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TITLE: Neoplastic transformation of peribiliary stem cell niche in cholangiocarcinoma arisen in primary sclerosing cholangitis

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List of Abbreviations: CCA: Cholangiocarcinoma; EMT: epithelial-to-mesenchymal transition; PSC: Primary Sclerosing Cholangitis; BTSC: Biliary Tree Stem/progenitor Cell; PBG: Peribiliary Gland; PAS: Periodic Acid Schiff; IHC: Immunohistochemistry; IF: Immunofluorescence; K: keratin; EpCAM: Epithelial Cell Adhesion Molecule; MVD: Microvascular density; vWF: von Willebrand factor; h: human; KM: Kubota's

Medium; LPS: lipopolysaccharide; PDT: Population Doubling Time; RT-PCR: Reverse-Transcription Polymerase Chain Reaction; WB: Western Blot; DYS: Dysplasia; PCNA: Proliferating Cell Nuclear Antigen; SOX: Sry-related HMG box; SALL4: Sal-like protein 4; OCT4A: octamer-binding transcription factor 4 A; VEGF: Vascular Endothelial Growth Factor; IL: Interleukin; pNF-kB: phosphorylated Nuclear Factor kB; TGF-β: Transforming Growth Factor β; αSMA: α smooth muscle actin; HDAC6: Histone deacetylase 6; IBD: inflammatory bowel disease; CFTR: Cystic Fibrosis Transmembrane conductance Regulator.

Conflict of interest: all the authors have nothing to disclose.

ABSTRACT

Primary sclerosing cholangitis (PSC) is a chronic inflammatory cholangiopathy frequently complicated by cholangiocarcinoma (CCA). Massive proliferation of biliary tree stem/progenitor cells (BTSCs), expansion of peribiliary glands (PBGs), and dysplasia were observed in PSC. The aims of the present study were to evaluate the involvement of PBGs and BTSCs in CCA which emerged in PSC patients. Specimens from normal liver (N=5), PSC (N=20), and PSC-associated CCA (N=20) were included. Samples were processed for histology, immunohistochemistry and

immunofluorescence. *In vitro* experiments were performed on human BTSCs, human mucinous primary CCA cell cultures, and human cholangiocyte cell lines (H69). Our results indicated that all CCAs emerging in PSC patients were mucin-producing tumors characterized by PBG involvement and a high expression of stem/progenitor cell markers. Ducts with neoplastic lesions showed higher inflammation, wall thickness, and PBG activation compared to non-neoplastic PSC-affected ducts. CCA showed higher microvascular density and higher expressions of nuclear factor-κB, interleukin-6, interleukin-8, transforming growth factor β, and vascular endothelial growth factor-1, compared to non-neoplastic ducts. CCA cells were characterized by a higher expression of epithelial-to-mesenchymal transition (EMT) traits and by the absence of primary cilia compared to bile ducts and PBG cells in controls and PSC. Our *in vitro* study demonstrated that lipopolysaccharide and oxysterols (PSC-related stressors) induced the expression of EMT traits, nuclear factor-κB pathway, autophagy, and the loss of primary cilia in human BTSCs. **Conclusion:** CCA arising in PSC patients is characterized by extensive PBG involvement and by the activation of the BTSC niche. In these patients, the presence of duct lesions at different stages suggests a progressive tumorigenesis.

Cholangiocarcinoma (CCA) represents the second most frequent type of primary liver cancer and comprises malignancies with high inter- and intra- tumor heterogeneities (1). CCA is currently classified into intrahepatic, perihilar and distal, based on the anatomical location (1). Typical features of CCA are high expression of cancer stem cell markers and epithelial-to-mesenchymal transition (EMT) traits (2). Moreover, CCA is characterized by a prominent desmoplastic stroma composed of cancer-associated fibroblasts, inflammatory and vascular cells (3, 4). CCA

development is strongly associated with chronic inflammatory conditions of the large bile ducts (5), such as primary sclerosing cholangitis and biliary liver fluke infestation (5, 6).

Primary sclerosing cholangitis (PSC) is a cholangiopathy characterized by chronic inflammation which primarily affects extra-hepatic and large intra-hepatic bile ducts, leading to concentric periductal fibrosis and obliterating strictures (7-9). In developed countries, PSC is the most common risk factor for CCA, and patients with PSC carry a 400-fold higher risk for CCA development compared to the general population (7). Among patients with PSC, the annual risk of cholangiocarcinoma is nearly 2% and the 30-year cumulative incidence is 20% (7).

Recently, the involvement of biliary tree stem/progenitor cells (BTSCs) has been demonstrated to play a role in the progression of typical PSC duct lesions (9). BTSCs represent a stem/progenitor cell niche located within the glands of the biliary tree (peribiliary glands: PBGs) (7, 10-12). A massive BTSC proliferation was revealed in PSC, and the subsequent PBG hyperplasia contributes to the bile duct wall thickening, leading to obliterative fibrosis and strictures (9, 12). In PSC, PBGs showed signs of dysplasia and have been suggested as a possible cell of origin for CCA and biliary intraductal papillary mucinous neoplasms (12).

Therefore, this study aimed to evaluate the involvement of PBGs and associated BTSCs in CCA emerging in PSC patients and, in particular, to: i) characterize PSC-associated CCA (PSC-CCA) in terms of histological subtypes, PBG involvement, and stem cell marker expression; ii) evaluate the presence of multifocal pre-neoplastic lesions in surrounding tissues; iii) study possible associations between CCA emergence and features of chronic damage in bile ducts and peribiliary vascular

plexus; and iv) investigate, *in vitro* and *in vivo*, the expression of EMT traits, and the loss of primary cilia, and autophagy in BTSCs and tumor cells.

MATERIALS AND METHODS

Human samples

Formalin-fixed and paraffin embedded liver tissue from patients with PSC were obtained from Norwegian PSC Research Center, Division of Cancer, Surgery and Transplantation, Oslo University Hospital Rikshospitalet, Oslo, Norway and from the ''Paride Stefanini'' Department of General Surgery and Organ Transplantation, Sapienza University of Rome, Rome, Italy. Livers with normal histology were obtained from organ donors from the Department of General Surgery and Organ Transplantation ''Paride Stefanini'', Sapienza University of Rome, Rome, Italy. Specimens included: (1) livers with normal histology from liver donors (N=5); (2) PSC (N=20) obtained from explant livers; (3) PSC-associated CCA (PSC-CCA) patients (N=20) obtained from explant livers. For liver specimens, the presence of at least 10 complete portal tracts was required. For large bile ducts, the complete transversal section of the wall was needed; tumor samples were considered adequate when at least 5 non-overlapping microscopic fields at 20x magnification could be examined in the same section. Written informed consent was obtained from each patient and the study protocol was conformed to the Ethical Guidelines of the 1975 Declaration of Helsinki. The research protocol was reviewed and approved by the Ethic Committees of Umberto I Policlinico of Rome, Italy, and the Regional committees for medical and health research ethics in South-Eastern Norway, Norway. No donor organs were obtained from executed prisoners or other institutionalized persons. Patients and tumor characteristics are indicated in **Supplementary Table 1**.

Light Microscopy, Histopathology and Immunohistochemistry

Tissue sections were stained with hematoxylin and eosin, Periodic Acid Schiff (PAS) and Sirius red, according to standard protocols. Immunohistochemistry (IHC) and immunofluorescence (IF) were performed as previously (9, 13), and complete methodology is provided in **Supplementary Methods**. List of primary antibodies is reported in **Supplementary Table 2**. All slides were scanned by a digital scanner (Aperio Scanscope CS System, Aperio Digital Pathology, Leica Biosystems, Milan, Italy) and processed by ImageScope software.

The area occupied by keratin (K) 7^+ PBGs was quantified by an image analysis algorithm and expressed as the percentage of the total area (9, 13). The expression of nuclear antigens and the membrane expression of Epithelial Cell Adhesion Molecule (EpCAM) were automatically calculated by specific algorithms on the entire section and expressed as percentage of positive cells. Microvascular density (MVD) was calculated as the area occupied by von Willebrand factor (vWF)$^+$ vessels quantified by an image analysis algorithm and is expressed as extension ($\mu m^2/mm^2$ of tissue).

For the other immunoreactions, the percentage of positive cells was automatically calculated by an algorithm on the entire section; furthermore, a semi-quantitative scoring system was applied (0= <5%; 1= 5–10%; 2= 11–30%; 3= 31–50%; 4= >50%) (9). In some figures, the semi-quantitative score was used to visualize data obtained from IHC stains and is reported in a color gradient heat map where each gradient represents statistically significant differences among groups (14). Percentage and scores of positive cells were included in **Table 1**.

Human Cells

Human (h) BTSCs were isolated from extra-hepatic biliary tree as previously described (15, 16). Human CCA cells were isolated from mucin-producing CCA samples as previously described (2, 17). H69 cells, an SV40-transformed (i.e. immortalized) human cholangiocyte cell line, were grown in H69 medium and used as positive control for the presence of primary cilia (18).

Human BTSCs were cultured in Kubota's Medium (KM), a serum-free medium developed for survival and expansion of endodermal stem/progenitors (2, 15). Then, hBTSC were conditioned by adding selected PSC-associated endogenous and exogenous stressors to KM for 10 days. The factors were lipopolysaccharide (LPS, 200ng/ml) or oxysterols (pro-inflammatory/oxidative bile compounds: cholest–4,6–dien-3-one, 0.14mM). Concentrations of tested compounds were chosen based on previous literature (19-22); cells were exposed for a prolonged period (10 days) to mimic chronic stimulation occurring in PSC. Enriched conditioned medium was changed every two days. After 10 days, cells were detached for analyses. In further experiments, the autophagy inhibitor Wortmannin (#W1628, Sigma-Aldrich; 100 nM) was added to above mentioned media for 4 hours (23). Media and solutions and complete methods for cell viability, colony counting, population doubling time (PDT), MTS assay, Reverse-Transcription Polymerase Chain Reaction (RT-PCR), and Western Blot (WB) are detailed in **Supplementary Methods**.

Statistical Analysis

Data are indicated as mean ± standard deviation. The Student t test or Mann–Whitney U test was used to determine differences between groups for normally or not normally distributed data, respectively. The Pearson correlation coefficient or the

Spearman nonparametric correlation were used. A *p*-value <0.05 was considered statistically significant. Analyses were performed using IBM SPSS software (IBM, Armonk, USA).

RESULTS

Histo-morphological features of CCAs and PBG involvement

The histo-morphological aspects of CCAs emerging in PSC patients were evaluated by hematoxylin and eosin, PAS and K7/19 stains (**Figure 1**). In all tumors, cells formed large glandular-like structures located within the lamina propria of extra-hepatic and large intra-hepatic bile ducts. Tumor glandular elements were composed of tall (cuboidal to cylindric) cells characterized by mucin production (PAS positivity) and K7/19 positivity. Tumor glands completely substituted PBGs residing in the lamina propria (periductal infiltrating growth) of bile ducts at the hilum (perihilar CCA) and of large intra-hepatic bile ducts (i.e. segmental ducts, intrahepatic CCA) both in the presence and in the absence of tumor cells within surface epithelium (**Supplementary Figure 1**). In some cases, normal PBGs could be found in between tumor elements (**Figure 1A**). In 4/20 cases, tumor glandular-like elements were revealed in smaller portal spaces within surrounding liver parenchyma as a sign of longitudinal growth/spreading along the intra-hepatic biliary tree (**Supplementary Figure 2A**); 3/20 tumors showed limiting plate infiltration and liver parenchyma invasion (**Supplementary Figure 2B**), a histological aspect that parallels the mass-forming growth pattern at gross examination. PAS positivity and cytokeratin profile were maintained by tumor cells invading the limiting plate, spreading along intra-hepatic biliary tree, or invading neighboring organs (**Supplementary Figure 2C/D**). In sum, based on histo-pathological features, all examined CCAs in PSC patients

could be classified as large bile duct-type (mucinous) CCAs, they invariably involved PBGs, and showed longitudinal growth (i.e. periductal infiltrating) along the biliary tree and/or infiltration of the surrounding liver parenchyma (i.e. mass-forming growth).

In all patients, the study of the surrounding liver parenchyma and biliary tree revealed that the large intra-hepatic (segmental and area) bile ducts were characterized by typical PSC lesions. Moreover, tissue specimens categorized as non-neoplastic at gross examination (so-called "surrounding tissue") obtained from PSC patients with CCA showed a complete spectrum of lesions in the same patient, ranging from ducts with no or minimal inflammation, to ducts with progressive inflammation and fibrosis, to ducts with dysplasia, and to neoplastic lesions. Remarkably, the presence of synchronous tumor and pre-neoplastic lesions (i.e. dysplasia in PBGs, biliary intraductal neoplasia or papillary intraductal lesions) was observed in large intra-hepatic ducts within surrounding tissue of all patients (**Supplementary Figure 3**).

Histo-pathological features in PSC and PSC-associated CCA

Normal large intra-hepatic and hilar ducts, and ducts affected by PSC, by PSC-associated dysplastic lesions (PSC-DYS), and by PSC-CCA were studied and compared (**Figure 1, Supplementary Figures 4/5** and **Supplementary Table 4**). The number of inflammatory infiltrates was increased in PSC-CCA compared to ducts without CCA (**Figure 1, panels B and D**; see also **Supplementary Figure 4**). Moreover, the thickness of bile duct walls was increased in PSC compared to normal specimens (**Figure 1** and **Supplementary Table 4**). Ducts affected by PSC-CCA had thicker walls when compared to ducts affected by PSC and by PSC-DYS. The

thickening in PSC-CCA samples was associated with the increased extension of neoplastic PBG acini in the stroma (**Figure 1, panels C and D**; see also **Supplementary Figure 5**). In keeping, PBG mass was progressively increased in PSC-CCA (14.4±6.4%) compared to PSC-DYS (8.7±2.5%; $p<0.05$) and to PSC ducts (5.4±1.5%; $p<0.05$). In PSC ducts, PBG mass showed a positive correlation with the entity of inflammatory infiltrate ($r=0.774$; $p<0.01$). Similarly, the proliferation index of PBG cells, calculated by IHC for Proliferating Cell Nuclear Antigen (PCNA), was progressively higher in neoplastic PBGs compared to dysplastic PBG, and to PBGs in PSC ducts (**Figure 2, panels A and C; Supplementary Table 4**).

Expression of stem cell markers in PSC and PSC-associated CCA

PSC-associated CCAs were investigated for typical markers of hepatic mature cells (K7, K19 for biliary lineage and Hepatocyte Paraffin-1 for the hepatocytic lineage) and putative stem cell markers. All CCAs in PSC patients were composed of cells which were almost all positive for biliary keratins (**Figure 1**) but were virtually negative for the hepatocyte marker Hepatocyte Paraffin-1 (**Supplementary Figure 6**).

In regard to stem cell markers, CCA tumor elements were characterized by the modification of EpCAM expression (**Figure 2B/C, Table 1**, and **Supplementary Figure 7**). In particular, PSC-CCA showed a lower percentage of cells with a membranous expression pattern (19.3±4.16%) compared to proliferating PBGs in PSC-affected ducts (74.7±4.04%; $p<0.05$); the EpCAM positivity was lower in CCA glandular elements invading deeply the lamina propria (20±9.4%) in comparison with tumor elements located near the surface epithelium (40.5±9.2%; $p<0.05$). Moreover, the expression of Sry-related HMG box (SOX) 9 was increased in PSC (39.4±4.4%)

and PSC-CCA (42.0±8.4%) compared to normal ducts (16±4.2%; p<0.05, **Figure 2D/F, Table 1,** and **Supplementary Figure 8**). Tumors were almost negative for Neural Cell Adhesion Molecule (**Supplementary Figure 6**).

CCAs were highly positive for Sal-like protein 4 (SALL4, **Figure 2E/F**), with more than 50% of neoplastic cells being positive; particularly, the nuclear expression of SALL4 was higher in PSC-CCA (19±8.2%) compared to normal (1.8.0±1.6%; p<0.01, **Supplementary Figure 8**) and PSC samples (8.6±1.9%; p<0.01). PSC-associated CCAs also diffusely expressed pluripotency markers. Octamer-binding transcription factor 4 A (OCT4A, **Figure 2D/F, Table 1**) was expressed at nuclear level in a subpopulation of CCA cells (15.75±4.4%); this percentage was higher compared to PBGs in PSC-affected (4.6±2.32%; $p<0.05$) and normal ducts (3.6±1.3%; $p<0.05$, **Supplementary Figure 8**). In addition, OCT4A was significantly more expressed in tumor cells invading limiting plate and in cells infiltrating smaller portal spaces (43.4±8.1%; $p<0.05$, **Supplementary Figure 9**). Moreover, Tra-1-60 was diffusely and strongly expressed in tumor cells with both a cytoplasmic and apical pattern (**Supplementary Figure 6**).

Microvascular expansion and inflammatory pathways in PSC and PSC-associated CCA

MVD resulted higher in PSC-affected ducts (7,663±4,663 $\mu m^2/mm^2$) and in PSC-CCA samples (7,395±5,108 $\mu m^2/mm^2$) compared to normal ducts (2,299±880 $\mu m^2/mm^2$; $p<0.05$, **Figure 3A**). In addition, MVD correlated with inflammatory infiltrate in PSC ($r=0.580$; $p<0.05$) and in PSC-CCA ($r=0.47$; $p<0.05$), and with neoplastic PBG area in PSC-CCA samples ($r=0.85$; $p<0.01$). Consistently, Vascular Endothelial Growth Factor (VEGF) expression by PBGs (**Figure 3B/E, Table 1**) was

higher in PSC and PSC-CCA (27.8±12.8% and 22.6±11.2%, respectively) compared to normal samples (1.2±0.4%; $p<0.05$, **Supplementary Figure 8**). In patients with CCA, VEGF expression by tumor glandular elements was correlated with MVD ($r=0.87$; $p<0.01$), PBG mass ($r=0.885$; $p<0.05$), PBG proliferation index ($r=0.62$; $p<0.05$), and with inflammation ($r=0.69$; $p <0.05$). Moreover, the expression of interleukin (IL)-8 (**Figure 3B and Table 1**), involved in both inflammation and angiogenesis, was higher in tumor glands (43.0±7.6%) and in PBGs in PSC-affected ducts (19.0±4.2%) compared to normal samples (1.0±1.4%; p<0.01, **Supplementary Figure 8**); PSC-CCA showed higher IL-8 expression compared to PBGs in PSC samples (p<0.05).

We further evaluated the expression of phosphorylated (p) Nuclear Factor κB (NF-κB), pIκB-α, IL-6, and Transforming Growth Factor (TGF)-β (**Figure 3C-E**, **Table 1**, **Supplementary Figure 8**). Proliferating PBGs in PSC-affected ducts and tumor glands in PSC-CCA showed a higher nuclear expression of pNF-κB (51.6±13.3% and 59.2±10.1%, respectively) compared to normal ducts (21.1±6.6%; $p<0.02$ and <0.001, respectively). Similarly, PBGs in PSC-affected ducts and tumor glands in PSC-CCA showed a higher expression of pIκB-α (37.0±6.1% and 36.8±3.8%, respectively) compared to normal ducts (1.6±1.1%; $p<0.001$).

Moreover, proliferating PBGs in PSC-affected ducts and tumor glands in PSC-CCA showed higher expression of IL-6 (30.0±14.7% and 56.3±4.8%, respectively) compared to normal ducts (6.0±2.7%; $p<0.05$, **Figure 3D**). IL-6 expression in PSC-CCA was higher compared to PBGs in PSC-affected ducts (p<0.05).

Finally, TGF-β expression was higher in PBGs in PSC-affected ducts and in tumor glands in PSC-CCA (17.8±5.3% and 22.0±7.7%, respectively) compared to normal ducts (1.2±0.4%; $p<0.05$, **Supplementary Figure 10**).

Expression of EMT traits in PSC and PSC-associated CCA

The phenotype of CCA in PSC patients was further characterized by analyzing the expression of EMT traits. CCA glandular elements expressed higher level of α smooth muscle actin (αSMA) and N-Cadherin (25.0±7.3% and 15.6±4.2%, respectively) compared to proliferating and dysplastic PBGs in PSC-affected ducts (9.2±2.4% and 6.2±2.9%, respectively; *p*<0.05, **Figure 4**). In keeping, CCA glandular elements also co-expressed Vimentin and K7 (**Supplementary Figure 11**). No or only occasional expression of the above-mentioned mesenchymal markers was present in normal specimens (**Figure 4 and Supplementary Figure 11**).

Primary cilium, autophagy and senescence in PSC and PSC-associated CCA

The presence of primary cilium in PBGs was evaluated (**Figure 5** and **Supplementary Figure 12**). In normal and in PSC-affected ducts, the percentage of PBG acini displaying primary cilia accounted for 30-50% (45.2±8.7% and 44.0± 9.4%, respectively); this percentage was significantly reduced in dysplastic PBGs in PSC-affected ducts (7.8±2.6%; p< 0.05); moreover, primary cilia almost completely disappeared in neoplastic glands in PSC-CCA samples (0.4±0.5%; p< 0.001 versus normal and PSC-affected ducts). Interestingly, both PSC-CCA and PSC samples were characterized by an extremely higher expression of Gli-1 (the effector of Sonic Hedgehog pathway) by PBG cells (71.6±5.9% and 67.4± 9.9%, respectively) compared to controls (6.4± 2.9%; p< 0.001) with no significant differences between PSC-CCA and PSC (**Figure 5F, Table 1,** and **Supplementary Figure 12**). Furthermore, Histone deacetylase 6 (HDAC6) expression was increased in PSC-CCA (36.6±7.1%, **Figure 5D**) compared to PSC-affected (7.8±1.9%; p< 0.05) and normal ducts (5.8±2.6%; **Supplementary Figure 8**).

In regard to autophagy markers **(Figure 5E, Table 1)**, neoplastic glands in PSC-CCA were characterized by increased expression of LC3 (40.0±7.1%) compared to PBGs in PSC-affected (12.5±5.0%; p< 0.05) and in normal ducts (3.2±1.3%; p< 0.05; **Supplementary Figure 8**); this was paralleled by p62 expression which was higher in neoplastic cells (42.6±6.0%) compared to PBGs in PSC-affected (22.8±5.7%; p< 0.05) and normal ducts (2.8±1.9%; p< 0.01; **Supplementary Figure 8**).

Finally, the senescence marker γH2A.x was investigated (**Figure 5F**; **Table 1**); neoplastic glands in PSC-CCA and PBGs in PSC-affected ducts were characterized by increased expression of γH2A.x (24.2±5.8% and 23.2±6.6%, respectively) compared to normal ducts (3.0±1.6%; p< 0.05; **Supplementary Figure 8**).

In vitro experiments in hBTSCs and primary cultures of human mucin-producing CCAs

Human BTSCs were *in vitro* exposed to endogenous (i.e. oxysterols) and exogenous (i.e. LPS) compounds already described as cellular stressors associated with PSC (7, 19). The prolonged (i.e. 10 days) exposure to LPS and cholest-4,6-dien-3-one (oxysterols) induced in hBTSCs a marked proliferative effect as demonstrated by the increase of proliferation index (MTS assay) and the significant reduction of Population Doubling Time compared to controls (i.e. KM alone; p<0.001, **Figure 6A**). This proliferative effect was further confirmed by WB for PCNA and IF for Ki67 (**Figure 6B/C**). Interestingly, the exposure to LPS and oxysterols increased the expression of the pluripotency genes (i.e. OCT4A, NANOG, and SALL4) (*p*<0.001 versus KM, **Figure 6D**) and the expression of pIκB-α and pNF-κB (*p*<0.001 versus KM, **Figure 6E**) in hBTSCs. In regard to EMT traits, the exposure of hBTSCs to LPS and oxysterols determined the increase of genes associated with EMT (i.e. TWIST

and αSMA, **Figure 6F**). Furthermore, the exposure to LPS and oxysterols increased the expression of the senescence marker (γH2A.x) compared to controls (**Supplementary Figure 13**). Primary cell cultures obtained from human mucinous CCA showed higher positivity for pIκB-α compared to hBTSCs in maintained in control medium (KM) and increased pNF-κB and γH2A.x expression compared to hBTSC in all culture conditions (**Figure 6E, Supplementary Figure 13**); furthermore, mucinous CCA cells showed higher expression of EMT genes (i.e. SNAIL1 and TWIST) compared to hBTSC in KM (**Figure 6F**).

To further investigate phenotypical changes in hBTSCs, we studied the eventual loss of apical primary cilium in hBTSCs after prolonged exposure to stressors (**Figure 7**). Interestingly, when hBTSCs were exposed to LPS and to oxysterols, they partially lost primary cilia as showed by IF for acetylated α-tubulin compared to the human cholangiocyte cell line H69 and hBTSCs in KM alone (**Figure 7A**). Human mucinous CCA cells were mostly devoid of primary cilia. Human BTSCs exposed to LPS and to oxysterols and CCA cells showed higher expression of HDAC6 gene and higher punctate LC3 expression compared to basal condition (i.e. KM; *p*<0.05; **Figure 7B**). Accordingly, hBTSCs exposed to LPS and oxysterols and CCA cells showed higher expression of p62 compared to hBTSC in KM (p< 0.05) as demonstrated both by IF and WB analyses (**Figure 7C**). The activation of autophagy pathway in hBTSC exposed to LPS and oxysterols and in CCA cells was further confirmed by the increase of LC3-II isoform by WB (**Figure 7D**). Notably, the administration of autophagy inhibitor (i.e. Wortmannin) to cultured cells determined a significant reduction of LC3-II (**Figure 7D**) and p62 (**Supplementary Figure 14**) compared to culture media without Wortmannin.

DISCUSSION

The main results in our study indicate that: i) CCAs which arise in PSC patients are mucin-producing carcinomas characterized by a high expression of stem/progenitor cell markers and by the primary involvement of PBGs; ii) tumorigenesis in PSC is a multifocal and multistep process which simultaneously involves several ducts and is preceded by pre-neoplastic lesions; iii) CCA onset in PSC is associated with chronic inflammation of the bile ducts affected by PSC and with the progressive thickening of duct walls mostly due to both the activation of the BTSC niche within the PBGs and the expansion of the peribiliary vascular plexus; iv) at the cellular level, the pathogenic hyperplasia-dysplasia-carcinoma sequence in PBGs is characterized by the acquisition of EMT features, the absence of primary cilia, and the increase of autophagy and senescence.

CCA development is a major risk factor in PSC and represents the predominant cause of PSC-related death[7]. Depending on the location[1], PSC-associated CCA involved hepatic ducts (perihilar CCA), segmental intrahepatic bile ducts (intrahepatic CCA), and distal bile ducts (distal CCA). The examination of the surrounding liver tissues and neighboring extrahepatic biliary tree revealed the presence of synchronous lesions and pre-neoplastic lesions in the same patient. Histologically, all tumors and synchronous lesions were composed of PAS+ large ductular structures lined by columnar cells; thus, all examined CCAs can be categorized histologically as mucinous large bile duct-type CCAs.

The multifocal synchronous CCA evolution in PSC patients seems to parallel the colorectal carcinogenesis in inflammatory bowel disease (IBD). IBD is clinically and pathogenetically associated with PSC. Patients with IBD have an increased risk of colorectal synchronous/metachronous malignancies arising from a field of marked

chronic inflammation and closely linked to the extent, duration and severity of inflammation (24). In keeping, the present study demonstrated that biliary malignancies in PSC patients were associated with the severity of bile duct inflammation, PBG hyperplasia, and duct wall thickening in the same duct. In PSC-affected ducts, chronic inflammation induces BTSC proliferation and subsequent mucinous metaplasia in PBGs (9); moreover, inflammation determines the emergence of SOX9$^+$/CFTR$^-$ (Cystic Fibrosis Transmembrane conductance Regulator) immature cells instead of normal mature cholangiocytes (CFTR$^+$/Secretin Receptor$^+$) in surface biliary epithelium (12). Translating the IBD tumorigenic model in PSC, these observations indicate that the biliary epithelium in PSC may show aspects of "field cancerization". The phenomenon of "field cancerization" represents the preconditioning of a large area of normal epithelium to the future development of neoplastic lesions and has been associated with synchronous carcinogenesis in IBD (24). Chronic inflammation is critical for field cancerization since it induces somatic mutations or epigenomic alterations and favors those cells (clones) best adapted to the inflamed microenvironment based on apoptosis resistance and replicative capability (24).

In IBD-related colorectal cancer, field cancerization derived from intestinal stem cells within crypts, given their role in epithelial regeneration and their long lifespan (24). A putative origin of field cancerization in PSC could be the Sox9$^+$ cell niche within PBGs. In PSC, BTSCs proliferate extensively, show few signs of cellular senescence, and have been proven to be able to escape immune response (25). A report based on lineage tracing in mice suggested that cells within PBGs could represent the origin of extrahepatic CCA, which supports our data in PSC patients (26). In keeping, CCAs in PSC patients diffusely expressed stem cell markers and

primarily involved PBGs which were largely substituted by neoplastic cells. A second mechanism at the basis of the reported aspects could reside in the de-differentiation of non-stem cells (i.e. mature cholangiocytes) (27). However, this possibility seems less probable considering that mature cholangiocytes in PSC showed features of cellular senescence, apoptosis, and impaired proliferative capabilities (19, 28-30). Furthermore, our results parallel evidence on the role of the pancreatic duct glands in pancreatic cancer (31). Pancreatic duct glands represent the anatomical counterpart of PBG within the pancreas (12) and share a unique stem/progenitor cell population with PBGs. Intriguingly, pancreatic duct glands, in response to chronic pancreatitis, can undergo a hedgehog-mediated mucinous metaplasia with features of pancreatic cancer precursor lesions (31) and form the basal crypt segments of intraductal papillary mucinous neoplasms (32). Thus, a key role for PDGs in early inflammation-mediated events of pancreatic carcinogenesis was identified and, remarkably, biliary neoplasms and pre-neoplastic lesions parallel pancreatic diseases both pathologically and pathogenetically (33).

In summary, our study indicates that carcinogenesis in PSC is a multistep and multifocal process characterized by a chronic inflammation – PBG hyperplasia – mucinous dysplasia – carcinoma sequence. PSC could represent a reliable model for the study of biliary tumorigenesis in humans since bile ducts affected by each consecutive step in the carcinogenesis sequence are present in the same patient. Furthermore, in the present study, we used PSC-associated CCA to explore the link between inflammation and BTSCs in carcinogenesis, and we confirmed our data in a parallel *in vitro* study model. Although etiologic factors associated with PSC are largely unknown, biologically relevant endogenous (e.g. oxysterols) and exogenous (e.g. LPS from bacterial translocation) molecules present in the bile have been

tested as stressors to mimic cholangiocyte damage in PSC (19, 34). In general, oxysterols were demonstrated to induce cholangiocyte apoptosis, to perpetuate inflammation in cholangiopathies, and to display mutagenic and carcinogenic properties (19, 34). Moreover, LPS play a role in the activation of the NF-kB pathway (35), can induce senescence and favoir the acquisition of a cytokine secretory phenotype in cholangiocytes. Therefore, we directly tested the effects of LPS and oxysterols on hBTSCs *in vitro*. The prolonged exposure to LPS and oxysterols triggered hBTSC proliferation and increased NF-κB pathway activation and senescence. In keeping, the high pIκB-α/pNF-κB expression in PBGs observed in tissue slides is associated with increased γH2A.x expression and the enhanced production of cytokines (i.e. IL-6, IL-8, and TGF-β), thus suggesting the acquisition of a senescence-associated secretory phenotype (19) and the existence of a pro-inflammatory loop. Proliferating BTSCs in PSC expressed elevated levels of VEGF and IL-8 which are associated with increased extension of the peribiliary vascular plexus. The presence of an expanded vascular plexus around PBGs in PSC can further support the tumor development and growth along the duct wall. Interestingly, in colitis-associated carcinogenesis, chronic inflammation could induce VEGF release by colonic epithelial cells, supporting the tumor development in autocrine and paracrine (angiogenesis) manners (36).

At a cellular level, the present study demonstrated the possible roles of EMT and cilium disarrangement in the progression from PBG hyperplasia to tumor development. In PSC, the increase of EMT-related markers in neoplastic compared to hyperplastic PBGs is paralleled by the progressive loss of membrane EpCAM positivity, thus indicating the acquisition of a mesenchymal phenotype favoring invasion. In keeping, low EpCAM expression was found in neoplastic glands distant

from the tumor mass (i.e. small portal tract and distant metastasis). The prolonged *in vitro* exposure of hBTSCs to LPS and oxysterols determined the up-regulation of EMT genes, confirming that PSC-associated endogenous and exogenous stressors can contribute to triggering EMT in hBTSCs. Similarly, CCA cells showed a higher expression of EMT-related genes *in vitro* as compared to hBTSCs.

The acquisition of the EMT trait phenotype is associated with the parallel absence of primary cilia in hBTSCs and CCA cells, as observed both in tissue slides and *in vitro*. In tissue slides, we demonstrated that primary cilia in PBGs were reduced in PSC samples and almost completely absent in PSC-CCA samples in parallel with an increased expression of HDAC6 and autophagy markers. *In vitro*, prolonged exposure to LPS and oxysterols reduced the number of ciliated hBTSCs while CCA cells rarely showed primary cilia; the loss/absence of primary cilia is associated with HDAC6 gene up-regulation and increased autophagy markers (p62 and LC3-II). In polarized epithelial cells, primary cilia are apical organelles and function as a cellular "antenna" by transducing extracellular signals to the cell body (37). Interestingly, previous reports have suggested that the loss of primary cilia has a major role in CCA pathogenesis and that its disruption in cholangiocytes is mediated by HDAC6 expression and by autophagy (i.e. ciliophagy) (38). Primary cilia are emerging as an important structure in influencing the proliferation and differentiation of stem cells by the modulation of signaling pathways (37, 39). Interestingly, neoplastic PBGs without primary cilia maintained high expression of Gli-1, suggesting a non-canonical activation of the hedgehog pathway (39, 40). Taken together, our data clearly indicate that PSC carcinogenesis is characterized by the loss of epithelial cell polarization and the acquisition of EMT traits in PBGs and associated BTSCs. Chronic inflammation in PSC could determine the loss of primary cilia in BTSCs by

stimulating HDAC6 expression and autophagy. In turn, deciliated BTSCs could be more prone to neoplastic transformation, thus representing a candidate cell of origin for CCA in these patients.

In conclusion, our results demonstrated that PBGs are profoundly involved in CCA which arises in patients affected by PSC. In these patients, CCA represents the final step of several progressive modifications involving PBG cells and including proliferation, mucinous metaplasia, loss of primary cilia, and acquisition of EMT-trait features. Furthermore, the present study identified autophagy and senescence as key processes in the neoplastic transformation of PBGs and in the expression of a secretory phenotype able to further trigger biliary inflammation.

Importantly, tumor appearance was anticipated by typical morphological modifications which resulted in the progressive thickening of the bile ducts and could support forthcoming radiological studies. In this light, bile duct wall thickness, PBG hyperplasia, and peribiliary vascular plexus remodeling could be investigated as surrogate markers to individuate PSC patients with higher PBG proliferation and, possibly, an increased risk of developing CCA. Finally, our results implicate the presence of a progressive tumorigenesis sequence in PSC patients which could represent a human model for biliary carcinogenesis and which could possibly identify specific molecular targets using a multi-omics approach.

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Table 1. Percentage and semi-quantitative score of positive cells within peribiliary glands for given antigens

	Normal	PSC	PSC-CCA
mEpCAM (score)	43.7 ± 6.1% (3)	74.7 ± 4.04%* (4)	19.3 ± 4.16%* (2)
SOX9 (score)	16.0 ± 4.2%* (2)	39.4 ± 4.4% (3)	42.0 ± 8.4% (3)
SALL4 (score)	1.8 ± 1.6% (0)	8.6 ± 1.9%* (1)	19.0 ± 8.2%* (2)
OCT4A (score)	3.6 ± 1.3% (0)	4.6 ± 2.32% (0)	15.75 ± 4.4%* (2)
VEGF (score)	1.2 ± 0.4%* (0)	27.8 ± 12.8% (2)	22.6 ± 11.2% (2)
IL-8 (score)	1.0 ± 1.4% (0)	19 ±4.2%* (2)	43.0 ± 7.6%* (3)
pNF-κB (score)	21.1 ± 6.6%* (2)	51.6 ± 13.3% (4)	59.2 ± 10.1% (4)
pIκB-α (score)	1.6 ± 1.1%* (0)	37.0 ± 6.1% (3)	36.8 ± 3.8% (3)
IL-6 (score)	6.0 ± 2.7% (1)	30.0 ± 14.7%* (3)	56.3 ± 4.8%* (4)
TGF-β (score)	1.2 ± 0.4%* (0)	17.8 ± 5.3% (2)	22.0 ± 7.7% (2)
α-SMA (score)	0.4 ± 0.6% (0)	9.2 ± 2.4%* (1)	25.0 ± 7.3%* (2)
N-Cadherin (score)	0.2 ± 0.5% (0)	6.2 ± 2.9%* (1)	15.6 ± 4.2%* (2)
Primary Cilium (score)	45.2 ± 8.7% (3)	44.0 ± 9.4% (3)	0.4 ± 0.5%* (0)

Gli-1 (score)	6.4 ± 2.9%* (1)	71.6 ± 5.9% (4)	67.4 ± 9.9% (4)
HDAC6 (score)	5.8 ± 2.6% (1)	7.8 ± 1.9% (1)	36.7 ± 7.1%* (3)
LC3 (score)	3.2 ± 1.3% (0)	12.5 ± 5.0%* (2)	40.0 ± 7.1%* (3)
p62 (score)	2.8 ± 1.9% (0)	22.8 ± 5.7%* (2)	42.6 ± 6%* (3)
γH2A.X (score)	3.0 ± 1.6%* (0)	23.2 ± 6.6% (2)	24.2 ± 5.8% (2)

*PSC: primary sclerosing cholangitis; CCA: cholangiocarcinoma; EpCAM: epithelial cell adhesion molecule; SOX9: Sry-related HMG box; OCT4A: octamer-binding transcription factor 4 A; VEGF: Vascular Endothelial Growth Factor; IL: interleukin; p: phosphorylated; NF-κB: Nuclear Factor κB; TGF-β: Transforming Growth Factor β; α-SMA: α Smooth Muscle Actin; HDAC6: Histone Deacetylase 6. Data are expressed as percentage (mean ± standard deviation) and relative semi-quantitative score in parentheses. The semi-quantitative scoring system was the following: 0= <5%; 1= 5–10%; 2= 11–30%; 3= 31–50%; 4= >50%. * p< 0.05 versus other groups.*

A

Normal · PSC · PSC-CCA

Nuclei αSMA

Nuclei K αSMA

B

Normal · PSC · PSC-CCA

N-Cadherin

C

	Normal	PSC	PSC-CCA
PC			
Gli-1			
HDAC6			
LC3			
p62			
γH2A.X			

IHC
*Score Legend
(% positive cells)*

< 5	
5-10	11-30
31-50	> 50