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

Mark Kalinich,1,4 Irun Bhan1,5, Tanya T. Kwan,4 David T. Miyamoto,6 Sarah Javadi,3,4 Joseph A. Lieu,6 John D. Milner,6 Xin Hong,6 Lipika Goyal,5,6,7 Srinjoy Sil,8 Melissa Chou,9,6 Uyen Ho,6 Ravi Kapur,6 Alona Muzikansky,9,6 Huidan Zhang,9 David A. Weitz,10 Lecia V. Sequist,10,11 David P. Ryan,3,4 Raymond T. Chung,10,11 Andrew X. Zhu,3,4 Kurt J. Isselbacher,5,11,12,13 David T. Ting,9,10 Mehmet Toner,4,14 Shyamala Maheswaran,6,11,12,13, and Daniel A. Haber,3,4,11,12,13

*Massachusetts General Hospital Cancer Center, Harvard Medical School, Charlestown, MA 02129; 1Division of Gastroenterology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114; 2Department of Radiation Oncology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114; 3Division of Hematology Oncology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114; 4Center for Bioengineering in Medicine, Massachusetts General Hospital and Harvard Medical School and Shriners Hospital for Children, Boston, MA 02114; 5Division of Biostatistics, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114; 6Department of Physics, School of Engineering and Applied Sciences, Harvard University, Cambridge, MA 02138; 7Department of Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114; and 8Howard Hughes Medical Institute, Chevy Chase, MD 20815

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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 demonstrated 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.

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

T 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.

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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.
(NAFLD), and nonalcoholic steatohepatitis (NASH) (9). Early-stage 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 patients 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, blood-based 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. 1A 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 depletion 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, CD45, CD16, and CD66b antibodies (5, 14). The high efficiency depletion of WBCs (4- to 5-log purification) enriches CTCs.

**Technical Validation of HCC-Specific RNA 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. 1C). 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 cell lines in 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. 1D and Fig. S2). Compared with unamplified cDNA derived from 1 ng of 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), glyceral-3-phosphate (GP3), retinol binding protein 4 (RBP4), and transferrin (TF)] were selected for developing the dPCR assay.

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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 μL 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 (R² = 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 = 9 HCC 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 i 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. 2A). As expected, intra-cohort 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
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 CTC 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 (\(P < 0.0001, \chi^2\); 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 (\(P = 0.56, \text{not significant}\)). 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 (\(APF, AHSG, APOH, FABP1, FGB, \) and \(FGG\)), yielding a multipredictor model with different features than the previous model (Fig. S7A 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 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 concurrent 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
...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 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 (26). An alternative blood-based detection strategy has been instrumental in population screening for infectious disease, such as HIV (21); it has the benefit of capturing 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).
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 seek AFP alone is unable to provide. Alternative CTC-scoring algorithms may also enhance the differentiability 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 tissues. Gene expression in other tissues and blood components (15–17). The low expression of candidate genes within WBCs persisting in the CTC-iChip 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 prepreations 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 molecular manipulation and spin-in studies, individual cells were micropipetted using an 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-Chip. 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 (S). 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). One-third 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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