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TITLE: Predicting Outcome of Transplant-Eligible Patients with Hepatocellular Carcinoma

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CONTRACTING ORGANIZATION: Johns Hopkins University, Baltimore, MD

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14. ABSTRACT

In recent years, significant advancements in systemic therapies for hepatocellular carcinoma (HCC) have markedly improved overall survival rates, expanding the scope of curative treatment options like resection and transplantation, which were previously limited to early-stage HCC. However, a key challenge in clinical practice remains the accurate identification of patients most likely to achieve successful long-term responses to these therapies. This study aims to address this challenge by developing a multi-modal prognostic algorithm that integrates radiographic data and innovative blood-based tumor markers to predict treatment responses and identify potential candidates for liver transplantation. The study cohort comprises HCC patients initially considered ineligible for transplantation due to their elevated baseline tumor burdens, but who subsequently undergo liver-directed treatments and immunotherapy. Our comprehensive approach involves the analysis of blood and urine samples for circulating tumor cells and circulating tumor DNA, as well as the detailed evaluation of MRI and CT images to extract valuable radiographic information. Over the course of one year, patients are closely monitored, and outcomes are assessed as they either achieve transplantation eligibility through down-staging or experience disease progression. This ongoing study holds the potential to pinpoint individuals who can benefit from early, more aggressive interventions, thereby acting as a bridge to liver transplantation and preventing further deterioration of liver function. It represents a significant stride forward in the realms of HCC treatment and transplantation eligibility assessment.

15. SUBJECT TERMS None listed.					
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1. INTRODUCTION:

Recent advancements in systemic therapies have enhanced survival rates for hepatocellular carcinoma (HCC) patients. These medical strides potentially extend curative options, like resection and transplantation, which were once reserved for only early-stage HCC cases. Yet, a critical gap persists: the absence of dependable clinical predictors for sustained therapeutic response. This research endeavors to construct a comprehensive prognostic algorithm, melding both radiographic insights with innovative blood-based tumor markers. The intent is to refine treatment outcome predictions and discern optimal candidates for liver transplantation. The study will concentrate on HCC patients who, given their substantial tumor load, are currently unsuited for transplantation but are undergoing specialized treatments and immunotherapy. Leveraging the detection of novel cancer biomarkers in blood and urine, and enhancing MRI and CT scan evaluations, the research aims to capture a more nuanced understanding of the disease trajectory. Over a 12-month span, patient conditions will be assessed to ascertain their readiness for transplantation or track any cancer exacerbation. The ultimate goal is to discern and promptly treat those poised to gain substantially from liver transplantation before their health further regresses.

2. KEYWORDS:

HCC, liver transplantation, prognostic model, liquid biopsy, circulating tumor cells, ctDNA

3. ACCOMPLISHMENTS:

Aim 1: To develop a prognostic algorithm using comprehensive clinical data and new radiographic parameters to predict responders to systemic treatments in patients with HCC and get listed for transplantation.

1) Objective 1: To establish a longitudinal registry that includes clinical and research data from patients with HCC.
   a) ACCOMPLISHMENT: With the no-cost extension approval, our goal for this year was to increase the patient recruitment for the study considering the negative impact we had with patient recruitment in the previous years during the pandemic. Between 9/1/2022 to 9/31/2023, we recruited 13 new patients from whom we obtained biospecimen including serial collection of blood, urine and tissue. Five patients also have post-treatment liver biopsy samples. We will continue enrollment until February 2024. Some of the blood and urine samples have begun analysis, as outlined below.
   b) ACTIVITIES: To achieve this goal with a higher number of patients, we started the process to include Precision Medicine analytics Platform (PMAP) offered by the institution to capture more data. PMAP allows automatic data collection from the electronic medical records and other research database (Transplant database) and will help identify the study target population who were referred to transplant for HCC but could not be a transplant

candidate because of their tumor burden and prognosis. This is currently in progress through our GI/Hepatology Clinical Translational Research Unit.

2) **Objective 2: Analysis of clinical risk factors and radiographic markers.** Subtasks include Collection of imaging data and gather clinical risk factors for prognostic model development and manuscript preparation.
   a) ACTIVITIES: Since the last publication on radiographic prognostics of HCC (year 2), we are continuing to collect data from the recent prospectively recruited patients. One of the major challenges initially was not having enough patient data for model development. Additional data pulled from the transplant database through PMAP will help to proceed with a multivariate regression analysis. I plan to complete the clinical risk factor analysis by the summer of 2024, followed by manuscript preparation.
   b) ACCOMPLISHMENT: During this course of work, we noted that MRI and CT imaging from HCC patients also capture esophageal varices that are prone to bleeding and are major clinical complications in this cohort. We hypothesized these varices may be captured by routine imaging and can predict bleeding diathesis. We conducted a retrospective study of 350 patients with and without HCC who had upper endoscopy for varices screening and with a high-resolution imaging within 3-month interval. We found that CT/MRIs have a high negative predictive value (NPV) of 92% in screening for high-risk esophageal varices and other bleeding sources. The manuscript is complete and pending re-submission for publication.

Aim 2: To determine if sequential liquid biopsies from patients with HCC can better predict treatment response.

1) **Objective 2: To isolate circulating tumor cell (CTC) from the patient's blood samples to use as a surrogate marker of HCC tissue.**
   a) ACCOMPLISHMENT: we completed our multiplex IF panel for CTC characterization. Single molecule FISH for stem cell characteristics in CTCs, first in HCC cells.
   b) ACTIVITY: We are continuing with immunostaining (IHC and IF) on cells from patients at this time. While most of the CTCs are cytokeratin 8/18 and Epcam+ (Fig 1), we also

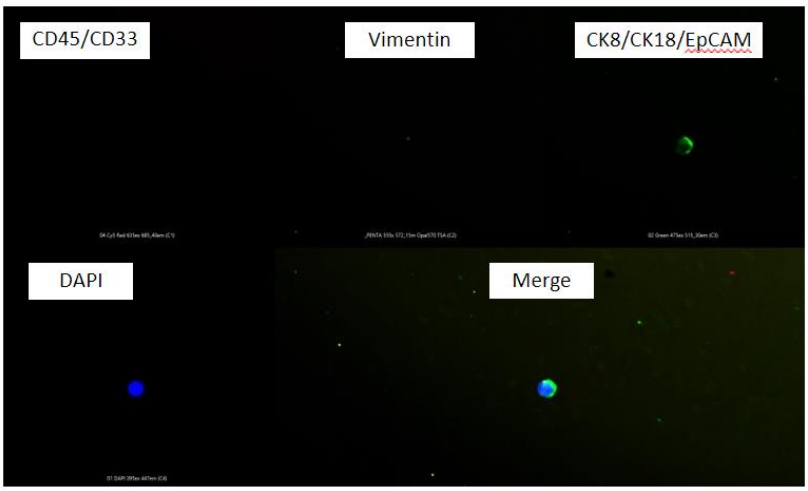

Figure 1a - cells from patient with CK/Epcam expression.

identified cells expressing only vimentin (Fig 2) , suggesting epithelial to mesenchymal transition.

d) We had some challenges with quantification because of variability of the sample collection platform and processing for consistency. We overcame this by equalizing the volume of blood as much as possible. We also also investigating stemcell characteristics of the cells with LGR5 smFISH and we will correlate with matched patient samples.

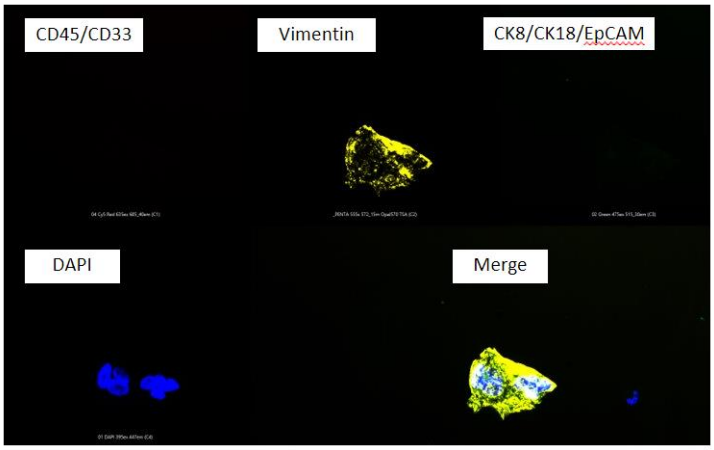

Figure 2 - CTC with vimentin expression. CD45(-)

2) **Major Task 2: circulating tumor DNA (ctDNA) and T-cell receptor (TCR) sequencing - sample collection, processing and analysis**

We are completing the serial sample collection with our last few enrollments. The available samples are being processed in the interim.

3) Objective : Tissue sample comparison

a) Immunostaining (IHC, IF) of tissue for comparison with CTC is in process.

b) DNA sequencing from tumor tissue (20 tissue samples) – this has not been started. We plan to do target sequencing of the HCC tissue and data will be compared to matched blood sample analysis

4) T-cell receptor sequencing from blood samples.
Activity: plans to use CyTOF using PBMC samples. We have not started the analysis yet.

What opportunities for training and professional development has the project provided?

Training – I have ongoing meeting with mentors, Dr. Goggins for ongoing basic science mentorship and research projects. Dr. Clark for career guide and advice on grantsmanship and writing.

Professional Development –
I continue to participate in K-to-R seminar series offered by the Office of Faculty Development with topics including running a lab and application process for independent grants by senior faculty. I was also accepted and enrolled in Department of Medicine Midcareer Women's Leadership conference this year which included biweekly conferences with colleagues on developing a successful career as a researcher and clinician in an academic medicine.
The research time allowed from the award and the work from the past two years allowed me to participate in national meetings as a co-chair/moderator of a session and presentation of my work.

How were the results disseminated to communities of interest?

ctDNA work in HCC was presented at the national meeting AASLD (Washington DC 11/2022). I also presented at Liver Talks webinar in Vietnam in collaboration with Hochiminh City International University 5/4/2023, and at a joint conference with Johns Hopkins and University of Maryland supported by the Maryland Cigarette Restitution Fund

The results of liquid biopsy in liver cancer generated much attention in the community as well. News media – (https://www.genengnews.com/news/liver-cancer-detected-by-ai-blood-test/) and NCI website news (https://www.cancer.gov/news-events/cancer-currents-blog/2023/liver-cancer-liquid-biopsy-fragmentomics).

What do you plan to do during the next reporting period to accomplish the goals?

I plan to submit abstracts to ASCO-GI (national clinical oncology meeting) and AASLD (national liver society) in 2024, while the manuscript is in process.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

We discovered that fragments of ctDNA is a robust diagnostic test that can significantly improve early detection of cancer. this finding is now being applied as a prognostic indication of HCC in this study. If ctDNA is found to be a reliable test to predict treatment response and prognosis, it can be applied to clinical management and decisions for patients.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

I have had difficulty in accurate quantification of CTCs using epithelial markers as there are variabilities depending on the blood collection method and cell enumeration despite standardizing

protocols. I have changed the marker to look into stem cell markers of liver cancer to characterize both CTC and the liver tissue.

Changes that had a significant impact on expenditures

I had hiring delays from May to August for lab specialist for this project. The no-cost extension that was granted helps to cover the expense of the staff to finish the work this year.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
None

Significant changes in use or care of human subjects
None

Significant changes in use or care of vertebrate animals
None

Significant changes in use of biohazards and/or select agents
None

6. PRODUCTS:

- **Publications, conference papers, and presentations**
  Journal publications.

1. Foda ZH, Annapragada A., Boyapati K, Bruhm DC, Vulpescu NA, Medina JE, Mathios D, Cristiano S Niknafs N, Luu HT, Goggins MG, Anders RA, Sun J, Mehta SH, Thomas DL, Kirk GD, Adleff V, Phallen J, Scharpf RB, …**Kim AK***, Velculescu VE***.** Detecting liver cancer using cell-free DNA fragmentomes. Cancer Disc. 2023 Mar 1;13(3):616-631. (*shared corresponding authorship) PMID: 36399356; PMCID: PMC9975663.
2. **Kim AK**, Lin SY, Wang Z, Luu H, Hamilton JP, Song W, Su YH. Impact of Cell-Debris and Room-Temperature Storage on Urine Circulating Tumor DNA from Hepatocellular Carcinoma. J Mol Diagn. 2023 Oct 9:S1525-1578(23)00221-0. doi: 10.1016/j.jmoldx.2023.08.006. Epub ahead of print. PMID: 3781329
3. Bruhm DC, Mathios D, Foda ZH, Annapragada AV, Medina JE, Adleff V, Chiao EJ, Ferreira L, Cristiano S, White JR, Mazzilli SA, Billatos E, Spira A, Zaidi AH, Mueller J, **Kim AK,** Anagnostou V, Phallen J, Scharpf RB, Velculescu VE. Single-molecule genome-wide mutation profiles of cell-free DNA for non-invasive detection of cancer. Nat Genet. 2023 Aug;55(8):1301-1310. doi: 10.1038/s41588-023-01446-3. Epub 2023 Jul 27. PMID: 37500728; PMCID: PMC10412448.

4. Gupta A, Zorzi J, Ho WJ, Baretti M, Azad NS, Griffith P, Dao D, **Kim A**, Philosophe B, Georgiades C, et al. Relationship of Hepatocellular Carcinoma Stage and Hepatic Function to Health-Related Quality of Life: A Single Center Analysis. *Healthcare*. 2023; 11(18):2571.

Books or other non-periodical, one-time publications.

Other publications, conference papers and presentations.

1. **Kim AK**, Urrunaga N, Zhu QF, Teferi L, Lee S, Rosenberg AZ, Rabiee A, Liddell RP, Georgiades C, Gurakar A, Ottmann S, Yarchoan M, Hong K, Anders RA. Transarterial chemoembolization enhances the tumor microenvironment of hepatocellular carcinoma. AASLD 2022. Washington DC. Published Abstract October 2022. [Selected as one of the four posters within Liver Cancer section for debriefing on Nov 6, 2022]
2. Foda ZH, Annapragada A., Boyapati K, Bruhm DC, Vulpescu NA, …**Kim AK**, Velculescu VE**.** Detecting liver cancer using cell-free DNA fragmentomes. American Association of the Study of Liver Disease. November 2022.

- **Website(s) or other Internet site(s)**
  NCI blog https://www.cancer.gov/news-events/cancer-currents-blog/2023/liver-cancer-liquid-biopsy-fragmentomics
  Science new media - https://www.genengnews.com/artificial-intelligence/liver-cancer-detected-by-ai-blood-test/

- **Technologies or techniques**
  None

- **Inventions, patent applications, and/or licenses**
  None

- **Other Products**
  NTR

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Example:

Name: *Boyoung Cha*

Project Role: *Research Specialist*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *3*

Contribution to Project: *bench related work for this project, including downstream analysis of the cells from human specimens including PCR, IHC/IF assays and FISH.*
Funding Support: *current award*

Name: *Harry Luu*
Project Role: *Clinical Research Coordinator*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *5*

Contribution to Project: *Patient recruitment and enrollment. Sample collection including blood and tissue if available. Maintenance of the electronic database*
Funding Support: *Division funding support and NIH K08 award.*

Name: *Kanako Yoshida*
Project Role: *Part-time research volunteer*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *3*

Contribution to Project: *Image review and sample collection*
Funding Support: *current award*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report

What other organizations were involved as partners?
Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* https://ebrap.org/ *for each unique award.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

CURRICULUM VITAE

The Johns Hopkins University School of Medicine

Signature  10-01-2023

AMY K. KIM

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Doctoral/Graduate:
7/03-6/07 MD, Medicine. Medical College of Virginia, Richmond, VA

Postdoctoral:
7/07-6/08 Internship. Internal Medicine, Duke University Medical Center, Durham, NC
7/08-6/10 Residency. Internal Medicine, Duke University Medical Center, Durham, NC
9/08-11/08 Professional Development. Comprehensive Introduction to Clinical Research, Duke University, Durham, NC
7/10-6/13 NIH T32 Fellowship. Gastroenterology & Hepatology, Yale School of Medicine, New Haven, CT
7/13-6/14 Fellowship. Transplant Hepatology, Yale School of Medicine, New Haven, CT

Professional Experience

9/14-present Assistant Professor. Division of Gastroenterology & Hepatology, Department of Medicine, JohnsHopkins University, Baltimore, MD

PUBLICATIONS

Original Research [OR]

Peer Reviewed Original Science Publications

1. Omuro AM, Ben-Porat LS, Panageas K, **Kim AK**, Correa DD, Yahalom J, DeAngelis LM, Abrey LE. Delayed Neurotoxicity in Primary Central Nervous System Lymphoma. Arch Neurol. 2005; 62:1595-1600.

2. Kallman J, Srivastava R, **Kim AK**, Younossi Z, "The impact of Chronic Liver Disease and Cirrhosis on Health Utilities Using SF-6D and Health Utility Index" Liver Transpl. 2008 Mar: 14(3):321-6.
3. Spirli C, Morell C, Locatelli L, Ferrero C, **Kim AK**, Okolicsanyi S, Fiorotto R, Strazzabosco M. "Paradoxical activation of Raf/MEK/ERK signaling in polycystin-2 defective treated with Sorafenib" Hepatology. 2012 Dec: 56(6):2363-74
4. Jakhete N, Saberi B, Jonassaint NL, Cosar AM, Luu H, **Kim AK**, Anders RA, et al. Microvascular Invasion in Hepatocellular Carcinoma and Liver Transplant. Exp Clin Transplant 2016; 14:14-18.
5. Chianchiano P, Pezhouh M, **Kim AK**, Luchini C, Cameron AD, Weiss M, He J, Voltaggio L, Oshima K, Anders RA, Wood LD. Distinction of Intrahepatic Metastasis from Multicentric Carcinogenesis in Multifocal Hepatocellular Carcinoma Using Molecular Alterations. Hum Pathol. 2018 Feb; 72:127-134. PubMed PMID: 29180252; PubMed Central PMCID: PMC6435273
6. Yarchoan M, Xing D, Luan L, Sharma R, Pawlik TM, **Kim AK**, Zhu Q, Jaffee E, Taube J, and Anders RA. Characterization of the Immune Microenvironment in Hepatocellular Carcinoma. Clin Cancer Res. 2017 Dec 1;23(23):7333-7339. PubMed PMID: 28928158; PubMed Central PMCID: PMC5881396.
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10. Chaudhari P, Tian L, **Kim AK**, Zhu Q, Anders R, Schwarz KB, Sharkis S, Ye Z, Jang YY. Transient c-Src Suppression During Endodermal Commitment of Human Induced Pluripotent Stem Cells Results in Abnormal Profibrotic Cholangiocyte-Like Cells. Stem Cells. 2019 Mar;37(3):306-317.
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14. Hammami MB, Garibaldi B, Shah P, Liu G, Jain T, Chen PH, **Kim AK**, Avdic E, Petty B, Strout S, Fine DM, Niranjan-Azadi A, Garneau WM, Cameron AM, Monroy Trujillo JM, Gurakar A, Avery R. Clinical course of COVID-19 in a liver transplant recipient on hemodialysis and response to tocilizumab therapy: A case report. Am J Transplant. 2020 Aug;20(8):2254-2259. PMID: 32359210
15. Abdalla A, Malangone-Monaco E, Noxon V, Henriques C, Benavente F, **Kim A.** Treatment patterns and direct medical costs among patients with advanced hepatocellular carcinoma. Curr Med Res Opin. 2020 Nov;36(11):1813-1823. PMID: 32969741.
16. Muhammad H, Tehreem A, Ting PS, Gurakar M, Li SY, Simsek C, Alqahtani SA, **Kim AK**, Ruhail Kohli RK, Gurakar A. Hepatocellular Carcinoma and the Role of Liver Transplantation: A Review Journal of Clinical and Translational Hepatology. J Clin Transl Hepatol 2021 Oct 28;9(5):738-748. PMID:34722189

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18. Li D, Jia AY, Zorzi J, Griffith P, **Kim AK,** Dao D, Anders RA, Georgiades C, Liddell RP, Hong K, Azad NS, Ho, WJ, Baretti M, Christenson E, Baghdadi A, Kamel IR, Meyer J, Ghabi E, Burkhart RA,Yarchoan, M. Impact of the COVID-19 Pandemic on Liver Cancer Staging at a Multidisciplinary Liver Cancer Clinic. *Annals of Surgery Open : Perspectives of Surgical History, Education, and Clinical Approaches*, 2022 Oct *3*(4), e207.
19. Krishnan, A., Prichett, L., Liu, Y., Ting, P.-S., Alqahtani, S. A., **Kim, A. K**., Ma, M., Hamilton, J. P., Woreta, T. A., & Chen, P.-H. (2022). Risk of Severe Illness and Risk Factors of Outcomes of COVID-19 in Hospitalized Patients with Chronic Liver Disease in a Major U. S. Hospital Network. *Canadian Journal of Gastroenterology & Hepatology*, *2022*, 8407990.
20. Baghdadi A, Luu HT, Shaghaghi M, Ghadimi M, Simsek C, Xu Z, Hazhirkarzar B, Motaghi M, Hammami M, Clark JM, Gurakar A, Kamel IR, **Kim AK.** Magnetic Resonance Imaging Predictors of Hepatocellular Carcinoma Progression and Dropout in Patients in Liver Transplantation Waiting List. Transplant Direct. 2022 Oct 18;8(11):e1365. PMID: 36284930; PMCID: PMC9584197.
21. Fan C, Kim, AY, … **Kim AK.** Outcomes of immunotherapy related hepatotoxicity from a multi-disciplinary toxicity team. Journal of Cancer Research and Clinical Oncology 2022 Feb;149(2):877-883. PMID: 36102989.
22. Foda ZH, Annapragada A., Boyapati K, Bruhm DC, Vulpescu NA, Medina JE, Mathios D, Cristiano S Niknafs N, Luu HT, Goggins MG, Anders RA, Sun J, Mehta SH, Thomas DL, Kirk GD, Adleff V, Phallen J, Scharpf RB, …**Kim AK***, Velculescu VE***.** Detecting liver cancer using cell-free DNA fragmentomes. Cancer Disc. 2023 Mar 1;13(3):616-631. (*shared corresponding authorship) PMID: 36399356; PMCID: PMC9975663.
23. **Kim AK**, Lin SY, Wang Z, Luu H, Hamilton JP, Song W, Su YH. Impact of Cell-Debris and Room-Temperature Storage on Urine Circulating Tumor DNA from Hepatocellular Carcinoma. J Mol Diagn. 2023 Oct 9:S1525-1578(23)00221-0. doi: 10.1016/j.jmoldx.2023.08.006. Epub ahead of print. PMID: 3781329
24. Bruhm DC, Mathios D, Foda ZH, Annapragada AV, Medina JE, Adleff V, Chiao EJ, Ferreira L, Cristiano S, White JR, Mazzilli SA, Billatos E, Spira A, Zaidi AH, Mueller J, **Kim AK,** Anagnostou V, Phallen J, Scharpf RB, Velculescu VE. Single-molecule genome-wide mutation profiles of cell-free DNA for non-invasive detection of cancer. Nat Genet. 2023 Aug;55(8):1301-1310. doi: 10.1038/s41588-023-01446-3. Epub 2023 Jul 27. PMID: 37500728; PMCID: PMC10412448.
25. Gupta A, Zorzi J, Ho WJ, Baretti M, Azad NS, Griffith P, Dao D, **Kim A**, Philosophe B, Georgiades C, et al. Relationship of Hepatocellular Carcinoma Stage and Hepatic Function to Health-Related Quality of Life: A Single Center Analysis. *Healthcare*. 2023; 11(18):2571. https://doi.org/10.3390/healthcare11182571
26. Utilization of radiomics features extracted from pre-operative medical images to detect metastatic lymph nodes in cholangiocarcinoma and gallbladder cancer patients: A systemic Review and Meta-Analysis. J Computer Assisted Tomography (accepted. Not published)

Review Articles [RA]

1. **Kim AK**, Dziura J and Strazzabosco M. Nonsteroidal anti-inflammatory drug use, chronic liver disease, and hepatocellular carcinoma. Hepatology 2013 Aug: 58(2):819-21
2. **Kim AK**, Schilsky, ML. Radiology in Transplant. Curr Transpl Reports. 2014 Dec; 1(4):238-245
3. **Kim AK**, Singal AG. Health disparities in diagnosis and treatment of hepatocellular carcinoma. Clinical Liver Disease. 2014 Dec; 4:143–145.
4. Grandhi MS, **Kim AK**, Kamel IR, Ghasebeh MA, Pawlik TM. Hepatocellular Carcinoma: Diagnosis to Treatment. Surg Oncol 2016 Jun; 25(2):74-85.
5. Su YH, **Kim AK,** Jain S, Liquid Biopsy in HCC. Transl Res. 2018 Nov; 201:84-97.
6. Scarlotta M, Simsek C, **Kim AK.** Liquid biopsy in Solid Tumors. Genetic Testing and Molecular Biomarkers. 2019 Apr;23(4):284-296. doi: 10.1089/gtmb.2018.0237. Epub 2019 Mar 27. Review. PubMed PMID: 30916594

Case Reports [CR]

1. Hammami MB, Garibaldi B, Shah P, Liu G, Jain T, Chen PH, **Kim AK**, Avdic E, Petty B, Strout S, Fine DM, Niranjan-Azadi A, Garneau WM, Cameron AM, Monroy Trujillo JM, Gurakar A, Avery R. Clinical course of COVID-19 in a liver transplant recipient on hemodialysis and response to tocilizumab therapy: A case report. Am J Transplant. 2020 Aug;20(8):2254-2259. PMID: 32359210; PMCID: PMC7267667.
2. Lin JS, Zaffar D, Muhammad H, Ting PS, Woreta T, **Kim A,** Kohli R, Oshima K, Cameron A, Philosophe B, Ottmann S, Wesson R, Gurakar A. Exertional Heat Stroke-Induced Acute Liver Failure and Liver Transplantation. ACG Case Rep J. 2022 Jul 12;9(7):e00820. PMID: 35919405; PMCID: PMC9278910.

Book Chapters [BC]

1. Jakhete N, **Kim AK**. (2017). Management of Hepatic Encephalopathy. *Current Surgical Therapy* 12th Edition (pp. 417-421) Philadelphia, PA: Elsevier

Other Publications

Research Letters/ White Papers [RL]

1. Yarchoan M, Agarwal P, Villanueva A, Rao S, Dawson LA, Llovet JM, Finn RS, Groopman JD, El-Serag HB, Monga SP, Wang XW, Karin M, Schwartz RE, Tanabe KK, Roberts LR, Gunaratne PH, Tsung A, Brown KA, Lawrence TS, Salem R, Singal AG, **Kim AK**, Rabiee A, Resar L, Hoshida Y, He AR, Ghoshal K, Ryan PB, Jaffee EM, Guha C, Mishra L, Coleman CN, Ahmed MM. Recent Developments and Therapeutic Strategies against Hepatocellular Carcinoma. Cancer Res. 2019 Sep 1;79(17):4326-4330. doi: 10.1158/0008-5472.CAN-19-0803. Erratum in: Cancer Res. 2019 Nov 15;79(22):5897. PMID: 31481419; PMCID: PMC8330805.
2. Baretti M, **Kim AK**, Anders RA. Expanding the immunotherapy roadmap for hepatocellular carcinoma, Cancer Cell. 2022 Mar 40(3):252-254. doi.org/10.1016/j.ccell.2022.02.017.

Media Releases or Interviews [MR]

1. Interview for Q&A column in New York Times on Reversal of Non-alcoholic fatty liver disease. 12/22/2015
2. Interview for Johns Hopkins *Inside Tract* on urine ctDNA test for HCC screening 4/12/2023
3. Interview for NCI Blog on ctDNA and Liquid Biopsy and HCC detection 4/25/2023

FUNDING

EXTRAMURAL Funding

Current:

04/01/2020 - 03/31/2025	Title: Improved early prognostic algorithm for hepatocellular carcinoma Identification number: 1K08CA237624 Sponsor: NCI Total direct cost: Role: PI, 75%
09/01/2020 - 08/31/2023	Title: Predicting outcome of transplant-ineligible patients with hepatocellular carcinoma Identification number: W81XWH-20-1-0605 Sponsor: Department of Defense, PRCRP-USAMRAA Total direct cost: Role: PI, 5%
02/01/2023 – 01/30/2028	Title: Impact of preanalytic procurement and processing variables on the detection of HCC DNA in urine

	Identification number: 01CA275648-01 Sponsor: NIH Total direct cost: Role: Co-investigator, 3%

Previous:

07/01/2015 – 07/01/2017	Title: Living Legacy Foundation of Maryland Research Grant Identification number: Sponsor: LLF Foundation Total direct cost: Role: PI, 2%
01/01/2017-12/30/2019	Title: Fibrolamellar HCC Cancer Foundation Grant Identification number: Sponsor: Cancer Research Institute Total direct cost: Role: PI, 10%
08/31/2018 – 08/31/2020	Title: Pathway to Specific functional biomarkers for the early detection of liver cancer Identification number: 1U01CA230690-01 Sponsor: NCI Total direct cost: Principal Investigator: Mishra Role: co-investigator 5%
09/25/2018 - 08/31/2021	Title: Development of a JBS Hi-LO urine DNA Kit Identification number: R44HG008700 Sponsor: NIH Total direct cost: Principal Investigator: Jain Role: Site-PI, 10%
05/16/2019-08/31/2022	Title: TARGET-HCC Identification number: Sponsor: TARGET-HCC Total direct cost: Role: Site-PI, 1%

INTRAMURAL Funding

Previous:

07/2016 - 06/2017	Title: Hopkins Conte Core Digestive Diseases Pilot Project Grant Identification number: P30DK089502 Sponsor: NIDDK Total direct cost: Your role, your percent effort; Notes: 0%

CLINICAL ACTIVITIES

Clinical Focus: My clinical focus is providing care for patients with end-stage liver disease, liver transplant patients and those with liver cancer. I co-lead the Multidisciplinary Liver Cancer Clinic at the Sidney Kimmel Comprehensive Cancer Center.

Certification

Medical, other state/government licensure

2014 - Present	Maryland #D0078043
2007 - 2010	North Carolina #142298 (expired)

Boards, other specialty certification

2010	American Board of Internal Medicine
2013	American Board of Internal Medicine, sub-board of Gastroenterology
2016	American Board of Internal Medicine, sub-board of Transplant Hepatology

Clinical (Service) Responsibilities

2014-2016	Inpatient service, 9 weeks. Gastroenterology/Hepatology Outpatient clinic, 2 days/week. Gastroenterology/HepatologyEndoscopy, 1 days/week. Gastroenterology/Hepatology
2016-2019	Inpatient service, 8 weeks. Gastroenterology/Hepatology Outpatient clinic, 1 day/week. Gastroenterology/Hepatology Endoscopy, 1 days/week. Gastroenterology/Hepatology
2019-2020	Inpatient service, 6 weeks. Gastroenterology/Hepatology Outpatient clinic, 1 day/week. Gastroenterology/Hepatology
2020-present	Inpatient service, 5 weeks. Gastroenterology/Hepatology Outpatient clinic, 1 day/week. Gastroenterology/Hepatology
2022-present	Inpatient service, 3-4 weeks. Gastroenterology/Hepatology Outpatient clinic, 1 day/week. Gastroenterology/Hepatology

Clinical Productivity

2022- present	Targeted clinical effort in outpatient and inpatient service is 19%

Membership in or examiner for specialty board

Clinical Program Building / Leadership

2020-present	Director of Hepatology in Multi-disciplinary Liver Cancer Clinic, SKCCC, JHMI

Clinical Demonstration Activities to external audience, on or off campus

Development of nationally/internationally recognized clinical standard of care

EDUCATIONAL ACTIVITIES

Educational Focus:

I have been a research or career mentor to our fellows, residents and students from both undergraduate and graduate schools. My primary educational activities include lectures, seminars, and mentorship of trainees. In my clinical practice, I precept gastroenterology and hepatology fellows, residents and medical students

Teaching

Classroom instruction

04/15/2013	GI cases. Teaching PA students. Yale University, New Haven, CT
11/20/2014	Hepatocellular carcinoma. Medical student course lecture. Johns Hopkins SOM, Baltimore, MD
11/15/2016	Hepatocellular carcinoma and Liver transplantation. Johns Hopkins SOM Fellows Lecture, Baltimore, MD
08/16/2016	Acute liver failure. Fellow didactic series. Johns Hopkins SOM, Baltimore, MD
09/21/2016	Management of Variceal bleeding, Fellow didactic series, Johns Hopkins SOM, Baltimore, MD
01/17/2017	HCC: Diagnosis and Management, Fellow didactic series, Johns Hopkins SOM, Baltimore, MD

Clinical instruction

CME instruction

11/14/2014	Topics in Gastroenterology and Hepato-Biliary Update: *Autoimmune Liver Disease*, Johns Hopkins SOM, Baltimore, MD
09/09/2015	Topics in Gastroenterology and Hepato-Biliary Update: *Hepatocellular Carcinoma*. Johns Hopkins SOM, Baltimore, MD
3/08/2016	16th Annual Gastroenterology and Hepatology Conference, Lecturer on Management of portal hypertension, and abnormal liver function.
11/09/2016	Topics in Gastroenterology and Hepato-Biliary Update: *Benign Liver Lesions*. Johns Hopkins SOM, Baltimore, MD
11/10/2017	Topics in Gastroenterology and Hepato-Biliary Update: *Multidisciplinary management of HCC*. Johns Hopkins SOM, Baltimore, MD
10/9/2019	Topics in Gastroenterology and Hepato-Biliary Update: *Advances in management of HCC*. Johns Hopkins SOM, Baltimore, MD
2014- present	Lecturer to Residents and fellows. Transplant Hepatology Didactics Series, Johns Hopkins University School of Medicine, Baltimore, MD Topics: Screening and treatment of hepatocellular carcinoma, Benign liver lesions, Autoimmune hepatitis, Pulmonary complications of liver disease

Workshops / seminars

Mentoring

Pre-doctoral Advisees / Mentees

2018 - 2020	Leya Teferi, BS Undergraduate student researcher from Johns Hopkins University. Awards/grants/degrees during mentorship: Dean's Undergraduate Research Award (DURA) 6/2018 Mentorship role: Career mentor, Research mentor
2022 - present	Ziyi Yu, BS

Graduate student from School of Public Health, involved in clinical research projects in HCC and maintaining the patient database
Mentorship role: Research mentor

Post-doctoral Advisees / Mentees

2020 - 2022 Chris Fan, MD
Position as of 1/1/2023: Assistant professor at Department of Medicine, Baylor School of Medicine, Houston, TX
Awards/grants/degrees during mentorship: Abstract poster presentation at American Association for the Study of Liver Diseases 2020. Manuscript published as first-author.

2021 – present Amanda Su, MD
Position as of 1/1/2023: Clinical fellow in Gastroenterology and Hepatology, Johns Hopkins School of Medicine, Baltimore, MD

2022 – present Nicole Rich, MD
Position as of 1/1/2023: Internal medicine resident, Johns Hopkins School of Medicine, Baltimore, MD.
Awards/grants/degrees during mentorship: Abstract accepted at American Association for the Study of Liver Diseases 2023

Thesis Committee

2018 Pooja Chauhari, PhD
Position as of 1/1/2023: Senior Manager, Guardant Health
Helped with doctoral thesis dissertation on pluripotent stem cells in human liver development

Educational Program Building / Leadership

Educational Demonstration Activities to external audiences, on or off campus

RESEARCH ACTIVITIES

Research Focus: My research is focused on developing liquid biomarkers (ctDNA from blood/ urine and CTCs) that reflect tumor biology, including the vascular network with immune system, to improve the clinical management and overall survival of patients with HCC.

Research Program Building / Leadership

2023- present Hepatology Pod Leader for GI Clinical Translational Research Center
I oversee the clinical and translational research activities within hepatology group supported by the GI CTRU program.

Research Demonstration Activities to external audience, on or off campus

Inventions, Patents, Copyrights

Technology Transfer Activities (e.g. Company Start-ups)

SYSTEM INNOVATION AND QUALITY IMPROVEMENT ACTIVITIES – N/A

ORGANIZATIONAL ACTIVITIES
Institutional Administrative Appointments

Editorial Activities

Editorial Board appointments

2015 - Present	Editorial advisory board member, Clinical and Translational Gastroenterology, Published by American Gastroenterological Association (AGA) AdHoc Review
2023 – Present	Editorial board, Clinical Liver Disease, Published by American Association of the Study of Liver Disease

Journal peer review activities

2015- Present	Regularly reviews for journals such as British Medical Journal of Cancer, Clinical Gastroenterology and Hepatology, Liver Transplantation

Other peer review activities [non-medico-legal]

2021 – Present	Abstract reviewer, The Liver Meeting, American Association for the Study of Liver Diseases

Advisory Committees, Review Groups/Study Sections

2023	Early Career Reviewer (ECR) for study sections, Center for Scientific Review (CSR), National Institute of Health.

Professional Societies

2004 - 2010	Member, American Medical Association
2011 - Present	Member, American Association for the Study of Liver Diseases (AASLD)
2010- 2017	Member, American Gastroenterological Association (AGA)

CONFERENCE ORGANIZER
Session Chair

International

9/30/2022	Moderator, International Cholangiocarcinoma Research Network and Cholangiocarcinoma Foundation (ICRN-CFF) joint webinar. “The role of PSC in Cholangiocarcinoma”.
11/6/2022	Moderator, American Association of the Studies of Liver Diseases. Novel Approaches for Risk Stratification and Surveillance for HCC. Washington DC.

National

11/06/2018	Moderator, NCI Working Group Workshop on Hepatocellular Cancer: New Indications and Directions, Biomarker session, Bethesda, MD
07/19/2019	Moderator, Princeton Workshop Hep B and HCC immunology. Princeton, NJ

Regional/JHMI

Consultantships

2019	Eisai – Advisor for HCC management roundtable discussion
2018	AstraZeneca – consultant to HCC epidemiology project
2020	Exelixis – advisor to HCC roundtable discussion
2022	AstraZeneca – consultation and advisory board

RECOGNITION

Awards, Honors

1998	Smithsonian Student Fellowship Grant
2000 - 2001	Johns Hopkins University: Dean's List
2000	Psi-Chi National Honor Society
2004	Memorial Sloan Kettering Cancer Center Research Grant Recipient

Invited Talks

International

8/3/2018	Speaker, The 8th Shanghai International Conference of Gastroenterology. "Updates on screening and management of hepatocellular carcinoma. Shanghai, China
4/27/2022	Speaker, Center of excellence for Liver Disease in Vietnam Webinar Series. Liver Talks: DELFI Score: From Discovery to Potential Clinical Application in Liver Cancer Management. Hochimihn City International Uuniversity and Center of Excellence in Liver Disease

National

11/02/2017	Speaker, Fibrolamellar Summit "Immune microenvironment of Fibrolamellar HCC" Stamford, CT
01/26/2022	Speaker, Surgical and Medical Management of HCC, *39th Annual Medical and Surgical Gastroenterology: A Multidisciplinary Approach.* Vail, CO.
10/26/2022	Speaker, Society for Advanced Body Imaging, Multidisciplinary Liver Tumor Case Reviews. New Orleans, LA

Regional

04/24/2014	Speaker, UTSW Liver conference, "HCC and Liver transplantation," Dallas, TX
12/02/2016	Speaker, Georgetown University "Fighting a smart war against cancer" Symposium," Washington D.C.
03/01/2018	Speaker, Blumberg Institute/ Hepatitis B Foundation "Novel Therapies in HCC" Doylestown, PA
06/07/2018	Speaker, University of Maryland Medical Systems/Cigarette Restitution Fund (CRF) Program, "Advances in HCC management," Baltimore MD
07/19/2019	Moderator, Princeton Workshop Hep B and HCC immunology. Princeton, NJ.
01/22/2020	Speaker, HCC screening and treatment, JHU AETC/CFAR Conference. Baltimore, MD
5/12/2023	Presenter, "Biomarkers of HCC" 21st Annual Research Matters Conference sponsored by the Maryland Cigarette Restitution Fund, Baltimore, MD

OTHER PROFESSIONAL ACCOMPLISHMENTS

Posters

1. Levy S; Su A; Anders RA; **Kim AK**. **"**Clinico-Pathologic Features of Severe Alcoholic Hepatitis Vs. Alcoholic Cirrhosis in Liver Transplantation". Web of Science accession number: WOS:000540349504599. Digestive Disease Week 2020, Published: May 2020 in Gastroenterology
2. Besharati, Sepideh; Bhaijee, Feriyl; Zhu, Qingfeng; **Kim AK**. "Early Liver Transplantation for Severe Alcoholic Hepatitis: Clinical and Pathologic Features in a Controversial Therapeutic Setting". Web of Science accession number: WOS:000518328903280, Published: Mar 2020 in Modern Pathology
3. **Kim AK.**; Luu, Harry; Anders, Robert A; et al. "Role of Circulating Tumor Cells and Circulating Macrophages In HCC Outcomes after Liver Transplantation". Web of Science accession number: WOS:000488653502027. AASLD 2019 San Francisco, Published: Oct 2019 in Hepatology
4. **Kim AK**, Urrunaga N, Zhu QF, Teferi L, Lee S, Rosenberg AZ, Rabiee A, Liddell RP, Georgiades C, Gurakar A, Ottmann S, Yarchoan M, Hong K, Anders RA. Transarterial chemoembolization enhances the tumor microenvironment of hepatocellular carcinoma. AASLD 2022. Washington DC. Published Abstract October 2022. [Selected as one of the four posters within Liver Cancer section for debriefing on Nov 6, 2022]

Oral/Podium Presentations [abstracts that were both presented orally and published]

1. Adams D, Lin S, Pass H, Chumsri, S, **Kim, AK** et al. "Circulating stromal cells as a potential blood-based biomarker for screening invasive solid tumors". JCO Vol. 38:15 Supplement S Meeting Abstract 3535, Meeting: American Society of Clinical Oncology 2020. Published 2020.
2. Vowles JV, **Kim, AK**, Hamilton JP, Lin SY, Shieh, FW, Luu H, Villafana G, Hu GT, Su YH. "Urine for noninvasive liquid biopsy for germline and somatic mutations" (Abstract 722) American Association of Cancer Research. August 2020.
3. Foda ZH, Annapragada A., Boyapati K, Bruhm DC, Vulpescu NA, …**Kim AK**, Velculescu VE**.** Detecting liver cancer using cell-free DNA fragmentomes. American Association of the Study of Liver Disease. November 2022. [Selected as the Best of 2023 AASLD Abstracts]

Military Service - none

Community Services - none

Humanitarian Activities - none

Philanthropic Activities - none

RESEARCH ARTICLE

Detecting Liver Cancer Using Cell-Free DNA Fragmentomes

Zachariah H. Foda[1,2], Akshaya V. Annapragada[1], Kavya Boyapati[1], Daniel C. Bruhm[1], Nicholas A. Vulpescu[1], Jamie E. Medina[1], Dimitrios Mathios[1], Stephen Cristiano[1,3], Noushin Niknafs[1], Harry T. Luu[2], Michael G. Goggins[1,2,4], Robert A. Anders[4], Jing Sun[5], Shruti H. Meta[5], David L. Thomas[2], Gregory D. Kirk[5], Vilmos Adleff[1], Jillian Phallen[1], Robert B. Scharpf[1,3], Amy K. Kim[2], and Victor E. Velculescu[1,2,4]

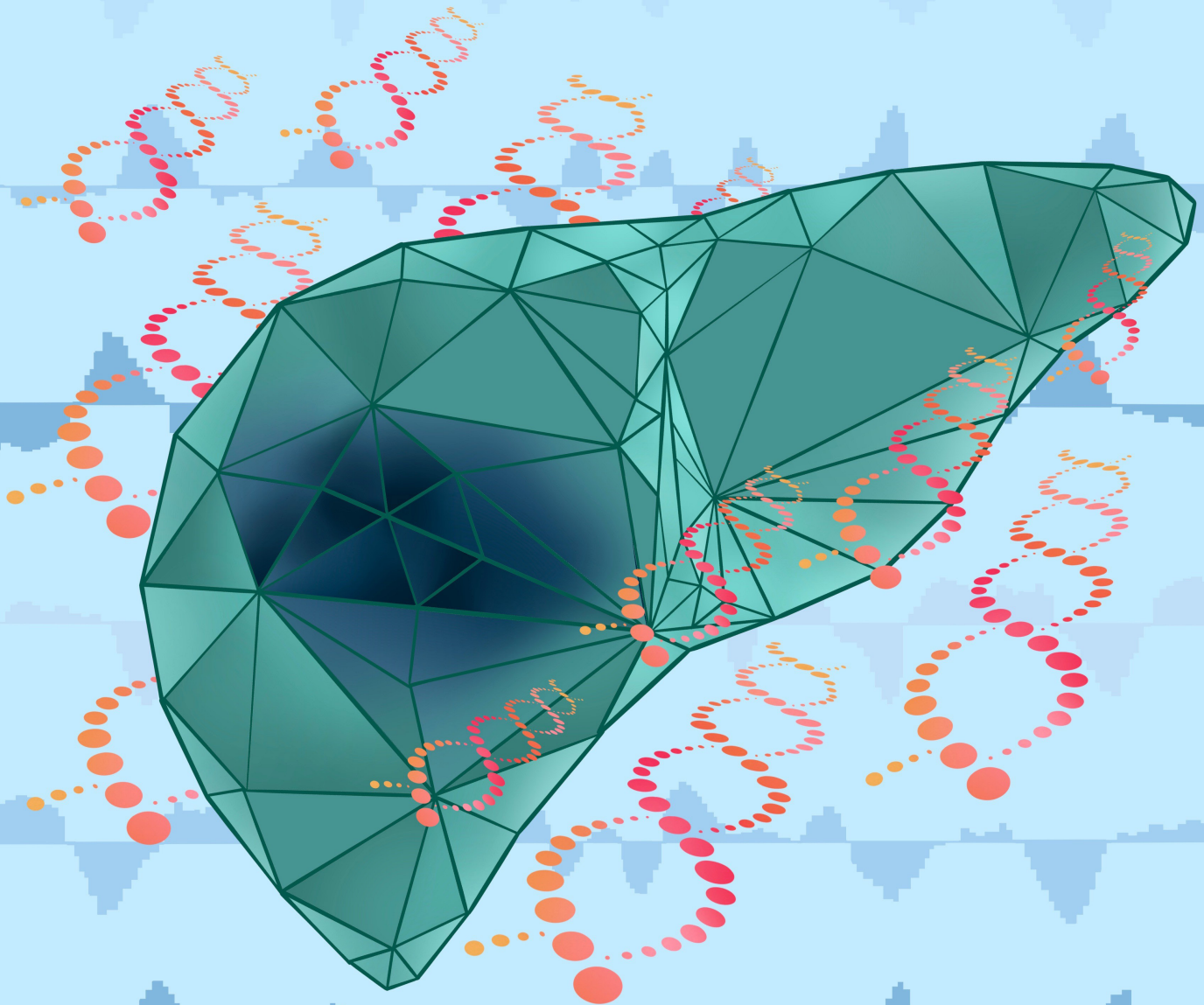

Illustration by Brianna Dunn

ABSTRACT Liver cancer is a major cause of cancer mortality worldwide. Screening individuals at high risk, including those with cirrhosis and viral hepatitis, provides an avenue for improved survival, but current screening methods are inadequate. In this study, we used whole-genome cell-free DNA (cfDNA) fragmentome analyses to evaluate 724 individuals from the United States, the European Union, or Hong Kong with hepatocellular carcinoma (HCC) or who were at average or high-risk for HCC. Using a machine learning model that incorporated multifeature fragmentome data, the sensitivity for detecting cancer was 88% in an average-risk population at 98% specificity and 85% among high-risk individuals at 80% specificity. We validated these results in an independent population. cfDNA fragmentation changes reflected genomic and chromatin changes in liver cancer, including from transcription factor binding sites. These findings provide a biological basis for changes in cfDNA fragmentation in patients with liver cancer and provide an accessible approach for noninvasive cancer detection.

SIGNIFICANCE: There is a great need for accessible and sensitive screening approaches for HCC worldwide. We have developed an approach for examining genome-wide cfDNA fragmentation features to provide a high-performing and cost-effective approach for liver cancer detection.

INTRODUCTION

Liver cancer causes a staggering amount of morbidity and mortality worldwide, with more than 900,000 newly diagnosed cases each year and more than 800,000 deaths (1). In the United States, liver cancer is one of the few cancers that has shown an increase in incidence and mortality over the last 20 years. Ninety percent of cases of liver cancer are hepatocellular carcinoma (HCC), and survival is highly dependent on the stage of the disease at diagnosis. The five-year survival rate is 34% when the cancer is localized (44% of patients), 12% when regional (27% of patients), and 3% when a distant disease is found (18% of patients; ref. 2). There is a large, well-defined population that is at significantly increased risk for HCC, including individuals with chronic hepatitis B (HBV) infection or with cirrhosis from various causes including hepatitis C (HCV; ref. 3), nonalcoholic fatty liver disease (NAFLD; ref. 4), heavy alcohol use (5), aflatoxin, and other conditions (6). Worldwide, there are 350 million individuals with chronic viral hepatitis infection and 50 million with cirrhosis (7). In the United States, 4.5 million individuals have chronic HCV and 29 million have been diagnosed with NAFLD. Up to one third of those with cirrhosis and between 25% and 40% with HBV will develop HCC over their lifetime, with an up to 8% annual risk for patients with cirrhosis (8). A growing group of individuals at risk for liver cancer, including 29 million in the United States, have NAFLD, and 20% of the HCC that develops in this population occurs without cirrhosis (9). Medical societies throughout the world recommend screening for the highest risk populations, currently with abdominal ultrasound imaging with or without alpha-fetoprotein (AFP). Overall adherence to international guidelines, however, remains low, with less than one in five eligible individuals worldwide receiving some level of surveillance and less than 2% following recommended screening (10–12). Many factors contribute to low adherence to screening guidelines, including the identification of high-risk individuals, the requirement of infrastructure, and personnel needed for imaging-based screening methods (11). Current screening tests that include ultrasound imaging, with or without AFP, have shown limited sensitivity, varying from 47% to 84% with specificities from 67% to over 90% (13). Additionally, the lack of noninvasive diagnostic approaches for NAFLD suggests that the population not currently covered by HCC screening recommendations is increasing. Therefore, there is a great need for the development of accessible and sensitive screening approaches for HCC worldwide.

One recent avenue for overcoming these challenges has been the development of novel blood-based cell-free DNA (cfDNA) biomarkers for the detection of cancer. Somatic mutation–based approaches have been used as biomarkers for liver cancer but are limited by the need for tissue-based mutation identification and by the few changes detectable in plasma (14). Methylation profiling, both at specific sites and throughout the whole genome, and copy-number changes have also provided feasible avenues for the detection of liver cancer, but their detection sensitivities in very early-stage disease remain suboptimal (15–20). Recently developed multicancer early detection tests appear useful for the detection of many

[1]The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, Maryland. [2]Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland. [3]Department of Biostatistics, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland. [4]Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, Maryland. [5]Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland.

Note: Z.H. Foda and A.V. Annapragada contributed equally to this article.

Corresponding Authors: Victor E. Velculescu, The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, 1550 Orleans Street, Room 544, Baltimore, MD 21287. Phone: 410-955-7033; E-mail: velculescu@jhmi.edu; and Amy K. Kim, Johns Hopkins University School of Medicine, 720 Rutland Avenue, Ross 918, Baltimore, MD 21205. Phone: 410-502-0418; E-mail: amy.kim@jhmi.edu

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cancers (including liver cancer) in an average-risk cohort (21), but there are no published reports of using these approaches in a population at high risk of HCC. Additionally, the cost of most cfDNA-based tests is much higher than estimates of what would be affordable for screening tests in the United States and worldwide (21). Combining these approaches with AFP has increased performance but requires two separate tests and still has limitations in early-stage disease (22). We have previously developed an approach called DNA evaluation of fragments for early interception (DELFI) that utilizes genome-wide fragmentation profiles to provide a high-performing and cost-effective approach to cancer detection (23, 24). Zhang and colleagues applied a variation of this approach to evaluate noninvasive detection of liver cancer in China, but the underlying source of fragmentation changes in these patients was not explored (25). Fragmentation and methylation information has also demonstrated the ability to differentiate patients with liver cancer from those without cancer (26), although such an approach requires two distinct methods of cfDNA library preparation and analysis. To date, no study has validated genome-wide approaches for detecting HCC in independent groups or across different high-risk populations.

Here we describe the development of a genome-wide fragmentome approach to detect individuals with liver cancer. We examine the molecular origins of cfDNA in these patients and identify genomic and chromatin features associated with fragmentation changes. Finally, we use this approach to detect liver cancer in the US population and validate this model in a separate Hong Kong cohort.

RESULTS

Clinical Cohorts and Genomic Analyses of cfDNA

We examined plasma samples from 501 individuals, including 75 individuals with HCC and 426 without cancer. Among individuals without cancer, 133 had conditions that increased HCC risk, including cirrhosis from all causes or viral hepatitis without cirrhosis. Blood samples were prospectively collected from patients with HCC at various cancer stages and from high-risk individuals at the Johns Hopkins Hospital, whereas the remaining samples were identified through screening efforts at other US or EU hospitals (US/EU cohort; Table 1; Supplementary Table S1). We isolated 0.5 to 5 mL of plasma from each of these individuals, generated genomic libraries, and sequenced the cfDNA fragments using low-coverage whole-genome sequencing (~2.6× coverage) with an average of 49 million high-quality paired reads per sample comprising 9 Gb of sequence data (Supplementary Table S2; refs. 23, 24). In addition to the US/EU cohort, we examined as a validation cohort whole-genome sequence data from 223 patients from Hong Kong, including patients with resectable early-stage HCC ($n = 90$, stage A = 85, B = 5), HBV ($n = 66$), and HBV-related cirrhosis ($n = 35$), as well as healthy individuals without liver disease ($n = 32$; Hong Kong cohort; Table 1; Supplementary Table S3; refs. 15, 27).

Genome-wide cfDNA Fragmentation Profiles Informed by Underlying Chromatin Structure

We evaluated the fragmentome and generated fragmentation profiles across the genome in 473 nonoverlapping 5-Mb regions, each region comprising ~80,000 fragments, and spanning approximately 2.4 Gb of the genome using the DELFI approach (23). The fragmentation profiles were consistent among individuals without cancer but highly variable among patients with HCC (Fig. 1A). Profiles of patients with cirrhosis were closer to noncancer individuals without cirrhosis than they were to those from patients with HCC (Fig. 1A). Likewise, patients with viral hepatitis had fragmentation profiles nearly identical to those of noncancer individuals without liver disease (Fig. 1A).

To examine the origins of cfDNA fragmentation patterns, we compared genome-wide fragmentome profiles with high-throughput sequencing chromosome conformation capture

Table 1. Patient demographics and clinical information

Patient characteristic	Noncancer individuals $n = 426$	Cancer patients $n = 75$	*P* value[a]
Age			
Mean	57.5	64.5	<0.001
Range	27–81	38–88	
Sex			
Male	235	63	<0.001
Female	191	12	
Liver disease			
None	293		
Hepatitis B	26	1	
Hepatitis C	29	1	
Cirrhosis	78	69	<0.001
HCV	53	41	
HBV	2	4	
EtOH	13	12	
NAFLD	3	11	
Child–Pugh stage			
A	20	49	<0.001
B	10	21	
C	10	5	
Unknown	38		
BCLC stage			
0		7	
A		17	
B		30	
C		21	
Previous treatment			
Yes		28	
No		47	
Validation cohort (Hong Kong)[b]	133	90	
Liver disease			
None	32		
Cirrhosis (HBV)	35	90	
Active HBV	66		
BCLC stage			
A		85	
B		5	

Abbreviations: BCLC, Barcelona Clinic Liver Cancer staging system; EtOH, alcohol associated.
[a]*P* values were calculated to compare data from individuals with and without liver cancer for the following variables: mean ages using Student unpaired two-tailed *t* tests, sex distribution, cirrhosis etiology, and Child–Pugh stage using a χ^2 test.
[b]Validation cohort data were obtained from Jiang et al. (15).

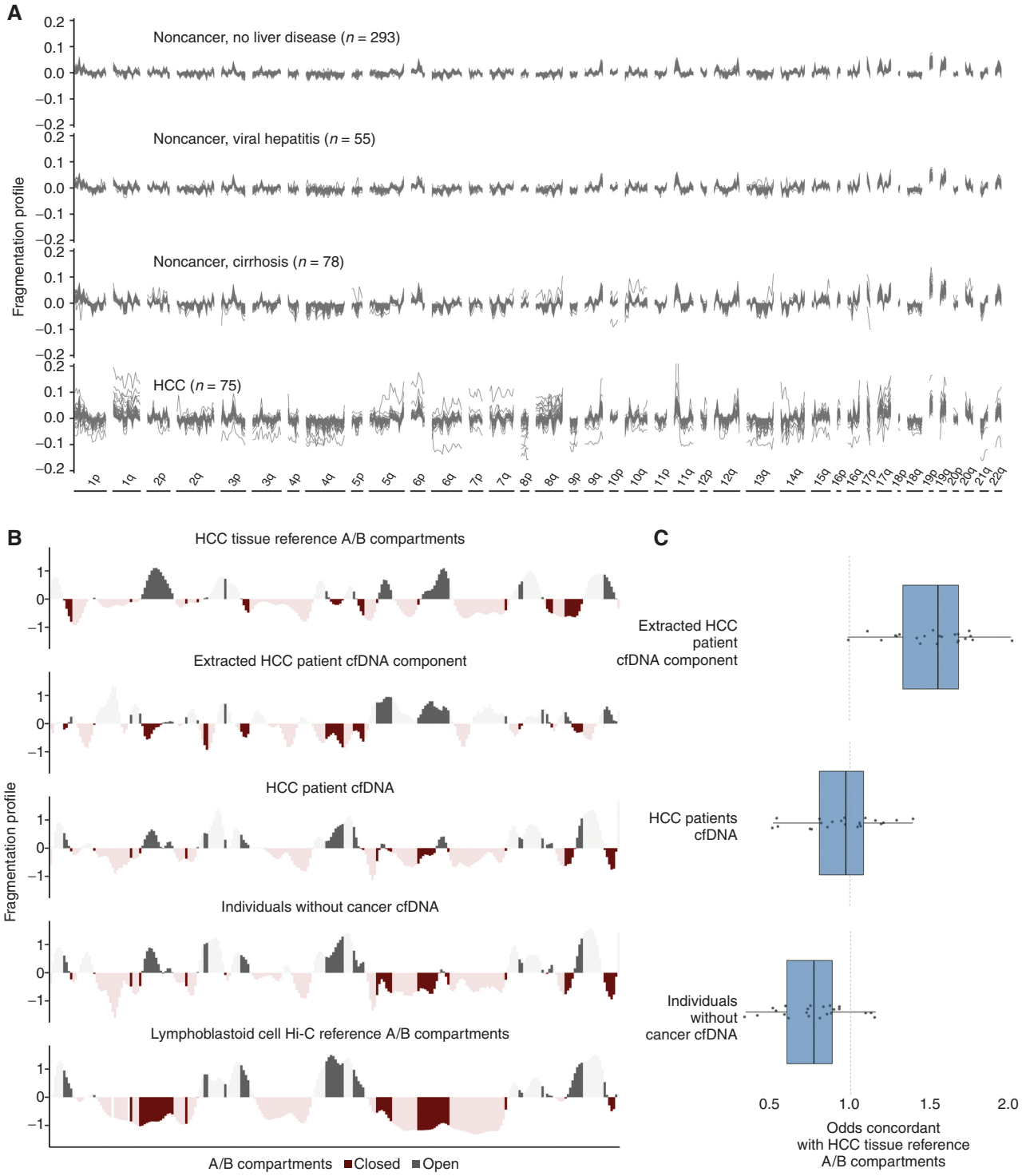

Figure 1. Genome-wide fragmentation profiles reflect underlying chromatin structure. **A,** Fragmentation profiles of 501 individuals in 473 nonoverlapping 5-Mb genomic regions. Fragmentation profiles for individuals with cancer show marked heterogeneity as compared with noncancer individuals with and without liver disease. **B,** Comparison of plasma fragmentation features to reference A/B compartments. Track 1 shows A/B compartments extracted from liver cancer tissue (28). Track 2 shows a median liver cancer component extracted from the HCC plasma samples of 10 liver patients with high tumor fraction by ichorCNA (56). Track 3 shows the median fragmentation profile in the plasma for these 10 HCC samples, and track 4 shows the median profile for 10 healthy plasma samples. Track 5 shows A/B compartments for lymphoblast cells (28). These five tracks show chromosome 22 as an example, with darker shading indicating informative regions of the genome where the two reference tracks differ in domain (open/closed) or magnitude. **C,** Among these informative bins, for each chromosome, the log odds of the plasma component matching the HCC reference track in domain. Log odds greater than 1 indicate more similarity to the HCC reference track, whereas log odds less than 1 indicate more similarity to the lymphoblast reference track. The extracted HCC component has the greatest similarity to the HCC reference track, and the noncancer plasma has the greatest similarity to the lymphoblast reference track; the HCC plasma track is intermediate to the two.

(Hi-C) open (A) and closed (B) compartments. We found that cfDNA patterns of healthy individuals were highly correlated to those of lymphoblastoid cells (Fig. 1B). Analysis of cfDNA profiles from 10 HCC patients with high ctDNA levels revealed that their fragmentome reflected two components: one resembling the profile of individuals without cancer and a separate cfDNA component that had high similarity to A/B compartments previously estimated from liver cancers (Fig. 1B; ref. 28). Additionally, when these two components were estimated, the cfDNA profiles of the predicted liver component had high similarity to genome-wide A/B compartments of liver cancer, whereas the profiles of patients with HCC were intermediate in similarity to liver cancer (Fig. 1B and C). In contrast, the profiles of individuals without cancer were closer to A/B compartments of lymphoblastoid cells (Fig. 1B and C). These analyses suggested that cfDNA fragmentomes from individuals with HCC represent a mixture of cfDNA profiles of chromatin compartments of cells from peripheral blood as well as those from liver cancer.

Disease-Specific Transcription Factors Inferred from Genome-wide cfDNA Fragmentation

As chromatin organization reflects underlying cellular transcriptional programs (29–31), we examined whether cfDNA fragmentation characteristics might reflect changes derived from altered DNA binding of transcription factors (TF) in liver cancer. To identify DNA binding sites for all known TFs, we analyzed 5,620 chromatin immunoprecipitation sequencing (ChIP-seq) experiments from the ReMap 2020 database (32). For each TF, we calculated the aggregate cfDNA coverage across all binding sites identified (4,000–490,000 per sample) compared with the overall adjacent genomic coverage, producing a single metric for each TF in each sample. We compared these TFs in patients with and without HCC to identify those TFs with the largest and smallest differences in genome-wide binding site coverage in cfDNA (Fig. 2A and B). Gene set enrichment analyses using the DisGeNET database of gene–disease associations revealed that differences in cfDNA TF binding coverages between individuals with HCC and individuals without cancer were predicted to be related to liver and other cancers (Fig. 2C and D). Additionally, the top-scoring individual TFs represented those with known biological relevance to chromatin organization and liver cancer, whereas the low-scoring TFs did not (Table 2; Supplementary Table S4). These included members of the activator protein 1 (AP1) complex, including JUN, JUND, ATF2, and ATF7 genes, which integrate extracellular signals (33) and have been linked to liver tumorigenesis (34, 35); Transcriptional Enhancer Factor Domain Family member 4 (TEAD4), which has been shown to have oncogenic roles in HCC (36, 37); Poly(C)-binding protein 2 (PCBP2) transcriptional coregulator, which when overexpressed is associated with a worse prognosis in patients with HCC (38); Prohibitin 2 (PHB), which promotes progression in HCC (39); and AT-rich interacting domain 3A (ARID3A), an oncogenic TF that when upregulated promotes liver cancer malignancy (40). A similar analysis of cfDNA fragmentation data from our recent study of patients in the LUCAS lung cancer diagnostic trial (23) revealed an enrichment of coverage differences in binding sites of TFs related to lung cancer (Fig. 2C and E). Altogether, these observations suggest that changes in cfDNA fragmentation in patients with liver and other cancers result from the multitude of altered transcriptional profiles present in the cancer cells.

Genomic Changes in HCC Are Revealed from cfDNA Fragmentomes

As the cfDNA fragmentome may comprise changes related to large-scale genomic alterations released from cancer cells (23, 24), we also examined chromosomal gains and losses in the circulation of these patients. In addition to the genome-wide fragmentation profiles resulting from chromatin and TF changes observed in patients with liver cancer (Fig. 3A), our analyses revealed an altered representation of chromosomal arms matching those commonly gained or lost in liver cancer as reported in previous The Cancer Genome Atlas (TCGA) large-scale genomic studies of HCC (n = 372; Fig. 3B). These included increased cfDNA representation of 1q, 7p, 7q, and 8q and decreased levels of 4q, 8p, 9p, 13q, and 21q, all known to be gained or lost, respectively, in HCC (41, 42). Importantly, these alterations were observed in the patients with HCC but not in individuals without cancer, even if they had cirrhosis or chronic liver disease (Fig. 3B).

DELFI Model for HCC Detection

Given the direct connection between genomic and chromatin changes in liver cancer and cfDNA fragmentation, we used a machine learning approach to determine if changes in cfDNA fragmentomes could distinguish patients with HCC from those without cancer. We previously used this approach to develop a robust classifier for lung cancer detection that was externally validated in an independent population (23). We determined the performance of this classifier in the US/EU cohort by repeated 5-fold cross-validation, generating a score for each individual that is an average over 10 cross-validation repeats (DELFI score). The resulting model included a combination of regional and large-scale fragmentation characteristics that were optimal for identifying individuals with liver cancer (Supplementary Fig. S1; Fig. 3C). These features comprised the majority of the informative chromosomal, chromatin, and local changes identified above, comprising >90% of the variance of the fragmentation profiles across samples.

As clinical characteristics may affect tumor biomarkers, we investigated whether measures of liver dysfunction or demographic parameters such as age, sex, race, or weight were associated with DELFI scores in individuals without cancer where this information was available (Supplementary Table S1). We observed no association of DELFI scores with age (R = 0.18, P = 0.08, Spearman correlation; Supplementary Fig. S2A) and no difference in DELFI scores between males and females (P = 0.58, Wilcoxon test; Supplementary Fig. S2B). Asians and African Americans have been shown to have a higher incidence of liver cancer that is diagnosed at later stages (43), and we observed small differences in fragmentation scores among high-risk individuals without cancer across these or other racial or ethnic groups, although these analyses are limited by lack of information on clinical covariates in some of these cases (P = 0.037 in patients with viral hepatitis and P = 0.026 in patients with cirrhosis, Kruskal–Wallis test;

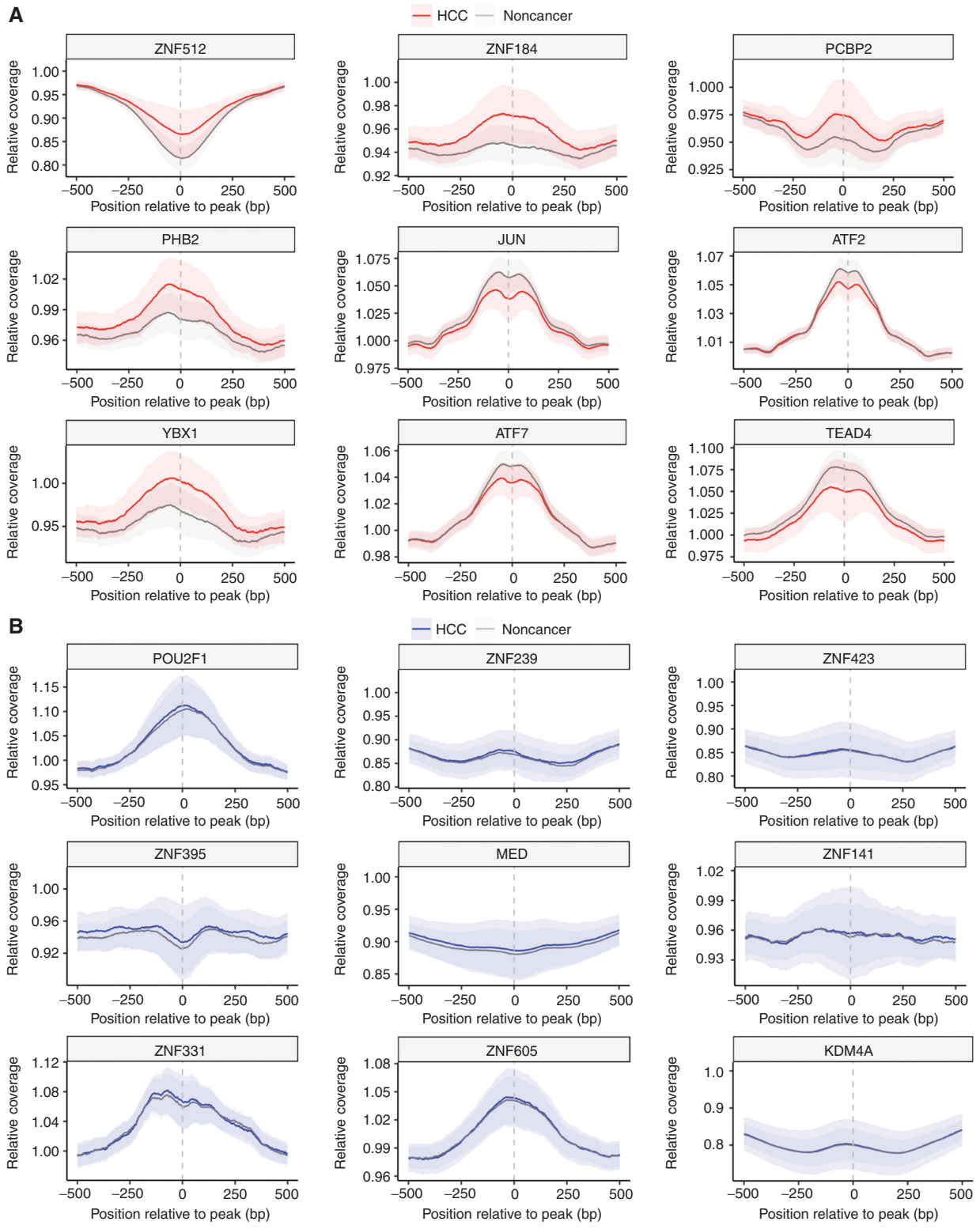

Figure 2. Fragmentation profiles in patients with HCC highlight liver-specific TFs. **A,** The coverage at and around the TF binding sites (TFBS) for the 9 TFs for which the relative coverage at the binding site had the highest separation of HCC from noncancer samples. The mean is plotted for each group, with ± 1 SD shown by shading. These confidence intervals (CI) show separation, highlighting that differences in coverage at a TFBS can provide information on cancer status. **B,** The coverage at and around the TFBS for the 9 TFs that had the lowest separation of HCC from noncancer samples in the US/EU cohort. These CIs are largely overlapping, reflecting their status as TFBS with poor discrimination. (*continued on next page*)

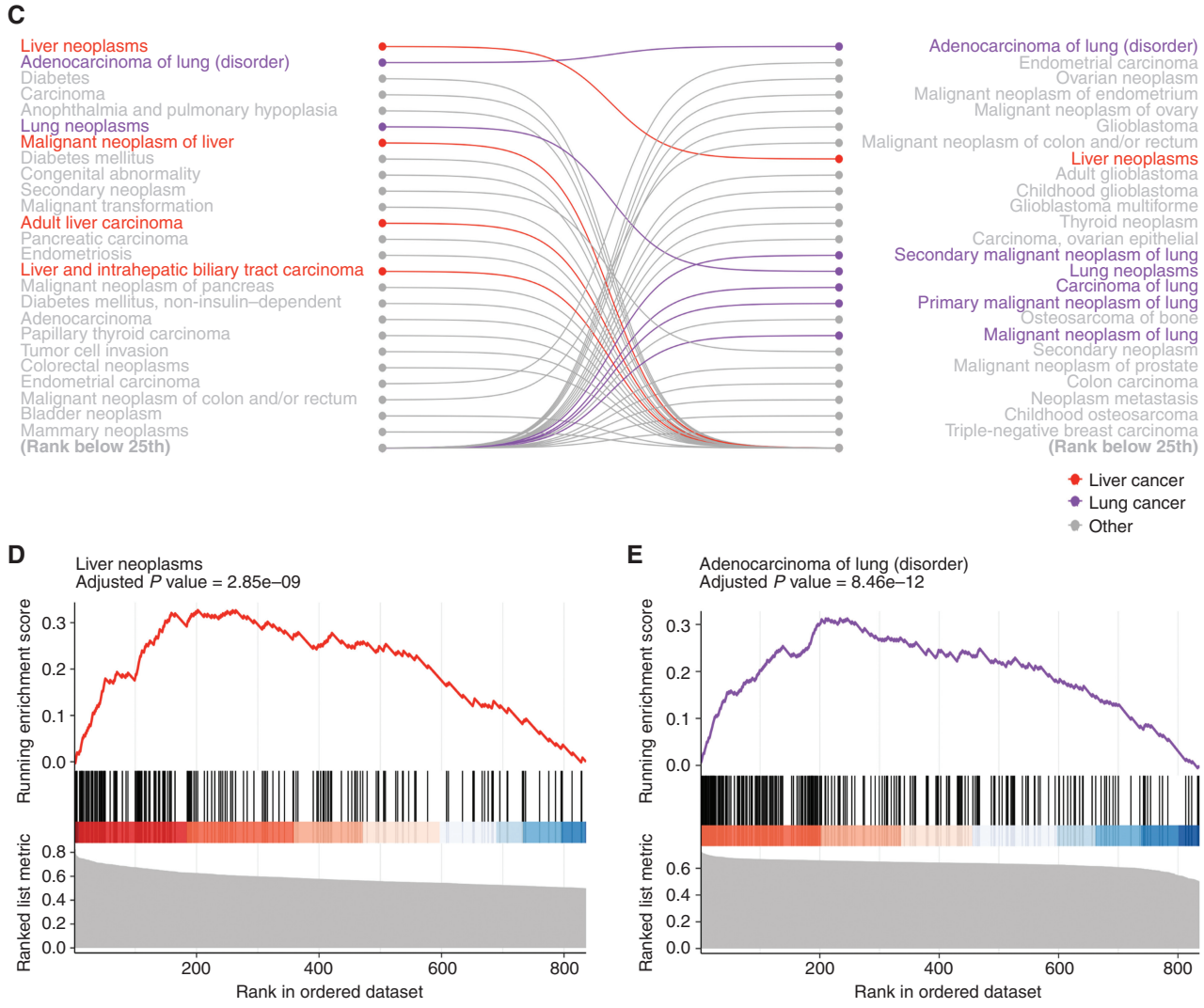

Figure 2. ***(Continued)*** Gene set enrichment analysis of TFs analyzed in both HCC and lung adenocarcinoma showed TFs are selectively enriched in numerous pathways related to liver and lung cancer, respectively (**C**), including adult liver carcinoma and adenocarcinoma of the lung (**D** and **E**).

Supplementary Fig. S3). Among individuals with cirrhosis, we observed a correlation between the degree of liver disease as measured by the Child–Pugh score and DELFI scores ($R = 0.58$ P = 8.6e–5, Spearman correlation; Supplementary Fig. S4). Increased body mass index (BMI), a risk factor for NAFLD and liver cancer, was not associated with changes in DELFI scores in patients with viral hepatitis ($R = 0.027$, $P = 0.85$, Spearman correlation); however, lower BMI in patients with cirrhosis was associated with higher DELFI scores, perhaps due to cachexia in patients with severe cirrhosis ($R = -0.23$, $P = 0.043$, Spearman correlation; Supplementary Fig. S5).

We next examined the relationship between DELFI scores and the presence and stage of liver cancer in a population at high risk for liver cancer. The DELFI scores for 133 individuals who were cancer-free were low, with median DELFI scores of 0.078 or 0.080 for those with viral hepatitis or cirrhosis, respectively. In contrast, the 75 patients with HCC had significantly higher median DELFI scores across all Barcelona Clinic Liver Cancer staging system (BCLC) stages, including stage 0 = 0.46, stage A = 0.61, stage B = 0.83, and stage C = 0.92 ($P < 0.01$ for stages 0, A, B, or C, Wilcoxon rank sum test; Fig. 4A). A receiver operator characteristic (ROC) curve of the DELFI approach to identify patients with HCC revealed an area under the curve (AUC) of 0.90 [95% confidence interval (CI), 0.86–0.94] among high-risk individuals (Fig. 4B). Performance remained robust for early-stage HCCs, with AUCs of 0.9 and 0.81 for BCLC stage 0 and A. Individuals with advanced-stage HCC (BCLC C) were almost perfectly detected among the individuals analyzed (AUC > 0.97; Fig. 4C).

To extend these analyses to individuals at low risk for developing liver cancer, we examined the ability of a DELFI model to distinguish between individuals with cancer and those from a general population (n = 293) without viral hepatitis or cirrhosis. In this larger cohort where additional features could be included in cross-validated training, we used the features of the model above and also included cfDNA coverage at ChIP-seq–derived TF binding sites from liver cell lines available in the ReMap database to create a DELFI model for a general

Table 2. Top scoring TFs in US/EU cohort samples

TF	Gene name	AUC	Cell type in ChIP-seq experiment	Gene function	Link to HCC
ZNF512	*Zinc Finger Protein 512*	0.836	K-562	Unknown	Undescribed
ZNF184	*Zinc Finger Protein 184*	0.826	K-562	Unknown	Undescribed
PCBP2	*Poly(C)-binding protein 2*	0.791	Hep-G2	Transcriptional coregulator	Overexpression contributes to poor prognosis and enhanced cell growth in HCC (38)
JUN	*Jun Proto-Oncogene, AP-1 Transcription Factor Subunit*	0.785	Hep-G2	TF	Promotes HBV-related liver tumorigenesis (59)
PHB2	*Prohibitin 2*	0.771	K-562	Transcriptional coregulator	Functions in mitophagy of HCC (39)
ATF2	*Activating Transcription Factor 2*	0.770	Hep-G2	TF, HAT	Mediates suppression of liver tumor formation (34)
ATF7	*Activating Transcription Factor 7*	0.765	MCF-7	TF	Regulates growth of liver cancer (60)
TEAD4	*TEA Domain Transcription Factor 4*	0.760	Hep-G2	TF	Oncogenic role in HCC (36)
ARID3A	*AT-rich interacting domain 3A*	0.756	Hep-G2	TF	Facilitates liver cancer malignancy (40)
JUND	*JunD Proto-Oncogene, AP-1 Transcription Factor Subunit*	0.754	HT29_DSMO	TF	Involved in PARγ signaling and NAFLD development (61)

Abbrevation: HAT, histone acetyltransferase.

population (Fig. 3C). This approach had high performance for cancer detection (AUC = 0.98) among these individuals. We evaluated the performance of this model at 98% specificity, a threshold appropriate for an average risk population (24), and observed an overall sensitivity of 88% in this setting (Fig. 4B), with sensitivity above 75% across all stages. Use of a model that did not incorporate TF binding sites led to a slightly reduced performance, and there was a high correlation among the rank-ordered scores using our DELFI models for high-risk and screening populations ($R = 0.48$, $P = 2e{-}5$; Supplementary Figs. S6A, S6B, and S7).

To examine the relationship between fragmentation profiles and liver cancer progression, we assessed whether the size, number, and characteristics of liver cancer lesions as well as the etiology of neoplasia were related to aberrant fragmentation profiles, where this information was available. We found that the tumor size and lesion number were positively correlated to DELFI scores ($R = 0.42$ and 0.31, $P = 0.00026$ and $P = 0.0064$, respectively, Spearman correlation; Supplementary Fig. S8A and S8B), consistent with the notion that the fragmentation profile was related to overall tumor burden. Among patients with liver cancer at resectable stages (0, A, and B), the cancer etiology, including viral hepatitis, or cirrhosis due to alcohol, NAFLD, or idiopathic sources, yielded similar DELFI scores ($P = 0.43$, Kruskal–Wallis test; Supplementary Fig. S9). These observations suggest that fragmentation profiles were a result of ongoing tumor-related cfDNA processes and were not affected by early events in tumorigenesis.

To examine the real-world impact of this method in the context of HCC detection, we compared the performance of the DELFI fragmentome with the current screening measurement of AFP levels. AFP levels were elevated above the recommended screening threshold of 20 ng/mL in 39 of 75 (52%) individuals with cancer, consistent with previous reports (44). Among individuals that had AFP levels below 20 ng/mL and who have been undetected by this approach, DELFI detected 30 of 36 (83%). The use of AFP measurements would have detected 8/24 (33%) stage 0/A patients, 17/30 (57%) stage B patients, and 14/21 (66%) stage C patients (Supplementary Fig. S10). In contrast, the DELFI approach detected 19/24 (79%) stage 0/A patients, 25/30 (83%) stage B patients, and 20/21 (95%) stage C patients. Overall, genome-wide cfDNA fragmentation analyses had improved performance compared with AFP detection of HCC, and the combination of DELFI and AFP may provide an improvement in detection over the DELFI approach alone, as we observed these to have a combined sensitivity of 92% at a combined specificity of 80%.

External Validation of DELFI Model in an East Asian Population with HCC

In addition to our cross-validated analysis of the US/EU cohort, we tested the fixed DELFI model in the 223 patients from the Hong Kong cohort. These included patients who had largely resectable early-stage HCC ($n = 90$, stage A = 85, B = 5) and 101 with cirrhosis or HBV infection. These samples were sequenced previously using a different sequencer (HiSeq 2000 vs. Novaseq; 76-bp vs. 100-bp read length), different library preparation, and a higher number of PCR cycles (14 vs. 4 cycles), but we observed similar genome-wide patterns to our earlier analyses (Supplementary Fig. S11). The fragmentation profiles of patients with viral hepatitis and cirrhosis, as well as healthy individuals, had highly consistent profiles throughout the genome, whereas those of patients with HCC were variable and disordered (Supplementary Fig. S12). Additionally, the chromosomal changes observed in plasma in the Hong Kong cohort were similar to those in the initial US/EU cohort, as well

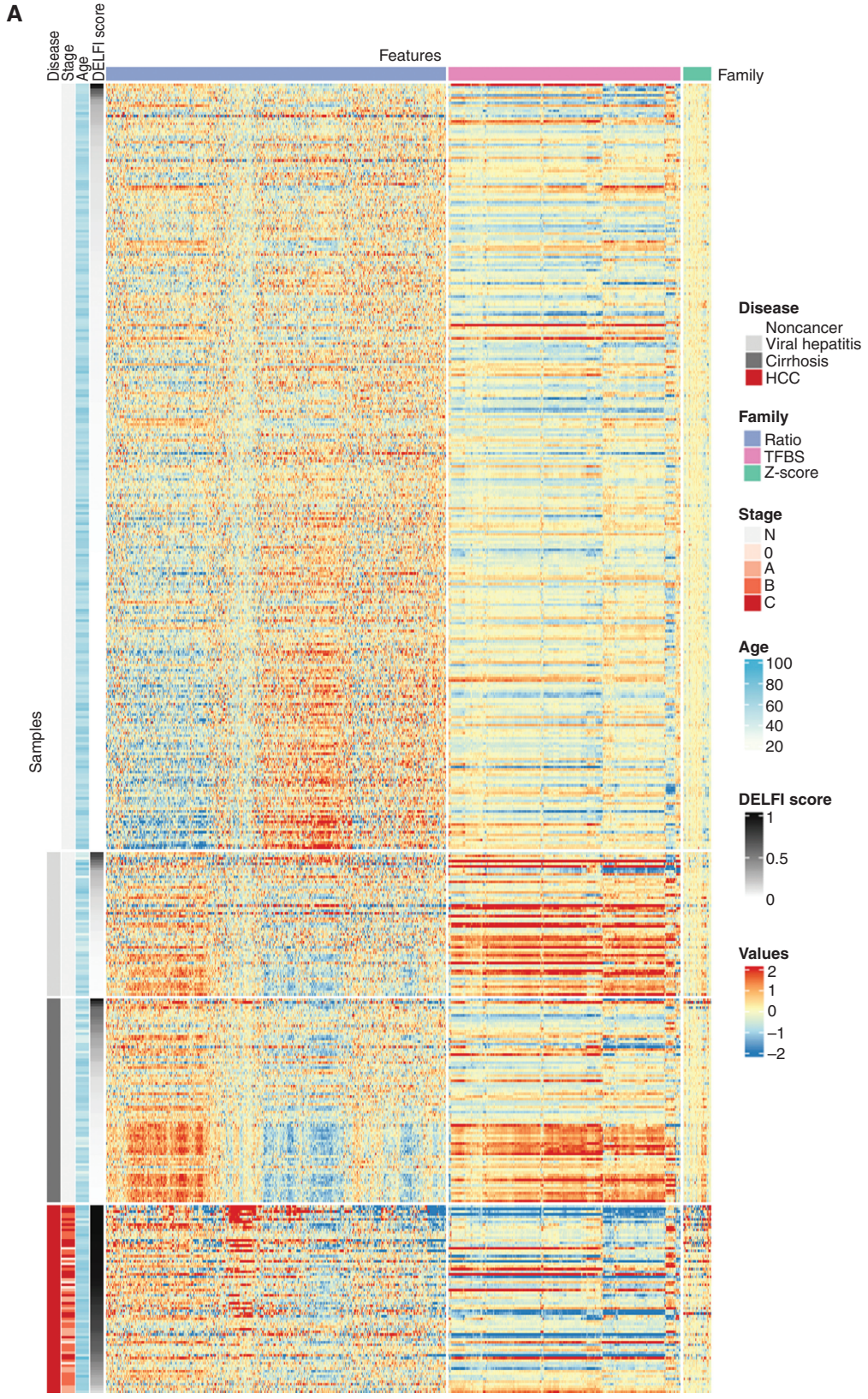

Figure 3. High-dimensional fragmentation features reflect liver cancer biology and are incorporated in DELFI machine learning approaches. **A,** A heat map reflecting the complexity of genome-wide fragmentation and TF binding site (TFBS) features utilized in the DELFI machine learning approach. Each row represents a sample, whereas columns show individual genomic features. (*continued on following page*)

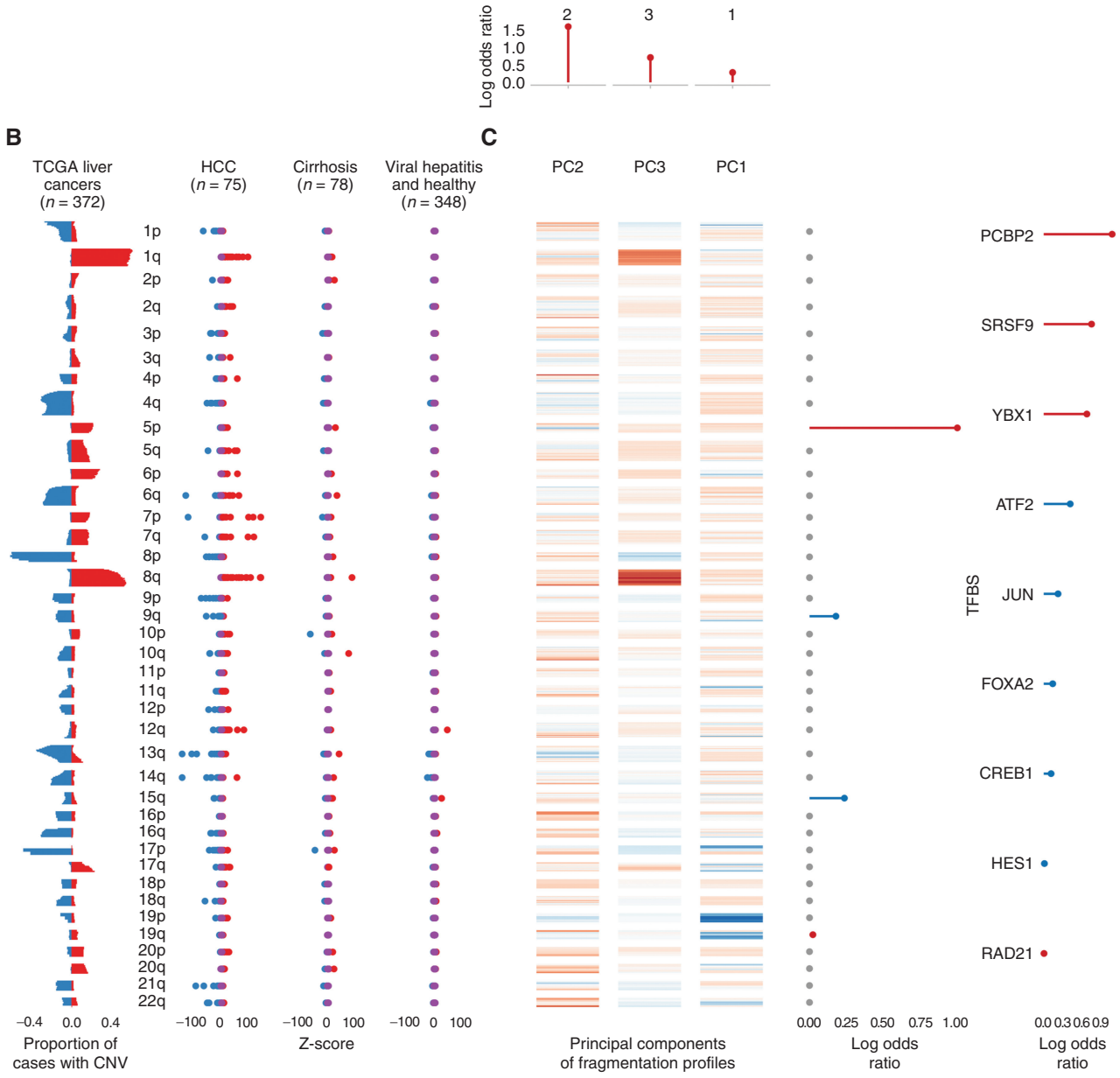

Figure 3. ***(Continued)*** **B,** Analysis of copy-number changes in tissue from 372 TCGA liver cancers and plasma from 501 individuals reflects biological consistency. Copy-number changes that occur in TCGA (red = gains, blue = losses) were also found at the chromosomal arm level in HCC plasma, but not in individuals without cancer. CNV, copy-number variation. **C,** Heat map depicting the contributions of individual genomic regions to the final trained DELFI model. The fragmentation features were summarized as three principal components (PC) in the model, whereas aneuploidy was summarized as arm-level z-scores. The top, middle, and right depict the coefficients of fragmentation components, arm-level z-scores, and TFBS, respectively, in the model.

as the cancers from the TCGA (Supplementary Fig. S13). Overall, in this validation cohort, the DELFI model distinguished HCC patients with an AUC of 0.97 from those with high-risk disease (Fig. 4D). These observations suggest that the underlying characteristics of cfDNA fragmentation were similar in this cohort, and that DELFI is a robust method to detect HCC and is generalizable across different high-risk populations.

Simulation of DELFI Performance at Population Scale

To evaluate how our approach would perform for surveillance and detection in patients at high risk for liver cancer, we evaluated the DELFI model in a theoretical population of 100,000 high-risk individuals using Monte Carlo simulations. Given the importance of the detection of early-stage cancers, we focused our modeling on the detection of stage 0/A disease. We compared the DELFI approach with the current standard of care, concurrent ultrasound, and AFP and modeled the uncertainty of sensitivity and specificity of these surveillance modalities in this theoretical population through probability distributions centered at empirical estimates from our cohort or previous reports (ref. 11; see Methods). Despite surveillance recommendations, the adherence to HCC surveillance in the United States is low, with the most

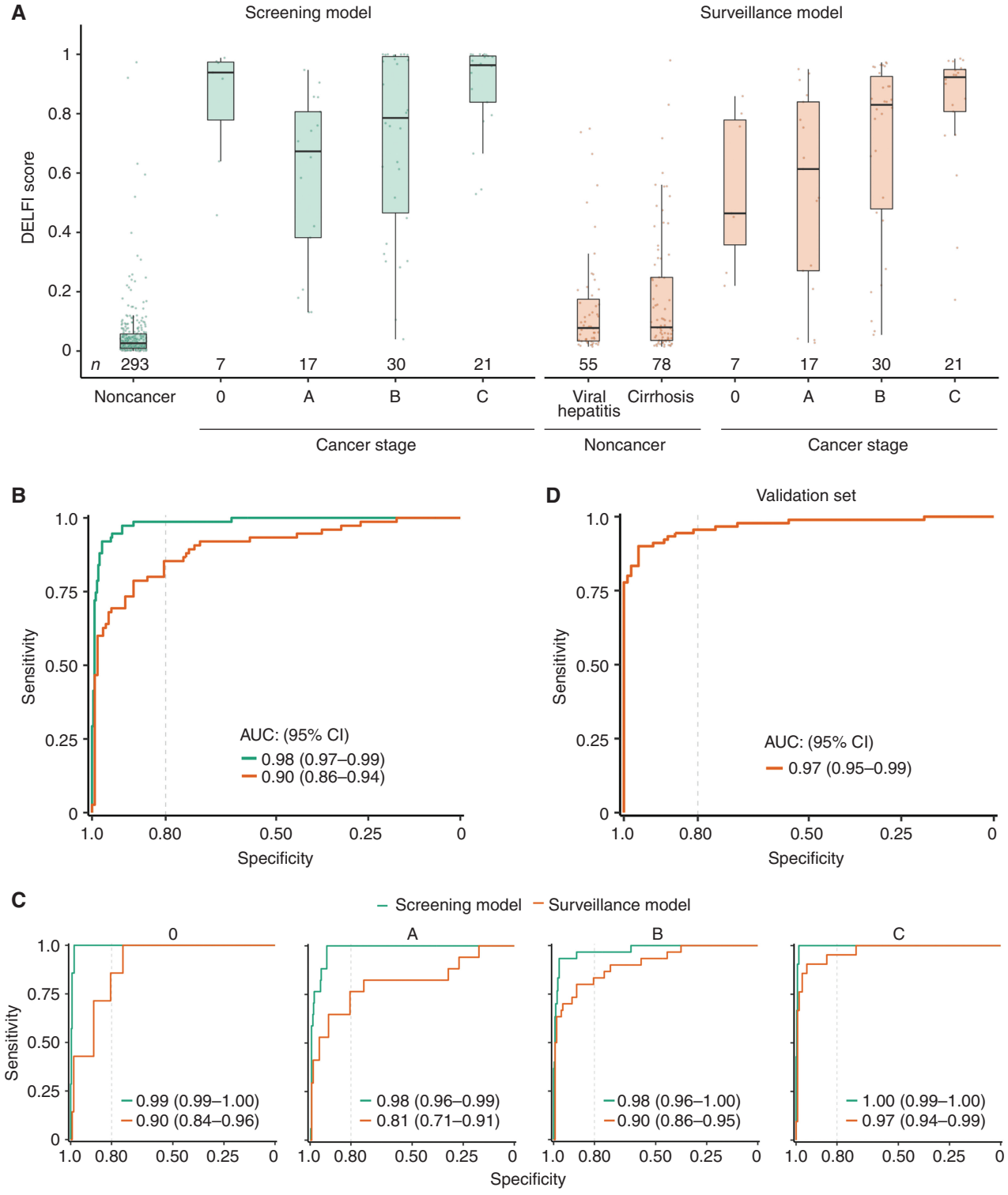

Figure 4. DELFI machine learning models detect liver cancer with high sensitivity and specificity. **A,** DELFI scores for the US/EU cohort across liver disease and cancer stage for the screening and surveillance models. Patients with cirrhosis have DELFI scores higher than individuals without cancer or with viral hepatitis on average, but lower than all stages of liver cancer. Patients with liver cancer across all stages have relatively high DELFI scores, with stage C individuals uniformly having the highest DELFI scores. **B,** ROC analyses of the US/EU general population cohort and the high-risk surveillance cohort. **C,** ROC analyses of the US/EU general population and surveillance cohorts separated by BCLC stage, showing high sensitivity and specificity across stages. **D,** ROC analyses for the fixed surveillance model applied to the Hong Kong cohort, which includes 90 individuals with HCC (85 with BCLC stage A cancer, and 5 with BCLC stage B cancer), 101 individuals with cirrhosis and viral hepatitis, and 32 individuals without cancer or liver disease.

generous estimates suggesting 39% adherence (45), resulting in an average of 40,042 individuals tested in this theoretical population (95% CI, 21,320–61,890). As blood tests offer high accessibility and compliance, with adherence rates of 80% to 90% reported for blood-based biomarkers (46, 47), we conservatively assumed an average of 75% (95% CI, 60%–90%) of this population would be tested using the DELFI approach. As the prevalence of cirrhosis, viral hepatitis, and the co-occurrence of these comorbidities with HCC could vary by region, we used a prior probability distribution to reflect our uncertainty of the composition of these diseases and possible regional differences. Monte Carlo simulations from these probability distributions (Methods) revealed that ultrasound with AFP detected an average of 2,233 (95% CI, 1,088–3,699) individuals with liver cancer. Using DELFI, we would detect on average 2,794 additional liver cancer cases, or a 2.46-fold increase (95% CI, 1.25–4.57-fold increase), compared with ultrasound with AFP alone (Supplementary Fig. S14A and S14B). The DELFI approach would not only substantially improve the detection of liver cancer but would be expected to decrease the false-negative rate, or fraction of cancers missed at testing, from 38% for ultrasound with AFP (95% CI, 25%–51.5%) to 24% for DELFI (95% CI, 9%–42.6%). Additionally, the negative predictive value of the test (NPV) would be expected to increase from 95.7% for ultrasound with AFP (95% CI, 93.8%–97.3%) to 97.1% for DELFI (95% CI, 94.8%–99.0%; Supplementary Fig. S14C and S14D). These analyses suggest a significant population-wide benefit for using a high-specificity blood-based early detection test as a tool for the detection of liver cancer.

DISCUSSION

Overall, in this study, we demonstrate the use of genome-wide cfDNA fragmentome features to detect HCC with high sensitivity and specificity. Furthermore, we show that the fragmentation profiles capture genomic and chromatin characteristics, including alterations known to be important in HCC. Our cfDNA fragmentome approach has robust performance in detecting HCC, including very early-stage disease, independent of disease etiology. To our knowledge, this is the first genome-wide fragmentation analysis that has been independently validated in a separate high-risk population, with stable and robust performance across different racial and ethnic groups from the United States and Hong Kong.

Our results also revealed that disease-specific TF signatures can be obtained through analysis of genome-wide cfDNA fragmentation profiles. Although such analyses have been performed using specific TFs to distinguish small cell from non–small cell lung cancers (23), this study suggests that analyses of disease-specific transcriptional regulation using genome-wide cfDNA fragmentation may improve the detection and identification of the tissue of origin in patients with cancer. With sufficient numbers of patients, cfDNA transcriptional profiles could further improve machine learning algorithms to detect HCC and other cancers.

HCC is unique in comparison with other solid cancers in that there is a large, well-defined high-risk population with an average 3% to 4% annual risk of developing HCC (48) recommended to have routine cancer screening every 6 months. Unfortunately, currently available tests have limited diagnostic utility, especially for early-stage disease (13). In our study, AFP had 52% sensitivity in detecting HCC, consistent with the known performance of this biomarker (13). Ultrasound-based surveillance also has technical limitations with its operator dependency and lower sensitivity in patients with cirrhosis and obesity (49). Most importantly, ultrasound has low compliance to established guidelines, less than 20% worldwide (10, 11), compared with much higher adherence to blood tests for other conditions (46). Despite these challenges, HCC screening provides overall survival benefit in patients with HBV (50) and cirrhosis (10), highlighting the major need to improve current screening tests. The high performance of cfDNA fragmentome analyses in HCC detection, along with its cost-efficient characteristics, would allow DELFI to be an accessible screening test for HCC and to increase the screening rates beyond the currently dismal levels. An interesting aspect of cfDNA analysis specific to HCC is that transplantation is the most curative treatment for early to intermediate stage HCC, and HCC surveillance of posttransplantation patients with a liquid biopsy approach could have a dual role in tracking recurrence and rejection, as studies of cfDNA in posttransplant patients have shown promise (51).

Although this study represents a potential improvement in current screening approaches, there are some limitations. For example, this study included a relatively small sample size of individuals with HCC. Although the independent validation cohort was performed with preanalytical differences in laboratory and sequencing methods, the fact that the DELFI approach performed well in this population suggests that the method will ultimately be able to be utilized in a range of different diagnostic laboratories. Larger validation studies will be needed before this approach can be useful clinically. Nevertheless, the observations that scalable and cost-effective noninvasive cfDNA fragmentome analyses can detect patients with liver cancer may provide an opportunity to screen high-risk and general populations worldwide.

METHODS

Study Population

For the US/EU cohort, samples from 208 patients, including 75 with HCC and 133 high-risk patients without HCC, were collected prospectively as part of the HCC biomarker registry and the AIDS Linked to the IntraVenous Experience (ALIVE) study at the Johns Hopkins University School of Medicine under protocols approved by the Johns Hopkins Institutional Review Board. HCC was defined by histologic examination or the appropriate imaging characteristics as defined by accepted guidelines. Tumor staging was determined by the BCLC. Detailed clinical data were extracted from the electronic medical record. High-risk patients were defined as individuals with cirrhosis from any etiology and/or individuals with chronic HBV or HCV who were recommended for routine HCC screening by expert society guidelines (49). In addition, we included 38 patients with HBV or cirrhosis retrospectively collected by BioIVT. AFP levels were quantified by partnering centers in their clinical laboratories using FDA-approved AFP tests.

The US/EU cohort also included samples from 293 individuals without cancer that were previously analyzed (23), originally from two screening clinical trial cohorts for colorectal cancer in Denmark (Endoscopy III) and The Netherlands (COCOS, Netherlands Trial Register ID NTR182946). The protocol for the Endoscopy III Project was approved by the Regional Ethics Committee and the Danish Data Protection Agency; for the COCOS trial, ethical approval was obtained from the Dutch Health Council. The inclusion criteria for both the Dutch and the Danish cohorts were any individuals of age 50 to 75 eligible for

colorectal cancer screening. All patients used had either a negative fecal immunochemical test result or a negative colonoscopy result.

For the Hong Kong cohort, all recruited subjects gave written informed consent, and the study was approved by the Joint Chinese University of Hong Kong and New Territories East Cluster Clinical Research Ethics Committee (15, 27).

Sample Collection and Preservation

The sample collection was performed as follows: Venous peripheral blood was collected in one K2-EDTA tube and two serum gel tubes. Within 2 hours from blood collection, tubes were centrifuged at 2330 × *g* at 4°C for 10 minutes, plasma was transferred to new tubes, and the samples were spun at 14,000 rpm (18,000 rcf) for 10 minutes at room temperature to pellet any remaining cellular debris. After centrifugation, EDTA plasma was aliquoted and stored at −80°C for cfDNA analyses.

Sequencing Library Preparation

Circulating cfDNA was isolated from 2 to 4 mL of plasma using the Qiagen QIAamp Circulating Nucleic Acids Kit (Qiagen GmbH), eluted in 52 μL of RNase-free water containing 0.04% sodium azide (Qiagen GmbH), and stored in LoBind tubes (Eppendorf AG) at −20°C. Concentration and quality of cfDNA were assessed using the Bioanalyzer 2100 (Agilent Technologies).

Next-generation sequencing cfDNA libraries were prepared for whole-genome sequencing using 15 ng cfDNA when available or entire purified amount when less than 15 ng (Supplementary Table S5). In brief, genomic libraries were prepared using the NEBNext DNA Library Prep Kit for Illumina (New England Biolab) with four main modifications to the manufacturer's guidelines: (i) the library purification steps followed the on-bead AMPure XP (Beckman Coulter) approach to minimize sample loss during elution and tube transfer steps; (ii) NEBNext End Repair, A-tailing, and adapter ligation enzyme and buffer volumes were adjusted as appropriate to accommodate on-bead AMPure XP purification; (iii) Illumina dual index adapters were used in the ligation reaction; and (iv) cfDNA libraries were amplified with Phusion Hot Start Polymerase. All samples underwent a 4-cycle PCR amplification after the DNA ligation step.

Low-Coverage Whole-Genome Sequencing and Alignment

Whole-genome libraries of patients with cancer and cancer-free individuals were prepared as in ref. 23 with the modification that they were sequenced using 100-bp paired-end runs (200 cycles) on the Illumina NovaSeq platform at 1 to 2× coverage per genome. Prior to alignment, adapter sequences were filtered from reads using the fastp software (52). Sequence reads were aligned against the hg19 human reference genome using Bowtie2 (53), and duplicate reads were removed using Sambamba (54). After alignment, each aligned pair was converted to a genomic interval representing the sequenced DNA fragment using bedtools (55). Only reads with a MAPQ score of at least 30 or greater were retained. Read pairs were further filtered if overlapping the Duke Excluded Regions blacklist (https://genome.ucsc.edu/cgi-bin/hgTrackUi?db=hg19&g=wgEncodeMapability). To capture large-scale epigenetic differences in fragmentation across the genome estimable from low-coverage whole-genome sequencing, we tiled the hg19 reference genome into nonoverlapping 5-Mb bins. Bins with an average GC base content < 0.3 and an average mappability < 0.9 were excluded, leaving 473 bins spanning approximately 2.4 Gb of the genome. Following Mathios and colleagues (23), GC correction was performed independently for short (<150 bp) and long (≥150 bp) cfDNA fragments using an external panel of 20 individuals without cancer sequenced on a NovaSeq to generate a target distribution.

Fastq files for patients in the Hong Kong cohort were obtained from The Chinese University of Hong Kong (CUHK) Circulating Nucleic Acids Research Group, as reported (ref. 15; #1645) and processed as described above and in Mathios and colleagues, to generate the DELFI features. GC correction was performed by normalizing to the target distribution provided in https://github.com/cancer-genomics/PlasmaToolsNovaseq.hg19, the same target distribution used for GC correction in the US/EU cohort. The validation set consisted of libraries constructed with 14 cycles of PCR and sequenced on the HiSeq 2000. These libraries were normalized to the 4-cycle NovaSeq target distribution to facilitate comparisons between studies. One sample each from the cirrhotic and HBV groups were excluded, as they were identified to have an HCC diagnosis.

Chromatin Structure Analysis

A/B compartments for liver cancer tissue and lymphoblastoid cells were obtained from https://github.com/Jfortin1/TCGA_AB_Compartments as well as from https://github.com/Jfortin1/HiC_AB_Compartments as described previously (28). The two reference tracks were compared to identify informative 100-kb bins, defined as bins where the chromatin domain differed between the two reference tracks or the magnitude difference in eigenvalues corresponded to a z-score greater than 1.96 or less than −1.96 ($P = 0.05$) across all eigenvalue differences.

The median fragmentation profile for 10 liver samples with high estimated tumor fraction by ichorCNA (56) and 10 randomly selected individuals without cancer was calculated. This information was used to extract an estimated median liver component in the plasma weighted by the ichor score of the individual plasma samples.

Genome-wide TF Analyses

ChIP-seq peaks from 5,620 experiments were downloaded from the ReMap 2020 database (32). This set was filtered for experiments with more than 4,000 peaks, resulting in 4,293 experiments. For each peak in the autosomes, we defined the center of the peak as position 0.

The mean of the coverages at each position (−3,000 to +3,000 with respect to the center of each peak) was computed across all peaks for each sample. For the ROC curves, relative coverage was computed for each sample as the mean coverage in a ±100-bp window surrounding the center of the binding sites divided by the mean coverage in a ±250-bp window surrounding 2,750 bp upstream and downstream of the binding sites. The ROC curve was generated using pROC 1.16.2 (57). The AUC for each peak set was ranked. Each TF was matched with its NCBI ID, leaving 797 unique TFs ranked by AUC. This ranked list was the input for the gseDGN function from the DOSE package in R. The output from this was ranked by the normalized enrichment score.

Whole-Genome Fragment Features

Fragmentation features were calculated as in Mathios and colleagues (23). Briefly, the ratio of short to long fragments was calculated for 473 nonoverlapping 5-Mb bins across the genome, and z-scores representing arm gains/losses were calculated for autosomal chromosome arms. The principal components of the ratios representing greater than 90% of variance and the z-scores were used to train machine learning models.

Machine Learning and Cross-Validation Analyses

Two machine learning models were developed: one for high-risk populations (a Gradient Boosting Machine using the Mathios et al. features) and the second for average-risk general populations (a penalized logistic regression with the Mathios et al. features as well as coverage from TF binding sites). These models were trained on the US/EU cohort in Caret with 5-fold cross-validation with 10 repeats, and scores for each sample were calculated by the mean across repeats and evaluated using AUC-ROC as in Mathios and colleagues (23). The first model used the high-risk noncancer and HCC patients, whereas the second model used the noncancer individuals without liver pathology. The locked high-risk model trained on the US/EU cohort was applied to the Hong Kong cohort to generate cancer predictions on an external validation set.

TCGA Analysis

Copy-number data from the HCC cancer cohort in TCGA [liver hepatocellular carcinoma (LIHC) $n = 372$] were retrieved using the package RTCGA v1.16.0 and were analyzed to determine the frequency of copy-number gains and losses in the 473 5-mb bins for this cohort (23). The somatic copy-number alteration threshold used in Mathios and colleagues (23) was used to call gains and losses in the HCC cohorts (23, 58).

Association of Clinical Covariates with DELFI Score

Potential associations between clinical covariates (for those patients for whom this information was made available) and the DELFI score were assessed with Spearman rank correlation coefficient (continuous variables) and Kruskal–Wallis one-way analysis of variance (categorical variables).

Simulation

Monte Carlo simulations were used to compare the DELFI approach to ultrasound and AFP in a theoretical surveillance population. We used estimated 95% CIs of sensitivity and specificity for DELFI and published 95% CIs for ultrasound with AFP (13). The R package epiR was used to derive prior predictive probability distributions (beta distributions) from these CIs (R package version 2.47, epiR; RRID:SCR_021673). Zhao and colleagues (45) reported that adherence to ultrasound and AFP surveillance was 39% (95% CI, 21%–65%). As other noninvasive blood-based tests have a reported adherence of more than 75% (46, 47), we assumed that adherence to DELFI would be 60% or greater with a probability 0.975 or higher. Using these confidence estimates, epiR was used to derive beta prior predictive distributions for adherence. We simulated multinomial probabilities for the prevalence of HBV, cirrhosis, HBV + HCC, cirrhosis + HCC, and HBV + cirrhosis + HCC from a Dirichlet with parameters 230, 680, 60, 23, and 7, respectively. For a single Monte Carlo simulation for ultrasound with AFP testing, we

(i) sampled the probability of adherence (η) from the prior predictive distribution,
(ii) simulated the number of 100,000 individuals (S) who participated in surveillance ($S \sim \text{Binomial}(\eta, 100{,}000)$),
(iii) sampled probabilities of comorbidities [Dirichlet (230, 680, 60, 23, 7)],
(iv) computed the prevalence of HCC (θ),
(v) simulated HCC cases ($P \sim \text{Binomial}(\theta, S)$) and computed the number of individuals without cancer ($N = S - P$),
(vi) sampled the sensitivity (se) and specificity (sp) from the corresponding prior predictive distributions, and
(vii) sampled the true positives ($TP \sim \text{Binomial}(P, se)$) and false positives ($FP \sim \text{Binomial}(N, 1 - sp)$).

Given TP and FP, we calculated the NPV as (true negatives)/(true negatives + false negatives), where true negatives = $N - FP$ and false negatives = $P - TP$. We repeated the above simulation 1,000 times, obtaining a distribution of TP, FP, and NPV. Using parameters for sensitivity, specificity, and adherence for the DELFI approach, we repeated the same Monte Carlo analysis to allow comparisons between these two surveillance methodologies.

Bioinformatic and Statistical Software

All statistical analyses were performed using R version 4.1.2. After trimming of adapter sequences using fastp (0.20.0), we used Bowtie2 (2.3.0) to align paired-end reads to the hg19 reference genome. PCR duplicates were removed using Sambamba (0.6.8), and the remaining aligned read pairs were converted to a bed format using Bedtools (2.29.0). We used the R package data.table (1.12.8) for manipulation of tabular data and binning fragments in 5-Mb windows along the genome. The R package Caret (6.0.84) was used to implement the classification by penalized logistic regression and resampling.

Data and Material Availability Statement

Sequence data and clinical variables used in this study are available at the European Genome-Phenome Archive (EGA) at accession EGAS00001005340, EGAD00001005093, and EGAS00001005340. Some data are not publicly available due to limitations in Institutional Review Board approval but are available upon reasonable request from the corresponding authors. The publicly available ChIP-seq data used in this study are available in the ReMap 2020 database (https://remap2020.univ-amu.fr/download_page). Segmented copy-number data, determined by analysis of the Affymetrix genome-wide human SNP array 6.0, were retrieved from the Broad Institute TCGA Genome Data Analysis Center (2016-01-28 release date, using RTCGA package, version 1.16.0). The remaining data are available within the article, Supplementary Information, or Source Data file. Computer code, software versions, and the computing environment for reproducing results from this study are available in the GitHub repository at https://github.com/cancer-genomics/reproduce_liver_final.

Authors' Disclosures

Z.H. Foda reports a patent for detecting liver cancer using cfDNA fragmentation pending. A.V. Annapragada reports a patent for detecting liver cancer using cfDNA fragmentation pending. D.C. Bruhm reports a patent for 63/423,003 pending, a patent for 63/290,017 pending and licensed to Delfi Diagnostics, and a patent for PCT/US2021/0646 pending and licensed to Delfi Diagnostics. D. Mathios reports a patent for detection of lung cancer using cfDNA fragmentation pending. S. Cristiano reports other support from Delfi Diagnostics outside the submitted work. R.A. Anders reports grants and other support from Bristol Myers Squibb, other support from Merck SD, AstraZeneca, and GSK, and grants from RAPT Therapeutics outside the submitted work. D.L. Thomas reports personal fees from Merck, Excision Bio, and UpToDate outside the submitted work. G.D. Kirk reports grants from the NIH during the conduct of the study. V. Adleff reports personal fees from Delfi Diagnostics outside the submitted work, as well as a patent for cfDNA for assessing and/or treating cancer and related patents pending, issued, licensed, and with royalties paid from Delfi Diagnostics. J. Phallen reports other support from Delfi Diagnostics during the conduct of the study, as well as a patent for cfDNA for assessing and/or treating cancer pending, licensed, and with royalties paid from Delfi Diagnostics. R.B. Scharpf reports grants and personal fees from Delfi Diagnostics outside the submitted work; a patent for US-2022-0325343 licensed to Delfi Diagnostics; and is a founder of and holds equity in Delfi Diagnostics, and serves as the head of Data Science. This arrangement has been reviewed and approved by Johns Hopkins University in accordance with its conflict-of-interest policies. A.K. Kim reports grants and personal fees from AstraZeneca and personal fees from Exelixis outside the submitted work. V.E. Velculescu reports grants, personal fees, and other support from Delfi Diagnostics during the conduct of the study; other support from Viron Therapeutics and Epitope outside the submitted work; patent applications related to early detection of cancer pending, issued, licensed, and with royalties paid from Delfi Diagnostics; and is a founder of Delfi Diagnostics, serves on the Board of Directors and as an officer of Delfi Diagnostics, and owns Delfi Diagnostics stock, which is subject to certain restrictions under university policy. Additionally, Johns Hopkins University owns equity in Delfi Diagnostics. V.E. Velculescu divested his equity in Personal Genome Diagnostics (PGDx) to LabCorp in February 2022. V.E. Velculescu is an inventor on patent applications submitted by Johns Hopkins University related to cancer genomic analyses and cfDNA for cancer detection that have been licensed to one or more entities, including Delfi Diagnostics, LabCorp, Qiagen, Sysmex, Agios, Genzyme, Esoterix, Ventana, and ManaT Bio. Under the terms of these license agreements, the

university and inventors are entitled to fees and royalty distributions. V.E. Velculescu is an adviser to Viron Therapeutics and Epitope. These arrangements have been reviewed and approved by Johns Hopkins University in accordance with its conflict-of-interest policies. No disclosures were reported by the other authors.

Authors' Contributions

Z.H. Foda: Conceptualization, data curation, formal analysis, validation, investigation, visualization, methodology, writing–original draft, writing–review and editing. **A.V. Annapragada:** Conceptualization, data curation, software, formal analysis, validation, investigation, visualization, methodology, writing–original draft, writing–review and editing. **K. Boyapati:** Formal analysis, investigation, methodology, writing–review and editing. **D.C. Bruhm:** Data curation, software, validation, investigation, methodology, writing–review and editing. **N.A. Vulpescu:** Software, validation, investigation, methodology, writing–review and editing. **J.E. Medina:** Validation, investigation, methodology, writing–review and editing. **D. Mathios:** Software, validation, investigation, methodology, writing–review and editing. **S. Cristiano:** Software, validation, methodology, writing–review and editing. **N. Niknafs:** Software, methodology, writing–review and editing. **H.T. Luu:** Resources, methodology, writing–review and editing. **M.G. Goggins:** Resources, writing–review and editing. **R.A. Anders:** Resources, writing–review and editing. **J. Sun:** Conceptualization, resources, investigation, writing–review and editing. **S.H. Mehta:** Resources, writing–review and editing. **D.L. Thomas:** Resources, writing–review and editing. **G.D. Kirk:** Conceptualization, resources, investigation, writing–review and editing. **V. Adleff:** Resources, data curation, methodology, writing–review and editing. **J. Phallen:** Software, validation, methodology, writing–review and editing. **R.B. Scharpf:** Resources, data curation, supervision, investigation, visualization, methodology, writing–review and editing. **A.K. Kim:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, investigation, methodology, writing–original draft, writing–review and editing. **V.E. Velculescu:** Conceptualization, resources, software, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing–original draft, writing–review and editing.

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