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Intrahepatic cholangiocarcinoma: a single cell resolution unraveling the complexity of the tumor microenvironment

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Cholangiocarcinoma (CCA) is one of the most aggressive epithelial cancer types with scarce therapeutic options, resulting in a still gloomy prognosis [1]. Even if the understanding of this disease has improved over the last decade, researchers and clinicians are still faced with an enigmatic and worrisome cancer, which marked heterogeneity makes therapeutic choices difficult to take. Beside the well-established anatomical criteria, by which CCA is categorized into three different tumor types (intrahepatic, perihilar and distal), the multifaceted tumor microenvironment (TME) is another factor that contributes to the complexity of CCA. TME is an intricate ecosystem able to exchange a multitude of signals with the tumor counterpart, which may exert either pro- and anti-oncogenic functions [2]. Taking advantage of single-cell RNA-sequencing (scRNA-seq) [3], Zhang *et al.* are the first to dissect intrahepatic CCA (iCCA) at the cellular level, and to demonstrate that indeed the CCA cellular landscape displays a strong diversity in the malignant, immune and stromal cells [4] (Figure 1). Although based on a relatively small sample set (five iCCA, of which three tumors also included the matched 'peritumoral' tissue), this study drives the field forward by providing a single-cell resolution transcriptomic landscape of iCCA. Moreover, the authors identify a novel mechanism underpinning the deleterious interplay between tumor and its microenvironment that can be harnessed for therapeutic targeting. In this study, Zhang *et al.* characterize distinct populations of immune and stromal cells, in particular tumor-infiltrating lymphocytes (TILs) and cancer-associated fibroblast (CAFs) in the context of a wide inter- and intra-tumor heterogeneity also involving malignant cholangiocytes, as anticipated by several studies performed in iCCA [1].

Within the tumor bulk, malignant cells form the core, and by definition, this population is classically regarded as heterogenous. Zhang *et al.* identified four different

clusters in the tumor cell compartment of iCCA, harboring patient-specific somatic mutations, but also sharing common activated signatures, including IL-6, Wnt, transforming growth factor (TGF) and tumor necrosis factor (TNF) pathways. Furthermore, this study pinpoints novel pathways that can be relevant in CCA progression, in particular the serine peptidase inhibitor Kazal type-1 (SPINK1, also called TATI or PSTI). SPINK1 is a kinase inhibitor of premature trypsin activation in the pancreas, and its overexpression is associated with poor prognosis in iCCA. *In vitro*, loss and gain function of SPINK1 in CCA cells, experiments indicate that SPINK1 contributes to stemness, cell invasion, and chemoresistance, which presumably involves a subset of tumor-promoting cells. Noteworthy, prognostic significance of SPINK1 overexpression has been reported in several cancers (lung, kidney, prostate, ovary, and breast) and determination of SPINK1 in serum has been proposed as a useful biomarker to identify patients at risk with a more aggressive disease [5].

Among TILs, CD4⁺ T regs are gaining special attention because of their strong immunosuppressive properties. T regs are mostly localized at the peritumoral region, whereby they dampen anti-tumor activity of NK and CD8⁺ cytotoxic T cells by secreting IL-10 and TGF- β 1 [6]. Moreover, overexpression of the transcription factor FoxP3, a distinct trait of their immunophenotype, up-regulates cytotoxic T lymphocyte antigen-4 (CTLA-4), which inhibits CD8⁺ T cell activation by binding to CD80 expressed by antigen presenting cells. Beside CTLA-4, Zhang *et al.* describe T regs expressing T cell Ig and ITIM domain (TIGIT), a co-inhibitory receptor that synergizes with CTLA-4 to suppress antitumor immune responses. Accordingly, they report a persistent cytotoxic or activated phenotype of NK and T cells supported by enriched signaling related to hypoxia, apoptosis, interferon response and oxidative phosphorylation. However, TIGIT also mediates intercellular communication with malignant

cholangiocytes that express the nectin-like protein, poliovirus receptor (PVR/CD155), which promotes tumor cell invasion, migration, proliferation, and immune escape to overall support tumor progression [7].

In iCCA, TME is characterized by an exuberant desmoplastic reaction sustained by a variety of stromal cells, laying within a rearranged and stiffer extracellular matrix (ECM). Several teams have already dissected the CCA microenvironment at tissue level by transcriptomics or proteomics [8-11], and all have emphasized the significant contribution of CAFs. Zhang *et al.* demonstrate the existence of five CAF subpopulations in iCCA. To overcome the limited yield provided by single cell analysis (only 1.6%) they turn to isolation of CAFs by a commonly used negative selection strategy. All CAF subsets express prototypal fibroblast genes, including *ACTA2* (α -SMA at different levels across subpopulations), *COL1a2* and *PDGFRb*. Of note, *PDGFRb* encodes the cognate receptor of platelet-derived growth factor-D (PDGF-D), which drives CAF recruitment and is secreted by the malignant cholangiocytes themselves [12]. Interestingly, the most abundant CAF subset (57.6%) displays a microvasculature signature (vCAF). Although CCA is regarded a hypo-vascular tumor type, it contains an extensive lymphatic vasculature likely involved in the early dissemination to regional lymph nodes, a critical step that may preclude curative effects of surgery [13]. Although not addressed by Zhang *et al.*, it is tempting to speculate that the vCAF subset is actively involved in generating the lymphatic vascularization of iCCA, since they are localized to the tumor core and in microvascular regions closely aligned with malignant cells. Indeed, upon PDGF-D stimulation, CAFs secrete the vascular endothelial growth factors (VEGF)-A and -C, which promote tumor-associated lymph angiogenesis and the propensity of CCA cells to invade across the endothelial wall [13]. Intriguingly, a subpopulation of myofibroblasts (named

portal myofibroblasts) with vascular properties have been identified in fibrotic livers [14] and thus, possibly they can be regarded as the cell source of vCAF in iCCA. Two CAF subclusters with low *ACTA2* expression were identified, as previously showed in other cancers, one cluster is enriched in ECM proteins and collagen fibril organization (mCAF), the second cluster is related to inflammatory responses and complement activation (iCAF). The last two CAF clusters comprise a subpopulation of CAFs that either expresses the major histocompatibility complex II (MHC-II) genes and were termed antigen-presenting CAF (apCAF), or a subpopulation of CAFs expressing the epithelial markers (KRT19 and KRT8), designated the epithelial-to-mesenchymal transition (EMT)-like CAF (eCAF). This phenotypic diversity of CAFs match the functional diversity that Zhang *et al.* have begun to unravel, starting with vCAF, the main CAF subpopulation identified. The authors explore the IL-6/IL-6R axis, a pro-invasive pathway in cholangiocytes that has been extensively studied in CCA. In the present study, the IL-6 axis represents a signature featuring malignant cholangiocyte subsets 1 and 2. Previously, IL-6 has been identified in stroma of CCA [8] and is now assigned to the vCAF subset, whereby it is upregulated when vCAFs lay in contact with tumor cells. The mechanism behind IL-6 stimulation in vCAF by malignant cells revealed a contribution of exosomes containing miR-9-5p. Once produced by vCAF, IL-6 targets tumor cells and increases their malignant nature through epigenetic alterations, specifically involving the polycomb group protein enhancer of zeste homolog 2 (EZH2), a type of histone methylation modification nuclear complex. Although the pleiotropic effects of EZH2 on proliferation, apoptosis, angiogenesis in CCA are well recognized [1], here the authors show its involvement in fostering the cancer stem cell niche since EZH2 inhibition by small molecules suppresses tumor sphere formation. Moreover, the authors provide further evidence supporting a 'yin-

yang' cooperation between CAFs and tumor cells, as previously shown with the heparin-binding epidermal growth factor-like (HB-EGF)/TGF- β axis [15]. Here, the cross talk is mediated by IL-6/IL-6R, which relevance previously was suggested for primary sclerosing cholangitis (PSC), the most common risk factor for CCA [16].

Taken together, the data outlined by Zhang *et al.* help to understand the multiple reasons behind the historical challenges in developing effective therapeutic strategies in iCCA. First, the common pathways found to be deregulated in tumor cholangiocytes are potentially druggable reaching broadly all malignant subsets (IL-6, Wnt, TGF, TNF), but unfortunately no inhibitor of these molecules has proven to be effective in humans despite rather promising preclinical studies [1]. Second, TIGIT targeting can fail given the presence of functional redundancy in the TME across coinhibitory receptor pathways that may compensate TIGIT deficiency. Third, as shown CAFs are a hugely heterogeneous population, displaying distinctive phenotypic traits, which reflect specific functions (angiogenesis, immune modulation, ECM synthesis, EMT). This important concept is consistent with the conflicting results generated by CAF depletion in experimental models of desmoplastic epithelial cancers. In fact, the experimental rat model of syngeneic iCCA deprived in CAF by inducing apoptosis with the BH3 mimetic navitoclax showed a reduction in the primary tumor growth, in the tumor lymphatic vascularization, and in the metastases to regional lymph nodes and to the peritoneum [13, 17]. Additionally, depletion of CAF can be achieved by photothermal therapy to normalize tumor stiffness in mouse xenografted CCA [18]. Conversely, in mouse models of pancreatic ductal adenocarcinoma, CAF depletion was associated with a more aggressive tumor phenotype and enhanced tumor spread, thus indicating the ambivalent roles of CAFs to either restrain or stimulate tumor growth [19, 20]. These issues need to be tackled before relying on TME targeting as a powerful

tool to fight iCCA invasiveness. Within this perspective, scRNA-seq may provide a valuable asset to capture the pronounced heterogeneity of iCCA at single-cell resolution and thus, lay the bases to develop novel and effective personalized treatment approaches.

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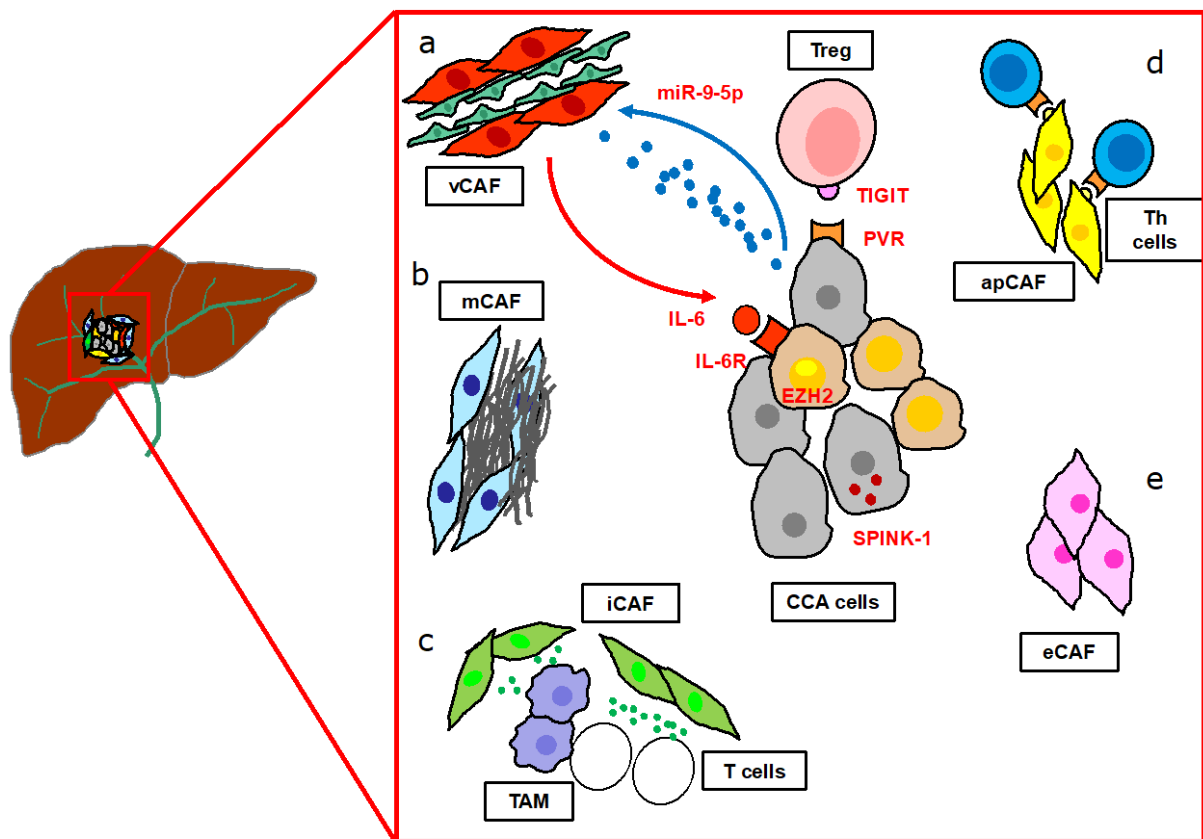


Figure 1 : The transcriptomic landscape of iCCA at single-cell resolution. iCCA is characterized by a marked heterogeneity affecting the tumoral, stromal and immune compartments. Within the tumor bulk, malignant cholangiocytes (CCA cells) show a high degree of cellular diversity (exemplified in different color and shape), whereby SPINK-1 expression identifies a more aggressive phenotype associated with poor prognosis. Among tumor-infiltrating lymphocytes, immunosuppressive T regs expressing TIGIT may interact with CCA cells expressing PVR further promoting immune escape, among other pro-invasive effects. Transcriptomic analysis of cancer-associated fibroblasts (CAF) leads to identify five subsets with distinctive functions: a) vascular CAF (vCAF, red), b) matrix CAF (mCAF, turquoise), c) inflammatory CAF (iCAF, green), d) antigen-presenting CAF (apCAF, yellow) and e) EMT-like (eCAF, purple). Each CAF subtype may variably affect tumor growth, acting on: a) lymphangiogenesis (vCAF), b) extracellular matrix remodeling (mCAF), c) regulation of inflammatory response (secreted cytokines in green dots), involving tumor-associated macrophages (TAM) and T cells (iCAF), d) regulation of adaptive immunity (apCAF), involving Th cells, and e) expression of epithelial markers (eCAF, depicted with a more cuboidal morphology compared with other CAF subtypes). Among them,

vCAF are engaged in an intensive cross-talk with CCA cells: they secrete IL-6 (red arrow), which induces epigenetic alterations in CCA cells by up-regulating EZH2, which enhances stemness properties (see orange subset of CCA cells, depicted with a cancer stem cell morphology). In turn, CCA cells release exosomal miR-9-5p (blue arrow with green dots), which locks IL-6 expression by vCAF in a self-perpetuating loop.