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Detection and Analysis of Circulating Epithelial Cells in Liquid Biopsies From Patients With Liver Disease

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Abstract

Epithelial cells in the circulation (circulating epithelial cells, or CECs) are analyzed as a non-invasive method to detect cancers; we investigated whether analysis of hepatocytes in the

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I.B., K.M., J.P., M.K., J.W.F., M.C. performed the experiments

L.G., A.X.Z., R.T.C. consented patients and provided clinical data

I.B., K.M., L.G., J.P., M.A., D.T.T. analyzed the data

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circulation can identify patients with chronic liver disease or hepatocellular carcinoma (HCC). We previously developed a cell-sorting device to isolate CECs from patient blood samples and combined it with an mRNA analysis system to identify CECs with liver-specific markers. We tested the ability of this device to detect CECs of hepatocyte origin in blood samples from healthy individuals (n=10), patients with chronic liver disease without HCC (n=39), and patients with HCC (n=54), using immunofluorescence. We confirmed hepatocyte origin using RNA-sequencing analysis. We found a similar concentration of circulating hepatocytes in blood samples from patients with chronic liver disease and HCC but an increased concentration from patients with advanced fibrosis compared to those without advanced fibrosis. Circulating hepatocytes isolated from patients with HCC had a different gene expression profile than those from patients with chronic liver disease. This system for detecting and analyzing circulating hepatocytes might be used in the evaluation of benign and malignant liver disease.

Summary:

We developed a system to detect liver cells in the circulation. These cells can be analyzed to identify patients with liver diseases, including cancer.

Keywords

biomarker; liver cancer screening; hepatitis; detection

Liquid biopsy refers to sampling cellular material that originated from a solid organ and has entered the bloodstream. Circulating epithelial cells (CECs) can be detected by liquid biopsy in the setting of localized cancer^{1, 2} and even preneoplastic pancreatic lesions,^{3, 4} suggesting their presence is not exclusive to carcinogenesis. Hepatic CECs or “circulating hepatocytes,” which have yet to be described in the absence of malignancy, could serve as a powerful biomarker in the diagnosis and monitoring of chronic liver disease and hepatocellular carcinoma.

Isolating CECs is a technological challenge due to their rarity in the bloodstream and the variable expression of antigens used for cell capture. For example, the EpCAM-dependent Veridex platform yielded HCC CEC detection rates of only 35% and 41% in two independent studies.^{5, 6} To overcome this limitation, we developed an antigen-agnostic cell sorting device called the iChip, which isolates CECs while preserving cell viability and high-quality RNA content. We previously combined the iChip with an RNA signature based on established liver-specific markers to create an assay for the enrichment and detection of CECs in HCC.⁷ In the current work, we aimed to use the iChip platform to detect CECs in CLD patients without HCC and to phenotypically discriminate between CECs in patients with and without HCC.

We first aimed to detect CECs by immunofluorescence (IF). Blood samples were obtained from 10 healthy blood donors, 39 CLD patients undergoing routine clinical surveillance for but had no evidence of HCC, 54 patients with HCC, and 10 HCC patients who underwent curative treatment and had no clinical evidence of disease (NED) (Supplementary Tables 1-4). The iChip performed size-based exclusion of red blood cells, platelets and plasma,

followed by magnetophoresis of labelled white blood cells (WBCs) (Figure 1A).⁸ CECs were then enumerated by IF staining for glypican-3, an oncofetal protein expressed in HCC but also in CLD liver tissue,⁹ or cytokeratin, an epithelial marker (Figure 1B). Using a threshold of 5 cells per 10 mL of whole blood, we identified CECs in a similar proportion of CLD (79%), HCC (81%), and NED patients (90%), but only in 5% of healthy donors (Figure 1C and Supplementary Figure 1A, 1B; $p < 0.01$, each group vs. healthy donors). iChip purification combined with immunofluorescent quantification demonstrated a high sensitivity for CEC detection with similar concentrations in HCC and CLD patients. Amongst CLD patients, those with advanced fibrosis (METAVIR F3 or F4) had a higher concentration of CECs (median 5.1 cells/mL) in comparison to those without advanced fibrosis (0.7 cells/mL, $p < 0.01$, Figure 1D). Because the CLD study population consisted only of patients with sufficiently high risk of HCC to undergo surveillance, the etiology of CLD for each patient in the subgroup without advanced fibrosis was hepatitis B infection. The difference in CEC concentration associated with fibrosis stage did not appear to be due to CLD etiology, as the trend persisted when the analysis was restricted to only those with hepatitis B-induced CLD (median 5.0 cells/mL with advanced fibrosis, 0.7 cells/mL without advanced fibrosis, $p = 0.06$, Supplementary Figure 1C). Otherwise, there was no difference in CEC concentration by CLD etiology (Supplementary Figure 1D).

As an orthogonal approach to detecting CECs, we used RNA-sequencing (RNA-seq). To determine the sensitivity of this approach, 0, 1, 3, 5, 10, or 50 HepG2 HCC cells were spiked into 4 mL of healthy donor blood and processed through the iChip for RNA-seq. HepG2 specific gene expression was detectable in whole blood from a single cell (Figure 2A). We then turned to identifying CECs in clinical blood samples from 64 CLD and 52 HCC patients. First, we created a 17 liver-specific gene signature based on Genotype Tissue Expression (GTEx) expression data. Liver-specific genes were identified in samples from both patient groups but were absent in WBC subtypes flow-sorted from iChip-processed blood (Figure 2B). Therefore, our data suggested that the liver-specific signature identified rare CECs rather than aberrant expression of these genes in contaminating WBCs.

As a proof of concept that CECs may phenotypically differ depending on the underlying disease state, we pursued gene expression profiling to identify qualitative rather than quantitative differences between CECs in the setting of CLD versus HCC. Our approach is outlined in Figure 2C. Using The Cancer Genome Atlas (TCGA) database, we identified 166 genes overexpressed in HCC compared to liver tissue and excluded genes expressed in WBCs. We then used a Random Forest (RF) machine learning approach to generate a classifier based on these genes to distinguish CLD from HCC CECs. In this approach, each decision tree in the random forest cast a “vote” classifying a sample as CLD or HCC. The final classifier used 25 genes (Supplementary Table 5). Notably, 3 of the most informative genes in the classifier (*TESC*¹⁰, *SLC6A8*¹¹, *SPPI*¹²) have been implicated in cancer metastasis and another (*E2F1*) is an established cell proliferation marker. The cross-validated classifier provided excellent separation between CLD and HCC samples, with a preliminary sensitivity of 85% at a specificity of 95% and with identification of both early and late stage HCC (by Milan criteria) (Figure 2D, Supplementary Figure 2). In comparison, recent work combining cell-free DNA and protein blood-based biomarkers had an accuracy of only 44% for predicting HCC likely due to the lack of common recurrent mutations and

specific protein markers inherent to HCC.¹³ Given these limitations, our CEC RNA approach would be complementary in the study of HCC.

Here we report the novel detection of cells from diseased livers circulating in the bloodstream both by immunofluorescence and RNA-seq and the potential to use these cells as biomarkers. Important applications of this liquid biopsy may include CLD etiology determination, fibrosis staging, and HCC surveillance. Further study of CECs could open a new field of biomarker development leading to a spectrum of non-invasive diagnosis and monitoring techniques for patients with liver disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: Immunofluorescence of hepatic CECs from peripheral blood. **(A)** The iChip depletes hematopoietic cells, leaving a sample enriched for CECs, which were analyzed by immunofluorescence. **(B)** Cells from iChip-processed blood from patients with HCC or CLD stained for DAPI (blue), CD45 (green), glypican-3 (GPC3, yellow), and wide-spectrum cytokeratin (CK-WS, red). A white blood cell (WBC) is shown for comparison. **(C)** Enumeration of CECs by immunofluorescence in iChip-processed blood samples from healthy donors (HD) or patients with CLD, HCC, or treated HCC with no evidence of malignant disease (HCC NED). **(D)** Enumeration of CECs in CLD patients with early stage disease or advanced fibrosis. P-values by Mann-Whitney test and significant at a Bonferroni-adjusted significance level of 0.05.

Figure 2:

RNA-seq of hepatic CECs from blood. **(A)** Heatmap of the HepG2 gene expression signature in control blood, control blood spiked with 1-50 HepG2 cells, and HepG2 single cell RNA-seq. **(B)** Heatmap of the liver-specific gene signature in CLD patients, HCC patient, or flow-sorted WBCs (B, B cells; C, cytotoxic T cells; H, helper T cells; M, monocytes; N, NK cells; G, granulocytes). Heatmap units are reads per million plus one, log2-transformed. **(C)** Schematic of the random forest algorithm. **(D)** HCC score (vote fraction from the random forest classifier) in CLD, early stage HCC, and late stage HCC. P-values by Mann-Whitney test and significant at a Bonferroni-adjusted significance level of 0.05.