

An RNA-based signature enables high specificity detection of circulating tumor cells in hepatocellular carcinoma

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Circulating tumor cells (CTCs) are shed into the bloodstream by invasive cancers, but the difficulty inherent in identifying these rare cells by microscopy has precluded their routine use in monitoring or screening for cancer. We recently described a high-throughput microfluidic CTC-iChip, which efficiently depletes hematopoietic cells from blood specimens and enriches for CTCs with well-preserved RNA. Application of RNA-based digital PCR to detect CTC-derived signatures may thus enable highly accurate tissue lineage-based cancer detection in blood specimens. As proof of principle, we examined hepatocellular carcinoma (HCC), a cancer that is derived from liver cells bearing a unique gene expression profile. After identifying a digital signature of 10 liver-specific transcripts, we used a cross-validated logistic regression model to identify the presence of HCC-derived CTCs in nine of 16 (56%) untreated patients with HCC versus one of 31 (3%) patients with nonmalignant liver disease at risk for developing HCC (P < 0.0001). Positive CTC scores declined in treated patients: Nine of 32 (28%) patients receiving therapy and only one of 15 (7%) patients who had undergone curative-intent ablation, surgery, or liver transplantation were positive. RNA-based digital CTC scoring was not correlated with the standard HCC serum protein marker alpha fetoprotein (P = 0.57). Modeling the sequential use of these two orthogonal markers for liver cancer screening in patients with high-risk cirrhosis generates positive and negative predictive values of 80% and 86%, respectively. Thus, digital RNA quantitation constitutes a sensitive and specific CTC readout, enabling high-throughput clinical applications, such as noninvasive screening for HCC in populations where viral hepatitis and cirrhosis are prevalent.

circulating tumor cells | early cancer detection | hepatocellular carcinoma | blood biopsy | predictive modeling

he shedding by epithelial cancers of circulating tumor cells (CTCs) into the bloodstream underlies the blood-borne dissemination of cancer, although only a small fraction of CTCs gives rise to metastases (1). Enumeration and analysis of CTCs may thus enable noninvasive monitoring of advanced cancers, as well as early detection of invasive but localized tumors before they give rise to viable metastases. Recent advances in CTC isolation provide sensitive and high-throughput platforms to enrich for these rare tumor cells within blood specimens, but antibody staining and microscopic imaging of captured cancer cells remain a critical bottleneck limiting broad application of the technology (2). Classical CTC staining criteria include the presence of cell surface epithelial cell adhesion molecule (EpCAM) and cytoplasmic epithelial cytokeratins and the absence of the hematopoietic CD45 marker (3), but epithelial marker expression is highly variable and extensive imaging criteria must be applied to score immunofluorescent signals reliably from

rare cancer cells surrounded by contaminating leukocytes (4). Emerging microfluidic CTC isolation technologies that effectively deplete leukocytes without manipulating tumor cells (5) preserve cell viability and ensure high-quality RNA content, as demonstrated by single-cell RNA sequencing studies (6–8). These CTC isolation platforms now enable the application of powerful RNA-based digital PCR (dPCR) technologies to score molecular signatures of cancer cells, thus providing a potentially robust and high-throughput readout for the presence of CTCs within blood specimens. To test the feasibility of RNA-derived digital scoring of CTC-enriched cell populations, we applied this strategy to hepatocellular carcinoma (HCC), a cancer that lacks defining gene mutations but originates in liver cells with unique tissue-specific expression profiles.

Liver cancer is the second highest cause of cancer mortality worldwide, leading to 765,000 deaths in 2015 (9). In the developing world, the high prevalence of hepatitis B virus (HBV) infection drives the incidence of HCC; worldwide, it is estimated that 248 million individuals are infected with HBV (10). The risk of developing HCC is calculated as 0.5–1% per individual per year in HBV carriers without cirrhosis and as high as 8% per individual per year in patients with cirrhosis (11). Developed countries are also witnessing a rise in HCC incidence, linked with cirrhosis due to chronic hepatitis C virus (HCV) infection, alcohol abuse, obesity-associated nonalcoholic fatty liver disease

Significance

The early detection of hepatocellular carcinoma (HCC) is of paramount importance for improving patient outcomes, yet an accurate, high-throughput screening methodology has yet to be developed. By combining microfluidic depletion of hematopoietic cells from blood specimens with absolute quantification of lineage-derived transcripts, we demonstrate the highly specific detection of circulating tumor cells, enabling noninvasive detection and clinical monitoring of HCC.

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(NAFLD), and nonalcoholic steatohepatitis (NASH) (9). Earlystage HCC is potentially curable by thermal ablation, surgical resection, or liver transplantation, with a 5-y survival of 50-80%following these therapies (9). Once the tumor disseminates within or outside the liver, however, therapeutic options are limited and 5-y survival declines to below 15% (11).

Although early detection of HCC in high-risk individuals offers a strategy for successful curative treatment, screening in patient populations with liver cirrhosis has been limited by the poor test characteristics of the primary biomarker, serum levels of alpha fetoprotein (AFP) (11). CTCs have been reported in patients with HCC, but the characteristically low EpCAM cell surface expression in this tumor type has limited the utility of standard CTC measurements (12, 13). To establish a high-throughput, bloodbased assay for HCC that would have broad applicability, we therefore adapted the microfluidic CTC-iChip isolation platform with a digital RNA-PCR readout combining liver-specific transcripts whose expression is retained in HCC.

We applied this molecular CTC assay as proof of principle in a pilot cohort of patients with HCC and high-risk patients suffering from liver disease.

Results

Establishment of the HCC-Specific RNA dPCR Assay. Fig. 1*A* outlines the RNA-based dPCR CTC-scoring assay. CTCs were isolated using the CTC-iChip microfluidic device, which depletes hematopoietic cells from blood by size-based exclusion of red blood cells, platelets, and plasma, followed by magnetic deflection of white blood cells (WBCs) tagged with magnetic bead-conjugated CD45, CD16, and CD66b antibodies (5, 14). The high efficiency depletion of WBCs (4- to 5-log purification) enriches CTCs, which are admixed with some contaminating WBCs (<500 WBCs per milliliter of processed whole blood) (5, 14). To replace CTC imaging with high-throughput detection of CTC-derived transcripts, we coupled whole-transcriptome amplification (WTA) of CTC-derived RNA with dPCR amplification, in which cDNA

molecules are encapsulated within individual aqueous droplets and multiple transcripts of interest are quantified by in-droplet PCR amplification (*Materials and Methods*).

We curated liver-specific transcripts whose expression is preserved in HCC cells, but virtually absent in blood components. Of the 20,000 genes measured from publicly available microarray datasets (15), the 100 most highly expressed genes in HCC were screened against expression profiles of hematopoietic cells and other normal tissues (16). These genes were then validated against a separate RNA-sequencing dataset, comparing 10 primary HCCs (17) with WBCs from eight healthy donor blood samples processed through the CTC-iChip (Fig. 1B). Quantitative RT-PCR (qRT-PCR) of cDNA from purified healthy donor WBCs (n = 3) was used to eliminate genes with low but detectable background signal (Fig. S1). Based on these results, 10 genes [AFP, alpha 2-HS glycoprotein (AHSG), albumin (ALB), apolipoprotein H (APOH), fatty acid binding protein 1 (FABP1), fibrinogen beta chain (FGB), fibrinogen gamma chain (FGG), glypican 3 (GPC3), retinol binding protein 4 (RBP4), and transferrin (TF)] were selected for developing the dPCR assay.

To technically validate this strategy, we determined the number of transcripts measured from the introduction of individually micromanipulated cells from the liver cancer cell line HepG2 known to express albumin into 4 mL of blood from healthy donors, followed by CTC-iChip processing and dPCR for the liver-specific transcript *ALB* (Fig. 1*C*). No *ALB* RNA-positive droplets were observed in unspiked blood processed through the CTC-iChip, whereas a range from 2 to 100 spiked HepG2 cells generated from 240 to 28,800 *ALB* transcripts. Given the heterogeneity of HCC cells within clinical specimens, we applied low-template WTA to maximize starting material and optimized the 10-gene liver-specific panel to ensure that this dramatic signal amplification preserved the relative distribution among multiple markers. We ensured the absence of amplification bias in three independent experimental replicates (Fig. 1*D* and Fig. S2). Compared with unamplified cDNA derived from 1 ng of



Fig. 1. dPCR quantitation of HCC cells after microfluidic enrichment from blood. (A) Schematic representation of the integrated platform for digital RNA-PCR scoring of CTCs. Hematopoietic components are depleted from whole blood through CTCiChip processing as previously described (5). RNA from the CTC-enriched product is subjected to WTA, encapsulation of cDNA molecules within lipid droplets, and PCR amplification for transcripts of interest. (B) Heat maps derived from microarray (Left) and RNA-sequencing (RNAseq) (Right) datasets comparing expression of 10 liver-specific transcripts in HCC versus other tissues. The microarray dataset compares fetal liver, adult liver, and 10 cases of HCC (JZR samples) versus normal tissues (15 samples shown of 79 tissues tested) and blood components (15, 16). RNAseg compares 10 cases of HCC (17), with WBCs collected from eight independent healthy donor (HD) blood samples processed through the CTCiChip. (C) dPCR quantitation of ALB transcripts from micromanipulated HepG2 spiked into whole blood and processed through the CTC-iChip. Each data point represents one-sixth of the CTC-iChip product. (D) Pie charts representing the distribution of transcripts for each of the 10 selected liver-specific genes, following dPCR analysis of 1 ng of HepG2-cell RNA. Samples were nonamplified or subjected to WTA (three independent reactions). (E) Total number of transcripts of interest after spiking increasing numbers of HepG2 cells into blood (n = 3), CTC-iChip processing, and dPCR. (F) Pie charts depicting the relative fraction of droplets for each of the 10 target transcripts after spiking increasing numbers of HepG2 cells into blood and CTC-iChip processing, as noted in E(n = 3).

HepG2 RNA, the increased overall signal resulting from WTA (25,000- to 100,000-fold amplification per gene) preserved the relative proportion of each transcript among the WTA replicates (Fig. S2 A and B).

To test the sensitivity of the 10-gene digital assay in rare tumor cells admixed with blood cells, we again micromanipulated either 1, 3, 5, 10, or 50 single HepG2 cells into 4 mL of healthy donor blood, which was then processed through the CTC-iChip, followed by WTA and dPCR. In two of three replicates, a single spiked HepG2 cell was detected, with an average 5,000-fold increase in signal over unspiked blood controls. All liver-specific genes were detected with progressively increasing signal and preservation of marker distribution as the numbers of input cells increased from 1 to 50 cells ($R^2 = 0.79$; Fig. 1 E and F). The high sensitivity of dPCR raised the possibility that CTC enrichment might not be required to detect tumor-derived transcripts in nucleated blood cell fractions. Past reports have suggested that standard RT-PCR amplification might identify PCR products comigrating with the expected ALB transcript from unpurified blood cells of patients with HCC (18); however, we were unable to reproduce this finding using the more sensitive and specific dPCR technology (n = 9HCC samples; Fig. S3). We therefore applied the combination of microfluidic CTC enrichment followed by dPCR detection of HCC-derived transcripts to clinical specimens from HCC patients.

Digital CTC Detection in Patients with HCC. We evaluated the performance of the optimized digital CTC-scoring assay in blood samples (5–15 mL) from six patient cohorts, per Institutional Review Board (IRB)-approved protocols at Massachusetts General Hospital. The cohorts studied included (*i*) healthy blood donors (n = 26, median age = 55 y); (*ii*) patients with high-risk nonmalignant chronic liver disease (CLD), including hepatitis virus-associated cirrhosis, who were being routinely monitored for HCC development (n = 31, median age = 58 y); (*iii*) newly diagnosed, untreated patients with HCC (n = 16, median age = 66 y); (*iv*) patients with HCC actively receiving therapy with radiographically evident disease (n = 32, median age = 67 y); (v) patients with HCC who had undergone curative-intent interventions, such as ablation, resection, or liver transplantation, and clinically had no evidence of disease (NED) any longer (n = 15, median age = 66 y); and (vi) patients with primary malignancies other than HCC, with or without liver metastases (n = 44, median age = 62 y). Patients in categories *ii* through *vi* were categorized by a clinician blinded to the dPCR data. Clinical characteristics of these cohorts are provided in Tables S1–S6.

dPCR analysis of CTC-iChip-processed blood specimens from patients with HCC generated high signal for individual transcripts, compared with healthy donors, patients with CLD, or patients with other malignancies (Fig. 24). As expected, intracohort variability was observed, with some HCC patient samples exhibiting high signal from multiple liver-specific transcripts and others containing few transcripts of interest. HCC cases in which no signal was detected may reflect the absence of CTCs within the single 5- to 15-mL blood sample or expression of transcripts that are not captured by the 10-gene panel.

To integrate the results of multiple genes into a statistically robust scoring model, we screened each transcript to determine if it served as a statistically significant single-gene predictor of HCC status (Fig. S4). Nine of the 10 genes met this selection criterion (all excluding *GPC3*). These genes were then combined into a single metric CTC score, using a leave-one-out cross-validated (LOOCV) multivariate logistic regression model (Fig. S5). The LOOCV allowed us to build and test the model using a single HCC patient cohort, although the high stringency associated with this approach may underestimate the predictive value of the model; Fig. 2C demonstrates the change in model performance with and



Fig. 2. CTC score from patients with HCC compared with at-risk patients. (*A*) Heat maps depicting relative signal intensities for each of the 10 liver-specific transcripts across different patient cohorts. Primary droplet numbers are log-10-transformed and scaled to the highest value for each transcript. (*Upper*) Healthy donors (blood bank, n = 26) and high-risk patients with CLD under active clinical surveillance for HCC (n = 31). Etiologies of CLD include HBV infection (n = 16), hepatitis C virus (HCV) infection (n = 6), alcohol (EtOH) (n = 6), or other causes (n = 3). (*Middle*) Patients with HCC, classified as untreated (newly diagnosed, n = 16) or receiving ongoing treatment (currently undergoing various therapies, n = 32). Patients are grouped according to Barcelona Clinic Liver Cancer (BCLC) criteria from early clinical stages (0 and A) to advanced clinical stages (B–D). Patients who have completed treatment and have NED are shown (n = 15). Four of these cases represent repeated analysis of patients initially tested before or during treatment (HCC-030_2, HCC-058_2, HCC-060_2, and HCC-064_2). (*Lower*) Patients with cancers of nonhepatic origin metastatic to the liver (MET). Clinical data are listed in Table 56. (*B*) Box plots representing the integrated CTC score for the patient cohorts above. **P < 0.01, ***P < 0.0001 (χ^2 , degrees of freedom = 5). (C) Receiver operator characteristic (ROC) curves for untreated HCC both without (*Left*) and with (*Right*) LOOCV. AUC, area under the curve; FPR, false-positive rate; TPR, true-positive rate.

without cross-validation. We tested the multigene model with 48 patients with HCC versus 57 patients without cancer (Fig. 2C).

Nine of 16 (56%) untreated patients with HCC were classified as positive by CTĆ score, compared with only one of 31 (3%) patients with at-risk nonmalignant CLD and two of 26 (7.6%) age-matched healthy blood donors (P < 0.0001, χ^2 ; Fig. 2B). Patients with HCC undergoing therapy but with radiographically detectable disease had a lower fraction of cases with positive CTC scores [nine of 32 (28%)], but this fraction was still significantly higher than the control population ($P = 0.004, \chi^2$). Patients with NED after curative-intent treatment were only positive in one of 15 (7%) cases, an incidence comparable to the incidence of the control population (P = 0.56, not significant). Together, these results demonstrate that the CTC score can identify patients with active disease while maintaining a high degree of specificity. Among all patients with HCC, the CTC score was not associated with specific underlying risk factors (P =0.73), but it was highly correlated with Barcelona Clinic Liver Cancer staging (P = 0.011) and trended toward significance when stratifying by vascular invasion (P = 0.06; Fig. S6).

We next determined the feasibility of differentiating between patients with HCC and patients with malignancies other than HCC using a separate logistic regression (Fig. S7). In this comparison, six of the 10 genes were statistically significant predictors of HCC vs. non-HCC status (*AFP*, *AHSG*, *APOH*, *FABP1*, *FGB*, and *FGG*), yielding a multipredictor model with different features than the previous model (Fig. S7 *A* and *B*). Among patients with pancreatic, prostate, breast, and non-small-cell lung cancers; cholangiocarcinoma; and melanoma, 39 of 44 (88%) cancers/carcinomas were correctly distinguished from HCC at a sensitivity of 50% for patients with HCC. Optimal differentiation of tissue of origin among CTCs will likely benefit from the inclusion of additional markers that are specific for other tumors, in addition to exclusion of HCC-associated transcripts.

Longitudinal Monitoring of Patients with HCC. The higher incidence of positive CTC scores in newly diagnosed, untreated patients with HCC (56%) compared with those patients with HCC who were undergoing active treatment (28%), and those patients with HCC who completed curative-intent therapy (7%) suggests a potential role for CTCs in longitudinal monitoring of tumor response. Furthermore, increasing tumor burden, as defined by the Barcelona Clinic Liver Cancer staging criteria, is associated with increased CTC score values (Fig. 2A and Fig. S6). A subset of patients with HCC in our study was monitored longitudinally for tumor response. Supporting the robustness of the assay, the CTC score remained high in two patients (HCC-041 and HCC-075), with no therapeutic intervention or change in clinical status between blood draws (Fig. 3A and Fig. S7E). Two other patients (HCC-058 and HCC-060) underwent surgical tumor resection and demonstrated a decrease in CTC score postoperatively (Fig. 3B). The CTC score for patient HCC-042 decreased impressively following an immune checkpoint inhibitor (nivolumab) treatment, and then showed a further reduction 3 wk after subsequent radioembolization of the tumor (Fig. 3C). Coincident computed tomography scans demonstrated a significant tumor response to radioembolization. Of note, for three of these five patients (HCC-042, HCC-058, and HCC-075), serum protein AFP measurements were below clinically informative values (<20 ng/mL) at all draw points. Although serum AFP protein measurements are often used for monitoring tumor response in patients with HCC, they are below detection in a significant fraction of cases. In such cases, CTC score monitoring may serve as a complementary marker to assess disease status.

Early Detection of HCC in High-Risk Populations. Although early detection of localized HCC in individuals with liver cirrhosis provides the only hope for curative therapy, serum AFP alone does not provide sufficient accuracy to enable screening of at-risk populations. Using a cutoff of 20 ng/mL, AFP has an estimated sensitivity of 53% with a specificity of 87%, leading to a positive



Fig. 3. Longitudinal monitoring of patients treated for HCC. (A) Serial blood measurements performed at 1-wk intervals in two patients (HCC-041 and HCC-075) in the absence of therapeutic intervention. Concurrent CTC score (red) and serum AFP (black) measurements are shown. (*B*) Longitudinal monitoring of two patients (HCC-058 and HCC-060), before (Pre) and after (Post) resection of localized HCC. HCC-060 had NED 1 mo after resection but then developed a recurrence of HCC (Rec). (C) Serial monitoring of a patient (HCC-042) initially treated with the immune checkpoint inhibitor nivolumab (Nivo), followed by radioembolization (Embo) of the residual mass. The tumor mass and postembolization changes are shown by computed tomography scan. Concurrent CTC score (red) and serum AFP (black) measurements are shown.

predictive value (PPV) of 6% in populations where the expected prevalence of HCC is 1% (19). Raising the AFP threshold to 100 ng/mL improves specificity to 99% but reduces the test sensitivity to 31% (20). Given these poor test characteristics, the American Association for the Study of Liver Diseases does not recommend using AFP as a screening tool for HCC (11).

To model the potential combination of AFP and CTC score in screening for HCC, we determined the correlation between these two biomarkers in all of the patients with HCC for whom concomitant assay results were available. CTC score and serum AFP levels were not significantly correlated ($R^2 = 0.0007$, P = 0.74), with concordance restricted to cases with high serum AFP levels (Fig. 4A). The discordance between AFP protein levels and the CTC score is consistent with the underlying basis for these assays: The multigene CTC score is a digital signature to quantify HCC cells that have invaded into blood, whereas serum AFP measures a single protein produced by HCC cells and released into blood from tumor deposits. As such, the two assays may be orthogonal and have added value as blood-based biomarkers for HCC.

Although our pilot study was not powered to test the accuracy of the CTC score directly in early detection of HCC, we modeled two strategies using either a high or low cutoff for AFP measurements. First, we tested the additive value of CTC score positivity and high-threshold AFP (100 ng/mL). Of the 15 patients with newly diagnosed HCC for whom both AFP and CTC scores were available, four (27%) were detected by CTC score alone, one (7%) by AFP alone, and five (33%) by both assays (Fig. 4B). Together, either AFP or CTC score was positive in 67% of patients, leaving only one-third undetected by either method. Importantly, among all 16 patients with newly diagnosed HCC, six (38%) patients met the Milan criteria for liver transplantation, a clinical indication that the disease is sufficiently localized to enable curative therapy. CTC scores were available for all six patients who met the Milan criteria, of whom two (33%) were positive (Fig. 4C). AFP levels were available for five of six patients, but none were above the 100-ng/mL threshold. Thus, a



Fig. 4. Modeling early detection of HCC using CTC score and AFP measurements. (A) Absence of correlation between CTC score and serum AFP levels in all patients with HCC with concomitant measurements. (B) Proportion of 15 newly diagnosed, untreated patients with HCC identified by CTC score alone, AFP (>100 ng/mL) alone, or both CTC score and AFP. No AFP measurement was available for one patient with untreated HCC. (C) Bar graphs representing all newly diagnosed HCC patients, showing those patients identified by serum AFP (>100 ng/mL), CTC score, or the combination of the two tests (Either). Six of these newly diagnosed patients met the Milan criteria for localized disease amenable to curative liver transplantation [Milan (+)]. Two of six Milan(+) patients were identified by CTC score, but none of five had an AFP level >100 ng/mL [one Milan (+) patient did not have AFP measurement]. (D) PPV and NPV calculations for CTC score alone. AFP (>20 ng/mL) alone, or both, as a function of HCC prevalence. The CTC score model assumes 56% test sensitivity and 95% specificity, as observed in untreated patients with HCC; for AFP (>20 ng/mL), the 53% test sensitivity and 87% specificity are established from a population-based study (19).

subset of patients identified as having HCC by digital CTC assay may have curable HCC.

As an alternative HCC detection strategy, we modeled the initial screening of patients with cirrhosis using the higher sensitivity 20-ng/mL AFP cutoff, followed by CTC score analysis as a confirmatory test to provide the required specificity. Such a sequential strategy has been instrumental in population screening for infectious disease, such as HIV (21); it has the benefit of capturing most patients at risk with a rapid primary test, thereby increasing the disease prevalence among the group tested with the higher specificity confirmatory test. In patients with HBV hepatitis without cirrhosis (associated with a 0.5-1% annual incidence of HCC), such sequential AFP/CTC screening leads to a calculated PPV of 36%, with a negative predictive value (NPV) of 98% (Fig. 4D). In higher risk patients with HBV-induced cirrhosis (8% annual incidence of HCC), the PPV rises to 86%, with an NPV of 83%. Although these calculations require confirmation in large population studies, these predicted values are within the range that would warrant cancer surveillance initiatives within appropriate clinical settings (22).

Discussion

We have described a sensitive and specific RNA-based readout for detection of CTCs following their microfluidic enrichment from blood specimens. Our approach combines the high efficiency depletion of hematopoietic cells, enabling isolation of CTCs with intact RNA and without bias for expression of tumor- or epithelial-specific cell surface epitopes, together with CTC quantitation using a high-throughput, tissue lineage-specific dPCR assay. Together, these approaches overcome the rate-limiting hurdle in CTC detection, namely, antibody staining and microscopic scoring of heterogeneous CTCs among an excess of contaminating WBCs. Moreover, the dramatic signal amplification and the molecular specificity derived from dPCR provide an effective way to detect the rare but highly biologically significant occurrence of intact tumor cells in the blood circulation. As an initial proof of concept, we applied this digital CTC measurement strategy to HCC, a highly lethal malignancy with worldwide impact, for which early detection strategies are currently inadequate (11).

The concept of liquid biopsies for noninvasive monitoring of cancer has emerged as one of the most promising approaches in cancer diagnostics, with applications ranging from early detection to treatment selection and monitoring response (23, 24). Three complementary technologies each interrogate different biological specimens and rely upon distinct technological assays. Circulating tumor DNA (ctDNA) is derived from small fragments of genomic DNA shed into the vasculature by dying tumor cells, amid the background of DNA released from normal tissues, and it provides powerful opportunities for targeted DNA-based genotyping (25). However, an inherent limitation of ctDNA-based genotyping is that it does not provide information as to the tissue of origin for mutations detected in the blood, in contrast to CTCs, which provide a source of intact RNA for lineage-based analysis. Genotype-based cancer detection is also of limited utility in tumors such as HCC, where highly prevalent mutations have not been identified. An alternative blood-based cancer detection technology takes advantage of exosomes, small membrane-bound cellular fragments encapsulating cytoplasmic RNA and other cellular components that are released by both tumor and normal cells. Although strategies for enrichment of tumor-derived exosomes are still being optimized, the analysis of pooled exosomes has allowed RNA-based detection of cancer-associated mutations (26). However, the fact that normal tissues also abundantly shed exosomes precludes the use of lineage and tissue markers to identify tumor-derived expression signatures in blood specimens. In contrast to exosomes, whole cells derived from normal tissues are extraordinarily rare in the blood circulation. Hence, the initial isolation of intact CTCs in the bloodstream, followed by their molecular quantitation, may provide a highly specific diagnostic assay that is amenable to large-scale clinical applications. HCC, which arises within well-defined at-risk populations, and in which early detection may be curative, is particularly appropriate to serve as a "proof of principle" for CTC-based screening. Of note, the sensitivity and specificity of this molecular CTC assay hinge on the successful identification of HCC-derived transcripts: Additional targets that capture the full heterogeneity of HCC may be identified by single-cell sequencing of HCC CTCs, and analysis of larger blood volumes may also improve the likelihood of CTC detection, especially in patients with small lesions.

The poor performance of AFP alone in HCC screening stems from the fact that only a minority of patients with liver cancer have very high elevations in this marker (>100 ng/mL), whereas low levels (>20 ng/mL) are common in conditions that predispose to HCC, including viral hepatitis. However, the combination of AFP screening with a second more specific assay has been shown to be effective in reducing disease mortality. In a large randomized trial, a 37% reduction in HCC-related mortality was reported among high-risk patients who underwent surveillance with AFP and liver ultrasound (27). Ultrasoundbased surveillance is now practiced in many centers, but image quality is operator-dependent and degraded in the setting of obesity or cirrhosis. Furthermore, access to high-quality ultrasound is limited in developing countries, which bear the greatest burden of HCC. The technology that we describe here, together with the analysis of pilot clinical cohorts and clinical modeling studies, raises the possibility that digital CTC scoring may provide an important new tool for HCC detection. The scalability of digital CTC monitoring may be particularly useful in underserved populations that lack access to MRI and ultrasound screening. Although large population-based studies are now required to test and validate this technology's performance against existing screening standards, we favor the combination of AFP

and CTC-scoring assays as orthogonal markers that, together, may provide both the sensitivity and specificity required for noninvasive and cost-effective HCC screening in high-risk populations that serum AFP alone is unable to provide. Alternative CTC-scoring algorithms may also enhance the differentiation of HCC from other hepatic lesions, an application of particular importance in the United States, where the use of imaging tests currently drives HCC detection and monitoring. Thus, quantitative analysis of multiple tissue-specific transcripts derived from CTCs may be optimized for distinct applications in the diagnosis and treatment of patients with HCC.

Finally, we note that digital scoring of CTCs for cancer monitoring is broadly applicable to other cancer types. Indeed, many cancers originate in tissues that express specialized gene transcripts that are absent in normal blood cells, and the curation and testing of these markers may enable high-sensitivity detection and monitoring of rare cancer cells in the blood. Such blood-based molecular monitoring for CTCs holds considerable promise for the early detection of cancer.

Materials and Methods

Gene Target Identification and Validation. Publically available RNA-sequencing and microarray datasets were used to identify the top 100 genes highly expressed in HCC with very low to no expression in other tissues and blood components (15– 17). The low expression of candidate genes within WBCs persisting in the CTCiChip output was confirmed by RNA-sequencing of processed blood from healthy donors, and qRT-PCR of WBCs purified from whole blood was used as an additional exclusion criterion. Ten genes were selected to establish a signature of HCC-derived CTCs, enriched within a background of normal blood cells.

Cell Culture and RNA Processing. HepG2 cells were cultured following American Type Culture Collection-recommended culturing conditions. RNA was isolated using the RNeasy Plus Micro kit (Qiagen), and cDNA was generated using the SuperScript III Reverse Transcriptase kit (Thermo Fisher). Buffy coat WBC preparations were generated using standard procedures, followed by TRIzol RNA extraction (Ambion). For qRT-PCR assays, 5 ng of cDNA generated from HepG2 cells or from WBCs (buffy coats) of three independent blood donors was amplified and compared with GAPDH using the Applied Biosystems 7500 RT-qPCR assay (40 cycles). Primer and probe combinations are provided upon request. For single-cell manipulation and spike-in studies, individual cells were micropipetted using an

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Eppendorf TransferMan NK2 micromanipulator and introduced into whole blood samples from healthy donors, before processing through the CTC-iChip.

Patient Cohorts and Blood Processing Through the Microfluidic CTC-iChip. Patient cohorts and clinical characteristics are provided in Tables S1–S6. Dana– Farber/Harvard Cancer Center IRB-approved protocols were used to obtain consent from all patients and healthy donors. Blood was processed using the CTC-iChip as previously described (5). A detailed description of each cohort and blood processing conditions can be found in *SI Materials and Methods*.

WTA and Droplet dPCR. WTA was performed on CTC-iChip-derived RNA using the SMARTer Ultra Low-input RNA kit, version 3 (Clontech Laboratory). Onethird of the RNA extracted from the CTC-iChip product was loaded into each reaction. The dPCR experiments were performed using an AutoDG automated droplet generator, C1000 Touch Deep-well thermocycler, and QX200 plate reader (Biorad) following manufacturer recommendations, with 1% of the WTA product. Thermocycling conditions and primers are provided upon request.

Statistical Calculations and Logistic Regression. Genes that served as statistically significant predictors of disease status were used to build a multipredictor logistic regression whose performance parameters were determined using LOOCV (Fig. 2C and Figs. S4, S5, and S7). For longitudinally collected samples or treated patients with HCC with NED, a single logistic regression was fit using the entirety of the aforementioned training set (Fig. S5). To calculate the PPV and NPV of the diagnostic assays at varying disease prevalences, we used published sensitivities and specificities for serum AFP at 20 ng/mL (19) and chose the CTC score sensitivity that yielded 95% specificity.

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Supporting Information

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SI Materials and Methods

The following methods were used to collect and process patient blood samples. Control samples (from healthy donors) were obtained from anonymized discarded specimens collected at a blood donation center. All patients with HCC or other cancers were enrolled at the Massachusetts General Hospital Cancer Center, and patients with CLD were enrolled through the Hepatology Unit of the Massachusetts General Hospital. Patients with CLD were at sufficiently high risk for HCC to warrant periodic screening as determined by their treating hepatologist. As such, patients with CLD suffered from either chronic HBV infection or advanced fibrosis (bridging fibrosis or cirrhosis) of any etiology. Following the receipt of informed consent, 5–15 mL of blood was isolated by standard venipuncture into K2EDTA BD Vacutainer Collection Tubes and processed through the CTCiChip as outlined previously (5, 20). Briefly, whole blood was incubated with a biotinylated antibody mixture (anti-CD45, anti-CD16, and anti-CD-66b; Jannsen Diagnostics) for 20 min, followed by the addition of DynaBeads MyOne Streptavidin T1 magnetic beads for an additional 20 min. The blood was then loaded onto the CTC-iChip at a flow rate of 10 mL·h⁻¹ using an automated processor, and the CTC-enriched cell product was collected on ice. The product was centrifuged at 5,200 × g for 5 min, resuspended in 200 µL of RNALater (Ambion), and flash-frozen before RNA isolation.



Fig. S1. WBC gene expression. Relative expression (qRT-PCR) of candidate liver-specific signature genes, amplified from 5 ng of cDNA from healthy blood donor WBCs (buffy coat), normalized to GAPDH (n = 3). The gene targets APOC1, HP, HPR, and SERPINA1 were eliminated from the liver signature due to their high relative expression.



Fig. S2. WTA characterization. (A) Total number of droplets derived from 1 ng of HepG2 cell RNA for all 10 liver-specific genes. Three independent WTA reactions are compared with a nonamplified cDNA control. (B) Consistent amplification ratio for each liver-specific gene following WTA (three independent reactions), relative to the nonamplified cDNA control.



Fig. S3. Buffy coat dPCR. Failure of ALB transcript droplet PCR amplification from buffy coat (WBCs and nonenriched CTCs) RNA extracted from blood specimens of nine HCC patients and three healthy donor (HD) controls. The ALB transcript is appropriately amplified from HepG2 RNA. Matched GAPDH transcript quantification is shown as a control for relative RNA content.



Fig. S4. ROC curves for individual genes. ROC curves were derived for each transcript within the liver-specific signature, using univariate logistic regression and all first-draw active HCC patient samples. AUC, area under the curve; FPR, false-positive rate; TPR, true-positive rate.

	Multi-gene Lo	Multi-gene Logistic Regression Results							
	AFP	0.406***							
		(0.107, 0.706)							
	AHSG	0.145***							
		(0.025, 0.266)							
	ALB	-0.033							
		(-0.140, 0.075)							
	APOH	-0.048							
		(-0.190, 0.094)							
	FABP1	0.005							
		(-0.103, 0.114)							
	FGB	0.044							
		(-0.095, 0.183)							
	FGG	0.092							
		(-0.058, 0.242)							
	RBP4	-0.020							
		(-0.127, 0.087)							
	TF	0.009							
		(-0.101, 0.118)							
	Constant	-1.124****							
		(-1.871, -0.377)							
	Observations	105							
	Log Likelihood	-52.065							
	Akaike Inf. Crit.	124.129							
	Note:	[*] p<0.1; ^{**} p<0.05; ^{***} p<0.01							
PPV =	sensitivity x prevalence								
	sensitivity x prevalend	ce + (1-specificity) x (1-prevalence)							
NPV =	specifi	city x (1-prevalence)							
	(1-sensitivity) x prevalence + specificity x (1-prevalence								

Fig. S5. Multigene model parameters and modeling equations. Coefficients and model statistics for are shown for the logistic regression model (table) using all first-draw active HCC patient samples. The formulae used for calculation of the PPVs and NPVs are shown below. Akaike Inf. Crit., Akaike information criteria.

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Fig. S6. CTC score clinical correlates. (A) Nonsignificant correlation between CTC score (all patients with HCC) and the etiology of underlying liver cirrhosis [alcohol-induced (EtOH), HBV infection, hepatitis C virus (HCV) infection and EtOH, and nonalcoholic steatohepatitis (NASH)]. All patients with HCV-induced HCC in our cohort also had significant alcohol exposure. (B) Significant correlation between CTC score and clinical stage (Barcelona criteria: early stage 0 and A versus advanced stage B–D). (C) Trend approaching significance between CTC score and imaging-based (macroscopic) evidence of vascular invasion.



Fig. 57. ROC curves, multigene model parameters, and model performance for HCC versus other malignancies. (*A*) Individual gene ROC curves with AUC and *P* values displayed. (*B*) Coefficients and model statistics for the logistic regression model using all first-draw active HCC patient samples. (*C*) Non-cross-validated and LOOCV logistic regression ROC curves for untreated HCC patient draws. (*D*) Comparison between scores of HCC patients and those patients with other cancers. P = 0.013, Mann–Whitney *U* test. (*E*) Transcript count variations across two blood draws on patients HCC.041 and HCC.075 in the absence of clinical intervention.

Patient	Age, y	Gender
HD1	21	F
HD2	23	F
HD3	25	F
HD4	26	F
HD5	51	F
HD6	53	F
HD7	54	F
HD8	55	F
HD9	23	М
HD10	25	М
HD11	25	М
HD12	43	М
HD13	46	М
HD14	53	М
HD15	55	М
HD16	57	М
HD17	62	М
HD18	62	M
HD19	63	M
HD20	66	М
HD21	67	М
HD22	69	M
HD23	72	М
HD24	72	M
HD25	73	M
HD26	74	M
HD27	50	M
HD28	40	M
HD29	51	F
HD30	53	F
HD31	42	F
HD32	57	М
HD33	62	М
HD34	67	М

Table S1. Healthy donor clinical information

F, female; HD, healthy donor; M, male.

PNAS PNAS

Table S2.	CLD	patient	clinical	information
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Patient	Age, y	Gender	Diagnosis	Cirrhosis	Serum AFP, ng/mL
CLD-002	56	М	NASH	Yes	NA
CLD-003	30	F	HBV	No	3.3
CLD-004	62	Μ	Alcohol	Yes	6.6
CLD-005	80	F	HBV	No	1.9
CLD-006	53	Μ	HBV	No	2.9
CLD-007	36	F	HBV	No	2
CLD-008	64	Μ	HBV	No	1.9
CLD-009	59	F	HBV	No	3
CLD-010	45	F	Alcohol	Yes	1.7
CLD-011	76	М	HBV	No	1.7
CLD-012	85	F	Alcohol	Yes	1.4
CLD-013	87	F	HCV	Yes	8.1
CLD-017	66	М	HCV	Yes	4.4
CLD-019	66	М	Alcohol	Yes	1.9
CLD-022	41	М	PSC	Yes	3.3
CLD-023	72	М	HCV	Yes	1.7
CLD-024	77	Μ	HBV	No	2.1
CLD-025	53	Μ	Alcohol/NASH	Yes	10.4
CLD-026	54	F	Alcohol	No	4
CLD-027	50	Μ	HBV	No	4.6
CLD-028	70	Μ	HBV	No	3.2
CLD-029	37	М	PSC	Yes	1.2
CLD-031	27	Μ	HBV	No	2.3
CLD-032	69	Μ	HCV	No	3.2
CLD-033	54	Μ	HCV	Yes	2.8
CLD-034	73	F	HBV	No	2.8
CLD-036	56	Μ	HCV/hemochromatosis	Yes	4.2
CLD-037	60	F	HBV	No	3.3
CLD-038	60	F	HBV	No	2.1
CLD-039	54	F	HBV	No	3.5
CLD-040	39	Μ	HBV	Yes	4

EtOH, alcohol; HCV, hepatitis C virus; NA, not available; NASH, nonalcoholic steatohepatitis; PSC, primary sclerosing cholangitis.

Table S3. Untreated HCC patient clinical information

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Patient	Age, y	Gender	Etiology	Cirrhosis	Multifocal disease	Diameter, cm	Metastatic disease	Macroscopic vascular invasion	Milan criteria	Barcelona stage	Serum AFP, ng/mL
HCC.015	70	F	PSC	Yes	No	2	No	No	Yes	0	3.8
HCC.018	82	М	NA	No	No	8.1	No	No	No	А	132,367
HCC.027	55	М	HBV	No	No	8.7	Yes	Yes	No	C, D	731.8
HCC.042	58	М	HCV/EtOH	Yes	Yes	NA	No	Yes	No	C, D	5.4
HCC.058	54	М	HBV	Yes	No	2.5	No	No	Yes	А	5.6
HCC.060	63	F	NASH	Yes	No	10	No	Yes	No	C, D	13,629
HCC.064	53	М	HCV	Yes	No	2.4	No	No	Yes	А	1.6
HCC.065	59	М	HBV	No	No	2.5	No	No	Yes	А	63.3
HCC.076	79	М	HBV	Yes	No	8.1	No	Yes	No	C, D	13,322
HCC.079	71	М	NA	No	Yes	NA	No	No	No	В	5.2
HCC.082	62	М	HCV/EtOH	Yes	No	1.9	No	No	Yes	А	5.9
HCC.090	69	М	EtOH	Yes	No	4.7	NA	Yes	No	C, D	19,960
HCC.091	72	М	HBV	No	No	15.2	No	No	No	А	3.2
HCC.098	66	М	EtOH	Yes	No	11	No	Yes	No	C, D	356
HCC.101	78	М	NASH	Yes	No	2.4	No	No	Yes	А	NA
HCC.102	61	М	EtOH/	Yes	No	1.4	No	No	Yes	А	9.6
			hemochromatosis								

Multifocal disease was defined as greater than three hepatic lesions based on Milan and Barcelona classifications. The listed diameter is the diameter of the largest lesion.

Table S4. Ongoing treatment HCC patient clinical information

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					Multifacal	Diamator	Motostatic	Macroscopic	Milan		Serum	Treatment
Patient	Age, y	Gender	Etiology	Cirrhosis	disease	cm	disease	invasion	criteria	BCLC	ng/mL	received
HCC.011	74	М	NA	No	Yes	NA	Yes	No	No	C, D	939.2	Ablation, resection
HCC.014	69	F	NASH	Yes	No	3.1	No	No	Yes	А	218	TACE
HCC.016	70	М	HBV	No	No	3.6	No	No	Yes	Α	43.1	TACE, RT, other
HCC.019	64	М	EtOH	Yes	Yes	NA	Yes	Yes	No	C, D	21	RT, sorafenib
HCC.021	68	М	NASH/EtOH	Yes	Yes	NA	No	Yes	No	C, D	4,891	Ablation
HCC.029	70	М	NASH	Yes	No	1.2	No	Yes	No	C, D	4,800	Ablation
HCC.030	78	М	HBV	Yes	No	1.9	No	No	Yes	0	26.9	TACE
HCC.035	82	F	NA	No	Yes	NA	No	No	No	В	2,043.5	SIRT, RT, sorafenib
HCC.037	54	М	EtOH	Yes	Yes	NA	No	No	No	В	5947	Ablation, SIRT, sorafenib
HCC.040	70	М	NA	No	No	NA	Yes	NA	No	C, D	5.6	RT, checkpt, resection
HCC.041	72	М	EtOH	Yes	Yes	NA	Yes	No	No	C, D	338	RT
HCC.046	66	М	HBV	Yes	No	2	No	No	Yes	0	5.5	Ablation
HCC.047	62	М	EtOH	Yes	No	1.8	No	No	Yes	0	12.7	Ablation
HCC.050	76	М	EtOH	Yes	No	1.3	No	No	Yes	0	5.4	Ablation, TACE, RT
HCC.052	23	М	Biliary atresia	No	Yes	NA	No	No	No	В	1.5	RT
HCC.059	66	М	HCV/EtOH	Yes	Yes	NA	Yes	Yes	No	C, D	4847	Ablation
HCC.061	67	F	NA	No	Yes	NA	No	No	No	В	60.6	Resection
HCC.062	63	М	NASH	Yes	No	1.2	No	No	Yes	0	185.2	TACE
HCC.067	74	F	NA	No	Yes	NA	Yes	No	No	C, D	2.5	Sorafenib
HCC.068	83	М	HBV	Yes	No	5.4	No	No	No	В	4.7	RT
HCC.069	62	М	NASH	Yes	No	1.8	No	No	Yes	А	20.8	TACE
HCC.074	64	М	NASH/EtOH	Yes	No	13	Yes	Yes	No	C, D	167,580	Sorafenib
HCC.075	81	М	HBV	Yes	Yes	NA	Yes	No	No	C, D	7.7	Ablation, sorafenib, chemotherapy, checkpt, other, resection
HCC.078	60	М	HBV	No	No	NA	Yes	No	No	C, D	4.7	Ablation, resection
HCC.083	59	М	HCV/EtOH	Yes	Yes	NA	Yes	No	No	C, D	8.5	TACE, SIRT, sorafenib, chemotherapy
HCC.084	81	м	NA	No	No	3.8	Yes	No	No	C. D	16.8	Sorafenib
HCC.087	57	M	EtOH	Yes	No	1.7	No	No	Yes	Α	16.7	Ablation, TACE
HCC.093	77	M	NA	No	Yes	NA	Yes	Yes	No	C. D	3.2	RT. resection
HCC.094	70	M	NA	No	No	1.2	Yes	No	No	C, D	156.4	SIRT, RT
HCC.095	64	M	HCV/EtOH	Yes	No	1.5	No	No	Yes	0	7.6	TACE
HCC.097	52	F	NA	No	No	NA	Yes	NA	No	C. D	1.3	Ablation.
										-, -		chemotherapy, other, transplant,
HCC.099	58	М	EtOH	Yes	No	2.3	No	No	Yes	А	254.9	TACE

checkpt, checkpoint inhibitor; RT, external radiation therapy; SIRT, selective internal radiation therapy; TACE, transarterial chemoembolization.

					Serum AFP,	
Patient	Age, y	Gender	Etiology	Cirrhosis	ng/mL	Treatment received
HCC.030_2	78	М	HBV	Yes	NA	Ablation, TACE
HCC.031	63	Μ	HCV	Yes	330.4	Ablation, TACE, transplant
HCC.031_2	63	Μ	HCV	Yes	660.7	Ablation, TACE, transplant
HCC.033	85	М	Hemochromatosis	No	1.6	TACE
HCC.051	68	М	NASH	Yes	2.5	Ablation, TACE, transplant
HCC.053	63	М	HCV/EtOH	Yes	2.3	Resection
HCC.055	51	М	EtOH	Yes	2.6	Ablation, transplant
HCC.057	67	F	NASH	Yes	5	Ablation
HCC.058_2	54	М	HBV	Yes	3.6	Resection
HCC.060_2	63	F	NASH	Yes	2,500	Resection
HCC.064_2	53	М	HCV	Yes	1.7	Ablation
HCC.085	71	М	NASH	Yes	7.4	TACE
HCC.086	75	М	HBV	Yes	2.9	Ablation
HCC.088	79	F	HCV	No	1.6	Ablation
HCC.089	62	М	HCV	Yes	6.8	Ablation
HCC.A001	62	М	HCV	No	NA	Transplant

Table S5. Postcurative intent treatment HCC patient clinical information

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Table S6.	Other cancer	patient clinical	information
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Patient	Age, y	Gender	Diagnosis	Metastasis to liver
GU-254	72	М	Prostate cancer	No
GU-256	62	М	Prostate cancer	No
GU-257	57	М	Prostate cancer	No
GU-259	54	М	Prostate cancer	No
GU-260	51	М	Prostate cancer	No
GU-261	69	М	Prostate cancer	No
GURa-01	69	М	Prostate cancer	No
GURa-02	61	М	Prostate cancer	No
GURa-03	73	М	Prostate cancer	No
BRX-169	79	F	Breast cancer	No
BRX-198	58	F	Breast cancer	No
BRX-202	62	F	Breast cancer	No
BRX-226	59	F	Breast cancer	No
BRX-237	65	F	Breast cancer	No
MEL-140	48	М	Melanoma	No
MEL-163	28	F	Melanoma	No
MEL-165	60	М	Melanoma	No
MEL-102	60	F	Melanoma	No
MEL-129	85	F	Melanoma	No
MEL-147	56	М	Melanoma	No
MEL-151	42	М	Melanoma	No
MEL-164	80	М	Melanoma	No
ICC-001	62	F	Intrahepatic cholangiocarcinoma	No
ICC-002	75	М	Intrahepatic cholangiocarcinoma	No
ICC-003	73	F	Intrahepatic cholangiocarcinoma	No
ICC-004	55	F	Intrahepatic cholangiocarcinoma	No
PDAC-2082	51	F	Pancreatic cancer	No
PDAC-2085	69	М	Pancreatic cancer	No
PDAC-2093	58	М	Pancreatic cancer	No
PDAC-2094	66	М	Pancreatic cancer	Yes
PDAC-2095	59	F	Pancreatic cancer	No
PDAC-A001	44	F	Pancreatic cancer	No
TH-159	71	М	Lung cancer	No
TH-173	73	М	Lung cancer	No
TH-215	79	М	Lung cancer	No
TH-248	80	М	Lung cancer	No
TH-255	69	М	Lung cancer	No
TH-257	70	F	Lung cancer	No
TH-179	46	F	Lung cancer	No
TH-262	50	F	Lung cancer	Yes
BRX-A001	63	F	Breast cancer	Yes
EAC.001	65	М	Hepatoid esophageal adenocarcinoma	Yes
CRC.001	52	F	Colon cancer	Yes
CRC.002	57	М	Colon cancer	Yes