



Experimental Models of Liquid Biopsy in Hepatocellular Carcinoma Reveal Clone-Dependent Release of Circulating Tumor DNA

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Liquid biopsy, the molecular analysis of tumor components released into the bloodstream, has emerged as a noninvasive and resourceful means to access genomic information from cancers. Most data derived from translational studies showcase its numerous potential clinical applications. However, data from experimental models are scarce, and little is known about the underlying mechanisms and factors controlling the release of circulating tumor DNA (ctDNA) and cells (CTCs). This study aimed to model liquid biopsy in hepatocellular carcinoma xenografts and to study the dynamics of release of ctDNA and CTCs; this included models of intratumoral heterogeneity (ITH) and metastatic disease. We quantified ctDNA by quantitative polymerase chain reaction (PCR) targeting human long interspersed nuclear element group 1; targeted mutation analysis was performed with digital droplet PCR. CTCs were traced by flow cytometry. Results demonstrated the feasibility of detecting ctDNA, including clone-specific mutations, as well as CTCs in blood samples of mice. In addition, the concentration of ctDNA and presence of tumor-specific mutations reflected tumor progression, and detection of CTCs was associated with metastases. Our ITH model suggested differences in the release of DNA fragments impacted by the cell-clone origin and the treatment. *Conclusion:* These data present new models to study liquid biopsy and its underlying mechanisms and highlighted a clone-dependent release of ctDNA into the bloodstream. (*Hepatology Communications* 2021;5:1095-1105).

Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer.⁽¹⁾ According to estimations for the United States, liver cancer is responsible for 42,000 and 30,000 new cases and deaths yearly, respectively.⁽²⁾ Unlike other cancers, mortality related to liver cancer has increased over the last decades. With a 5-year survival of 18%, it is the second most lethal malignancy only after pancreatic cancer.⁽²⁾

Liquid biopsy, the molecular analysis of tumor components released to the bloodstream, has emerged as a convenient source for biomarker development as it allows for noninvasive sample collection.⁽³⁻⁵⁾ In addition to reflecting the genomic aberrations identified in solid tumors,⁽⁶⁾ liquid biopsy appeared as a polyvalent tool allowing for an early diagnosis,^(7,8) the detection of minimal residual disease after curative therapy,⁽⁹⁾ or to guide decision making for systemic treatment,

Abbreviations: APOB, apolipoprotein B; CD, cluster of differentiation; CTC, circulating tumor cell; ctDNA, circulating DNA; ddPCR, digital droplet polymerase chain reaction; EDTA, ethylene diamine tetraacetic acid; FGA, fibrinogen alpha chain; GFP, green fluorescent protein; HCC, hepatocellular carcinoma; hLINE-1, human long interspersed nuclear element group 1; ITH, intratumor heterogeneity; miR/miRNA-517, microRNA-517; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

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such as the need for adjuvant chemotherapy in colon and pancreatic cancers.^(10,11) Liquid biopsy has even allowed studying complex biological traits, such as drug resistance or tumor heterogeneity.⁽¹²⁻¹⁵⁾ However, many fundamental questions related to the biology of circulating analytes remain unanswered, and this underscores the need to develop new experimental models to study liquid biopsy.⁽¹⁶⁾ For instance, it is assumed that DNA fragments are released by all cancer cells into the bloodstream by apoptosis and necrosis, but there is no evidence on whether and how DNA release might vary between cancer clones. Studies exploring the underlying mechanisms of release of circulating tumor cells (CTCs) or DNA (ctDNA) are scarce, particularly in HCC. As noted by a joint European Association for Cancer Research–American Association for Cancer Research conference on liquid biopsy, basic research is needed to address some unanswered questions regarding the biology of circulating tumor material.⁽¹⁶⁾ Our study aimed to investigate the use and applications of HCC xenografts to monitor ctDNA and CTC dynamics. We demonstrated the feasibility of detecting ctDNA and CTCs in murine blood samples. In addition, the release of ctDNA correlated with tumor progression and appeared to depend on the cell clone of

origin, whereas the detection of CTCs correlated with the number of liver nodules and was identified as a predictor of lung metastases.

Materials and Methods

ANIMALS AND CELL LINES

The animal study protocol was approved by the institutional animal care and use committee (IACUC) of the Icahn School of Medicine at Mount Sinai (IACUC-2016-0039). Female athymic nude mice (Fox1nu) of 6–10 weeks were purchased from Envigo. Three different human HCC cell lines were used that were derived from previous reports: Huh7, HepG2, and green fluorescent protein (GFP)-micro-RNA (miR)-517a-Huh7.^(17,18) Cell lines were cultured as monolayers in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% L-glutamine, at 37°C. IMPACT I (It is a test that includes PCR evaluation for: Ectromelia, EDIM, Hantaan, K virus, LCMV, LDEV, MAV1, MAV2, mCMV, MHV, MNV, MPV, MTV, MVM, Mycoplasma pulmonis, Mycoplasma sp.,

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Polyoma, PVM, REO3, Sendai, TMEV) was tested by IDEXX BioResearch in each cell line to exclude contamination. DNA was extracted from the cell lines with the DNeasy Blood and Tissue Kit (Qiagen) and submitted to ultra-deep DNA sequencing of a target panel of 58 frequently mutated genes in HCC, as described.⁽⁶⁾

SUBCUTANEOUS MODELS

In order to recapitulate intratumor heterogeneity (ITH), two different cell lines (Huh7 and HepG2) were used. A total of 5×10^6 cells of each cancer cell line were diluted in 0.1 mL of phosphate-buffered saline (PBS) and subcutaneously injected in each flank of 24 nude mice (Fig. 1). Tumor size was monitored daily and measured with a handheld caliper. Tumor volume was calculated as length \times width² \times 0.5. Once a tumor reached 200 mm³, mice were randomized according to a 1:1 ratio to either receive placebo or sorafenib (Fig. 1). Sorafenib was dissolved by sonication in 95% ethanol, cremophor, and sterile water (12.5:12.5:75). The treatment arm received daily oral administration of sorafenib

(30 mg/kg body weight) until the tumor reached a volume of 1,000 mm³ when mice were euthanized.

ORTHOTOPIC METASTATIC MODEL

To study the contribution of CTCs in predicting metastatic disease, we used a xenograft model where GFP-miR-517a-Huh7 cancer cells were orthotopically injected in 11 nude mice. These cells were previously engineered to express GFP and have shown a high metastatic potential in mice.⁽¹⁷⁾ Inhaled isoflurane was used for anesthesia. A bilateral subcostal incision was performed. A total of 2.5×10^6 cells suspended in 50 μ L of PBS were slowly injected in the left lobe of the liver. Hemostasis was controlled by a gentle compression of the puncture site. The wound was closed with sterile 3-0 silk sutures. Liver ultrasound was performed every 2 weeks in three randomly selected mice to monitor tumor development. In order to capture the disease at different stages, mice were euthanized at two different time points, either after 4 (n = 6) or 5 weeks (n = 5).

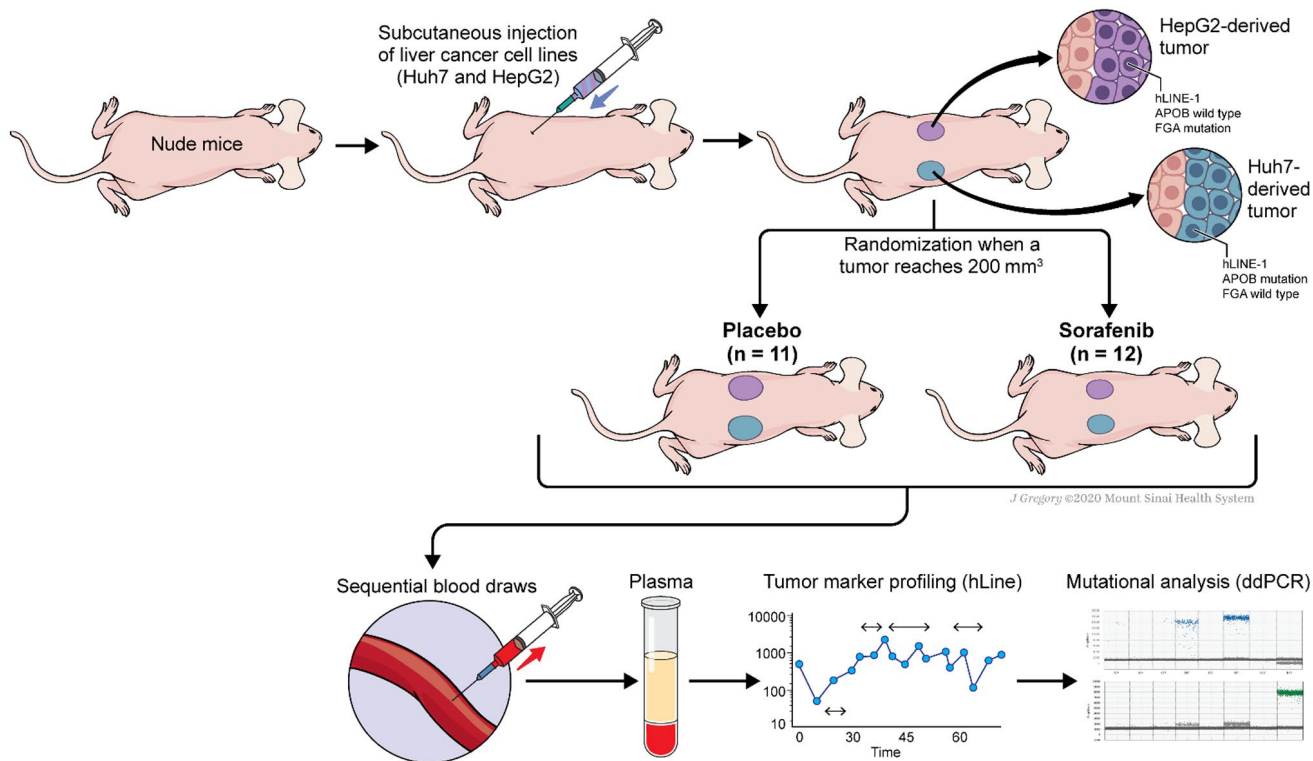


FIG. 1. Analysis of ctDNA to monitor tumor progression and to trace cancer clonal composition. Design of the experiment. *J Gregory* © 2020 Mount Sinai Health System.

BLOOD COLLECTION, PLASMA PREPARATION, AND DNA EXTRACTION

Whole venous blood was serially collected by sub-mandibular vein puncture into a disposable ethylene diamine tetraacetic acid (EDTA) tube. When mice were euthanized, whole venous blood (0.5–1 mL) was collected by heart puncture. We added 100 μ L to 200 μ L PBS (1 \times) in an EDTA-containing 1.5-mL polypropylene tube. Samples were centrifuged at 1,500g for 15 minutes at room temperature. Plasma was collected, aliquoted, and stored at -80°C . Circulating DNA was extracted from plasma samples using the QIAamp circulating nucleic acid kit (Qiagen), following the recommendations of the manufacturer.

Extracted DNA was immediately stored at -20°C .

HISTOPATHOLOGICAL ANALYSES

For subcutaneously xenografted mice, tumors were surgically resected postmortem. In the orthotopic model, autopsy was performed on the euthanized orthotopic models and macroscopic evaluation was made for the detection of primary and metastatic tumors. Tumor tissue and representative sections from grossly uninvolved organs (including lungs, kidney, and brain) were harvested. All specimens were fixed in 10% formalin, processed, and embedded in paraffin blocks. Five-micrometer sections were cut and stained for hematoxylin and eosin. Histopathological analyses included confirmation of carcinoma morphology, analysis of lymphovascular invasion on the liver nodules, and screening for microscopic lesions in organs that were not affected by tumors on gross examination.

HUMAN LONG INTERSPERSED NUCLEAR ELEMENT GROUP 1 QUANTIFICATION BY QUANTITATIVE POLYMERASE CHAIN REACTION

The following primers were used to amplify specifically human long interspersed nuclear element (hLINE) retrotransposons: forward, 5'-TCACTCAAAGCCGCTCAACTA-3'; reverse, 5'-TCTGCCTTCATTTTCGTTATGTACC-3'. All reactions were performed in triplicate. Each reaction was performed in a final volume of 25 μ L with the

following composition: 13.75 μ L H_2O , 2.5 μ L 10 \times polymerase chain reaction (PCR) buffer, 2.5 μ L 10 mM deoxyribonucleotide triphosphate mix, 1.5 μ L dimethyl sulfoxide, 0.5 μ L SYBR Green I dye, 0.5 μ L forward hLINE primer, 0.5 μ L reverse hLINE primer, 0.25 μ L Platinum Taq DNA polymerase, and 3 μ L template DNA. A standard curve was calculated for each plate from DNA-purified control human peripheral blood mononuclear cells (PBMCs). The cycling parameters were as follows: (a) stage 1: activate enzyme, one cycle (94° , 2 minutes); (b) stage 2: touchdown 1, three cycles (94° , 15 seconds; 67° , 20 seconds; 72° , 30 seconds); (c) stage 3: touchdown 2, three cycles (94° , 15 seconds; 64° , 20 seconds; 72° , 30 seconds); (d) stage 4: touchdown 3, three cycles (94° , 15 seconds; 59° , 20 seconds; 72° , 30 seconds); (e) stage 5: quantification, 35 cycles (94° , 15 seconds; 59° , 20 seconds; 72° , 30 seconds); (f) stage 6: melting curve, one cycle.

DIGITAL DROPLET PCR

Droplets were generated using a QX100 Droplet Generator (QuantaLife/Bio-Rad) to partition the DNA and fluorescent-labeled target-specific primers into thousands of droplets. This was followed by a PCR in each single droplet, which accounts for the ultra-high sensitivity of this approach. Digital droplet PCR (ddPCR) mutation assays were designed by Bio-Rad to target clone-specific mutations of the respective cell lines, as previously confirmed by our next-generation sequencing data (apolipoprotein B [*APOB*] for Huh7 and fibrinogen alpha chain [*FGA*] for HepG2; sequences are displayed in Supporting Table S1). The experiments were carried out following the manufacturer's protocol. Readout was done on a QX100 Droplet Reader (QuantaLife/Bio-Rad) in "rare event detection" mode. Cell-line DNA with the respective mutation status served as positive and wild-type controls (Huh7 for *APOB* mutations, HepG2 for *FGA* mutations, and Hep3B for wild-type status of *APOB* and *FGA*). Mutation calling was done with QuantaSoft Analysis Pro Software, version 1.0.596, according to the manufacturer's best practice guidelines.

CTC ENRICHMENT AND IDENTIFICATION

CTC isolation was conducted in the mouse model of orthotopic metastatic HCC. Whole blood was

withdrawn by heart puncture at euthanasia and immediately transferred into a 1.5-mL EDTA disposable tube. The blood was immediately processed for CTC enrichment by Ficoll gradient, adapting the manufacturer's protocol for the amount of blood collected. The blood was diluted in 2 mL of PBS and carefully layered on 3 mL of Ficoll Paque reagent (GE Healthcare). After centrifugation (400g at room temperature according to the manufacturer's instructions), the PBMC layer was collected. If evident red cell contamination was detected at macroscopic examination, a red cell lysis step was added by using a commercial red cell lysis buffer (e-Bioscience). After two washing steps with 2.5% fetal bovine serum–PBS, the cells were immediately stained with a fluorescent anti-mouse monoclonal cluster of differentiation (CD)45 antibody (AF700CD45; BioLegend), according to the manufacturer's instructions, and then analyzed by flow cytometry using DIVA software. We identified miRNA-517 cells by flow cytometry as GFP-positive cells.

DATA ANALYSIS

Categorical variables were provided as frequencies with valid percentages and compared with Fisher's exact test. Continuous data were provided as median with interquartile range and compared with the Mann-Whitney U test. Correlations were assessed with Pearson's test (r). Receiver operating characteristics were generated to calculate the area under the curve. Recurrence and survival rates were plotted with Kaplan-Meier curves and analyzed with the log-rank test. An exact binomial z test of proportions was used to compare mutation detection rate. Statistical significance was defined as $P < 0.05$. All statistical analyses were conducted with IBM SPSS Statistics, version 25.0.

Results

A ctDNA MODEL TO MONITOR TUMOR PROGRESSION AND TRACE CANCER CLONAL COMPOSITION

The design of this experiment aimed to recapitulate the clinical context of patients with advanced HCC treated with systemic therapies (Fig. 1). In order to reproduce ITH, two different cancer cell lines (Huh7

and HepG2) were injected in each flank of 24 mice. Tumor volumes were monitored daily, and mice were randomized with a 1:1 ratio to receive either placebo or sorafenib when tumors reached 200 mm³. Blood samples were sequentially collected until the tumor reached 1,000 mm³. Among the 24 injected mice, 23 (96%) developed at least one tumor in one flank. Both tumors developed in 20 mice (87%), whereas two mice (8.7%) and one mouse (4.3%) only developed Huh7-derived and HepG2-derived tumors, respectively. Tumor profiles of the mice are detailed in Supporting Figs. S1-S4. As predicted, sorafenib was associated with better outcomes,⁽¹⁹⁾ showing significantly slower tumor progression (Fig. 2A) and a prolonged median survival (median survival of 7 days vs. 4 days in mice treated with placebo; $P = 0.002$) (Fig. 2B).

We first evaluated whether ctDNA can be detected in HCC xenografts. To test this, blood was processed to plasma samples and submitted to both qPCR and ddPCR (Fig. 1). The qPCR was performed in 23 samples of seven mice with sequential blood collections. It targeted hLINE-1, a human-specific DNA product that can be used as a proxy of ctDNA because no rodent cells will express it and all should derive from the implanted human cell lines.⁽²⁰⁾ We confirmed amplification of hLINE-1 and how its expression correlated with tumor progression or specific clinical events in 4/7 mice. For examples, hLINE-1 correlated with tumor burden in mouse #799. Mice #252 and #260 developed macroscopic necrosis in HepG2-derived tumors, which was followed by a prompt decrease in hLINE-1 levels in plasma. Mouse #271, which exhibited a tumor response following sorafenib, also had a coincidental reduction of hLINE-1 expression, which subsequently increased following progression of the tumor (Supporting Figs. S1-S4). Thereafter, we aimed to validate ctDNA detection by using a different readout. We analyzed cell line-specific single-nucleotide variants in plasma samples of the 22 mice, using ddPCR (i.e., *APOB* for Huh7 and *FGA* for HepG2). *FGA* mutations were detected in six mice (Fig. 2C).

CANCER CELL CLONES UNEVENLY RELEASE DNA FRAGMENTS INTO THE BLOODSTREAM

ITH has emerged as a major driver of cancer resistance.⁽²¹⁾ Different studies suggest how liquid biopsy

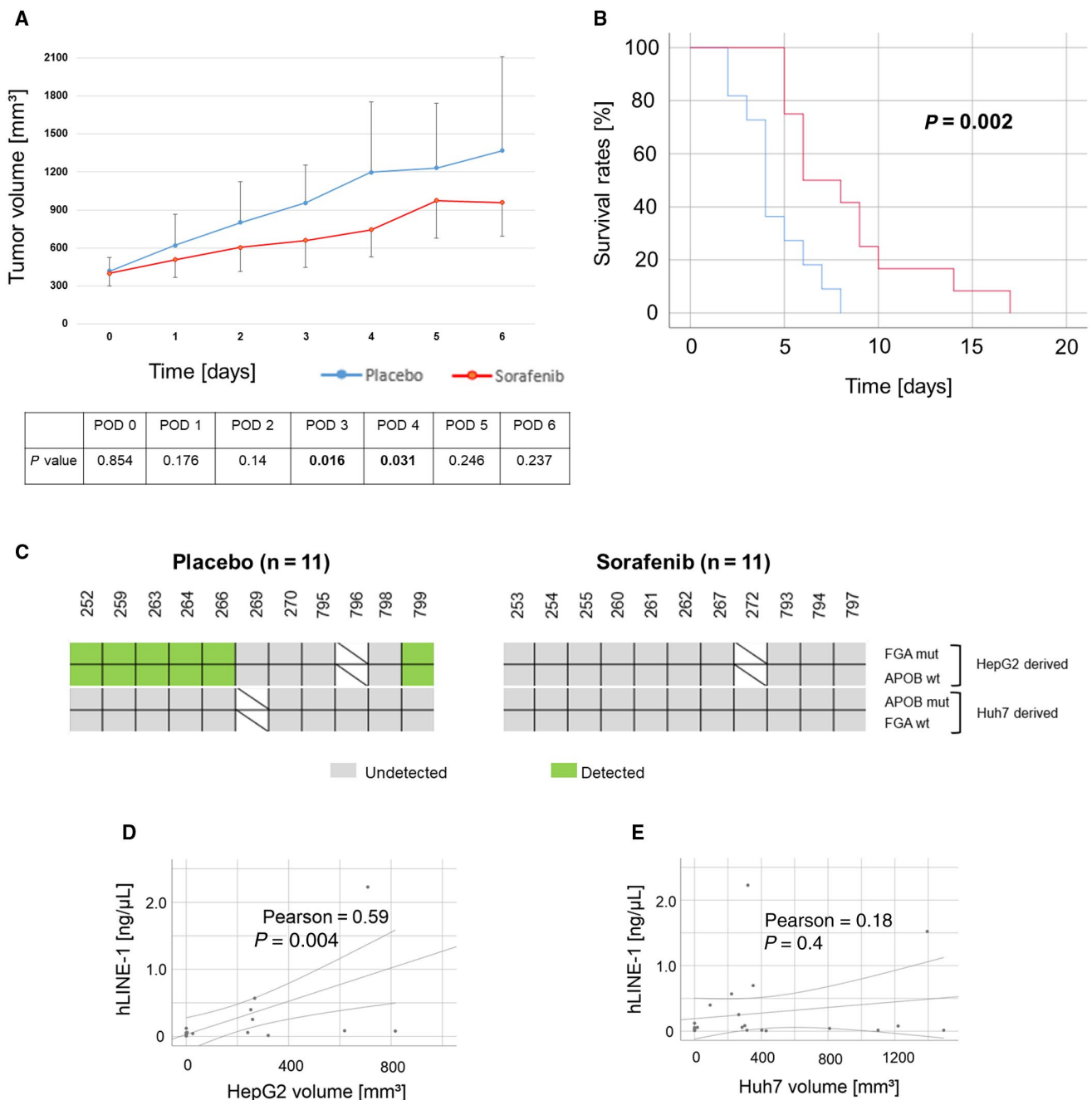


FIG. 2. ctDNA is detected in plasma, and its release is cell clone dependent. (A) Tumor progression (total volume) in mice treated with placebo (blue curve) and sorafenib (red curve). Data show mean \pm SD. (B) Kaplan-Meier curves for survival (time to reach the endpoint: total tumor volume of 1,000 mm³). Blue and red curves illustrate the groups treated with placebo and sorafenib, respectively. (C) Mutational analysis with ddPCR targeting *APOB* and *FGA*. (D) Correlation analysis between HepG2 tumor volume and hLINE-1 concentration. (E) Correlation analysis between Huh7 tumor volume and hLINE-1 concentration. Abbreviations: mut, mutant; PID, post-injection day; wt, wild type.

could capture ITH based on the assumption that all cancer cells release DNA into the bloodstream at a similar rate. Thus, we sought to test this hypothesis using our ITH murine model. Mice with marginal

(#254) or no development of HepG2-derived tumors (#272 and #796) showed low concentrations of hLINE-1 compared to those mice that developed HepG2-derived tumors (0.015 vs. 0.391 ng/uL,

$P < 0.001$). In addition, in HepG2-derived tumors, hLINE-1 was not correlated with tumor burden. We next studied the correlation between tumor volume and hLINE-1 concentration for each cell line and found a moderate correlation for HepG2 ($r = 0.59$, $P = 0.004$; Fig. 2D) but nonlinear correlations with Huh7 ($r = 0.18$, $P = 0.4$; Fig. 2E) or when we combined tumor volumes from both Huh7 and HepG2 cells ($r = 0.40$, $P = 0.07$).

The detection of mutant DNA in plasma with ddPCR showed striking results (Fig. 2C). Overall, *APOB* mutations were captured in none of 21 mice, while mutant *FGA* was detected in six of 20 mice ($P < 0.001$). HepG2-derived tumors harbor an *FGA* mutation and are wild type for *APOB*, as was detectable in six of 10 mice treated with placebo. In contrast, we did not detect *APOB* mutations or *FGA* wild-type DNA in any of the plasma DNA samples, suggesting that Huh7 tumors, which harbor *APOB* mutations and are wild type for *FGA*, have a substantially lower release of DNA fragments compared to HepG2 (Fig. 2C). We also assessed the impact of sorafenib treatment on mutation detection. Among 20 mice that developed HepG2-derived tumors, *FGA* mutations were detected in six of 10 versus none of 10 animals in the placebo and sorafenib groups, respectively ($P = 0.011$). This indicates that (a) treatment impacts mutation detection and (b) the difference in ctDNA release between different clones is not due to pharmacologic factors because it was observed in the placebo group. These results of positive and negative controls allowed us to reasonably exclude a technical issue related to poor sensitivity of our assay (Supporting Fig. S5). Altogether, qPCR and ddPCR results consistently suggested that the release of DNA fragments into the bloodstream is different in the different cancer cell lines.

CTC DETECTION IS A PREDICTIVE FACTOR OF LUNG METASTASES AND CORRELATES WITH THE NUMBER OF LIVER NODULES

We next sought to explore if CTC detection predicts metastatic disease and whether the number of detected CTCs correlates with tumor burden. We previously derived a particularly aggressive clone of Huh-7 by using stable transfection of miRNA-517.

This cell is particularly aggressive and can induce metastases in 40% of mice after orthotopic injection.⁽¹⁷⁾ Thus, we injected this cell line orthotopically in 11 nude mice (Fig. 3). Tumor progression was monitored by liver ultrasound, and autopsy was performed after 4 ($n = 6$) or 5 ($n = 5$) weeks, where whole blood and organs (liver, lungs, kidneys, cerebrum, peritoneum, and heart) were collected. Blood was processed and submitted to flow cytometry to detect CTCs.

Four weeks after injection, ultrasound identified HCC liver nodules in nine of 11 (82%) mice, with five of nine (56%) and four of nine (44%) mice showing single or multiple nodule(s), respectively (Table 1; Fig. 4A). Pathologic examination of the specimens confirmed macroscopic nodules in nine of 11 mice in which HCC nodules had been detected by ultrasound (Fig. 4B). Microscopic disease was detected in the two mice without visible nodules on ultrasound; therefore, 11 of 11 (100%) mice developed HCC. Histologic analyses also highlighted vascular invasion in seven of 11 (64%) mice (Fig. 4C). Dissemination to the lungs was reported in four of 11 (36%) mice (Fig. 4D), among which two mice (18%) also showed peritoneal implants.

As miRNA-517-Huh7-injected cells were tagged with GFP, flow cytometry targeting GFP and CD45 allowed us to easily trace CTCs. Overall, CTCs were detected in four of 11 (36%) mice (Fig. 4E). These were the mice that harbored lung metastases, whereas no CTCs were detected in the seven of 11 mice without metastasis ($P = 0.003$). The amount of detected CTCs correlated with tumor volume ($r = 0.70$, $P = 0.01$) but not with number of nodules ($r = 0.30$, $P = 0.36$) or the largest nodule diameter ($r = 0.54$, $P = 0.08$).

Discussion

This study demonstrates the feasibility of modeling liquid biopsy in HCC xenografts and allowed us to identify cell clone origin as a potential major determinant of DNA fragment release into the bloodstream. Numerous studies have shown how liquid biopsy has become a promising source for biomarker development in oncology. However, there are few experimental models designed to evaluate ctDNA dynamics or the main mechanisms involved

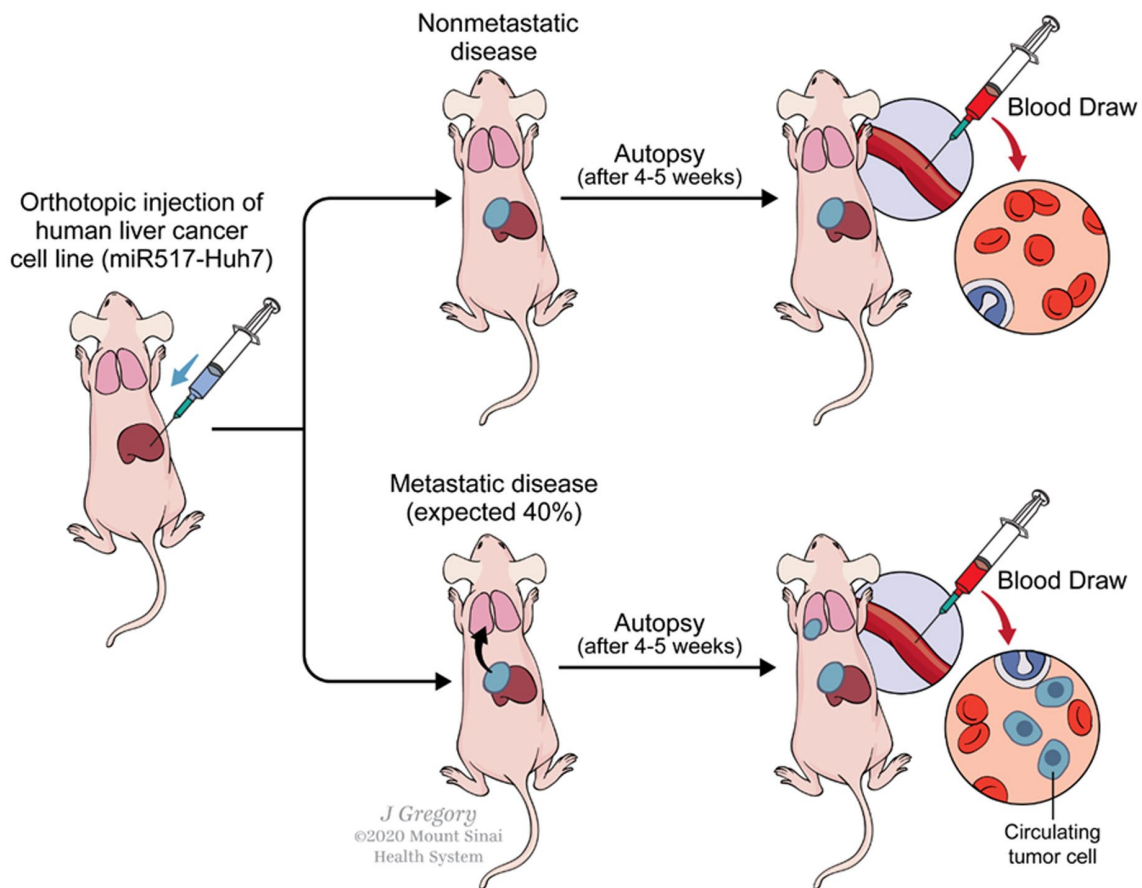


FIG. 3. Analysis of CTCs to predict metastatic disease. Design of the experiment. *J Gregory* © 2020 Mount Sinai Health System.

TABLE 1. SUMMARY OF THE CTC EXPERIMENT

	Orthotopic HCC Xenografted Nude Mice (n = 11)
Imaging	
Liver nodules on US (4 weeks after injection)	9/11 (82%)
Pathology	
Evidence of HCC development	11/11 (100%)
Macroscopic nodules	9/11 (82%)
Microscopic nodules	2/11 (18%)
Vascular invasion	7/11 (64%)
Dissemination to lungs	4/11 (36%)
Peritoneal implants	2/11 (18%)
Blood	
CTC detection	4/11 (36%)

Abbreviation: US, ultrasound.

in ctDNA release. In 2007, Rago et al.⁽²⁰⁾ published a pioneering study on liquid biopsy in xenograft models. Using hLINE-1, they were able to track ctDNA in serial measurements, highlighting its correlation with tumor burden. Our study expands on this initial report and offers additional insights on how ctDNA and CTC quantification are impacted by ITH, systemic therapies, and cancer dissemination. Although hLINE-1 is an elegant proxy of ctDNA, its monitoring is unfortunately not applicable in patients because hLINE-1 in humans may also be released into the bloodstream by noncancer cells.

Numerous reports underscore the prognostic value of CTC enumeration in multiple tumor types, including HCC.^(22,23) More sophisticated genomic characterization of CTCs using single-cell RNA

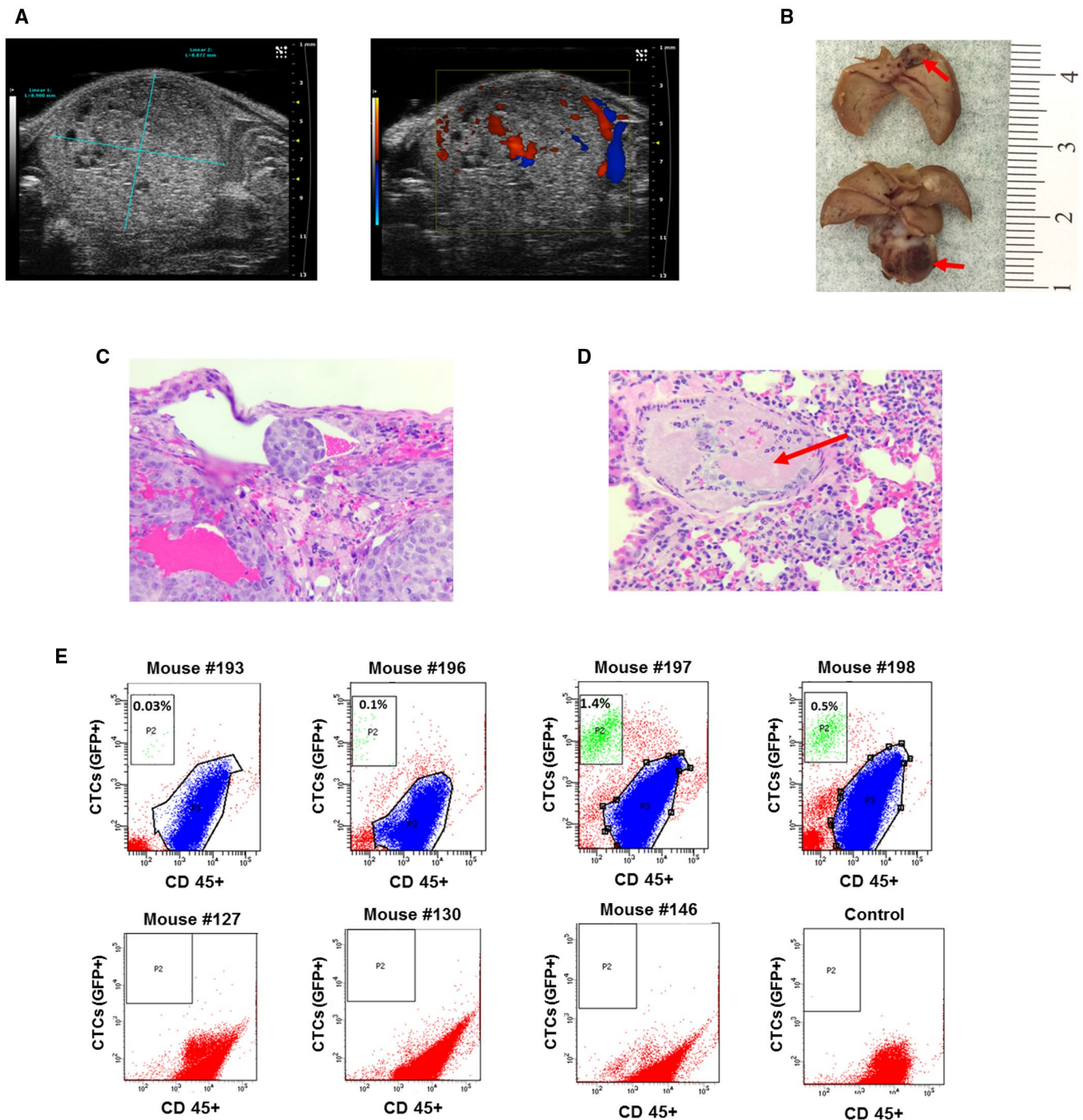


FIG. 4. CTC detection predicts lung metastases and correlates with hepatic tumor burden. (A) Liver ultrasound at 4 weeks. This exemplificative image shows a hypervascular intrahepatic nodule 0.8×0.8 cm. (B) Pathologic analysis of livers and HCC nodules (arrows) (macroscopy). (C) Pathologic analysis of vascular invasion (histology, hematoxylin and eosin staining). (D) Pathologic analysis of lung metastasis (arrow) (histology, hematoxylin and eosin staining). (E) Flow cytometry of blood from mice with HCC and lung infiltration (mice 193, 196, 197, and 198), mice with no lung infiltration (127, 130, 146), and a control mouse.

sequencing enhanced our ability to track their tissue of origin and identify potential driver genes.⁽²⁴⁾ Detection of CTCs in plasma of animal models

has been reported in several cancers. As an example, studies in pancreatic cancer demonstrated that CTC analyses in animal models may offer valuable

opportunities to investigate the seed and soil theory.^(25,26) In HCC, however, data remain scarce.⁽²⁷⁾ Likewise, genetic analyses on ctDNA has emerged as a potent source for minimally invasive detection of DNA copy number variations, mutations, and methylation alterations of a tumor.^(6,28,29) Other than studies where blood samples of different animals were pooled to obtain a sufficient volume of plasma, there are few reports on circulating mutant DNA in plasma of animal models,⁽³⁰⁾ such as mutation calling using ddPCR in lung cancer xenografts.⁽³¹⁾ Our study helps fill this gap and demonstrates how the application of highly sensitive detection techniques (e.g., ddPCR) enable accurate monitoring of ctDNA in HCC xenografts.

Recent studies have mapped the extent and implication of ITH in HCC.⁽²¹⁾ While ITH cannot be adequately assessed using a single tissue biopsy, data suggest that liquid biopsy could be a good proxy for ITH as it can capture tumor components from different areas.⁽¹²⁾ Thus, we tried to evaluate whether liquid biopsy could detect DNA fragments from different cell lines in our xenografts, which will recapitulate ITH as a result of clonal evolution. We targeted specific mutations that were exclusive to each cell line (i.e., *APOB* to Huh7 and *FGA* to HepG2). This allowed us to easily trace cancer clonal composition, and we found an uneven release of DNA fragments between these two clones. Our approach does not allow us to rule out differences in DNA fragment degradation rate, but considering that both cell lines were implanted in the same animal, we believe this is improbable. The ddPCR results were also robust. Assumption of false-positive results is unlikely because HepG2-derived DNA was identified by both *FGA* mutant and *APOB* wild-type pairwise detection. False negatives are also improbable because Huh7-derived DNA readouts (i.e., *APOB* mutant and *FGA* wild type) were not captured in any animals despite large tumors and positive controls working well. Our data question the assumed notion that ctDNA release is a linear process and that all tumor clones release DNA fragments to the bloodstream at a similar pace. This suggests that liquid biopsy may have some limitations when designed to accurately map the complete landscape of mutations present in a given patient. A recent study using colorectal cancer (CRC) xenografts showed similar results. Authors analyzed different CRC cell lines and found that DNA fragments were released differently

in vivo and *in vitro*. In addition, the likelihood of detecting a mutation in plasma was dictated by its cell clone of origin rather than by its genomic location.⁽³⁰⁾ Some differences with our approach include that those researchers had to pool plasma samples of different animals in order to perform sequencing analysis and their xenografts were derived from a single cell so that ITH could not be assessed in the same animal.

One limitation of our study is that the analyses included only two liver cancer cell lines. It would be interesting to increase the number of different cell lines to assess how DNA release may vary between other cell clones. There is no reason to believe that the difference in DNA release we found is limited to Huh7 and HepG2. Despite our model only discriminating between two liver cancer cell lines, our successful execution certainly paves the way to study more complex scenarios of ITH, including other cancer types. Our models also lack the typical tumor microenvironment (TME) of human HCC (e.g., immunodeficient mice, subcutaneous injection). Thus, we cannot estimate the impact of TME in ctDNA release. Also, although our approach discriminated ctDNA derived from two very distinct liver cancer cell lines (Huh7 and HepG2), we have not tested its performance in scenarios where molecular differences among tumor clones are more subtle, which is likely more common when dealing with human ITH. On a technical perspective, we controlled for a number of injected cells and location of injection, but we cannot conclusively rule out other potential technical issues impacting ctDNA release (e.g., precise location of injection). Another limitation could be the modest sample size, particularly for the metastatic mouse model.

In summary, our study paves the way to intensify the effort and to conduct future studies on liquid biopsy in animal models to further unveil the underlying mechanisms of tumor by-product release to the bloodstream. These results demonstrate the feasibility of modeling liquid biopsy in HCC xenografts. We show tumor clone-dependent rates of ctDNA detection, ctDNA dynamics following systemic therapy, and the role of CTC detection to predict clinical endpoints, such as the development of distant metastasis.

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