TITLE: Circulating tumor cells and cholangiocarcinoma

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LIST OF ABBREVIATIONS

CCA, cholangiocarcinoma

iCCA, intrahepatic cholangiocarcinoma

pCCA, perihilar cholangiocarcinoma

dCCAs, distal cholangiocarcinoma

HCC, hepatocellular carcinoma

CTC, circulating tumor cell

PSC, primary sclerosing cholangitis

TEXT

Cholangiocarcinoma (CCA) is the second most common primary hepatobiliary malignancy, with solid epidemiologic data suggesting that its incidence has steadily increased in the last two decades. CCA is generally classified according to its anatomical location along the biliary tree into intrahepatic (iCCAs), perihilar (pCCAs) and distal (dCCAs) (1). Histological, clinical and molecular data indicate that in addition to their different anatomical location, there may be inherited differences in terms of biological behavior among theses CCA subtypes. Indeed, their clinical course and invasive behavior is quite different. Unfortunately, most patients are still diagnosed at advanced stages where palliative treatments provide limited survival benefits (1). In terms of molecular prognosis prediction, a recent study showed how nuclear expression of S100A4 in neoplastic ducts was associated with poor response to surgical therapy in CCA patients (2). In terms of circulating markers, there has been some refinements including the diagnostic role of CYFRA 21-1 (a fragment of CK19), which has been suggested to correlate with circulating tumor cells in biliary tract cancers (3). Recent reports using deep-sequencing technologies identified candidate oncogenic drivers in iCCA. In particular, gene fusions involving *FGFR2* (4) have been shown to induce tumor formation in experimental models of iCCA, what highlights FGF inhibition as a potential new therapeutic strategy in these patients. Other common genetic defects include mutations in *IDH* and *KRAS*. Similar to hepatocellular carcinoma (HCC), clinical practice guidelines for CCA don't include any molecular from the tumor in their recommended management strategy.

The study of circulating tumor cells (CTCs) has emerged as a potential source of novel biomarkers in oncology. CTCs are cancer-derived cells released from solid tumors into the bloodstream; they bear tumor-initiation properties and can eventually drive metastasis formation, particularly when they group in CTC clusters (5). In this context, the cell junction component plakoglobin seems to participate in CTC cluster formation and stabilization, being

a potential new target to prevent metastatic cancer spread. A number of studies have evaluated the role of CTC as biomarkers to predict recurrence, survival or response to therapy in different tumor types. Most of them use CTC enumeration as a surrogate of metastatic potential, and hence a marker of poor outcome. CTCs are relatively rare in the bloodstream and more likely to be detected in patients with metastatic disease, what questions its role as a biomarker at early tumor stages. The majority of CTCs isolation methods rely on capturing cells that express cytokeratin and EpCAM, and that are also CD45 negative so immune cells are discarded. Other enrichment methods use physical properties of malignant cells such as size. The prognostic performance of CTCs in other liver tumors such as HCC has been thoroughly explored, mainly in studies using EpCAM-based capture methods. Data from gene expression studies suggest that only a small fraction (10-20%) of HCC expresses EpCAM, so it is feasible that CTC enumeration may be underestimated in these studies. Interestingly, some of these reports even analyzed specific genetic traits within these CTCs, to ascertain their potential as drivers of metastasis formation.

Data on the prognostic role of CTCs in CCA are scarce. In this issue of Hepatology, Yang et al. (6) reports the association of the number of CTCs with more aggressive tumor characteristics and lower survival in CCA patients. Despite this is not the first analysis of CTC in CCA (7), it is the largest of this kind performed so far that included correlation with clinical outcomes. The study included CTC enumeration in a cohort of 88 patients using the semi-automated CellSearch (Janssen Diagnostics), the first CTC enumeration system to achieve US Food and Drug Administration approval. Study population was markedly heterogeneous, what somehow limits extrapolation of the outcome results. Authors found an association between CTC number and baseline tumor features suggestive of increased tumor burden such as size, multinodularity and loco-regional lymph node invasion. It was also correlated with overall survival using 2 different cut-offs, CTCs ≥ 2 and CTCs ≥ 5, and it was

an independent predictor of survival in this dataset along with other well know clinical variables. CTC enumeration allowed to stratify patients with extrahepatic disease, since those with higher CTC number also had shorter survival. Similarly, subgroup analyses according to stage (i.e., AJCC/UICC TNM system) confirmed the prognostic role of CTCs across stages, particularly when using the cut-off of CTC ≥ 5. Unfortunately, number of patients is relative low in these sub-analyses, so these results should be considered with caution. Also, the optimal CTC cut-off value has been under some debate in the oncology field, but solid data suggest that healthy individuals and those with non-malignant conditions rarely have more than 2 circulating epithelial cells. There is not much data on CTCs in patients with primary sclerosing cholangitis (PSC), a well-known risk factor for CCA. None of the PSC-CCA patients in Yang's study had detectable CTCs, but numbers are still small to drive a definitive conclusion on the presence of circulating epithelial cells as a potential confounding factor in patients with PSC-CCA.

The study has some limitations. First one relates to the capture method, that relies on EpCAM expression and could eventually miss non-EpCAM CTCs, so the actual CTC number could be underestimated. Paired analysis of EpCAM expression in tumor tissue with CTC enumeration could help understand the extent of this limitation. Also, the patient population included is quite heterogeneous, ranging from patients at early stages treated with surgery to those with metastatic disease that received chemotherapy. Along with this, some of the proposed sub-analyses might be underpowered to accurately capture prognostic differences. Despite this, the study provides initial evidence of the prognostic role of CTCs in CCA, and further reinforces the notion that circulating tumor biomarkers could have a role in decision-making for patients with liver malignancies.

CTC enumeration is framed within the concept of 'liquid biopsy', that encompasses the analysis of tumor by-products in peripheral blood as a source of novel prognostic and

predictive biomarkers (8). In addition to CTC, the predictive value of circulating cell-free DNA and RNA is being thoroughly evaluated in different malignances. Of note, a recent report that compared the performance of both circulating tumor DNA (ctDNA) and CTCs to detect tumor-specific molecular events suggests that the level of ctDNA tends to be higher than of CTCs (9). It has also been suggested that tumor cells may actively release cell-free nucleic acids to promote metastasis formation. For instance, exosomes released by pancreatic cancer cells induce liver pre-metastatic niche formation and increase metastatic burden (10). Exosomes are small membrane vesicles capable of transferring DNA, mRNA and proteins between cells. High expression of macrophage migration inhibitory factor (MIF) within pancreatic cancer cell-derived exosomes activates fibrotic and inflammatory pathways via Kupffer cell uptake, what constitutes a favorable milieu for metastatic deposit. Ultimately, a combined vector integrating molecular information from plasmatic nucleic acids coupled with data from CTCs may maximize prognostic and predictive performance of liquid biopsy. Potential applications are endless, including patient stratification or evaluation of molecular tumor response after palliative treatments. As with any other potential new biomarker, confirmatory data generated on well-designed studies will be critical, but certainly, liquid biopsy could become a very useful tool in the clinical management of patients with CCA.

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