Mutations in circulating cell-free tumour DNA: Predictors of survival in hepatocellular carcinoma

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Abstract

Background: Hepatocellular carcinoma (HCC) incidence is increasing worldwide and prognostic biomarkers are urgently needed to guide treatment and reduce mortality. Circulating cell-free DNA of tumour origin (ctDNA) is a novel, minimally invasive means of determining genetic alterations in cancer. We determined the utility of ctDNA as a prognostic biomarker of survival in HCC.

Methods: Plasma cell-free DNA and matched germline DNA were isolated from patients with HCC (n=51) and cirrhosis (n=10). Targeted, multiplex PCR ultra-deep sequencing was performed using a liver cancer-specific primer panel for genes ALB, AMPH, APC, ARID1A, ARID2, ATM, AXIN1, BAZ2B, BRAF, CSMD3, CTNNB1, DSE, ERBB2, HNF1A, IGF2R, IGFBP3, KEAP1, MET, TP53, UBR3, USP25, ZIC3 and ZNF226. Associations between mutations in ctDNA and overall survival were analysed using Cox proportional hazards modelling.

Results: 114 putative mutations (70 unique) in were detected in plasma ctDNA in 35 of 51 patients with HCC (69%). On univariable analysis, CSMD3 gene mutations were associated with shorter overall survival (Logrank HR 3.18, 95% CI 1.14-8.86, P = .027). The median survival time was 15.5 months (IQR 7.77-16.5 months) in patients with CSMD3 mutations compared with the median survival of 26.5 months (IQR 16.93-46.07 months) in patients without CSMD3 mutations. Other factors associated with overall survival were advanced BCLC stage (HR 16.52, 95% CI 2.22-122.94, P = .006) and Child-Pugh Class (CPC HR 7.98, 95% CI 2.31-27.61, P = .001). Cox proportional hazards modelling showed mutations in CSMD3 remained a significant independent risk for shorter overall survival in HCC when adjusted for age, BCLC stage and Child-Pugh class (HR 4.91, 95% CI 1.60-15.02, P = .005).

Conclusion: Detection of CSMD3 mutations in plasma ctDNA is associated with reduced overall survival in HCC patients, adjusted for potential confounding factors.

Keywords
cell-free DNA, hepatocellular cancer, survival

[Correction added on August 27, 2021 after first online publication: Prof Shahid A Khan is included as fourth author.]

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the seventh most common cancer and fourth most common cause of cancer-related death worldwide and incidence continues to increase in parallel with the global epidemics of viral hepatitis and non-alcoholic fatty liver disease. Despite important advances in HCC management over the last decade, survival remains poor, contrasting sharply with improvements in mortality for most other cancer types. Prognostic biomarkers are urgently needed to optimise treatment allocation to improve survival, particularly in the era of the rapidly expanding armoury of systemic therapies being approved for advanced stage HCC.

The genetic pathways of HCC carcinogenesis have been well characterised and it is now appreciated that there is considerable genetic tumour heterogeneity, even within the same tumour. This genetic heterogeneity, coupled with low rates of tumour biopsy because of radiological criteria for HCC diagnosis, means identification of prognostic mutations has been a major challenge to the field.

Circulating cell-free DNA (cfDNA) is an exciting potential alternative to tissue biopsy that may overcome the need for tumour biopsy, cfDNA is released through apoptosis and necrosis from both healthy and malignant cells into the blood stream. Tumour-derived cfDNA (ctDNA) can be distinguished from wild-type cfDNA by identification of somatic alterations present in the tumour of interest, but not in matched somatic tissue. ctDNA quantification and the presence of genetic and epigenetic variants within ctDNA are therefore attractive biomarkers in cancer, facilitating dynamic assessment of genetic mutations before, during and after treatment without the need for repeated tumour biopsies.

Several studies have demonstrated the clinical utility of tumour-derived plasma ctDNA for guiding prognosis and treatment decisions in various cancer types, including breast, ovarian and lung cancer. Preliminary studies have also demonstrated the potential utility of cfDNA levels and specific genetic mutations in ctDNA as clinical biomarkers in HCC. For example, Kirk and colleagues demonstrated that the Ser249 TP53 mutation could be detected in ctDNA from West African patients with advanced-stage HCC and this mutation had good discriminative accuracy for distinguishing HBV-related HCC from chronic hepatitis B infection and HBV-related cirrhosis. We have previously shown that mutations in classic carcinogenic pathways described in HCC can be detected in ctDNA in patients at all BCLC stages of the disease and that these mutations can be found in matched HCC tumour tissue. However, to date, there have been few studies investigating the detection of mutations in genes with potential prognostic and therapeutic utility in ctDNA, particularly across all BCLC stages of HCC.

The aim of this study was to determine whether the detection of mutations in well-established carcinogenic driver genes in ctDNA are associated with overall survival in HCC patients.

METHODS

2.1 Study design and participants

Methods for this study have been described elsewhere. In brief, 51 adult patients (over 18 years of age) with confirmed radiological diagnosis of HCC were consecutively recruited from two specialist liver cancer clinics (Imperial College NHS Trust, n = 19 (37.3%), UK; and University of Piemonte Orientale, Italy, n = 32, 62.8%) and 10 adult patients with cirrhosis but no HCC were recruited as controls from a specialist liver clinic (Imperial College NHS Trust, UK). Patients were recruited prior to commencing treatment for HCC; prior treatment was not a preclusion. Controls were screened for HCC with liver ultrasound within six months of recruitment to the study. The study was approved by the local Institutional ethics committees and was conducted in accordance with the 1975 Declaration of Helsinki.

2.2 Sample collection

A 20 mL blood sample was collected from all subjects on the day of recruitment in an EDTA tube and 1 mL aliquots of plasma and buffy coat were stored in a minus 80°C freezer until further use (Supplementary Methods). Plasma samples from the UK were collected within 12 months of ctDNA analysis, whereas samples from Italy were archival (Biobank), collected more than 12 months prior to ctDNA analysis.

Demographical data (including age, gender and ethnicity), clinical data (including aetiology of underlying liver disease, presence of cirrhosis determined by Fibroscan™ or liver biopsy, Child-Pugh score [CTP], liver function tests) and HCC stage (including BCLC stage, alpha-fetoprotein [AFP] level, presence of portal venous thrombosis and metastases) were also collected on the same day as the blood sample. Survival outcomes were prospectively collected after recruitment to the study and recorded prior to analysis of cfDNA samples.
2.3 DNA extraction from FFPE tissues, PBMC and plasma specimens

cfDNA was extracted from plasma using the QIAsymphony platform (Qiagen Inc) and QIAsymphony Circulating DNA Kit according to the manufacturer’s recommendations. DNA from PBMC samples was extracted on a Maxwell platform (Promega GmbH) using the Maxwell DNA Blood and the Maxwell DNA FFPE Plus Tissue purification kit, respectively, according to the manufacturer’s instructions.

2.4 DNA quantification, library preparation and next-generation sequencing (NGS)

Quality control was performed and PCR amplification of a 173 bp region of the HFE gene was used to quantify amplifiable DNA in all samples. Real-time PCR was performed in triplicate (Supplementary Methods). Primer sets of the Human Liver Cancer GeneRead DNAseq-targeted panel V2 from QiAgen were used for multiplex PCR-based target enrichment, including primer sets for 2052 amplicons targeting 191 KB (up to 99% coverage) in 33 liver-specific genes (Supplementary Methods). Multiplex PCR, using the GeneRead DNAseq Panel PCR Kit, was performed and the amplicons of each sample were pooled, purified and analysed by capillary electrophoresis using the Fragment Analyser (Advanced Analytical). After additional purification and size selection steps, targeted DNA was PCR enriched and library pools prepared using GeneRead DNA Library I Amp Kit (Qiagen), prepared according to the MiSeq System User Guide (Illumina, Inc). NGS sequencing was performed on a MiSeq instrument (Illumina, Inc) using v2 chemistry as recommended by the manufacturer (Supplementary Methods).

2.5 Data analysis

Analysis of matched plasma ctDNA and HCC DNA sequences was performed using Mutect v.1 (Broad Institute) using matched germline PBMC DNA as a reference, as outlined previously. Genetic variants were identified after filtering with additional quality control steps including removal of all variants with low read coverage and samples with low GC quality (outlined in Supplementary Methods, Figure S1). Non-coding variants were excluded to focus on variants of potential clinical significance. Mutations identified in ctDNA, but not in matched germline DNA were determined. We then applied additional filters to remove variants in order to maximise specificity for clinically significant variants in ctDNA. Non-coding genetic variants, variants that were also identified in cirrhotic controls, and recognised variants listed in human SNP databases (dbSNP, 1000 Genomes) were excluded from analysis, unless these variants were also listed in the COSMIC cancer mutation database (see Supplemental Methods and Figure S1). Finally, we excluded variants identified in the cirrhosis controls.

2.6 Statistical analysis

Clinical variables were described using mean ± standard deviation or median (IQR) as appropriate to distribution. Associations between categorical variables were assessed by chi-square test. Associations with overall survival were assessed using Cox proportional hazards modelling, including all variables with $P < .05$ in association with overall survival. We included genetic markers associated with survival on univariable analysis ($P$ value $.05$), adjusted for BCLC stage, Child-Pugh class and age (known determinants of survival in HCC). Treatment was not included in the model because (a) numbers for individual treatment modalities were small, (b) treatment modality was not found to be independently associated with survival when adjusted for BCLC stage and (c) sample size precluded more than five parameters in the model. Proportional hazards assumption was tested using log-log plots and Schoenfeld residuals. The significance threshold was a two-sided $P$-value of $<.05$. All statistical analyses were performed using STATA version 12.1 (Statacorp).

| Clinical variable | Number (%)
|-------------------|----------------|
| Gender (n = 51)   | Male 45 (88.2%) Female 6 (11.8%) |
| Mean age (years ± SD) (n = 52) | 70.3 ± 9.3 years |
| Cirrhosis (n = 50) | 46 (92.0%) |
| Hepatitis B (n = 51) | 6 (12.0%) |
| Hepatitis C (n = 51) | 19 (38.0%) |
| Alcohol liver disease (n = 50) | 21 (42.0%) |
| Median number of nodules (n = 51) | 2 (IQR 1-5) |
| Median size largest nodule (cm; n = 51) | 3.5 cm (IQR 2.7-5.5 cm) |
| Portal vein thrombosis (n = 51) | 7 (14.0%) |
| Extra-hepatic metastases (n = 51) | 6 (12.0%) |
| Median AFP (n = 50) | 19.1 (IQR 4.0-140.2) |
| BCLC (n = 51) | A 20 (39.2%) B 11 (21.6%) C 16 (31.4%) D 4 (7.8%) |
| CTP Class (n = 50) | A 31 (62.0%) B 15 (30.0%) C 4 (8.0%) |

Abbreviations: AFP, alpha-foetoprotein; BCLC, Barcelona Clinic Liver Cancer score; CTP, Child Turcotte Pugh.
3 | RESULTS

Matched plasma and PBMC samples were collected simultaneously from 51 HCC patients and 10 cirrhotic controls. The mean age of HCC patients was 70 years and the majority were male (88%) with cirrhosis (92%) and CTP class A stage liver function (62%). Fifty percent (n = 25) had hepatitis B or C. A significant proportion of patients had early-stage HCC (39% BCLC stage A, Table 1).

3.1 | Mutations in key drivers of hepatic carcinogenesis can be identified in plasma ctDNA from HCC patients

Plasma ctDNA samples from HCC patients (n = 51) were analysed with a median read depth of 423 (IQR 183-889; n = 51) and a median mutant allelic depth of 318 (IQR 147-576). The median mutant allelic frequency was 0.07 (IQR 0.05-0.13). Putative mutations in ALB, AMPH, APC, ARID1A, ARID2, ATM, AXIN1, BAZ2B, BRAF, CSMD3, CTNNB1, DSE, ERBB2, HNF1A, IGF2R, IGSF10, KEAP1, MET, TP53, UBR3, USP25, ZIC3 or ZNF226 were detected in plasma ctDNA in 35 of 51 patients with HCC (69%). The median number of gene mutations was 1 (IQR 0-3) per patient. Across the whole cohort of patients with HCC, analysis of matched plasma ctDNA and germline DNA identified 114 mutations, 70 of which were unique (Table 2). 25 of the 70 unique mutations identified (36%) were listed in the COSMIC cancer-related mutation database, 13/70 (19%) were listed in both COSMIC and dbSNP databases and 45/70 (64%) were novel mutations. Most genetic variants identified in ctDNA from patients with HCC were missense SNP mutations (60/70, 86%); 8 (11%) were nonsense stop codon SNPs and two were splice site mutations (3%). The most commonly mutated genes in ctDNA from HCC cases were CSMD3 (17/51 patients, 33%), DSE (9/51, 18%), IGF2R (7/51, 14%), ARID1A (5/51, 10%), CTNNB1 (4, 8%), MET (4/51, 8%) and TP53 (4, 8%; Table 2). Correlation between gene mutations identified in ctDNA and matched HCC tissue in the eight patients in whom HCC tissue DNA samples were available have been published previously.15

3.2 | CSMD3 gene mutations detected in ctDNA are prognostic for survival in HCC

We then assessed whether mutations in gene pathways identified in ctDNA were prognostic for survival in HCC, restricting our analysis to genes with mutations present in four or more of the HCC patients (8% or more). During the study period, only one patient was lost to follow-up; the remaining 50 provided 851.2 person-months at risk. The median overall survival was 22.7 months (IQR 14.7, 46.7 months) and there were 22 deaths. The median survival by BCLC stage was 22.9 months (IQR 19.6, 46.1 months) for stage B, 15.1 months (IQR 6.2, 26.5 months) for stage C and 7.8 months (IQR 3.5, 8.3 months) for BCLC stage C HCC; all patients with BCLC stage A disease were still alive at the end of the study.

On univariable analysis, the presence of CSMD3 gene mutations was associated with shorter overall survival (Logrank HR 3.18, 95% CI 1.14-8.86, P = .027; Table 3 and Figure 1). The median survival time was 15.5 months (IQR 7.77-16.5 months) in patients with CSMD3 mutations compared with a median survival of 26.5 months (IQR 16.93-46.07 months) in patients without CSMD3 mutations. Other factors associated with overall survival were advanced BCLC stage (HR 16.52, 95% CI 2.22-122.94, P = .006) and Child-Pugh Class (CPC HR 7.98, 95% CI 2.31-27.61, P = .001; Table 3). Importantly, Cox proportional hazards modelling showed mutations in CSMD3 remained a significant independent risk for shorter overall survival in HCC when adjusted for age, BCLC stage and Child-Pugh class (HR 4.91, 95% CI 1.60-15.02, P = .005; test for proportional hazards assumption 0.748; Table 3). CSMD3 mutations were seen in 17 patients with cirrhosis; 4 (24%) because of hepatitis B, 7 (41%) because of hepatitis C and 7 (41%) because of alcohol-related liver disease.

Interestingly, we noted that the number of mutations were significantly higher in HCC patients with detectable CSMD3 mutations in ctDNA (mean 3.8 mutations ± 1.87 vs 1.4 ± 2.58, P < .0001). There was also a positive association between the presence of CSMD3 mutations and having two or more genes affected by mutations in ctDNA (Chi-square P < .012). The overall concentration of circulating cfDNA in plasma was also higher in patients with CSMD3 mutations (mean concentration 86.8 pg/mL ± SD 70 pg/mL) compared with those without CSMD3 mutations (mean concentration cfDNA 52.2 pg/mL ± 61.6 pg/mL, P = .03). Mutation number was not significantly associated with survival.

4 | DISCUSSION

Plasma ctDNA has the exciting potential to provide real-time information about HCC biology to guide treatment choice and optimise responses to improve survival outcomes in HCC, which otherwise would not be possible without repeated tumour biopsies.10 While the clinical utility of ctDNA for prognosis, early detection of tumour recurrence and detection of mutations affecting drug efficacy is well described in other cancers,16-18 the clinical experience with ctDNA mutation detection in HCC has been limited.15 Building on our previous work,15 in this pilot study we have identified putative gene mutations in ctDNA from HCC patients with early and advanced stage HCC comparing to PBMC germline DNA without the need for tumour DNA. We also show for the first time that CSMD3 mutations are associated with shorter overall survival in patients with HCC, independent of age, liver disease stage and HCC stage.

In contrast to other tumour types, HCC often remains a well-differentiated tumour and has a low rate of metastasis,6 characteristics that may theoretically reduce ctDNA release into the bloodstream. This may explain the relatively low levels of ctDNA and low number of mutations detected in ctDNA in our study, which has also been reported by others.12,13 The mutations detected were not observed in cirrhotic control samples or matched germline DNA and were mostly putative mutations in known carcinogenic gene
| Gene   | Position | Exon | DNA change | Protein change | Number (% of HCC patients with mutation (n = 51)) | Number (% of HCC patients with gene affected (n = 51)) |
|--------|----------|------|------------|----------------|-------------------------------------------------|--------------------------------------------------|
| ALB    | chr4:74285311 | 11   | c.1290G>T  | E430D          | 1 (2%)                                           | 1 (2%)                                           |
| AMPH   | chr7:38433726 | 18   | c.1487A>C  | K496T          | 1 (2%)                                           | 1 (2%)                                           |
| APC    | chr5:112173992 | 16   | c.2701C>A  | Q901K          | 1 (2%)                                           | 3 (6%)                                           |
| ARID1A | chr1:27087479 | 5    | c.2503A>C  | T685P          | 4 (8%)                                           | 5 (10%)                                          |
| ARID2  | chr12:46230776 | 8    | c.e8->2    |                | 1 (2%)                                           | 2 (4%)                                           |
| ATM    | chr11:108123551 | 13   | c.1810C>T  | P604S          | 1 (2%)                                           | 3 (6%)                                           |
| AXIN1  | chr16:348233  | 6    | c.1273G>A  | G425S          | 1 (2%)                                           | 2 (4%)                                           |
| BAZ2B  | chr12:46230776 | 8    | c.e8->2    |                | 1 (2%)                                           | 2 (4%)                                           |
| BRAF   | chr7:140449185 | 16   | c.1894C>T  | P632S          | 1 (2%)                                           | 3 (6%)                                           |
| CTNNB1 | chr3:41266110 | 3    | c.107A>C   | H36P           | 1 (2%)                                           | 3 (6%)                                           |
| CSMD3  | chr8:113243844 | 16   | c.1075B>A  | D358E          | 1 (2%)                                           | 16 (32%)                                         |
| DSE    | chr6:116720487 | 3    | c.74C>T    | T25I           | 2 (4%)                                           | 9 (18%)                                          |
| DSE    | chr6:116720534 | 3    | c.121G>T   | D41Y           | 1 (2%)                                           |                                                  |
| DSE    | chr6:116720786 | 3    | c.373A>C   | M125L          | 1 (2%)                                           |                                                  |
| DSE    | chr6:116747816 | 4    | c.496T>C   | Y166H          | 1 (2%)                                           |                                                  |
| DSE    | chr6:116747843 | 4    | c.523G>A   | E175K          | 2 (4%)                                           |                                                  |
| DSE    | chr6:116747909 | 4    | c.589T>C   | Q197R          | 2 (4%)                                           |                                                  |
| DSE    | chr6:116756896 | 7    | c.1265T>C  | L422P          | 1 (2%)                                           |                                                  |
| DSE    | chr6:116756905 | 7    | c.1274G>A  | R425H          | 1 (2%)                                           |                                                  |
| DSE    | chr6:116756914 | 7    | c.1283A>G  | Y428C          | 2 (4%)                                           |                                                  |
| DSE    | chr6:116757243 | 7    | c.1612C>T  | R538*          | 2 (4%)                                           |                                                  |
| DSE    | chr6:116757271 | 7    | c.1640C>T  | P547L          | 1 (2%)                                           |                                                  |
| ERBB2  | chr17:37855834 | 1    | c.22C>A    | P8T            | 1 (2%)                                           | 3 (6%)                                           |
| HNF1A  | chr12:121435427| 7    | c.1460G>A  | S487N          | 1 (2%)                                           | 2 (4%)                                           |
| IGF2R  | chr6:160466851 | 14   | c.1840G>A  | A614T          | 1 (2)                                            | 7 (14%)                                          |
| IGFBP3 | chr6:160480061 | 22   | c.3022G>T  | E1008*         | 1 (2%)                                           |                                                  |
| IGFBP3 | chr6:160482952 | 25   | c.3574C>A  | Q1192K         | 5 (10%)                                          |                                                  |
pathways, supporting their clinical relevance. We have previously reported the presence of mutations in ctDNA in several genes that are well described in HCC. In this study, we additionally report mutations in genes that are less common but described in HCC, including KEAP1, MET, AMP and APC, and in several genes that are described in other malignancies but have not frequently been reported in HCC, including CSMD3, IGF2R, BAZ2B and DSE. Whilst putative mutations in these genes have been described in the COSMIC database and in other studies, their role in HCC is less certain. It has been previously reported that the mutational rate in HCC increases with advancing tumour stage. Thus, the propensity for mutations to accumulate as checkpoint and DNA repair mechanisms are lost to non-driver mutations also become more common at mutation ‘hotspots’ that may still serve as useful biomarkers in HCC. 

In this study, we additionally report mutations in genes that are less common but described in HCC, including CSMD3, IGF2R, BAZ2B and DSE. Whilst putative mutations in these genes have been described in the COSMIC database and in other studies, their role in HCC is less certain. It has been previously reported that the mutational rate in HCC increases with advancing tumour stage. Thus, the propensity for mutations to accumulate as checkpoint and DNA repair mechanisms are lost to non-driver mutations also become more common at mutation ‘hotspots’ that may still serve as useful biomarkers in HCC.

It is important that the biological significance of CSMD3 in HCC is determined in future studies and further work exploring whether these mutations are key drivers of HCC progression or mere bystanders reflecting loss of the usual DNA replication checkpoint genes must be undertaken before conclusions regarding the role of CSMD3 in HCC can be made. Interestingly, all HCC patients with a mutation at position chr11:113529446 also had a mutation at chr11:113529449, suggesting these mutations are linked. However, these mutations occurred independently of other mutations in CSMD3 or other genes. Importantly, none of the CSMD3 mutations were recorded in know SNP databases and were not present in cirrhotic controls, further evidence that these mutations were true mutations in HCC patients. Moreover, all gene sequencing was performed in triplicate and our sequencing pipeline (Figure S1) was deliberately conservative to reduce sequencing error.

Only a few previous studies have identified CSMD3 mutations in cancer, including melanoma, lung, thyroid, prostate and gastric cancer. The first study utilised an cDNA microarray-based comparative genomic hybridisation technique to identify chromosomal gains and upregulation of the gene. More recently, Gylfe et al identified non-synonymous variation in CSMD3 in a cohort of patients with familial colorectal cancer. Huang et al also reported non-synonymous

### Table 2

| Gene   | Position | Exon | DNA change | Protein change | Number (%) of HCC patients with mutation (n = 51) | Number (%) HCC patients with gene affected (n = 51) |
|--------|----------|------|------------|----------------|---------------------------------------------------|---------------------------------------------------|
| chr6:160500621 | 38 | c.5488G>T | D1830Y | 1 (2%) | |
| chr6:160525736 | 48 | c.7096G>T | E2366* | 1 (2%) | |
| chr3:151155571 | 6 | c.6778G>T | D2260Y | 1 (2%) | 3 (6%) |
| chr3:151164746 | 4 | c.3203G>T | R1008I | 1 (2%) | |
| chr3:151166912 | 4 | c.857C>T | S286L | 1 (2%) | |
| chr1:1171439 | 3 | c.448T>G | Y150D | 1 (2%) | |
| chr1:7577098 | 8 | c.840A>T | R280S | 1 (2%) | |
| KEAP1 | chr19:10597401 | 6 | c.1802G>T | R601L | 1 (2%) | 2 (4%) |
| chr19:10602359 | 3 | c.1219G>A | A407T | 1 (2%) | |
| MET | chr7:116395482 | 6 | c.1775G>A | R592K | 1 (2%) | 4 (8%) |
| chr7:116403158 | 11 | c.2473C>T | Q825* | 2 (4%) | |
| chr17:7577098 | 8 | c.840A>T | R280S | 1 (2%) | 4 (8%) |
| UBR3 | chr2:170799128 | 20 | c.2789T>C | F930S | 2 (4%) | 2 (4%) |
| USP25 | chr21:17205690 | 17 | c.2017C>G | Q673E | 1 (2%) | 1 (2%) |
| TP53 | chr17:7577539 | 7 | c.742C>G | R248G | 1 (2%) | |
| chr17:7577543 | 7 | c.738G>T | M246L | 1 (2%) | |
| chr17:7577565 | 7 | c.716A>G | N239S | 1 (2%) | |
| chr17:7578257 | 6 | c.592G>T | E198* | 1 (2%) | |
| chr17:7578433 | 5 | c.497C>A | S166* | 1 (2%) | |
| ZIC3 | chrX:136649170 | 1 | c.320C>T | A107V | 1 (2%) | 2 (4%) |
| chrX:136649823 | 1 | c.93G>T | E325* | 1 (2%) | |
| ZNF226 | chr19:44680524 | 7 | c.1109G>A | R370K | 4 (8%) | 5 (10%) |
| chr19:44680527 | 7 | c.1112C>T | A371V | 3 (6%) | |
| chr19:44680907 | 7 | c.1492A>G | T498A | 1 (2%) | |

*Stop codon.
TABLE 3 Association between gene mutations identified in ctDNA in at least three or more HCC patients and overall survival

| Clinical factor                  | HR  | 95% CI      | P-value |
|----------------------------------|-----|-------------|---------|
| **Univariable analysis (Log-rank)** |     |             |         |
| APC                              | 0.39| 0.05-2.93   | .360    |
| ARID1A mutation                  | 1.04| 0.24-4.48   | .962    |
| CSM3D mutation                   | 3.18| 1.14-8.86   | .027    |
| CTNNB1 mutation                  | 1.46| 0.19-11.51  | .718    |
| DSE                              | 1.00| 0.33-2.99   | .998    |
| IGFR2                            | 1.99| 0.66-6.02   | .220    |
| MET                              | 0.57| 0.08-4.26   | .584    |
| TP53 mutation                    | 2.71| 0.79-9.32   | .114    |
| ZNF226                           | 0.40| 0.05-2.97   | .368    |
| Two or more gene mutations       | 1.72| 0.80-3.72   | .163    |
| Advanced BCLC stage (B/C/D)      | 16.52| 2.22-122.94 | .006    |
| Age                              | 1.00| 0.96-1.05   | .906    |
| Child-Pugh class                 |     |             |         |
| A                                | 1.00| N/A         | N/A     |
| B                                | 1.89| 0.75-4.78   | .180    |
| C                                | 7.98| 2.31-27.61  | .001    |
| **Cox proportional hazards analysis** |     |             |         |
| CSM3D                            | 5.66| 1.79-17.87  | .003    |
| Advanced BCLC stage (B/C/D)      | 22.29| 2.59-192.02| .005    |
| Child-Pugh class                 |     |             |         |
| B                                | 0.80| 0.24-1.94   | .479    |
| C                                | 4.27| 1.14-18.38  | .032    |
| Age                              | 0.98| 0.92-1.03   | .341    |

*A Model adjusted for CTP class, Age and BCLC stage and proportional hazards assumption assessed graphically using log-log plots and using test for proportional hazards assumption. Bold values indicate P < .05.

mutations in hepatitis B-related HCC in a small number of patients. To our knowledge, this is the first study to identify variants in CSM3D in patients with HCC that were associated with survival. There have been several papers reporting whole-genome sequencing data for HCC, none of which identified CSM3D as a driver mutation in HCC.2,6,7,28,29 This may be because liver tissue sequencing studies have traditionally focussed on HCC tissue specimens, not adjacent liver tissue specimens. Circulating cell-free DNA is an admixture from both cancer and adjacent liver cells and it is feasible that CSM3D mutations may be present in adjacent non-cancerous liver cells in patients with HCC, but not in those with cirrhosis and no HCC. This requires further exploration via sequencing of gene mutations present in adjacent liver cells without malignant transformation in HCC.

CSMD3 and the other genes in the targeted gene panel used in this study were analysed because they have a proven putative role in other malignancies,22-25 and non-silent mutations have been reported in HCC.27,30,31 Interestingly, of the five unique CSM3D mutations detected in our study, four of those mutations were very frequent, with one of those mutations present in plasma ctDNA in 30% of all HCC cases (Table 2), making CSM3D more likely to be useful clinically given the relatively high frequency of mutation detection in HCC cases. TERT promotor mutations, one of the most common genetic alterations found in HCC,7 were not assessed in this study and would likely add greater sensitivity to HCC mutation detection in ctDNA in future studies.21,22

There are several limitations in this pilot study of ctDNA prognosis in HCC. Matched HCC tissue DNA was only available for a small subset of patients. However, we have previously validated the technique of using PBMCs and matched germline DNA against HCC tissue DNA.15 Our small sample size and the considerable heterogeneity of the sample-limited study power to detect associations between mutations in gene pathways and survival in HCC and this is reflected in the large error margins for the hazard ratios in our multivariable model (Table 3); our findings are therefore hypothesis generating and not generalisable to other HCC populations. Adequately powered, larger studies should be undertaken prospectively to evaluate the prognostic value of plasma ctDNA in HCC. Our patient cohort had survival in the lower end of the expected range reported in the literature, which likely reflects the comorbidities and socioeconomic circumstances of patients with HCC in our health service. The role of cfDNA in the stratification of patient therapy or as a marker of drug resistance has not been addressed in this study and these remain important questions for future studies. Nonetheless, this exploratory study provides important data demonstrating a significant association between CSM3D mutations and survival and these mutations were relatively common among the HCC patients in this study, suggesting ctDNA could be used for survival prognosis in the clinic. As sequencing costs reduce over time, it is highly likely that ctDNA will become an exciting, accessible and affordable prognostic biomarker in HCC in the future.
5 | CONCLUSION

Detection of CSMD3 mutations in plasma ctDNA was associated with reduced overall survival in our pilot HCC patient cohort. These preliminary data highlight the exciting potential of ctDNA as a prognostic biomarker in HCC and further studies evaluating its clinical utility in large prospective cohorts of HCC patients are warranted.

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CONFLICT OF INTEREST

The Institute for Pathology (University Hospital of Cologne, Germany) is an official testing institution of Qiagen Inc (Hilden, Germany) and Reinhard Büttner is a member of the advisory board of Qiagen Inc. Furthermore, Qiagen Inc has supported the study by providing reagents for cfDNA extraction and NGS library construction.

AUTHORS’ CONTRIBUTIONS

JH lead the study, contributed to study design, recruited UK study participants, collected UK clinical samples and data, contributed to bioinformatics pipeline development and analysis of genetic sequencing data, performed all statistical analyses and wrote the manuscript. SA performed the bioinformatics, contributed to the analysis of genetic sequencing data and contributed to the manuscript. DP provided all Italian samples and data and contributed to the manuscript. RB and MO contributed to study design and performed DNA extraction, library construction and ultra-deep targeted sequencing from all PBMC, plasma and tissue samples. RM, MEB, ML and MP processed all Italian samples. RS contributed to study design, provided UK samples and clinical data, contributed to the manuscript and co-supervised the project.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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