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Molecular profiling of hepatocellular carcinoma using circulating cell-free DNA

Ahmed O. Kaseb^{1,*}, Nora S. Sánchez^{2,*}, Shiraj Sen³, Robin K. Kelley¹⁰, Benjamin Tan^{11,12}, Andrea G. Bocobo¹⁰, Kian H. Lim^{11,12}, Reham Abdel-Wahab^{1,9}, Marc Uemura³, Roberto Carmagnani Pestana³, Wei Qiao⁴, Lianchun Xiao⁴, Jeffrey Morris⁸, Hesham M. Amin⁵, Manal M. Hassan⁶, Asif Rashid⁷, Kimberly C. Banks¹⁴, Richard B. Lanman¹⁴, AmirAli Talasaz¹⁴, Kenna R. Mills-Shaw², Bhawana George⁵, Abedul Haque⁵, Kanwal P.S. Raghav¹, Robert A. Wolff¹, James C. Yao¹, Funda Meric-Bernstam^{2,13}, Sadakatsu Ikeda¹⁵, Razelle Kurzrock¹⁵

¹Department of Gastrointestinal Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas;

²Sheikh Khalifa Bin Zayed Al Nahyan Institute for Personalized Cancer Therapy, The University of Texas MD Anderson Cancer Center, Houston, Texas

³Division of Cancer Medicine, The University of Texas MD Anderson Cancer Center, Houston, Texas

⁴Department of Biostatistics, The University of Texas MD Anderson Cancer Center, Houston, Texas

⁵Department of Hematopathology, The University of Texas MD Anderson Cancer Center, Houston, Texas

⁶Department of Epidemiology, The University of Texas MD Anderson Cancer Center, Houston, Texas

⁷Department of Pathology, The University of Texas MD Anderson Cancer Center, Houston, Texas

⁸Department of Investigational Cancer Therapeutics-The University of Texas MD Anderson Cancer Center, Houston, Texas

⁹Arizona Clinical Oncology Department, Assiut University Hospital, Assiut, Egypt

Corresponding Authors: Ahmed O. Kaseb, MD, Department of Gastrointestinal Medical Oncology, Unit 426, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, Texas 77030, USA (akaseb@mdanderson.org; Tel: (713) 792-2828; fax: (713) 745-1163). Razelle Kurzrock, MD, Center for Personalized Cancer Therapy and Division of Hematology and Oncology, University of California San Diego, Moores Cancer Center, La Jolla, CA (rkurzrock@ucsd.edu).

*Equally contributed to this work

Author contributions:

Conception and design of the study: Ahmed O. Kaseb, Nora S. Sánchez, Jeffrey S. Morris, Hesham M. Amin, Manal M. Hassan, Asif Rashid, Kenna R. Mills-Shaw, Funda Meric-Bernstam, Razelle Kurzrock

Generation, collection, assembly, analysis and/or interpretation of data: Ahmed O. Kaseb, Nora S. Sánchez, James T. Link, Shiraj Sen, Roberto Carmagnani Pestana, Robin K. Kelley, Benjamin Tan, Andrea G. Bocobo, Huat C. Lim, Reham Abdel-Wahab, Marc Uemura, Wei Qiao, Lianchun Xiao, Jeffrey S. Morris, Hesham M. Amin, Manal M. Hassan, Asif Rashid, Kimberly C. Banks, Richard B. Lanman, AmirAli Talasaz, Bhawana George, Abedul Haque, Sadakatsu Ikeda, Razelle Kurzrock

Drafting or revision of the manuscript: Ahmed O. Kaseb, Nora S. Sánchez, James T. Link, Shiraj Sen, Roberto Carmagnani Pestana, Robin K. Kelley, Benjamin Tan, Reham Abdel-Wahab, Hesham M. Amin, Bhawana George, Kanwal P.S. Raghav, Robert Wolff, James C. Yao, Razelle Kurzrock

¹⁰Helen Diller Family Comprehensive Cancer Center-University of California San Francisco

¹¹Department of Medicine, Washington University School of Medicine, St. Louis, MO

¹²Department of Radiology-Washington University School of Medicine, St. Louis, MO

¹³Arizona Cancer Center, Tucson, AZ

¹⁴Guardant Health, Inc., Redwood City, CA

¹⁵Center for Personalized Cancer Therapy and Division of Hematology and Oncology, University of California San Diego, Moores Cancer Center, La Jolla, CA.

Abstract

Purpose: Molecular profiling has been used to select patients for targeted therapy and determine prognosis. Noninvasive strategies are critical to hepatocellular carcinoma (HCC) given the challenge of obtaining liver tissue biopsies.

Experimental Design: We analyzed blood samples from 206 HCC patients using comprehensive genomic testing (Guardant Health, CA) of circulating tumor DNA (ctDNA).

Results: 153/206 (74.3%) were men; median age, 62 years (range, 18–91 years). 181/206 patients had 1 alteration. The total number of alterations was 680 (non-unique); median number of alterations/patient was 3 (range, 1–13); median mutant allele frequency (% cfDNA), 0.49% (range 0.06 – 55.03%). *TP53* was the common altered gene (>120 alterations (non-unique)) followed by *EGFR*, *MET*, *ARID1A*, *MYC*, *NF1*, *BRAF*, and *ERBB2* (20–38 alterations (non-unique)/gene). Of the patients with alterations, 56.9% (103/181) had 1 actionable alterations, most commonly in *MYC*, *EGFR*, *ERBB2*, *BRAF*, *CCNE1*, *MET*, *PIK3CA*, *ARID1A*, *CDK6*, and *KRAS*. In these genes, amplifications occurred more frequently than mutations. Hepatitis B (HBV)-positive patients, were more likely to have *ERBB2* alterations, 35.7% (5/14) versus 8.8% HBV- negative (p=0.04).

Conclusions: This study represents the first large-scale analysis of blood-derived ctDNA in HCC in USA. The genomic distinction based on HCC risk factors and the high percentage of potentially actionable genomic alterations suggests potential clinical utility for this technology.

Keywords

cell-free DNA; hepatocellular carcinoma; molecular profiling

INTRODUCTION

Recent technological advances in molecular diagnostics have allowed for the study of solid malignancies through non-invasive blood sampling. Importantly, intact circulating tumor cells (CTCs) and cell-free DNA (cfDNA) [leukocyte-derived and tumor-derived (circulating tumor DNA; ctDNA)] can now be interrogated through advanced sequencing methods in order to identify somatic mutations that may be druggable targets for future therapies^{1, 2}. Although cfDNA and ctDNA are similar in that they both derive from cell lysis and apoptosis³, ctDNA is the fraction of cfDNA, which can range from <0.1% to >10%⁴, specifically derived from primary or metastatic tumors⁵. Therefore, profiling the

mutational landscape of ctDNA from solid tumors may represent a particularly attractive method for identifying tumor-associated somatic mutations. Applications that can be envisioned to be of clinical utility for hepatocellular carcinoma (HCC) include detection of genomic changes, mutational analysis, prognostication, oncogenic pathway determination, prediction/monitoring of treatment response, drug resistance alterations, and identification of mechanisms of malignant/metastatic transformation based on underlying risk factor and genetic predisposition.

CtDNA has been found to be inversely correlated with HCC prognosis⁶ and shorter overall survival⁷. Although several studies have investigated the global alterations in cfDNA of HCC^{8–13}, the small number of patients and heterogeneity of demographics and geographic locations posed a challenge to the application of this methodology in the US^{12, 13}.

Therefore, the purpose of the current study was to examine a large multi-institutional cohort of HCC tumors with a CLIA-certified cfDNA technology that is currently used in practice and to identify the mutational landscape that may be contributing to HCC tumorigenesis for the purpose of selecting patients for targeted therapy trials. This investigation represents the largest reported series of HCC patients analyzed to date for the genomic portraits derived from blood-derived cfDNA.

METHODS

Patients:

During the period extending from November 2014 to July 2016, 219 patients with advanced HCC underwent blood-derived cfDNA testing by Guardant Health (Redwood City, CA). The diagnosis of HCC was established either by histopathological examination or by characteristics imaging findings, following clinical guidelines by the American Association for the Study of Liver Disease (AASLD)¹⁴. The majority of the patients were from the following 4 institutions: MD Anderson Cancer Center (MDACC; 37 patients), University of California San Diego, Moores Cancer Center (UCSD; 36 patients), Washington University School of Medicine-Siteman Cancer Center (18 patients), and University of California San Francisco-Helen Diller Family Comprehensive Cancer Center (UCSF) (11 patients). The remaining patients were from other US institutions. From the 219 patients, 12 had serial testing. For patients who had more than one test, only the first test result was considered. The analysis and consent of patients in this cohort followed guidelines of various institutional review boards and clinical protocols, as follows: MDACC: [NCT01772771](#)- Molecular Testing for the MDACC Personalized Cancer Therapy Program; UCSD: [NCT02478931](#) (PREDICT)-Study of Personalized Cancer Therapy to Determine Response and Toxicity; Washington University School of Medicine-Siteman Cancer Center: WU IRB protocol 201606097, A retrospective analysis of circulating cell free DNA in patients with gastrointestinal malignancies; UCSF, UCSF IRB# 12–09576 Hepatobiliary Tissue Bank and Registry. The latter two institutional IRB approvals were for non-therapeutic/non-interventional use and hence are not registered in [clinicaltrials.gov](#). The study was conducted in concordance with the Declaration of Helsinki.

Demographic information (i.e., gender, age) and date of blood collection were available for all patients. Additional patient information was requested from the institutions that contributed more than 10 patients for cfDNA testing (UCSD, MDACC, Siteman Cancer Center, and UCSF). The information requested was as follows: (i) confirmation of HCC diagnosis; (ii) risk factor information, such as hepatitis A, B, and C status, non-alcoholic steatohepatitis (NASH), alcoholic liver disease, and metabolic syndrome; (iii) relevant clinicopathologic information including Child-Pugh score, Cancer of the Liver Italian Program (CLIP), Barcelona Clinic Liver Cancer (BCLC), Tumor-Node-Metastasis (TNM) Staging, and treatment status at time of cfDNA testing. Metabolic syndrome as established as a clinical diagnosis as per guidelines by the 2001 National Cholesterol Education Program ATP III¹⁵. The diagnosis of NASH was established by pathologic diagnosis of NASH in patients with available clinical data.

Comprehensive genomic testing in plasma:

cfDNA was extracted from whole blood collected in 10 mL Streck tubes. Samples were shipped to a Clinical Laboratory Improvement Act (CLIA)-certified, College of American Pathologists-accredited laboratory (Guardant Health, Redwood City, California). After double ultracentrifugation, 5 ng – 30 ng of cfDNA was isolated for digital sequencing as previously described^{12, 13, 16}.

Cell-free DNA fragments, both leukocyte- and tumor-derived, were simultaneously sequenced. The variant allele fraction (VAF) was calculated as the proportion of cfDNA harboring the variant in a background of wild-type cell-free DNA. The analytical sensitivity allowed detection of 1–2 mutant fragments in a 10 ml blood sample (0.1% limit of detection) with analytic specificity > 99.9999%. Twelve CNAs were reported as the absolute gene copy number in plasma. Since most cfDNA is leukocyte-derived, the gene copy number is generally 2.0. Tumor-derived DNA shed into the bloodstream raises this value but, due to the relative proportions of tumor-derived versus leukocyte-derived cfDNA, it is typically a minor contributor. Gene copy number in plasma is thus a function of both copy number in tissues and the degree to which tumor DNA is shed into circulation. Plasma copy number of 2.5–4.0 is reported as ++ amplification and >4.0 as +++, representing the 50th-90th and >90th percentile, respectively of all CNA calls in the Guardant360 database^{12, 13, 16}.

Over the course of the study, the panel composition expanded from 54 to 68 to 70 genes. The initial 54-gene panel consisted of complete exon coverage or critical exon coverage in 54-cancer related genes, and amplifications in 3 genes. In the 68-gene panel, 8 genes were retired from the single nucleotide variation (SNV) gene set, while coverage of gene amplifications expanded from 3 to 16 genes, and detection of fusions in 4 genes, and insertions or deletion of bases (indels) in 1 gene. The 70-gene panel includes all NCCN somatic genomic targets, including complete or critical exon coverage in 30 and 40 genes, respectively; amplifications in 18 genes, fusions in 6 genes, and indels in 3 genes [Supplementary Table 1]. Seven patients were tested on the 54-gene panel was used, eighty-six on the 68-gene version of the original, and one-hundred-forty-two on the 70-gene panel version [Supplementary Table 2].

Actionable genes and variants defined

To determine whether an alteration was actionable or not, we referenced the actionable gene list set in place by the Institute for Personalized Cancer Therapy-Precision Oncology Decision Support (PODS) team at MD Anderson Cancer Center¹⁷. By these standards, a gene is considered actionable if there is supporting evidence that such gene is a driver of tumorigenesis, wherein actionability of the gene can refer to either sensitivity and/or resistance to a drug(s), and sensitivity and/or resistance can be inclusive of all alterations types or specific to alteration classes, i.e., applicable to amplifications, but not mutations. Furthermore, there must be a clinically available agent targeting such gene, and for a specific agent, there must be at least pre-clinical evidence that supports its role in targeting the specific gene. While the PODS team aims at maintaining the actionable gene list as comprehensive and up to date as possible, we relied heavily on the availability of a drug in the context of clinical trial at MDACC. However, we acknowledge that drug availability and trials varies greatly by institution. Finally, the actionability of a gene can be in the context of tumor type(s). For example, in colorectal cancer, the use of the EGFR monoclonal antibodies cetuximab and panitumumab is contraindicated in tumors with activating KRAS mutations, specifically those in codons G12 and G13 (FDA label)^{18–20}. Hence, KRAS is deemed actionable for treatment with MEK inhibitors in all tumor types. However, in the case of colorectal cancer, it is also deemed actionable for resistance to cetuximab and panitumumab²¹. At the moment, there are no HCC-specific actionable gene.

Based on the aforementioned criteria, the following genes were considered actionable based on their sensitivity towards respective targeted agents: *AKT1*, *ALK*, *ARAF*, *ARID1A*, *ATM*, *BRAF*, *BRCA1*, *BRCA2*, *CCND1*, *CCND2*, *CDK4*, *CDK6*, *CDKN2A*, *CDKN2B*, *EGFR*, *ERBB2*, *FGFR1*, *FGFR2*, *FGFR3*, *HRAS*, *IDH1*, *IDH2*, *JAK2*, *JAK3*, *KIT*, *MAP2K1*, *MAP2K2*, *MET*, *MPL*, *MYC*, *NF1*, *NOTCH1*, *NRAS*, *NTRK1*, *PDGFRA*, *PIK3CA*, *PTEN*, *PTPN11*, *RET*, *ROS1*, *SMO*, *STK11*, and *TSC1*.

The following genes were deemed actionable based on context-specific criteria: *CCNE1* (sensitivity to CDK2 inhibitors; resistance to CDK4/6 inhibitors); *ESR1* (presence is sensitizing to hormone therapy, mutations cause resistance to anti-hormone therapy); *KRAS* (sensitivity to MEK inhibitors; resistance to cetuximab and panitumumab in colorectal and erlotinib, and gefitinib in NSCLC; *NRAS* (sensitivity to MEK inhibitors, resistance to cetuximab and panitumumab); *RAF1* (activating alterations cause sensitivity to MEK inhibitors and resistance to RAF inhibitors; inactivating alterations cause resistance to dasatinib). Only *AR* and *RBI* were deemed actionable solely because of their resistance to anti-hormone therapy and to CDK4/6 inhibitors, respectively.

At the time of analysis, several genes included in the Guardant panel were denoted as non-actionable including: *TP53*, *CTNNB1*, *APC*, *GNAS*, *NFE2L2*, *MLH1*, *RIT1*, *SMAD4*, *HNF1A*, *CDH1*, *GATA3*, *VHL*, *FBXW7*, and *RhoA*. To note, some investigators, including co-authors on this manuscript, have considered *TP53* actionable for Wee-1 or VEGF/VEGFR inhibitors^{22–24}. Since this remains a matter of debate, *TP53* was not considered actionable in this paper. Likewise, since the initial analysis, *MLH1* has also become actionable based on FDA-approval of nivolumab and pembrolizumab in 2017. Finally, variants found in actionable genes but which lacked any supporting, functional evidence

were designated as “variants of unknown functional significance.” A comprehensive list of all genes considered and their actionability is included in Supplementary Table S1b.

Statistical analysis

The distribution of each continuous variable was summarized by its mean, standard deviation, and range. The distribution of each categorical variable was summarized in terms of its frequencies and percentages. Continuous variables were compared between groups by Wilcoxon rank sum test, and for categorical data the comparison was conducted by Fisher Exact test. Heat map with dendrogram was generated to explore and visualize the gene mutation/amplification relationship along with the corresponding various risk factors. The relationship between gene types with regard to mutation and amplification as well as synonymous and targetable status was evaluated with Spearman’s rank correlation and displayed in the correlation matrix in which the non-significant correlations are marked with “blank” in the graph [Figure 6]. All computations were carried out in SAS version 9.3 and R version 3.13.

RESULTS

Patient Characteristics:

A cohort of 219 patients was enrolled in the study. The median age was 62.3 years (range: 18–91; 160 males and 59 females). Irrespective of gene actionability, the median number of alterations per patient was 3 (range: 1–13); the median allele frequency (AF) of altered cfDNA (%cfDNA) was 0.49% (range: 0.06–55.03).

After further evaluation, it was determined that 13 of these patients had a diagnosis other than HCC, namely, fibrolamellar HCC, cholangiocarcinoma or mixed HCC/ cholangiocarcinoma. This determination was based on Liver Imaging Reporting and Data System (LI-RADS) and tumor markers for HCC, while fibrolamellar and mixed HCC/ cholangiocarcinoma were mostly confirmed by biopsy. These patients were excluded from further analysis. In addition, for the patients who had more than one cfDNA test ordered (n=12 patients), only the first order was considered for further analysis. After exclusion of these unique cases, as well as the non-HCC cases, the initial data set was reduced from 219 to 206 patients, and from 777 to 680 alterations [Figure 1a–d].

Overall distribution of alteration types

The majority of patients (87.8%=181/206) had at least one alteration; only 12.2% (25/206) had no alterations [Figure 1a]. A total of 680 alterations (non-unique) were detected among the former patient population, of which 10.5% (72/680) were synonymous/silent in nature, and 16% were amplifications (109/680). The remaining 73.3% of alterations (499/680) consisted of indels, missense, nonsense, and splice variants. A fraction of these alterations (214/499), were classified as “variants of unknown functional significance” (VUS) due to lack of supporting evidence for their functionality. The remaining variants (285/499) were broadly categorized as “mutations”, and made up 42% (285/680) of the total alterations identified [Figure 1b].

Further analysis of the 42% of alterations that made up the “mutation” category revealed that 53% of these alterations occurred within actionable genes (actionable mutations) while the remaining 47% occurred within genes not considered actionable (non-actionable mutations) at the time of analysis [Figure 1c]. On the other hand, all of the reported amplifications occurred within actionable genes. After accounting for synonymous alterations, variants of unknown significance (VUS), and mutation in non-actionable genes, the total number of actionable alterations was 209 out of the initial 680 (non-unique) alterations identified [Figure 1d]. Of the patients with alterations, 56.9% (103/181) had 1 actionable alterations.

Most frequently mutated genes identified

We assessed the overall molecular landscape of the 181 HCC patients with detected alterations, both in terms of number of alterations reported per gene and number of patients per gene. Additionally, to assess the true utility of cfDNA findings, in terms of their potential for guiding clinical decisions, genes were parsed into “actionable” and “non-actionable genes”, as defined above (see Methods section for details). Inclusive of all alteration types (i.e., synonymous, VUS), the most frequently altered genes (20 events/gene) were as follows: within actionable genes--*EGFR*, *MET*, *ARID1A*, *MYC*, *NF1*, *BRAF*, and *ERBB2*; among non-actionable genes--*TP53*, followed by *CTNNB1* and *APC* [Figure 2a]. Overall, *TP53* was the most commonly altered gene, with >120 alterations. By comparison, *EGFR* had 38 alterations. Of note, the gene panel evolved over the course of this study, hence the number of patients evaluated for each of the genes varied depending on the panel available at that time. Moreover, detection of copy number variations was limited to amplifications in a subset of genes (see Material and Methods and Supplementary Table S1a and S2 for details).

Exclusion of non-actionable alterations, i.e., VUS and synonymous alterations, further reduced the total number of alterations from 680 alterations to 209 alterations. Moreover, the patient population was also reduced from 181 to 103 patients, as many patients only had alterations detected within non-actionable genes, or all alterations were synonymous or VUS. Among the 103 patients with actionable alterations, the most commonly altered genes (10 patients/gene) were *MYC*, *EGFR*, *ERBB2*, *BRAF*, *CCNE1*, *MET*, *PIK3CA*, *ARID1A*, *CDK6*, and *KRAS*. Likewise, amplifications were more common than mutations among the top genes [Figure 2b].

Gene correlation matrices to visualize co-expression and mutual exclusivity of genes with alterations detected

Two correlation matrices were generated, independent of the availability of risk factor information: the first matrix considered all confirmed HCC patients with alterations detected, and included all alteration types (synonymous, VUS, mutations, amplifications) in both actionable and non-actionable genes. This amounted to 181 patients and 62 genes analyzed [Figure 2c]. The premise of this matrix was to allow an overall appreciation of *all* alterations identified. For more hypothesis-generating analysis, the second matrix, which focused only on mutations and amplifications within actionable genes (see S1b), and excluded synonymous and VUS alterations [Figure 2d] was generated. Application of

these exclusion criteria reduced the initial correlation matrix from 181 to 103 patients, and from 62 to 36 actionable genes. Concordance estimates and 95% intervals (Cohen's kappa) for each pair of genes were generated to better understand any association in terms of co-expression or mutual exclusivity of genes analyzed. In this analysis, any value <0 indicated no agreement, 0 – 0.20 slight agreement, 0.21–0.40 as fair agreement, 0.41–0.60 as moderate agreement, 0.61–0.80 as substantial agreement, and 0.81–1 as almost perfect agreement [Figure 2c–d]. Firm conclusions were not possible with these matrices, given the small sample size, but hypothesis-generating observations could be deduced.

Identifying patients with alterations in actionable genes and available risk factor data

To understand if molecular findings correlated with any of the risk factors commonly observed among patients diagnosed with HCC, we requested additional patient information from institutions having >10 HCC participants, as detailed under Methods. These included: University of California San Diego Moores Cancer Center: 36 patients, 48 orders; MD Anderson Cancer Center: 37 patients, 37 orders; Siteman Cancer Center: 18 patients, 19 orders; and University of California San Francisco Comprehensive Cancer Center: 11 patients, 11 orders) for a total of 103 patients.

The 13 non-HCC cases previously excluded were identified from within this population. Hence, the final number of patients from participating institutions was 89 patients, of which 82 patients had alterations detected while 7 had none [Figure 3]. We again parsed this subset of alterations into actionable or non-actionable groups; the latter included synonymous alterations, VUS, and non-actionable genes. We identified a subset of patients ($n=34$) who only had alterations detected in non-actionable genes or the alteration type was non-actionable, in the case of synonymous or VUS. Ultimately, this reduced the patient population with alterations from 82 to 48 patients contributing, for a total of 98 alterations (non-unique) in 31 actionable genes. This small subset of patients was statistically evaluated for their correlation with risk factors contributing to HCC, and consisted of 14 women, 34 men, mostly Caucasian patients ($n=30$) followed by Hispanic ($n = 8$), Asian ($n = 5$), Black ($n = 2$), Native American ($n = 1$) and other ($n = 2$). Nineteen patients were reported to have alcoholic liver disease, 8 had confirmed non-alcoholic steatohepatitis (NASH) and 10 had metabolic syndrome. Staging information as classified by Child-Pugh Score (CPS), Barcelona Clinic Liver Cancer staging system (BCLC), CLIP, and/or TNM staging was requested. There were 27 patients with Child-Pugh Score of A: (5–6, well compensated), 13 with stage B: (7–9, significant functional compromise), and 1 with C: (10–15, decompensated). Six patients had an unknown BCLC classification, while 1 was classified as stage A, 4 as stage B and 37 as stage C. Hepatitis A was reported in 12 patients; hepatitis B in 14 patients; and hepatitis C in 17 patients. There were only a handful of cases with both hepatitis A & B ($n=4$), hepatitis B & C ($n=6$) or hepatitis A & C ($n=7$). [Supplementary Table 3, Supplementary Figure 1a–d].

Although this smaller subset (only patients with available risk factor information and only actionable genes) included only 48/206 patients, it maintained the overall molecular landscape of the larger subset (see Figure 2a–b). Whereas in the overall population the top 10 most frequently altered actionable genes were *MYC*, *EGFR*, *ERBB2*, *BRAF*,

CCNE1, MET, PIK3CA, ARID1A, CDK6, and KRAS, in this subset of patients (n= 48) the top 10 most frequently altered actionable genes were: *EGFR, ERBB2, MET, CCNE1, MYC, BRAF, CCND1, CDK6, FGFR2, and ARID1A* [Figure 4a–b]. Hence, this subgroup should be considered a suitable and representative model of the molecular profile of circulating cell-free DNA in patients with hepatocellular carcinoma.

Correlation of risk factors with alterations identified

Significant differences were noted in the molecular profile of patients based on their underlying hepatic reserve and risk factors for HCC. Among patients with metabolic syndrome, 30% (3/10) had alterations detected in *PDGFRA* (p=0.0113), while patients without metabolic syndrome (n=30) had no alterations in this gene. In terms of hepatitis status, 35.7% (5/9) of patients with hepatitis B had alterations in *ERBB2* (p=0.0365), and 16.7% (3/18) of patients with hepatitis C had alterations in *BRCA1* (p=0.0472). Hepatitis B and Hepatitis C negative patients, did not have any alterations in these respective genes. In addition, 23.1% (3/13) of patients with Child-Pugh score B had alterations in *PIK3CA* (p=0.0341) versus 0% (0/27) Score A (0/27) or C (0/1) (p=0.03). Heat map with dendrogram was generated to better visualize and study the gene mutation/amplification relationships along with the corresponding various risk factors. The relationships between gene types, with regard to mutation and amplification as well as synonymous and targetable status, was evaluated with Spearman's rank correlation and displayed in the correlation matrix in which the non-significant correlations are marked with "blank" in the graph. All computations were carried out in SAS version 9.3 and R version 3.13. [Figure 5–6; Supplementary figure 2a–c].

DISCUSSION

In the vast majority of patients, HCC comprises a two-disease state; HCC tumors and concomitant liver disease. Therefore, HCC staging/prognostication systems take into account tumor parameters in addition to hepatic reserve grade, manifested by Child-Pugh classification. While mounting evidence in the literature suggests different molecular pathogenesis pathways based on the degree of liver fibrosis and underlying HCC risk factors, this has not been translated into defining molecular subclasses in HCC. The challenge to defining molecular nosology is largely due to lack of access to tissue sampling in HCC, given the risk of bleeding due to concomitant coagulopathy and thrombocytopenia in patients with advanced fibrosis and portal hypertension. This bleeding risk led to the development and wide acceptance of guidelines to diagnose HCC without tissue sampling²⁵. Therefore, despite recent advances in our understanding of HCC molecular profiling based on tissue sampling²⁶, firm conclusions and biomarker integration into HCC staging were not achieved due to major challenges in prospective validation studies that are powered to study risk factor-specific HCC. Liquid biopsies could bridge this gap, given the practicality of obtaining them in this patient population, and are expected to help advance the field of molecular prognostication and patient selection in targeted therapy trials. The current study represents a step towards achieving this major goal given our important observations related to specific common risk factors in HCC. This study also represents the largest US cohort ever reported. Hence, our correlative findings of molecular alterations, as they relate to

specific risk factors and demographics, highlight the importance of cfDNA methodology in HCC and warrant future validation and exploration on its utility.

Chronic infection with hepatitis B with its incorporation into the human genome is a common HCC risk factor, particularly in Asia. The pathogenesis can be due to oncogenic viral proteins or insertional mutagenesis. The mutational spectrum in HCC arising from hepatitis B infection is known to be enriched with *TERT*, *CCNE1*, and *MLL4* due to insertional events, while hepatitis C infection is typically chronic and leads to chronic inflammatory cascade events in the microenvironment and mutations.

Interestingly, our study showed statistically significant associations between certain alterations and specific risk factors, such as metabolic syndrome and *PDGFRA* (p=0.0113), HBV and *ERBB2* (p=0.0365), HCV and *BRCA1* (p=0.0472), and Child-Pugh score B and *PIK3CA* (p=0.0341). This relationship between gene types with regard to alteration type, risk factor, and targetable status was evaluated with Spearman's rank correlation and displayed in the correlation matrix. However, given the small number of patients in each category, we consider these findings as hypothesis-generating associations that should be studied in future validation studies. However, given the small number of patients in each category, we consider these findings as hypothesis-generating associations that should be studied in future validation studies. For example, while *ERBB2* dysregulation is not known to be a significant driver or hallmark of HCC, upregulation, correlation with HCC poor outcome, and cell line data suggests that HBV interacts with *ERBB2* and *ERBB3* resulting in protein stabilization [20,21]. Similarly, dysregulation of *BRAF*, *BRCA1*, *MET*, *CDK6*, *ARID1A*, *CCNE1*, *EGFR*, *FGFR1*, *MYC*, and *NOTCH1* correlated with a history of HCV in our study [Supplementary Figure 4a-d]. In the case of *BRCA1*, while there is an exhaustive amount of literature supporting the role of *BRCA1* in breast and ovarian tumor types, less is known about the role of *BRCA1* in HCC, much less in regards to its role in HCV affected HCC patients. Nonetheless, one whole genome-sequencing analysis of 88-matched HCC tumor/normal tissue samples, reported that *BRCA1* alterations were identified in 1.1% of HCC patients²⁷. By comparison, our study using cfDNA assay identified *BRCA1* alterations in 4 of 212 patients (1.8%) by ctDNA. Although this is not direct evidence for *BRCA1* in HCV in HCC, overall these findings are consistent with NGS results in solid tumor testing. Furthermore, increasing evidence supports the role of common molecular signatures across distinct tumor types²⁸⁻³⁰. Hence, there is the potential that *BRCA1* and/or other genes that have specifically been implicated in other tumor types, may also be relevant to HCC.

Notably, alterations in *TERT* promoter and in *CTNNB1* gene were reported among the most frequent alterations in HCC³¹, and in particular viral hepatitis-related HCC³². Similarly, our data indicated the high prevalence of *CTNNB1* mutations but did not confirm the hepatitis status correlation, most likely because of the relatively small sample size of patients with available risk factors for analysis³³⁻³⁸.

One of the major strengths of our study is the large number of patients representing numerous US institutions driven by samples submitted prospectively to aid in therapeutic decision making using a CLIA-certified assay approved for clinical practice. Moreover,

almost half of the patients had risk factors. Another major advantage to our study is the critical importance to assessing ctDNA in HCC patients, where, obtaining initial and repeat tissue sampling may be challenging as previously mentioned. However, the study has some limitations. First, firm conclusions of the correlation with risk factors were not possible given the small number of patients with available risk factors from five institutions. However, the pattern of the alterations was similar to the overall cohort. Second, the cfDNA panel is not as comprehensive as some tissue molecular profiling panels. However, the panel used is clinically driven in that it is designed to identify somatic alterations in 73 genes, primarily focused on genomic alterations that are associated with 1) FDA-approved targeted therapies, 2) targeted therapies in late stage clinical trials, 3) known predictive or prognostic value, or 3) informative of the presence of cfDNA. It is also important to note that the Guardant360 assay reports out only somatic alterations. Mutant allele fractions (MAF) are reported quantitatively for somatic single nucleotide variants (SNVs) of clinical significance and distinguished from heterozygous and homozygous germline variants by their low concentrations, which are filtered out by the Guardant360 molecular tumor board³⁹. Another study limitation was that there was no direct comparison with tumor tissue, in large part because of the difficulty in doing biopsies in HCC; therefore, our work reflects current clinical practice, in which the imaging diagnosis of HCC in the appropriate setting without tissue diagnosis is widely adopted in both academic and community centers. However, surgical resection may be a source of tissue in patients - this procedure is more frequent in South East Asia where HBV patients with HCC may not have cirrhosis. Finally, we acknowledge the definition of actionability is dynamic and institution-dependent, and data on enrollment on targeted trials based on cfDNA results was not available. Prospective studies using ctDNA profiling to guide therapy decisions are warranted.

Notwithstanding the fact that further validation with solid tumor testing results is necessary, we conclude that cfDNA testing is a valid methodology for HCC molecular profiling given the abundance of ctDNA observed in this, the largest collection of US-based blood samples. While this direct comparison was not possible within this patient population, as few patients had solid tumor testing results available, comparison to the findings reported recently by the Cancer Genome Atlas initiative⁴⁰ and other prior studies^{34, 41}, are greatly encouraging, considering the limitations of the current sample population and the limited comprehensiveness of the cfDNA gene panel. In both the TCGA results and this study, the most commonly altered genes were *TP53* and *CTNNB1*, while amplifications were most commonly observed in *MET* and *CCND1*. Moreover, the interesting molecular alteration findings related to specific HCC risk factors, if validated, could also pave the way for developing a molecularly driven HCC classification, and could aid in stratification for clinical trials. Indeed, genomically driven trials, both ongoing and completed, have begun to allow enrollment based on cfDNA test results. In HCC specifically, Ikeda *et al* recently published their findings on the clinical utility of cfDNA testing in HCC, reporting 79% of tested patients having at least one clinically actionable alteration identified via cfDNA testing, with two patients showing good clinical response to matched targeted therapies⁴². Nonetheless, future validation studies with larger numbers of patients with available patient characteristic data are essential for directing HCC translational efforts. Such data is expected to carry critical importance in using mutational signatures for prognostication and for

predicting response to targeted therapies. In addition, blood-derived ctDNA analysis is amenable to serial assessment while on therapy in order to investigate acquired mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflicts of Interest:

R.K. receives consultant fees from Actuate Therapeutics, XBiotech and Loxo, Speaker fees from Roche, has an ownership interest in CureMatch, and receives research funds from Genentech, Pfizer, Merck Serono, Sequenom, Guardant, Foundation Medicine, and Incyte.

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R.B.L is Chief Medical Officer at, and has an ownership interest (including patents) in Guardant Health, Inc.

K.C.B has an ownership interest (including patents) in Guardant Health.

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Abbreviations:

cfDNA	Cell-free DNA
HCC	hepatocellular carcinoma
TACE	transarterial chemoembolization
MDACC	MD Anderson Cancer Center
UCSD	University of California San Diego
UCSF	University of California San Francisco
NASH	non-alcoholic steatohepatitis
CLIP	Cancer of the Liver Italian Program
BCLC	Barcelona Clinic Liver Cancer

TNM	Tumour-Node-Metastasis
CLIA	Clinical Laboratory Improvement Act
VAF	variant allele fraction
SNV	single nucleotide variation
PODS	Personalized Cancer Therapy-Precision Oncology Decision Support
AF	allele frequency
LI-RADS	Liver Imaging Reporting and Data System
VUS	variants of unknown functional significance
CPS	Child-Pugh Score

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STATEMENT OF RELEVANCE:

Molecular subgrouping of hepatocellular carcinoma (HCC) is challenging due to lack of tissue sampling, and profiling the HCC mutational landscape by cfDNA may be particularly attractive given the challenge of obtaining liver biopsies. We report the largest USA cohort, using a CLIA-certified assay approved for clinical practice. Our results demonstrate significant trends between alterations and risk factors - metabolic syndrome and *PDGFRA*, HBV and *ERBB2*, HCV and *BRCA1*. In addition, we observed a high percentage of potentially actionable genomic alterations, suggesting clinical utility for this technology in HCC.

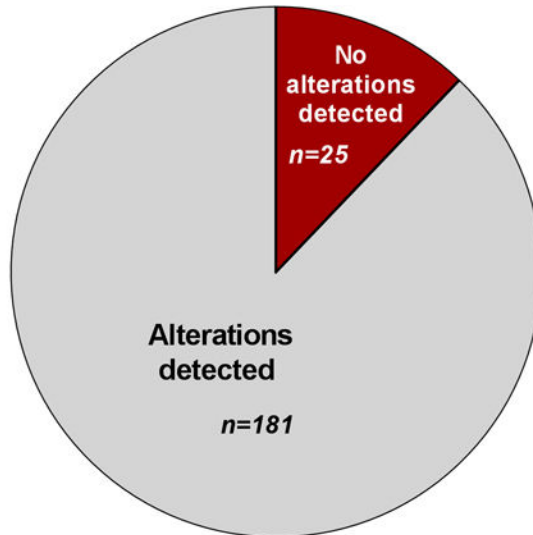
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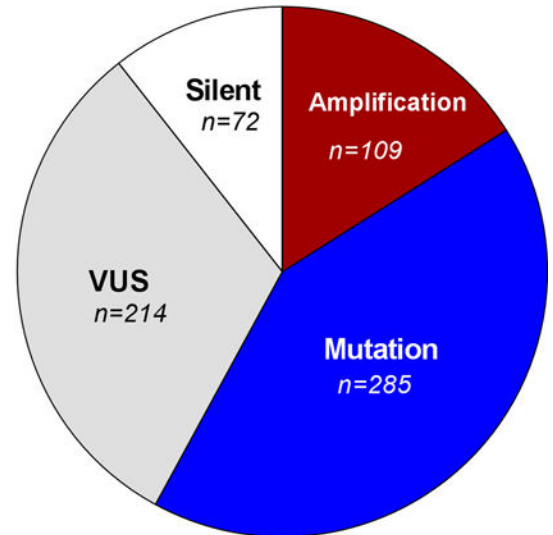
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1a.



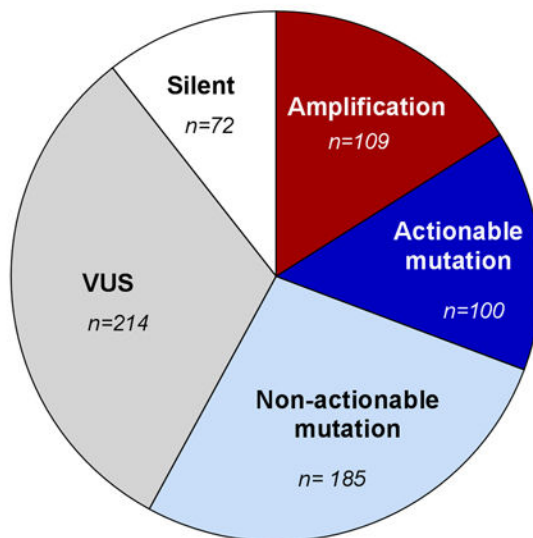
Total HCC patients, n = 206

1b.



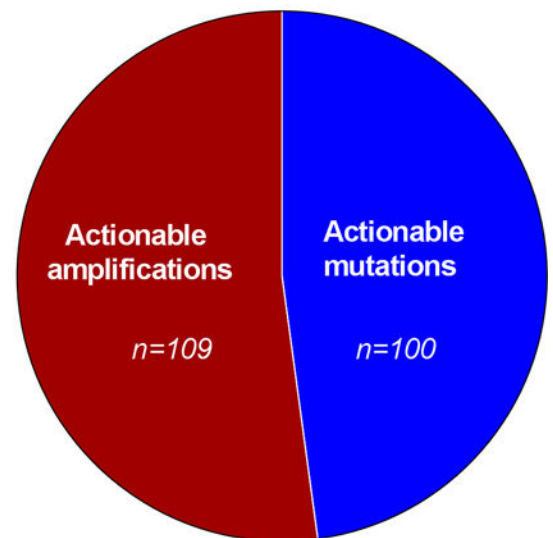
Total alterations, n = 680

1c.



Total alterations, n = 680

1d.



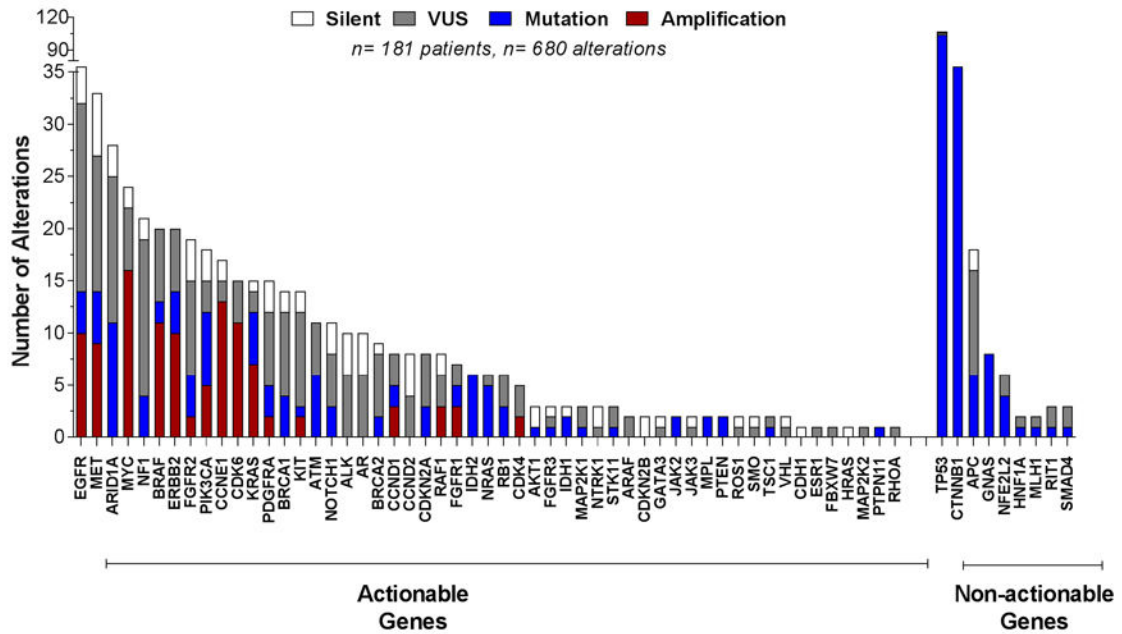
Total alterations, n = 209

Figure 1:

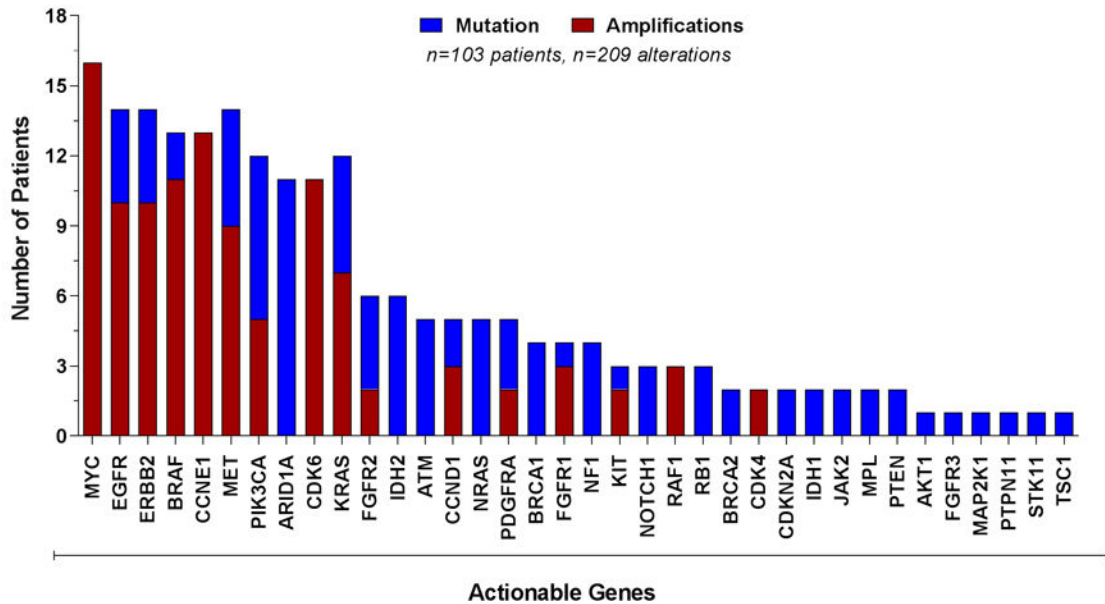
Overview of Findings

- Number of HCC confirmed cases with alterations detected through cfDNA.
- Total number of alterations identified among the 181 patients with alterations, and the proportion of alteration types identified.
- Action ability among mutations identified.
- Total number of actionable alterations, after removing silent, VUS, and non-actionable mutations.

2a.



2b.



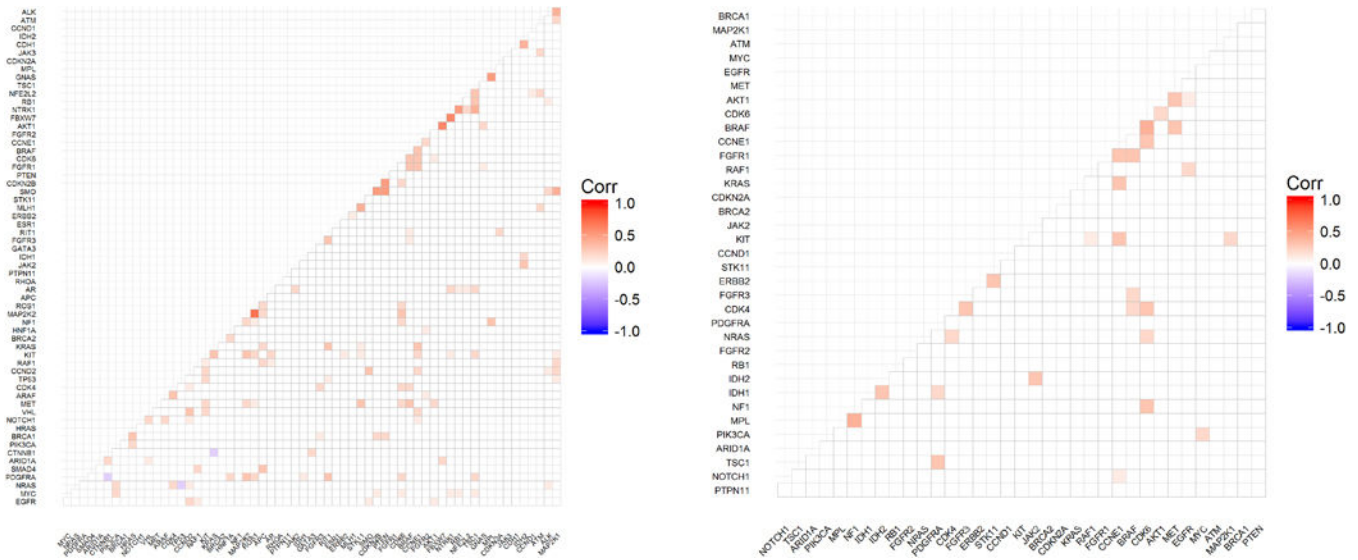


Figure 2:

Most frequently mutated genes identified in cfDNA among HCC patients.

- a) Distribution of all alterations identified, actionable and non-actionable genes.
- b) Proportion of patients with alterations in actionable genes only.
- c) Gene correlation matrix among all patients with alterations detected. All patients with alterations detected, irrespective of the actionability of the alteration (i.e. variant in non-actionable genes, VUS, and/or synonymous) were considered for this matrix. In all from 181 patients and 62 genes were evaluated with Spearman’s rank correlation and displayed in the correlation matrix above. Non-significant correlations are marked with “blank” in the graph. Values < 0 indicate no agreement, 0 – 0.20 slight agreement, 0.21 – 0.40 fair agreement, 0.41 – 0.60 as moderate agreement, 0.61 – 0.80 as substantial agreement, and 0.81 – 1 as almost perfect agreement.
- d) Gene correlation matrix among HCC patients with alterations in actionable genes (synonymous and VUS alteration excluded). Exclusion of non-actionable genes, VUS, and synonymous alterations reduced the patient population size considered for this matrix from 181 to 103 patients and from 62 genes to only 36 actionable genes. The correlation between mutated genes was evaluated with Spearman’s rank correlation and displayed in the correlation matrix above. Non-significant correlations are marked with “blank” in the graph. Values < 0 indicate no agreement, 0 – 0.20 slight agreement, 0.21 – 0.40 fair agreement, 0.41 – 0.60 as moderate agreement, 0.61 – 0.80 as substantial agreement, and 0.81 – 1 as almost perfect agreement.

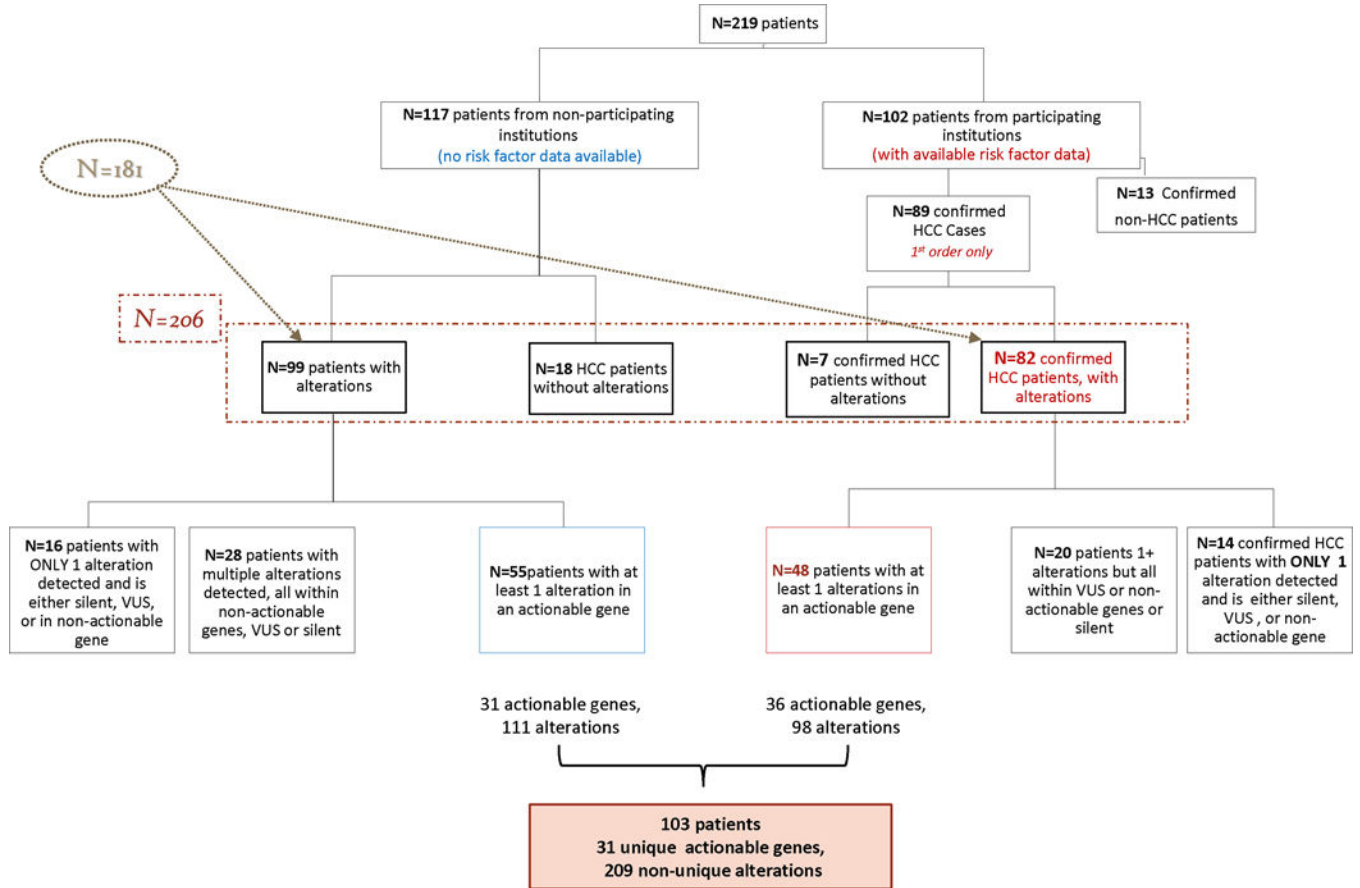


Figure 3: Stratification of patients for analysis over a period of approximately 2 years. 219 patients from various institutions underwent biopsy-free, next generation sequencing. We collected additional risk factor information for 102 patients, irrespective of alterations detected or not. Two correlation matrices were generated, independent of risk factor information. The first considered all patients, and all alteration types (Fig 3a, n=181 patients), and the second considered only patients with at least 1 alteration in an actionable gene and excluded VUS and synonymous alterations (Fig 3b, n=103 patients)

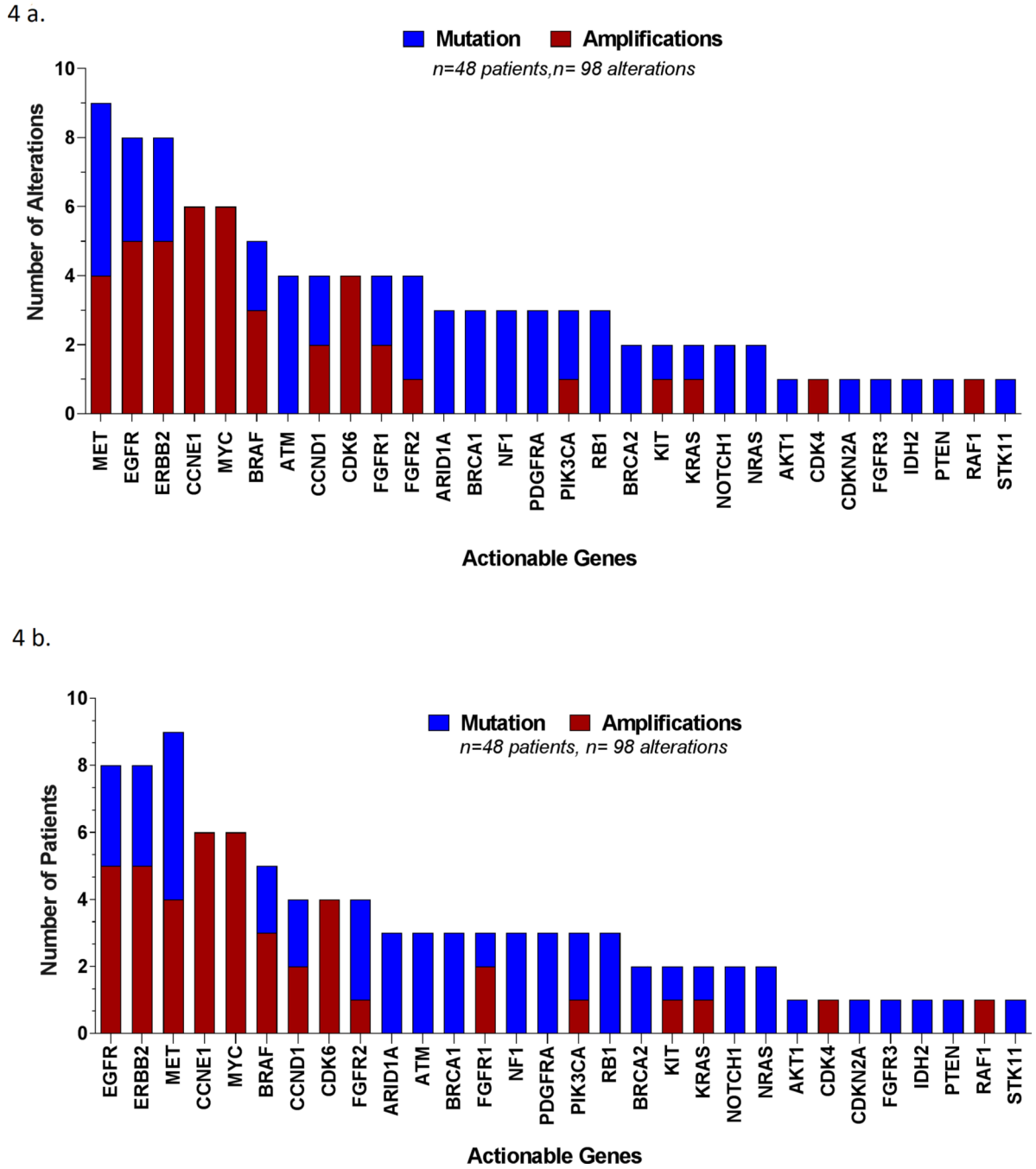


Figure 4:
Representative molecular landscape among patients with available risk factor data.
a) Distribution of all alterations identified, actionable genes only, by alteration.
b) Distribution of all alterations identified, actionable genes only, by patient.

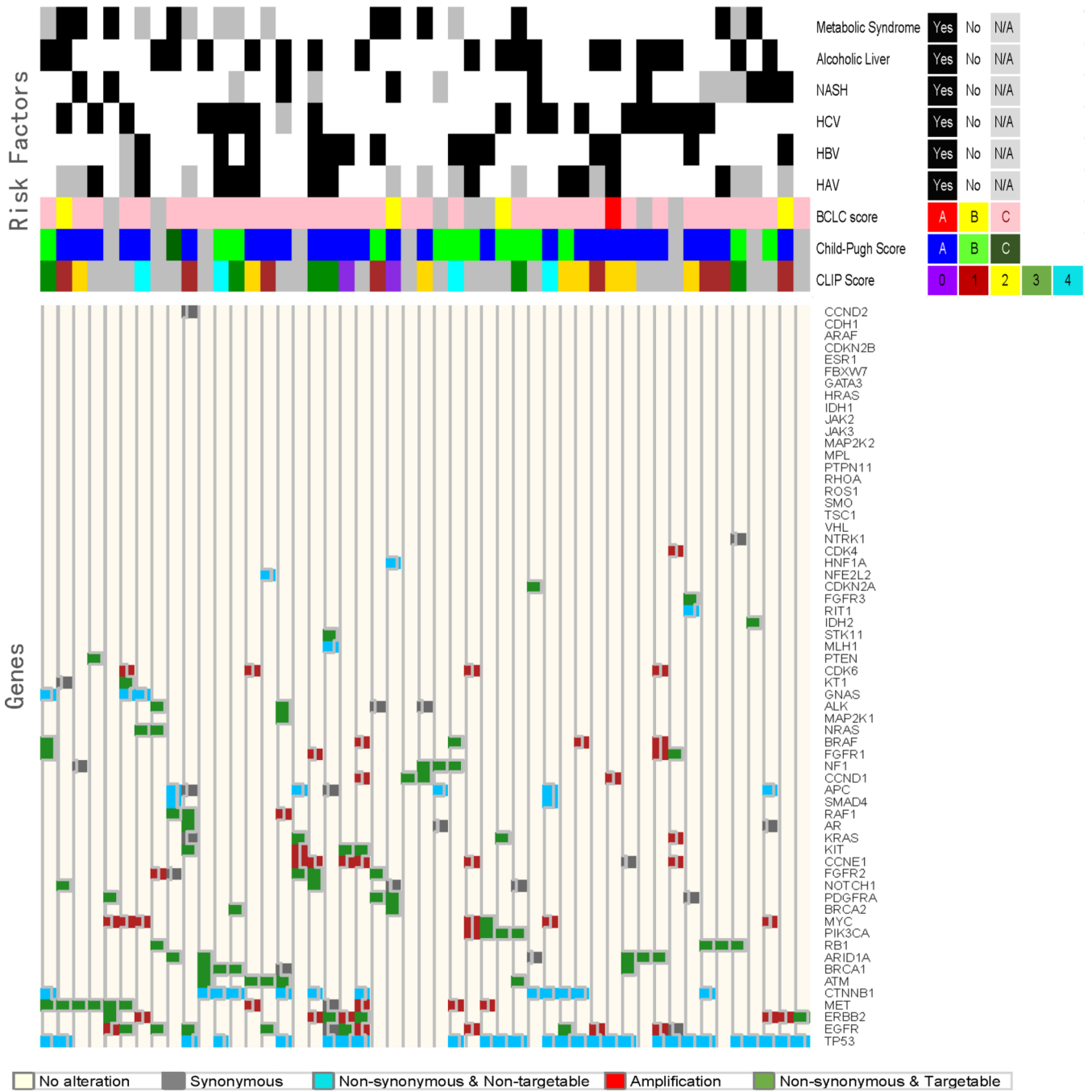


Figure 5: Correlation between molecular landscape and risk factors contributing to HCC. Heat map dendrogram was generated to explore and visualize the gene mutation/amplification relationship along with the corresponding various risk factors. The relationship between gene types with regard to mutation and amplification, synonymous and targetable status was evaluated with Spearman’s rank correlation and displayed in the correlation matrix in which the non-significant correlations are marked with “blank” in the graph.

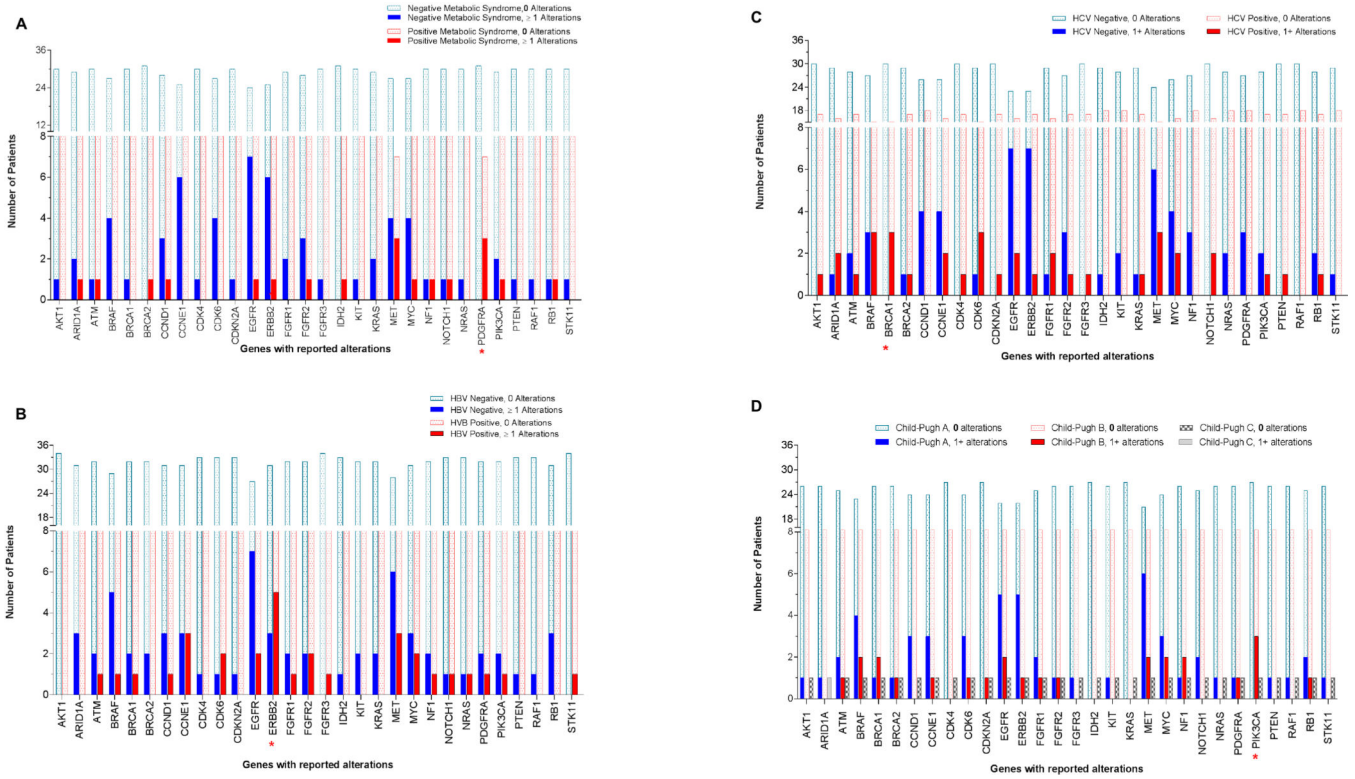


Figure 6: Correlation between molecular landscape and metabolic syndrome and/or history of hepatitis.

a) Correlation with metabolic syndrome: A significant number of patients with metabolic syndrome, 30% (3/10) had alterations detected in PDGFRA (p=0.0113), while patients without metabolic syndrome (n=30) had no alterations in this gene.

b) Correlation with hepatitis B status: A significant number of patients with hepatitis B 35.7% (5/9) had alterations in ERBB2 (p=0.0365), while HBV-negative patients had none.

c) Correlation with hepatitis C status: 16.7% (3/18) of patients with hepatitis C (HCV) had alterations in BRCA1 (p=0.0472), while HCV-negative patients had none.

d) Correlation with Child-Pugh score: 23.1% (3/13) of patients with Child-Pugh score B had alterations in PIK3CA (p=0.0341), versus 0% (0/27) Score A or C (0/1) (p=0.03).

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