is a commercial cell line that is isolated from Caucasian colon adenocarcinoma grade II. It was used as the internal control for each experiment and cultured in H69 medium. The H69 cell line, a cholangiocyte cell line, is a commercial immortal nonmalignant cell line that was isolated from BEC and cultured in H69 medium.

Other materials and methods are described in the Supporting Information.

Results

EXPRESSION *IN VITRO*

Subpopulations of CSCs were immune-sorted from pCCA or iCCA and investigated by real-time quantitative PCR and western blot (WB) to analyze the presence of DCLK1.

In the pCCA, the DCLK1 expression was evaluated by real-time quantitative PCR. The highest DCLK1 gene expression was found in the pCCA^{LGR5+} subpopulation ($1.15*10^{-5} \pm 3.23*10^{-6}$; n = 6; $P < 0.01$), whose mRNA levels were significantly higher than DCLK1 mRNA levels in unsorted pCCA cells ($1.35*10^{-7} \pm 1.76*10^{-8}$) (Fig. 1A). Moreover, other subpopulations showed significantly enhanced DCLK1 gene expression such as pCCA^{CD90+} ($4.99*10^{-6} \pm 9.08*10^{-7}$; n = 6; $P < 0.01$), pCCA^{EpCAM+} ($4.87*10^{-7} \pm 7.09*10^{-8}$; n = 6; $P < 0.001$), and pCCA^{CD13+} ($3.98*10^{-6} \pm 9.23*10^{-7}$; n = 6; $P < 0.01$) with respect to unsorted pCCA cells (Fig. 1A). Instead, the pCCA^{CD133+} subpopulation did not show significantly different DCLK1 mRNA levels as compared with unsorted pCCA (n = 6). Similar results were observed in the protein analysis by WB. In all populations, a 82-kDa band was observed, corresponding to the most studied isoform from previous studies.[23] Figure 1A shows the expression of DCLK1 protein in the pCCA subpopulation compared with 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; $P < 0.01$;), and pCCA^{CD13+} (1.3 ± 0.03; n = 6; $P < 0.01$) expressed
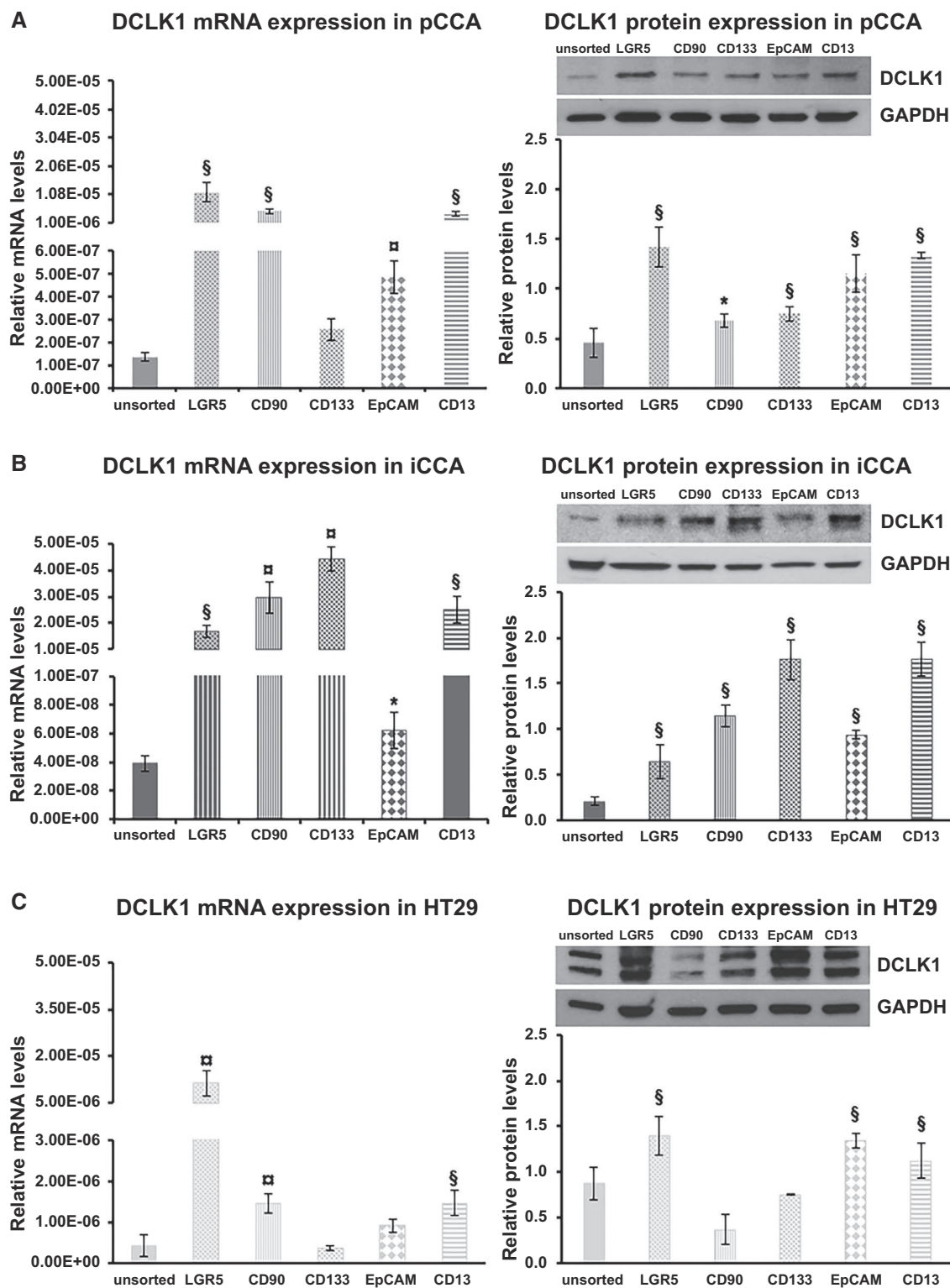
FIG. 1. DCLK1 expression in the subpopulation of pCCA-CSC and iCCA-CSC primary cell culture. Relative DCLK1 mRNA level expression analysis by real-time quantitative PCR (left) and DCLK1 relative protein-expression analysis by WB (right) of pCCA (A), iCCA (B), and HT29 (C) whole and subpopulation. Data expressed as mean ± SD of n = 6 experiments (*$P < 0.05$ versus unsorted cells; §$P < 0.01$ versus unsorted cells; ¤$P < 0.001$ versus unsorted cells). Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

higher protein levels compared with the unsorted control (0.45 ± 0.14; n = 6). In pCCA, the higher genetic and protein expression of DCLK1 results in a LGR5$^+$ subpopulation.

Notably, pBECs used as physiologic control of pCCA did not express DCLK1 by real-time quantitative PCR and WB analysis (data not shown).

In the iCCA, the DCLK1 expression in all sorted subpopulations (LGR5$^+$ [$1.69*10^{-5} \pm 2.22*10^{-6}$; n = 6; $P < 0.01$], CD90$^+$ [$2.95*10^{-5} \pm 6.03*10^{-6}$; n = 6; $P < 0.001$], CD133$^+$ [$4.44*10^{-5} \pm 4.61*10^{-6}$; n = 6; $P < 0.001$], EpCAM$^+$ [$6.24*10^{-8} \pm 1.25*10^{-8}$; n = 6; $P < 0.05$], or CD13$^+$ [$2.51*10^{-5} \pm 5.06*10^{-6}$; n = 6; $P < 0.01$]) was significantly higher than unsorted iCCA cells ($3.93*10^{-08} \pm 5.46*10^{-09}$; 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 with the unsorted iCCA (0.2 ± 0.05; n = 6) (Fig. 1B). In iCCA, the higher genetic and protein expression of DCLK1 results in CD133$^+$ and CD13$^+$ subpopulations. Interestingly, iBECs used as the physiologic control of iCCA did not express DCLK1 by real-time quantitative PCR and WB analysis (data not shown).

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

The DCLK1 protein expression was higher in the 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; $P < 0.05$) subpopulations compared with the unsorted HT29 population (0.87 ± 0.17; n = 6) (Fig. 1C). Instead, the HT29^{90+} and HT29^{133+} tumor cell subset did not show a significant difference with respect to the unsorted HT29 cells.

LOCALIZATION OF DCLK1 IN PRIMARY CCA CELL CULTURES

Next, we investigated the localization of DCLK1 protein in the unsorted population and in pCCA^{LGR5+}, iCCA^{CD133+}, or HT29^{LGR5+} sorted tumor cells, which have shown the highest DCLK1 mRNA and protein expression.

DCLK1 was localized in the pCCA and iCCA primary cell line by immunofluorescence (Fig. 2). DCLK1 was observed in cytoplasmatic localization in all unsorted and sorted analyzed populations (Fig. 2), as previously shown in other tumors.[12,24] DCLK1 was not detected in iBECs and in pBECs (Fig. 2), confirming the gene expression and WB results that we previously presented.

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

EFFECTS OF DCLK1 INHIBITION ON VIABILITY OF 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 (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) 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 72 hours.

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

The HT29^{LGR5+} subpopulation showed a higher IC$_{50}$ (5.48 μM; n = 4) with respect to unsorted HT29 (3.02 μM; n = 4) (Fig. 3C). Furthermore, the viability of iBECs and pBECs was not influenced by LRRK2-IN-1 treatment (Supporting Fig. S1).
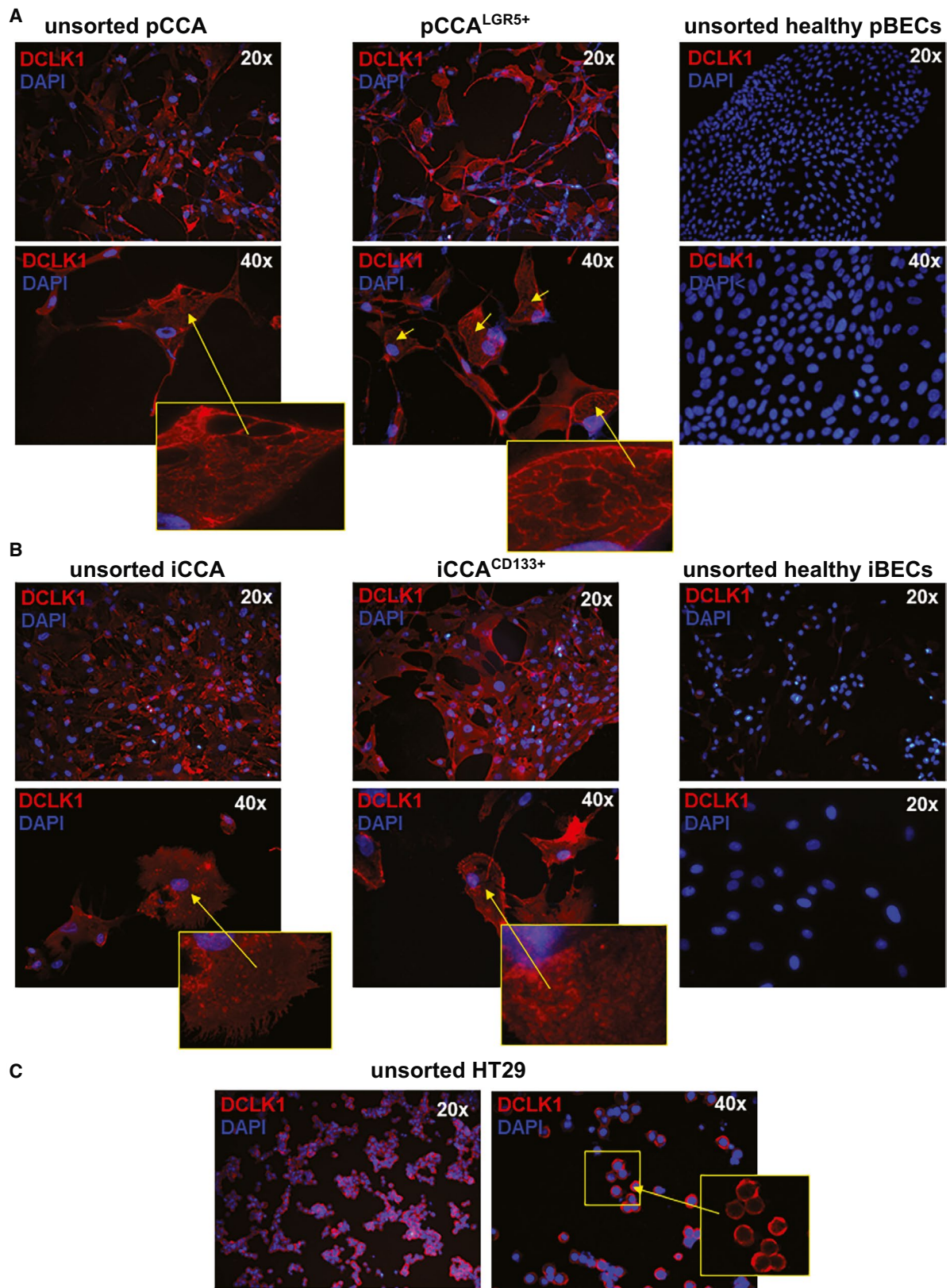
FIG. 2. DCLK1 localization in subpopulation of pCCA-CSC and iCCA-CSC primary cell culture. (A) Cytoplasmic localization of DCLK1 (red) in LGR5⁺ sorted and unsorted pCCA; nuclei are stained with DAPI (blue). No difference between pCCA^LGR5+ and unsorted pCCA cells were observed. No DCLK1⁺ observed in unsorted healthy perihilar biliary duct (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 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). Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

Supernatant from CCA cultures was collected and used to culture the H69 nonmalignant cholangiocyte cell line. MTS assay showed that all conditioned mediums increased the H69 proliferation compared with control. H69 grown in conditioned medium from CCA cell culture with LRRK2-IN-1 for 72 hours at the 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 (Supporting 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, Annexin V fluorescein isothiocyanate/propidium-iodide double-labeling flow cytometry was carried out.

We treated the pCCA and iCCA cells with LRRK2-IN-1 for 72 hours at the 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 with the respective untreated control cells (Fig. 3D).

Moreover, LRRK2-IN-1 increased apoptosis in unsorted iCCA when compared with 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 with 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 with 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

To analyze the population doubling (PD) index, we treated the pCCA and iCCA cells with LRRK2-IN-1 for 72 hours at the concentrations of IC_{50} for each subpopulation.

The PD index of the unsorted pCCA cells treated with LRRK2-IN-1 was significantly reduced (1.99 ± 0.32; n = 6; $P < 0.01$) compared with untreated pCCA cells (3.74 ± 0.07; n = 6) (Fig. 4A). Similarly, pCCA^LGR5+ cells treated with LRRK2-IN-1 underwent a reduction of proliferation rate (0.99 ± 0.54; n = 6; $P < 0.01$) when compared with pCCA^LGR5+ untreated cells, which showed a stable growth over time (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 with untreated cells, which exhibited a stable growth over time (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 reduced compared with untreated iCCA^CD133+ cells (3.80 ± 0.10; n = 6) (Fig. 4B).

In HT29 cells treated with inhibitor, the PD rate was reduced (HT29 unsorted: 3.74 ± 0.20; and HT29^LGR5+: 3.22 ± 0.32; n = 6; $P < 0.01$) compared with 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 was analyzed 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 with controls cells ($P < 0.05$) (Fig. 4D). Moreover, we observed a trend toward a decrease in the size of colonies, measured by a colony dimension index in pCCA, iCCA, and HT29 cultures, treated with LRRK2-IN-1 compared with untreated controls, respectively ($P < 0.05$) (Fig. 4E).
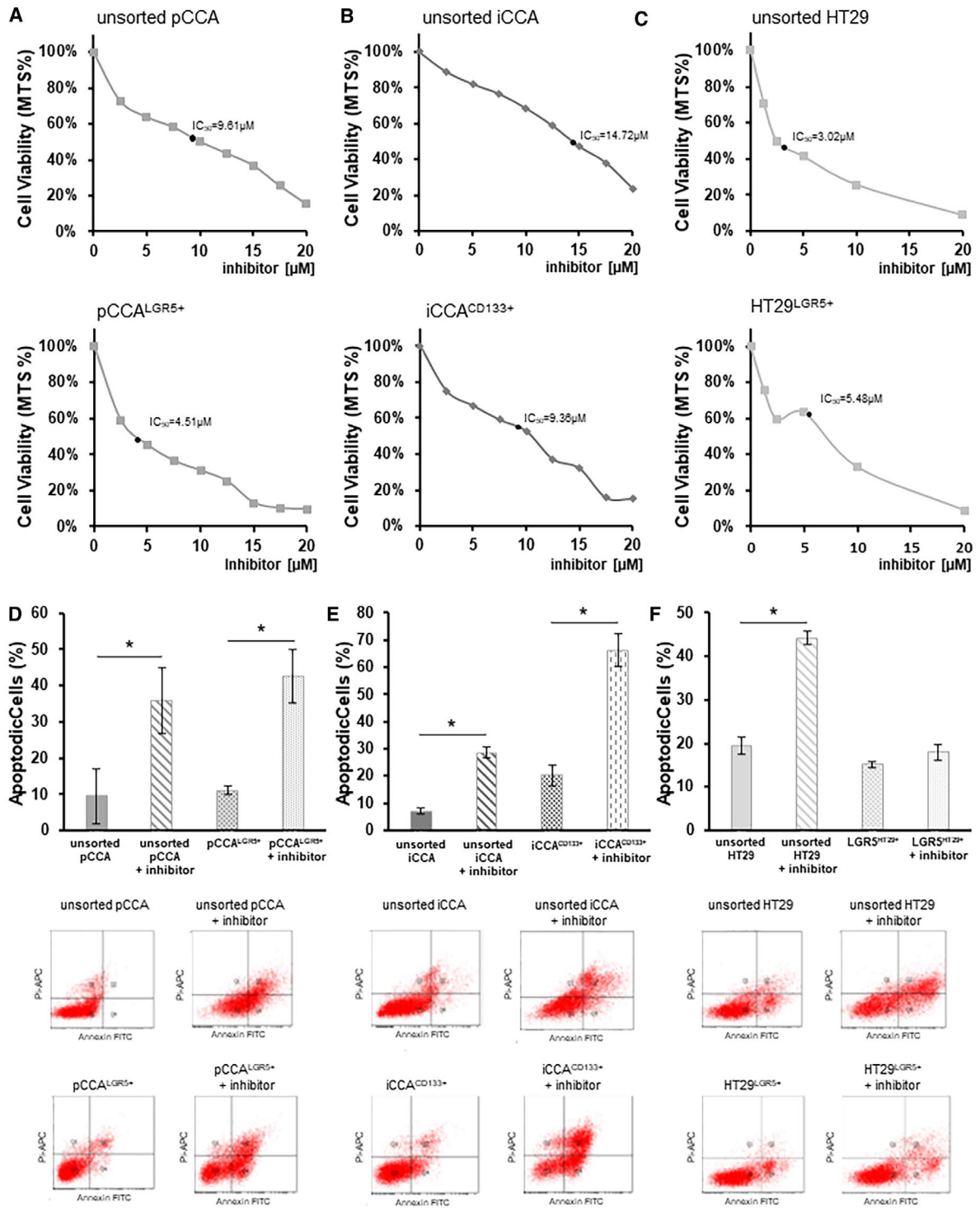
FIG. 3. DCLK1 inhibition decreases cell viability and enhances apoptosis of the subpopulation of pCCA-CSC and iCCA-CSC primary cell culture. Viability and apoptosis analysis by MTS assay and flow cytofluorimetric assay, respectively, after treatment with LRRK2-IN-1, a specific DCLK1 inhibitor. (A) Curve dose-response of pCCA (up) and pCCA^{LGR5+}(down) cells with 0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, and 20 μM of LRRK2-IN-1 concentrations. Data are expressed as mean ± SD of n = 6 experiments. The IC$_{50}$ is indicated by a bold black dot. (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, and 20 μM LRRK2-IN-1 concentrations. Data are expressed as mean ± SD of n = 6 experiments. The IC$_{50}$ is indicated as a bold black dot. (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, and 20 μM LRRK2-IN-1 concentrations. Data are expressed as mean ± SD of n = 6 experiments. The IC$_{50}$ is indicated by a bold black dot. (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 are 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 are 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 are expressed as mean ± SD of n = 6 experiments; *P < 0.05.

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 [IBDs]) and extrahepatic bile ducts (hepatic ducts at the hilum) were mostly negative (less than 5% of epithelial cells) for DCLK1 in healthy organs (Supporting Fig. S2). In the hepatic duct, peribiliary glands were also present and were mostly DCLK1-negative. In CCA samples, DCLK1 was expressed by tumor 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 tumor subtypes (Fig. 5A,B).

We then used double immunofluorescence to evaluate whether CSC subpopulations co-expressed DCLK1 in the same cell (Fig. 5C,D). 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 real-time quantitative PCR (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 with healthy hepatic bile ducts at hilum region (HepD) ($2.26*10^{-8} ± 9.65*10^{-9}$; n = 6) and healthy liver parenchyma ($7.39*10^{-5} ± 1.68*10^{-5}$; n = 6), respectively (Fig. 6B).

DCLK1 AS PUTATIVE DIAGNOSTIC SERUM BIOMARKER

Furthermore, we evaluated the DCLK1 concentration in human serum samples of patients with pCCA or iCCA, in patients with HCC, cirrhosis, and primary sclerosing cholangitis (PSC), and in healthy subjects (Supporting Table S1). Higher DCLK1 levels were detected both in pCCA (4.35 ± 1.76 ng/mL; n = 10; *P* < 0.01 vs. patients with cirrhosis, HCC, and PSC) and iCCA patients (3.21 ± 1.98 ng/mL; n = 10; *P* < 0.01 vs. patients with cirrhosis and PSC; *P* < 0.05 vs. patients with HCC) when compared with patients with cirrhosis (0.90 ± 0.39 ng/mL; n = 10), HCC (1.20 ± 0.39 ng/mL; n = 10), and PSC (0.96 ± 0.23 ng/mL; n = 10). The analysis of the variance confirmed that DCLK1 concentration in human serum of patients affected by iCCA and/or pCCA was sensibly higher compared with all 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 IL-6 expression in DCLK1$^+$ cells (Supporting Fig. S3A). However, we did not find any significant
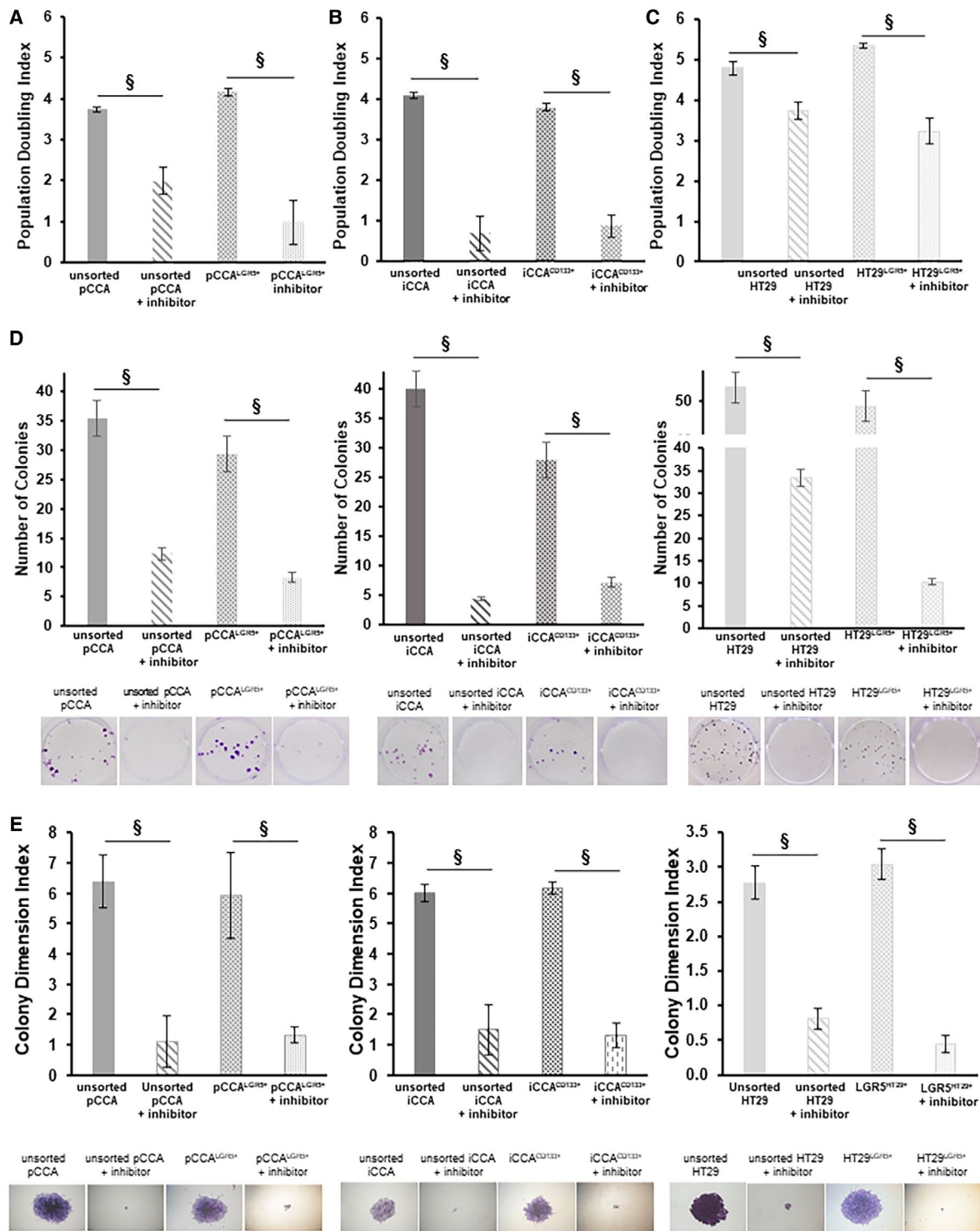
FIG. 4. DCLK1 inhibition exerts an effect on proliferation and colony formation capacity of subpopulation of pCCA and iCCA-CSC primary cell culture. Proliferation rate of unsorted pCCA and pCCA[LGR5+] (A), unsorted iCCA and iCCA[CD133+] (B), and unsorted HT29 and HT29[LGR5+] (C) were analyzed after 3 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 compared with untreated cells. The results are expressed as mean ± SD of n = 6 experiments ([§]$P < 0.01$ versus untreated control). (D) Number of colonies of unsorted pCCA, pCCA[LGR5+], unsorted iCCA, iCCA[CD133+], and 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 with untreated cells. Data are 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+], and 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 shows a significant decrease in the colony dimension index compared with untreated cells ([§]$P < 0.01$ versus untreated control). Data are expressed as mean ± SD of n = 6 experiments ([§]$P < 0.01$).

correlation between macrophage number and DCLK1 expression, nor did we observe a significant overlap (co-expression) between IL-6^{+} and DCLK1^{+} CCA populations (Supporting Fig. S3B). Furthermore, we analyzed several tumors (CA19-9) and inflammation (IL-6 and tumor necrosis factor α) biomarkers using enzyme-linked immunosorbent assay (ELISA) and observed a marked increase in all serum biomarkers analyzed, similar to DCLK1 in serum samples (Supporting Fig. S4).

Discussion

We have demonstrated the DCLK1 overexpression in the CSC-CCA populations, particularly in pCCA[LGR5+] and iCCA[CD133+], and the DCLK1 functional role in tumor proliferation and viability.

The results reveal that (1) the expression of genetic and proteic DCLK1 changed based on expression of different pluripotent/multipotent markers analyzed 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 real-time quantitative PCR and WB, respectively, and this expression was significantly high when compared with the whole tumor cell population; (3) DCLK1 is located in the cytoplasm of CSC-CCA, without any relevant differences between sorted and whole populations, whereas in healthy tissues, pBECs and iBECs were not detectable; (4) the specific inhibition of DCLK1 by LRRK2-IN-1 in CCA cells demonstrated that 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, and decreased colony formation capacity and colony size in both pCCA and iCCA treated with LRRK2-IN-1 when compared with untreated CCA cells; (6) tumoral cells within iCCA and pCCA tissue samples expressed DCLK1 using immunohistochemical analysis; and (7) using ELISA, DCLK1 was only found in the serum of CCA, patients with cirrhosis, HCC, and, importantly, was never detected in healthy control serum samples.

Currently, there are no techniques for early diagnosis of CCA; moreover, no specific CCA-CSC markers are known.[1] Furthermore, many research groups are studying several strategies to block CSC activity, the cells responsible for tumor growth, recidivism, and chemoresistance.[1,9,25] Currently, the drug therapy using radio-chemotherapy is intended primarily for palliative purposes, because it does not target CSCs.[19,26] In fact, surgical resection is the only effective therapy, but it has low applicability given the numerous late diagnoses.[1] Many research groups are trying to identify specific CSC markers to formulate a targeted therapy that would be able to block the CSCs.[9,24,25] A new protein that can be expressed at low levels in gastrointestinal non-CSCs 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 leads to tumor 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
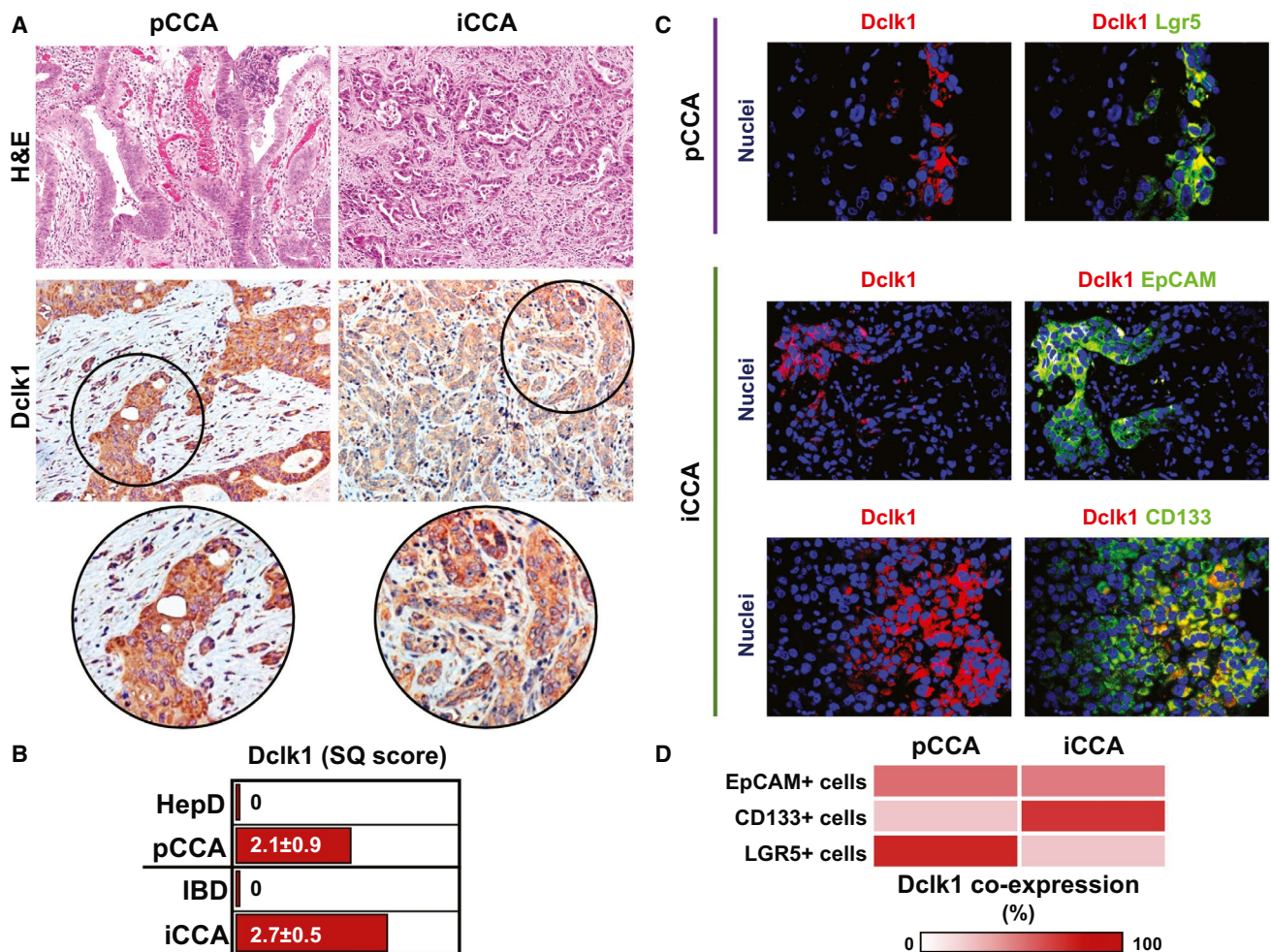
FIG. 5. *In situ* expression of DCLK1 and CSC markers in CCA samples. (A) Hematoxylin and eosin (H&E) staining (upper panels) and immunohistochemistry for DCLK1 (lower panels) in pCCA and iCCA. H&E staining shows histological heterogeneity between the two tumor subtypes. Immunohistochemistry for DCLK1 shows that both tumor subtypes express DCLK1. Original magnification: ×20. Areas in the circles are magnified below. (B) The graph reports the mean and SD of the SQ score for DCLK1 expression in pCCA and iCCA compared with the expression in cholangiocytes lining the HepD and IBDs, respectively ($P < 0.05$ vs. normal samples). See also Supporting 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 CSC markers (i.e., Lgr5, EpCAM, and CD133, in green). Separate channels are also provided. Nuclei are displayed in blue. Original magnification: ×20. (D) Heat map showing the percentage of CSC+ cells co-expressing DCLK1 in pCCA and iCCA.

(CD133). These data confirm that 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 tumors 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, in which DCLK1 was found to be associated with microtubules.[18,29-31] However, no difference in cytoplasmic localization was found between the analyzed subpopulations and the total population.

In many studies, it was observed that inhibition of DCLK1 by a specific inhibitor (LRRK2-IN-1)[28] or by small interfering RNA[24,32,33] leads to a
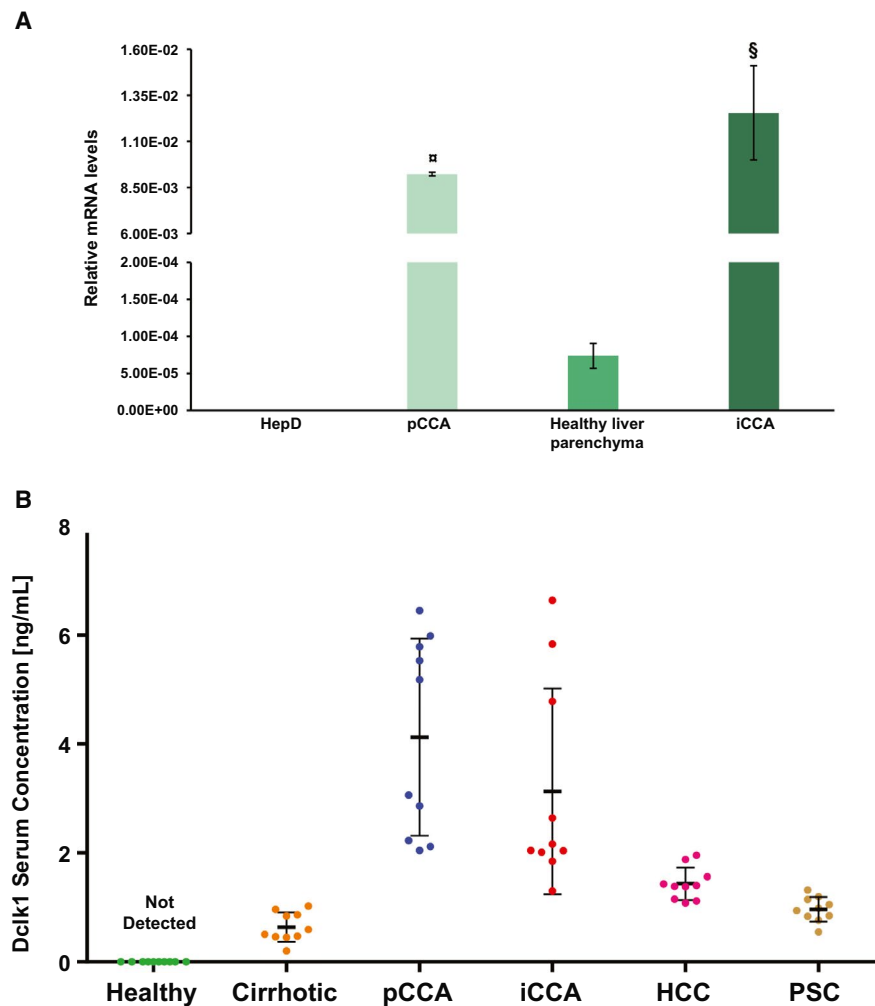
FIG. 6. Putative role of DCLK1 as a diagnostic biomarker. (A) DCLK1-relative mRNA levels in total extracts of human tumors are statistically higher compared with healthy human tissue from perihilar biliary ducts (healthy pBDs) and small biliary ducts (healthy sBDs), which were the healthy controls of pCCA and iCCA, respectively. Data are expressed as mean ± SD of n = 6 experiments ([¤]$P < 0.001$ versus healthy pBDs; [§]$P < 0.01$ versus healthy sBDs). (B) DCLK1 serum concentrations in patients with pCCA, iCCA, HCC, and PSC were analyzed by ELISA. DCLK1 was not detected in healthy controls, and its average concentration increased in the serum of patients with CCA ($P < 0.01$ vs. healthy samples). Data are expressed as mean ± SD of n = 5 experiments.

reduction of colorectal and pancreatic tumor mass *in vivo*, and carcinogenesis and viability *in vitro*. Our data demonstrate that in the subpopulations with the higher DCLK1 expression, in both gene and protein levels, DCLK1 inhibition leads to a significant decrease of cell viability and proliferation compared with whole populations. In fact, we observed high inhibitor efficiency in specific CSC-CCA subpopulations when compared with whole CCA cells. Furthermore, the sensitivity to treatment in pCCA[LGR5+] cells was increased. In previous studies, the DCLK1 pathway was hypothesized to be in HCC[11] with a direct effect on c-myc and an indirect effect on nuclear factor kappa B (NF-kB) through the S100A9 factor, enhancing the tumor proliferation and cell mobility. We hypothesized 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 of an effect on cell viability, until the vitality was reduced to below about 80%. In addition, the inhibition of DCLK1 led to an

increase of apoptosis in the CSC-CCA subpopulations. This indicates an involvement of DCLK1 protein in the resistance to apoptosis in the CSC-CCA; confirming this evidence, it was recently shown 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 (Supporting 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 the subject of future research.

Our data showed a strong reduction in epithelial-mesenchymal transition gene expression and an impairment of cell migration after DCLK1 inhibition (Supporting Figs. S5 and S6). Consistent with these results, several research teams have demonstrated an important role of DCLK1 in the progression of different gastroenterological tumors.[35-38]

Additionally, we assessed the correlation between DCLK1 and inflammation markers in tissue and serum. However, at this time we do not have enough data to support a functional role of DCLK1 in tumor inflammation; therefore, 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 patients with CCA, 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 the findings observed in other tumor studies, in which the precancer condition showed lower DCLK1 levels than the tumor. 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 esophageal adenocarcinoma and HCC, respectively) was higher than precancer conditions (Barrett's esophagus and liver cirrhosis, respectively). Many research teams have associated DCLK1, analyzed in different tumors, with metastasis, tumor invasion, and prognosis.[18,38,40,41] Moreover, Nakanishi et al.[14] demonstrated that the ablation of DCLK1 in tumor 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, PSC) should be confirmed in larger investigations assessing the clinical use of DCLK1 as a serum biomarker in CCA.

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

Author Contributions: L.N. and S.D.M. were responsible for the study concept and design, the generation, collection, assembly, analysis, and interpretation of the data, the manuscript draft and revision, and approval of the final manuscript. G.C., I.Z., and P.M.G. were responsible for the data analysis and interpretation, and the drafting, revision, and approval of the final manuscript. V.C., V.A., D.C., O.V., and S.S. were responsible for the data collection, generation and analysis, and approval the final manuscript. F.M. and P.B.B. were responsible for the collection of biliary trees from organ donors. A.G., M.M., D.B., and P.B.P. were responsible for the collection of fetal biliary trees from therapeutic abort. V.D.P., A.O., A.M.D., G.G., and F.G. were responsible for the collection of CCA surgical resection. S.M. and P.I. were responsible for the collection of PSC serum

samples. G.C., E.G., and D.A. were responsible for the study concept and design, the drafting and revision of the manuscript, and approval of the final manuscript.

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Supporting Information

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