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Integrative genomics highlights opportunities for innovative therapies targeting the tumor microenvironment in gallbladder cancer

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Abbreviations: BTC, biliary tract cancer; CAFs, cancer-associated fibroblasts; CCA, cholangiocarcinoma; GBC, gallbladder cancer; ICIs, immune checkpoint inhibitors; MDSCs, myeloid-derived suppressor cells; OS, overall survival; TGF β , transforming growth factor beta; TIDE, tumor immune dysfunction and exclusion; TIME, tumor immune microenvironment.

Gallbladder cancers (GBC) together with intrahepatic and extrahepatic cholangiocarcinoma (CCA) constitute rare malignancies of the biliary tract [1]. Although all biliary tract cancers (BTC) are associated with dismal outcome, GBC is the most common and aggressive one, with an estimated incidence of 2.5 per 100,000 individuals worldwide and a 5-year overall survival (OS) rate of less than 5% [2, 3]. According to the GLOBOCAN 2018 data, GBC accounts for 1.2% of all diagnosed cancers, but 1.7% of all cancer-related deaths [2, 3]. GBC exhibits a dismal outcome due to a high frequency of late diagnosis and a lack of long term curative therapeutic options. Striking geographical and ethnicity-related variations in the incidence rate of GBC are observed, suggesting that genetic variations contribute to GBC [1]. GBC is also one of the few cancers more common in women than in men. Besides geographical and gender bias, chronic inflammation is the most important risk factor for GBC [1].

Surgery is currently the only curative option but few patients are eligible for surgery being usually diagnosed at an advanced stage. GBC onset and progression are usually asymptomatic at early stages. Cytotoxic chemotherapy remains the mainstay of treatment in first-line metastatic BTC, including GBC, but with modest efficacy regarding to long-term survival [4]. Combined chemotherapy did not improve OS compared with single-agent chemotherapy as second-line treatment in patients with advanced GBC [4]. A better morpho-molecular characterization of GBC is urgently needed to better stratify GBC patients into clinically relevant subgroups and to identify new actionable alterations. A deep and comprehensive characterization of GBC gene expression profiles constitutes also an important prerequisite to identify potential companion biomarkers to stratify patients, especially for first-line therapies [4].

In this issue of *Journal of Hepatology*, Nepal and colleagues address this issue by establishing a comprehensive landscape of genetic, epigenetic and transcriptomic alterations in a large cohort of patients with GBC [5]. Importantly, by integrating various data from DNA methylation changes, copy number analysis, RNA sequencing and whole exome sequencing,

they highlight clinically-relevant tumor subgroups associated with prognosis (**Figure 1**). The study further suggests that targeting the tumor immune microenvironment (TIME), alone or in combination with cytotoxic drugs, may provide effective therapeutic opportunities in GBC.

To date, only few large-scale studies focusing on GBC have been performed. In some studies GBC were included together with CCA revealing that GBC and CCA exhibit some distinct and common molecular alterations [6, 7]. Common mutations included *TP53*, *ARID1A* and *PI3KCA*. In contrast, mutation frequencies of *IDH1*, *BAP1* and *FGFR* fusions were very low or absent, while mutations of the ERBB family of proteins were higher in GBC compared to CCA [8, 9]. Thus, morphologically and molecularly GBC and CCA are clearly separate entities, suggesting also significant differences in molecular pathogenesis. A large integrative study of GBC patients from Korea, India and Chile analyzing exomes and transcriptomes found that mutated genes not previously linked to GBC include the ETS domain containing genes, *ELF3* and *EHF* [10]. Interestingly, *ELF3* mutations were predominantly frame-shift alterations resulting in several neoantigens which may activate CD8+ T cells, indicating that they may serve as potential cancer vaccine candidates [10].

Alterations in the Wnt pathway have been observed at the genomic or transcriptomic level in both GBC and CCA [10-12]. Mutations in the central Wnt pathway genes, *CTNNB1* and *APC*, have been detected in GBC and may lead to constitutively increased Wnt signaling [10]. Moreover, a study focusing on DNA methylation profiling revealed gradual hypermethylation and repressed gene expression of *APC* from non-neoplastic epithelia via dysplasia to invasive GBC [13]. Highlighting the importance of Wnt signaling, the integrative analysis by Nepal and colleagues on a large cohort of GBC patients demonstrated that Wnt signaling is among the most significantly affected pathways in GBC. Furthermore, this study identified three GBC subtypes based on the transcriptional profiles of which subtype 2 had relatively long survival, whereas subtypes 1 and 3 had poor survival (**Figure 1**).

Besides patient survival and gene expression, multiple differences in histomorphology and the tumor microenvironment were observed between the three subtypes. However, no significant differences in mutation profiles were observed in the three subtypes possibly due to low mutation frequencies. Histomorphological analysis by Nepal and colleagues showed an enrichment of biliary, gastric foveolar or intestinal growth patterns of subtype 1, subtype 2 and subtype 3, respectively [5]. Interestingly, this morpho-molecular subtyping resembles the pattern of intracholecystic papillary neoplasm, the known premalignant lesion of the gallbladder. This may open further interesting questions regarding tumor differentiation and malignant transformation processes. However, independent validation of this finding and identification of tissue biomarkers associated with the histological subtypes would provide additional support for association of gastric foveolar GBC with good prognosis. Furthermore, subtype 2, which is associated with good outcome, exhibited high levels of T cell infiltration, high tumor cell content and increased metabolism, whereas subtypes 1 and 3 (poor outcome group) showed a hypoxia signature, high levels of cancer-associated fibroblasts (CAFs), high levels of MDSCs (myeloid-derived suppressor cells), and high Tumor Immune Dysfunction and Exclusion (TIDE) scores indicating T cell exclusion and dysfunction (**Figure 1**) [5]. MDSCs have been shown to exhibit strong immunosuppressive effects by upregulation of regulatory T cells and immune suppressive cytokines and to support the conversion of fibroblasts to CAFs [14]. Therefore, the study by Nepal and colleagues sheds new light on the tumor microenvironment of GBC in poor and good outcome subgroups supporting the hypothesis that immunosuppression and microenvironment remodeling may account for poor patient survival and possibly poor response to immune checkpoint inhibitors (ICIs) (**Figure 1**).

Immunotherapy phase 2 trials studying anti-PD-(L)1 as monotherapy or combined with anti-CTLA-4 previously demonstrated disappointing response rates (5 to 16%), suggesting that only a minority of patients could benefit from immunotherapy [15, 16]. PD-L1 expression was not

suggested as a strong predictive factor of efficacy. Despite these disappointing results, large phase 3 trials were launched, based on a combination of standard of care chemotherapy with ICIs. These trials, run in an unselected population, are unlikely to identify the subgroup of patients that might benefit. The work of Nepal and colleagues, aiming at deciphering the different TIME present in GBC, could thus represent a major step forward for future clinical trial development [5]. Similar analyses were previously done in CCA, highlighting clinically relevant subgroups of patients who may benefit from immunotherapies based on specific TIME [17]. The subgroup of GBC with a better prognosis identified by Nepal and colleagues would be at first glance a good target for evaluating ICIs. In contrast, poor prognosis GBC were associated with dysregulation of immune-related pathways, including dysfunctional T cells and T cell exclusion [5]. In agreement with the presence of an immunosuppressive microenvironment, poor prognosis GBC also exhibited expression of markers linked to the activation of transforming growth factor beta (TGF β), a potent immunosuppressive cytokine (**Figure 1**). This again could be of value in the current effort of bringing TGF β inhibitors to the clinics. Indeed, bifunctional antibodies targeting TGF β with either CTLA-4 or PD-L1 were suggested to improve results of anti-CTLA-4 or anti-PD-L1 antibodies, due to adequate local sequestration of TGF β within the TIME [18]. Interestingly, preliminary results of a clinical trial testing bispecific anti-PD-L1/anti-TGF β suggested activity with a 23% response rate [19]. Once again, selecting the appropriate population might greatly improve predictive testing and achieving positive results in clinical trials. Finally, the author studied the TIDE signature of potential response to immune checkpoint inhibitors [20]. This analysis suggested that subtype 2 GBC might be the focus of interest for the implementation of ICIs.

Taken collectively, the study by Nepal and colleagues suggests that different prognosis in GBC depends on the tumor cell properties and/or the TIME. The study further suggests that a comprehensive analysis of the TIME might represent the best approach to identify the population

of interest for the development of immuno-oncology drugs in GBC (**Figure 1**). Combined therapies targeting actionable alterations in tumor cells as well as in the TIME are emerging as relevant strategies to treat GBC, and also liver cancer. We believe that testing combination therapies should be considered together with relevant biomarkers for specific GBC subtypes. Biomarker-driven clinical trials would further help in better disease management and appropriate therapy selection.

Figure 1. Molecular stratification of GBC: implication for therapy.

In this issue of *Journal of Hepatology*, Nepal and colleagues report an integrative molecular analysis of a large cohort of GBC. Genomic alterations in the WNT, MAPK and p53 pathways were observed. The study highlights three GBC subtypes with differential prognosis. GBC with a better prognosis exhibit increased metabolism and infiltration of functional T cells, suggesting that this subtype may benefit from ICIs. On the contrary, GBC with a worse prognosis exhibit an immunosuppressive stroma and activation of the TGF β pathway, suggesting that TGF β inhibitors may offer potential in this subtype. TILs, tumor infiltrating lymphocytes.

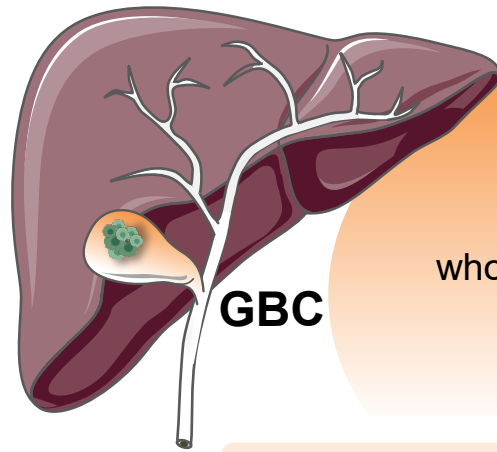
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Author names in bold designate shared co-first authorship

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integrative genomics

whole exome sequencing
DNA methylation
RNA sequencing

genomic alterations

WNT pathway
MAPK pathway
p53 pathway

clinically relevant GBC subtypes

