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Commentary

Combination immunotherapy: The new roadmap for the treatment of intrahepatic cholangiocarcinoma

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Abstract

Intrahepatic cholangiocarcinoma (iCCA) is a catastrophic malignancy of the intrahepatic bile ducts and one of the neoplasms with incidence rates that have been rising faster than almost any other cancer. This steady increase combined with the high mortality rates underscore the fact that optimal management of iCCA remains a challenge. While immune checkpoint inhibitors (ICIs) as single agents have elicited discouraging results in patients with iCCA, the combination of chemotherapy *plus* anti-PD-L1 blockade has recently become the new standard of care, underscoring the importance of designing rational combination strategies able to increase the therapeutic efficacy of ICIs. To do so, a thorough understanding of the tumor-immune interactions is becoming critical. This commentary aims to discuss recent advancements in our understanding of the complex relationship between tumor cells and the surrounding immune microenvironment of iCCA, with particular emphasis on our recent manuscript published in the journal *Gut* (*Martin-serrano et al., 2022*) [1].

Body Text

With an occurrence of 1–2 new cases per 100,000 persons annually in the US, intrahepatic cholangiocarcinoma (iCCA) – a cancer that forms in the small bile ducts within the liver – is relatively rare but highly fatal, accounting for less than 3% of all gastrointestinal cancers, yet nearly 20% of all deaths [2]. Despite the low prevalence of iCCA in most high-income countries (global age standardized incidence rate of 1.4 cases per 100,000 person-years) [3], a steady increase in its incidence rates has been recorded over the past decades. In the US the incidence of iCCA has increased by 128% over the past 40-years [4]. iCCAs are rarely diagnosed at early stage due to the lack of specific symptoms. Late diagnosis compromises the efficacy of hepatic resection, which represents the only curative option for early stage iCCA[2], and palliative chemotherapy with gemcitabine *plus* cisplatin (GemCis) has been the standard of care (SOC) for advanced disease for the past 12 years, with a median overall survival (OS) of just 12 months [5]. Therefore, patients with iCCA face a highly dismal prognosis, with 95% of patients dying within five years [6].

In the past decade, the high incidence and mortality rates have sparked renewed interest in this long-forgotten disease. As a result, multi-institutional collaborations as well as international consortia have assembled human tissue collections and provided detailed information on the molecular landscape of human iCCA, ultimately laying the groundwork for the evaluation of targeted therapies. In this regard, our group and others identified *recurrent* and *targetable* genetic alterations (i.e., gene fusions in FGFR2, NRTK, mutations in IDH, etc) [7-13]. Despite such breakthroughs and the increase in clinical trials investigating targeted therapies, progress has been modest with the most promising drugs, FGFR and IDH inhibitors, conferring a survival benefit of only 2-4 months [14-16].

Immune checkpoint inhibitors (ICIs) have revolutionized the management of several solid cancers [17,18]; yet, results in iCCA have been modest. ICIs are a new class of immunotherapeutic antibodies that block T cell surface-expressed inhibitory receptors or their ligands to unleash the patient's own immune response against the tumor [16]. As a result, monoclonal antibodies (moAb) blocking the inhibitory receptor programmed cell death protein 1 (PD1) or its ligand PD-L1 have quickly become the new SOC in many cancers [19]. Unfortunately, despite the great success and long-lasting clinical

benefit, only a small fraction of patients responds to PD1/PD-L1 checkpoint blockade. ICIs are particularly effective in patients with mismatch repair (MMR) deficiency, with an observed clinical response in up to 40% of patients [20]. Unfortunately, MMR deficiency occurs in only 3% of iCCA patients [21-24], and initial clinical results in unselected CCA populations treated with the anti-PD1 moAb pembrolizumab as single agent have been disappointing, with only 6% to 13% of clinical responses [25]. Recently, the TOPAZ-1 trial found that the addition of durvalumab, a moAb targeting PD-L1, to the SOC chemotherapy significantly prolongs overall survival and increases objective response rates from 19% to 27% in patients with iCCA [26]. Based on these results, the Food and Drug Administration (FDA) approved this combination as new SOC for advanced iCCA in September 2022. Despite this breakthrough, these results are far from optimal given the mild improvement in median overall survival (from 11.5 to 12.8 months26). Hence, the identification of biomarkers for optimal patient selection as well as the design of rational combination strategies able to increase the efficacy of ICIs in iCCA remain top priorities.

The lack of understanding of the interaction between tumor cells and the immune microenvironment represents one of the main reasons for unsatisfactory clinical trial outcomes in iCCA. The first pan-cancer immunogenomic analyses characterized the intratumoral immune landscape of over 20 solid cancers (so-called Cancer Immunome Atlas) and suggested that tumor-intrinsic genomic alterations determine the tumor immunophenotype and escape mechanisms [27,28]. Unfortunately, no iCCA cases were included in these studies. To fill this knowledge gap, our group virtually deconstructed the tumor immune microenvironment (TIME) of a large collection of human iCCA samples [1]. By considering elements of the **S**troma, **T**umor and **I**mmune **M**icroenvironment, our algorithm successfully classified iCCA tumors into 5 robust groups, called **STIM** classes, encompassing both *immune inflamed* (or *hot*) and *non-inflamed* (or cold) profiles [1]. Compared to previous studies which described distinct iCCA classes based on either tumor [29,30] or immune-only [31] related features, our unsupervised approach unveiled actionable genotype-immunophenotype relationships, with each class being defined by a unique TIME associated with distinct underlying genetic alterations. In line with emerging single cell RNA-sequencing-based analyses [32,33] and the immunotolerant nature of the liver [34], we observed that more than half of iCCAs were non-inflamed tumors with abundance of immunosuppressive components (i.e. macrophages, regulatory T cells, etc.). The non-inflamed tumors entail 3 distinct classes with the largest being the "*hepatic stem-like*" class, so called because of the enrichment of stem cell features along with high rate of genetic alterations in driver genes, including mutations in *BAP1*, *IDH1/2* and *FGFR2* fusions. Immunophenotypically, the tumors in this class are characterized by low cytolytic activity and an abundance of immunosuppressive macrophages. Interestingly, a recent study found that pharmacologic inhibition of mutant *IDH1* reactivates $CD8^+$ T cells in an iCCA subcutaneous murine model [35] confirming the validity of the genotype-immunophenotype relationships identified by our algorithm, and that the immunogenicity of this neoplasm is indeed influenced by its underlying genotype. Similarly, recent studies confirmed the association between *FGFR2* fusions and reduced immune infiltration [36]. Evidence from other tumors points to an important immunosuppressive role for oncogenic FGFR signaling in shaping the TIME (i.e., upregulation of PD-L1, inhibition of IFNγ signaling, increase of myeloid-derived suppressive cells, T cell exclusion) [37-40]. In this regard, emerging experimental [41-43] and clinical [44] evidence from lung, renal and head and neck carcinoma showed promising results with the addition of FGFR inhibitors to ICIs. As an example, in a *FGFR2*-driven autochthonous mouse model of lung cancer [41] treatment with the FGFR inhibitor erdafitinib elicited unique changes in the TIME (i.e., reduction of regulatory T cells, increase of T cell infiltration, etc.), and synergized with anti-PD1 ICI by inducing tumor cell killing that enhanced tumor antigen presentation and antitumor T-cell responses. Unfortunately, similar studies in iCCA are missing. Major hurdle to these clinical goals is that current preclinical models of *FGFR2* fusion-driven iCCA include human cell lines [7,45-47] and patient-derived xenografts (PDX) models [48], which albeit useful, lack an immunocompetent host environment and therefore, do not fully mimic the immunobiology of iCCA patients. In a recent study, a series of murine organoid lines have been engineered from *TP53* null mice with *FGFR2* fusions constructs [49], which could be potentially injected into immunocompetent syngeneic hosts (*i.e.*, same genetic background as the organoids). Nonetheless, loss of *TP53* and *FGFR2* fusion rarely co-occur in human iCCA [50] while orthotopic transplantation of fully developed tumor bypasses the initial steps of tumorigenesis, potentially leading to aberrant inflammatory responses. Future studies in iCCA aimed at establishing clinically-relevant *FGFR2* fusion-driven models are anxiously awaited since they will offer the valuable opportunity to understand the impact of this alteration on the TIME of iCCA and provide the rationale for more effective ICI combinations able to enhance the extent of initial responses in FGFR2-driven iCCA patients. The other two non-inflamed classes, named *Tumor classical* and *Desert-like*, showed enrichment of co-occurrent mutations in *KRAS* and *TP53* genes, and were significantly associated with abundance of regulatory T cells. Cooperation between *TP53* and *KRAS* in driving immune evasion has been described in ovarian cancer [51], although no studies have been reported in iCCA.

The 'inflamed class' represents less than 35% of this cohort and encompassed two classes named "*Immune classical*" and "*Inflammatory Stroma*". Despite that both classes were enriched in immune infiltration, great heterogeneity was observed in terms of their stromal characteristics, with the *Inflammatory Stroma* being enriched in myofibroblastic CAFs, signatures of resistance to ICIs and *KRAS* mutations. In this regard, emerging evidence suggests that gain-of-function *KRAS* mutations contribute to the immunosuppressive TIME that supports cancer growth through various mechanisms and thus, compromise clinical responses to ICIs [52-55]. Accordingly, treatment of colorectal cancer murine models with sotorasib, the first FDA-approved $KRAS^{G12C}$ inhibitor, results in an increase of actively proliferating T cells and produces durable responses in combination with PD1 moAb [56]. In line with this, we demonstrated that *KRAS* inhibition with the potent BI3406 was able to sensitize *KRAS*-mutant iCCA to anti-PD1 in a subcutaneous model of the disease and in a *KRAS*-mutant iCCA patient-derived organoid co-culture with autologous T cells, further confirming a key immunosuppressive role for mutant *KRAS* in iCCA.

Considering the emerging data with selective inhibitors in both *KRAS* [1]- and *IDH1* [35]-mutant iCCA, it is clear that targeting tumor-intrinsic vulnerabilities that modulate anti-tumor T cell responses may identify new combinations for improving the

efficacy of ICIs in this malignancy. To do so, preclinical models able to reproduce the underlying genotype and tumor immune cell interactions of iCCA are urgently needed. In our study, we performed a comprehensive immunogenomic cross-species analysis between human iCCAs and the most commonly used hydrodynamic gene delivery-based mouse models [1]. These models offer several advantages compared to traditional germline-based genetically engineered mouse models since they are able to quickly develop autochthonous and mosaic liver tumors harboring specific and customizable genetic alterations. In these models, plasmid DNA either targeting tumor suppressors or inducing overexpression of oncogenes are delivered to the hepatocytes within the liver by hydrodynamic tail vein injection [57]. Transient transduction of the hepatocytes induces their trans-differentiation into cholangiocytes and subsequent neoplastic transformation as demonstrated in previous lineage tracing studies [58-60]. The hepatocytic origin of iCCA is consistent with genomic studies of human samples [61] suggesting not only that iCCA can derive from both hepatocytes and cholangiocytes, but also that hepatocytes may actually represent the main source, ultimately generating a lot of controversy in the field. Interestingly, in further support of genomic studies in human samples [61,62], we identified hydrodynamic gene delivery-based mouse models able to closely recapitulate the immunobiology of two largest STIM classes, *Inflammatory Stroma* and *Hepatic Stem-like*, suggesting that, regardless of their cell-of-origin, hepatocyte-derived iCCAs are indeed bone fide iCCA and highly resemble the human disease. In particular, we described that murine tumors driven by activating *KRAS* mutations and p19 loss closely resemble iCCA tumors of the *Inflammatory Stroma*, which are enriched in *KRAS* mutations with abundant stroma and immune infiltration. Similar to their human counterpart, *KRAS*-driven murine tumors showed a significant increase in in $CD8^+$ T cells and enrichment of dysfunctional $CD8^+PD1^+$ T cells. On the other end, murine tumors carrying NOTCH1 or YAP1 activation in combination with AKT1 activation resembled cold tumors of the *Hepatic Stem-like*. Overall, this analysis demonstrates validity of these mouse models in general, and for pre-clinical studies of ICIs in particular.

The discovery of targetable *FGFR2* fusions and *IDH1* mutations has accelerated the use of personalized treatment approaches in iCCA. However, whether our STIM classifications of iCCA could effectively guide the management of the diseases still remains unclear. Similarly, the predictive role of tumor mutational burden (TMB), neo-antigen burden (TNB), and chromosomal aberration load in predicting response to ICI in iCCA requires further elucidation. Interestingly, while we did not observe any association between our STIM classes and TMB or TNB [1], a recent study reported that highly infiltrated tumors are associated with significantly higher TNB, suggesting that the immunogenicity of the underlying tumor mutations might direct immune infiltration [36]. Similarly, we observed that focal chromosomal losses impacting genes of the interferon pathway were more frequent in the non-inflamed tumor classes, potentially impacting their immunogenicity [1]. The use of immunogenomic information in clinical practice would require validation in prospective clinical trials using pre- and post-treatment biopsies and high-resolution technologies, such as single cell profiling. However, great challenges exist to guarantee routine access to tissue biopsies and overcome the limitation of high intratumor genomic and immune heterogeneity. A valid alternative would be to rely on the use of liquid biopsies to detect circulating biomarkers [63]. Given the emerging association between the genotype and immunogenicity of iCCA, an alternative would be the detection of oncogenic mutations (i.e. *KRAS*, *TP53*, *IDH1*) or mutation/neoantigen load using circulating tumor DNA to guide the selection of patients for ICI combination strategies. To achieve these clinical goals, improved pre-clinical models that faithfully recapitulate diverse patient-specific and tumor-intrinsic genotypes will significantly advance our understanding of the mechanisms underpinning how the underlying genotype influences immunogenicity as well as the efficacy of ICI in iCCA.

While extremely valuable, murine tumors can feasibly model only few genetic alterations and therefore reproducing the genetic heterogeneity of human iCCA in mice remains difficult. Given that the crosstalk between oncogenic pathways instructs the composition of the TIME and dictate therapeutic efficacy [51,64], precision modeling of tumor genetic heterogeneity in a patient-specific manner is pivotal for the discovery of optimal treatment strategies. To overcome these limitations, recent advances in 3D cell cultures have led to the development of cancer patient-derived organoid models (PDO) that reproduce the underlying (epi)genetic, proteomic, morphological and pharmacotypic features of primary patient tumors [65-68], including CCA [69]. Comparison between drug responses in patients with gastrointestinal cancers and matched PDOs showed >80% accuracy in predicting positive treatment response and 100% accuracy in predicting no response [70]. Thereby, PDOs have been extensively used in drug screens and have shown feasibility for drug discovery in different cancers [71-73]. A significant improvement in PDO modeling is the incorporation of immune cells to study tumor-immune cell interactions and responses to ICIs in a patient-specific manner. However, only few approaches leverage PDO/immune cell co-cultures. In air-liquid interface cultures, PDOs are grown in collagen in an inner trans-well from minced tumor fragments [74] and preserve diversity of endogenous immune cells for a maximum of ~4-6 weeks [75]. Alternatively, a key study demonstrated the feasibility of generating PDO-reactive T cells from peripheral blood of colorectal and lung cancer patients [76], and CD8 T cell responses were observed in few samples upon treatment with PD1 ICI [76]. In iCCA, the only study that attempted PDO/T cell co-cultures from three patients used allogeneic blood–derived T cells, which were polyclonally stimulated to elicit a response [77]. Such PDO/T cell interactions do not recapitulate tumor-specific T cell (TST) interactions and immune synapse formation, which are key to functional T cell responses to ICI. Furthermore, the latter studies do not use intratumoral T cells that have been exposed to the TIME of the primary tumor and lack functional readouts that capture the breadth of anti-tumor T cell responses. Lastly, major challenge in these approaches is reproducing the full spectrum of cellular interactions within the TIME. Nevertheless, T cells are key effectors of anti-tumor immunity and optimizing autologous PDO/T cell co-cultures is advantageous in modeling tumor-T cell interactions, identifying TST and testing strategies targeting tumor vulnerabilities in combination with ICI.

In conclusion, despite the great advancements in our understanding of the immunobiology of iCCA at the bulk and single cell level, a lot more work remains to be done. Future studies should focus on dissecting the mechanisms underpinning immune evasion and immunosuppression driven by different tumor-intrinsic alterations. At the same time, more accurate preclinical platforms

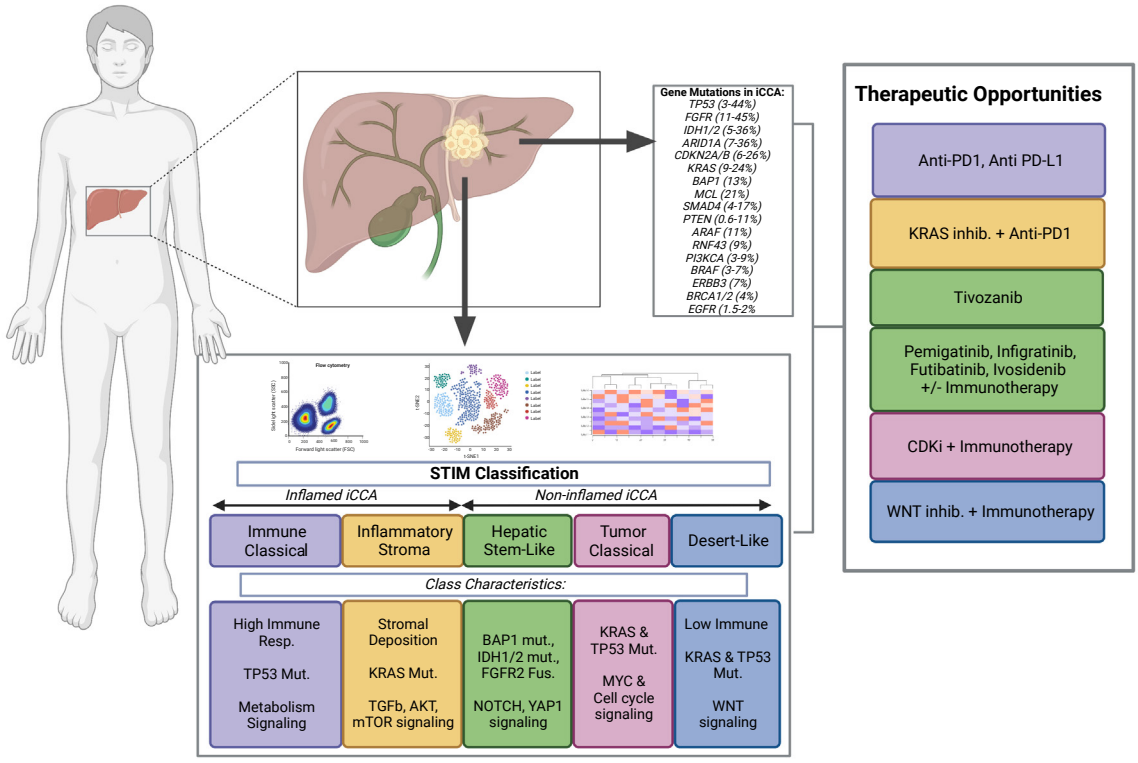

Figure 1: **A novel immune microenvironment-based classification of iCCA**. The STIM classification is based on the integrative analysis of genomic, transcriptomic and immunophenotypic analysis of a large cohort of human iCCA samples. Characteristics of each class are indicated. Potential therapeutic opportunities include small-molecule inhibitor and immunotherapy combinations targeting characteristics of specific tumor profiles. BAP1: BRCA1-Associated Protein 1; CDKi: Cyclin Dependent Kinase inhibitor; CNV: Chromosomal Number Variation; FGFR: Fibroblast Growth Factor Receptor; IDH1/2: Isocitrate Dehydrogenase 1/2; KRAS: Kirsten Rat Sarcoma; TP53: Tumor Protein 53.

for large ICI screenings are anxiously awaited. Clinical trials should include genomic analyses of pre-treatment biopsies to shed light on potential biomarkers of response and resistance to ultimately optimize patient selection and improve the outcome of patients affected by this fatal malignancy.

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Disclosure

The authors declare no conflict of interest.

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