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ORIGINAL ARTICLE

Applications of molecular barcode sequencing for the detection of low-frequency variants in circulating tumour DNA from hepatocellular carcinoma

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Abstract

Purpose: Liquid biopsy has emerged as a promising tool for minimally invasive and accurate detection of various malignancies. We aimed to apply molecular barcode sequencing to circulating tumour DNA (ctDNA) from liquid biopsies of hepatocellular carcinoma (HCC).

Study Design: Patients with HCC or benign liver disease were enrolled between 2017 and 2018. Matched tissue and serum samples were obtained from these patients. Plasma cell-free DNA was extracted and subjected to targeted sequencing with ultra-high coverage and molecular barcoding.

Results: The study included 143 patients: 102 with HCC, 7 with benign liver tumours and 34 with chronic liver disease. No tier 1/2 or oncogenic mutations were detected in patients with benign liver disease. Among the HCC patients, 49 (48%) had tier 1/2 mutations in at least one gene; detection rates were higher in advanced stages (75%) than in early stages (26%–33%). *TERT* was the most frequently mutated gene (30%), followed by *TP53* (16%), *CTNNB1* (14%), *ARID2* (5%), *ARID1A* (4%), *NFE2L2* (4%), *AXIN1* (3%) and *KRAS* (1%). Survival among patients with *TP53* mutations was significantly worse ($p = 0.007$) than among patients without these mutations, whereas *CTNNB1* and *TERT* mutations did not affect survival. ctDNA testing combined with α-fetoprotein and prothrombin induced by vitamin K absence-II analyses improved HCC detection, even in early stages.

Conclusions: ctDNA detection using molecular barcoding technology offers dynamic and personalized information concerning tumour biology, such information can guide clinical diagnosis and management. This detection also has the potential as a minimally invasive approach for prognostic stratification and post-therapeutic monitoring.

KEYWORDS
circulating tumour DNA, hepatocellular carcinoma, liquid biopsy

Hye Won Lee and Esl Kim equally contributed to this work.

1 | INTRODUCTION

Since 2018, liver cancer has been the sixth most commonly diagnosed cancer and the fourth-leading cause of cancer-related deaths worldwide.[1] The main types of primary liver cancer are hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma. These constitute 75%–85% and 10%–15% of all liver cancer respectively. Chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections, heavy alcohol use, obesity, aflatoxin-contaminated foodstuffs, smoking and type 2 diabetes are the main risk factors for HCC.[1,2] The incidence and prevalence of HCC are both high, and affected patients have a poor prognosis and low rate of survival.[2–4] However, estimated survival is ≥5 years for patients diagnosed during early stages, as defined using the Barcelona Clinic Liver Cancer (BLCL) staging system.[2,5]

Surveillance for early detection of HCC involves liver ultrasonography and serum markers, including α-fetoprotein (AFP) and prothrombin induced by vitamin K absence-II (PIVKA-II).[6–8] However, these serum biomarkers have limitations in terms of sensitivity, specificity and defining the cut-off for clinical decision-making.[9] Tissue biopsy is invasive and has limited utility for monitoring disease progression and therapeutic response.[2] The genetic characterization of cancer cells has become a central component of treatment planning and prognosis prediction. Although they are useful in clinical practice, genetic analyses of tissue biopsy specimens can lead to underestimation of cancer genome complexity because of tumour heterogeneity.[10]

Liquid biopsy has emerged as a promising tool for minimally invasive and accurate detection of various cancers.[11–13] Liquid biopsy markers include exosomes, circulating tumour cells and circulating tumour DNA (ctDNA). ctDNA has emerged as a prominent target in this context, reflecting the rapid development of molecular technologies such as droplet digital polymerase chain reaction (ddPCR), BEAMing and next-generation sequencing (NGS). Cell-free DNA (cfDNA), predominantly derived from apoptotic and necrotic cells, is short (~160 bp) and has a limited half-life (~15 min to 2.5 h). The concentration of cfDNA extracted from blood samples is very low, particularly in patients with early cancers. Thus, sophisticated technologies are required for the detection of cancer mutations in ctDNA.[14–16] Current methods for detecting cancer mutations in ctDNA include NGS, which can target numerous cancer genes with high sequencing coverage.[13] Molecular barcoding, which tags each DNA molecule with oligonucleotide adaptors that contain randomly generated unique molecular identifier (UMI) sequences, allows the removal of polymerase chain reaction (PCR) errors and duplicate reads by determining the origin of each DNA fragment. Consequently, molecular barcoding is effective for the elimination of sequencing errors and enhancement of sensitivity.[13,17,18]

Unlike other cancers, data for ctDNAs in HCCs have been limited to small studies, focused on a few hotspot mutations or DNA methylation[19–21]; multi-gene NGS panels are rarely used.[22] Accordingly, we examined ctDNA from a large HCC cohort using a custom NGS panel, specifically designed for HCC, along with advanced molecular barcoding technology. We also investigated the clinical applicability of liquid biopsy for predicting clinical outcomes in Korean HCC patients.

Lay summary

- Although the prevalence of liver cancer is still high, it is known that the prognosis of patients is good if detected early.
- Liver biopsy is invasive, whereas liquid biopsy is a promising tool for minimally invasive and accurate detection of malignancy.
- This study showed that circulating tumour DNA detection using molecular barcoding technology offers personalized information concerning tumour biology in liver cancer.

2 | METHODS

2.1 | Patients

In total, 143 patients with HCC or benign liver disease at our centre were enrolled between 2017 and 2018. Inclusion criteria were age >19 years, histologically or radiologically confirmed HCC or other liver diseases and consent for tissue or blood sampling. If histological findings were unavailable, compensated cirrhosis was clinically defined as either platelet count <150 000/μl and ultrasonographic findings suggestive of compensated cirrhosis (e.g. a blunted, nodular liver surface accompanied by splenomegaly [>12 cm]) or oesophageal or gastric varices. Exclusion criteria were age ≤18 years, history of HCC diagnosis or treatment or a history of transplantation. HCC diagnosis and treatment were performed in accordance with current guidelines,[23] and matching tissue or serum samples were obtained from all patients. The study protocol was approved by the Severance Hospital Institutional Review Board (IRB No. 4-2015-0184).

2.2 | Samples, reference materials and DNA extraction

Peripheral blood (8 ml) was collected from each patient into a Cell-Free DNA BCT tube (Streck, Inc.). Within 48 h of blood collection, plasma was processed and stored at −80°C. cfDNA was extracted using a NextPrep-Mag cfDNA Isolation Kit (Bio Scientific, PerkinElmer, Inc.). For a comparison of mutations between plasma and matched tissues, 27 fresh tissue samples were collected from patients during surgery or tissue biopsy.

Commercial reference materials with varying mutation allele frequencies (MAFs), including the Multiplex I cfDNA Reference Standard (Horizon Discovery) and the Seraseq ctDNA Mutation Mix v2 (SeraCare Life Sciences, Thermo Fisher Scientific), as well as nine cell lines containing oncogenic mutations were used to validate the limit of detection (LoD) and accuracy of testing procedures (Table S1).

2.3 | Library preparation and target enrichment

The NGS library was constructed using a ThruPLEX Tag-seq 96D Kit (Takara Bio, Inc.), which has adapters that contained UMI sequences. For tissue samples, an NGS library was constructed using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs), in accordance with the manufacturer's instructions. A review of comprehensive genomic studies performed in HCCs[24–29] was used to select key mutated genes, including 200 genes for HCC tissue panel analysis and 11 genes for HCC ctDNA panel analysis (Tables S2 and S3). The ctDNA panel included *ALB*, *APOB*, *ARID1A*, *ARID2*, *AXIN1*, *CTNNB1*, *KRAS*, *NFE2L2*, *PIK3CA* and *TP53* as well as the *TERT* promoter. The custom panels were designed and obtained commercially (Dxome); hybridization capture was performed for each pool of eight libraries using the probes. The *TERT* promoter region, which exhibits high GC content and low sequencing coverage according to hybridization capture, was PCR-amplified. Sequencing was performed on a NextSeq 550Dx instrument using a NextSeq 500/550 High Output Kit v2.5 (2 × 150 bp; Illumina, Inc.).

2.4 | Data processing and analyses

Unique molecular identifier sequences in each sequencing read were extracted from NGS data using our custom algorithm and a modified version of an established protocol.[30] To avoid index hopping errors associated with dual UMIs in the Illumina library system,[31] we designed an additional algorithm for the condensation of hopped indexes (Chi-Seq; Dxome Co., Ltd.), which corrected errors from swapped UMIs (Figure 1). Briefly, hopping groups were constructed based on the assumption of swapped dual UMIs on one side that matched ≥2 different UMIs on the other side within the same genomic region were considered swapped; the swapped UMIs were merged into and regarded as a single group of UMIs. Variants present in all reads within a specific UMI group were considered true positives, whereas variants present in a small proportion of reads were considered false positives that had resulted from PCR errors or sequencing errors. The final construction of the consensus sequence was mapped to the human reference sequence (GRCh37). Annotation was performed using the DxSeq Gene Analysis software (Dxome), based on population and cancer databases, as well as preliminary classification of oncogenicity. The final classification was performed by molecular pathologists in accordance with the standards and guidelines of the Association for Molecular Pathology, American Society of Clinical Oncology and College of American Pathologists.[32] For tier 1 and 2 mutations with allele frequencies of approximately 50%, both in plasma and tissue, the consequences of the mutations were manually reviewed to distinguish germline and somatic mutations.

2.5 | Statistical analyses

Statistical analyses were performed using R Statistical Software (v 4.0; R Foundation for Statistical Computing). Comparisons of

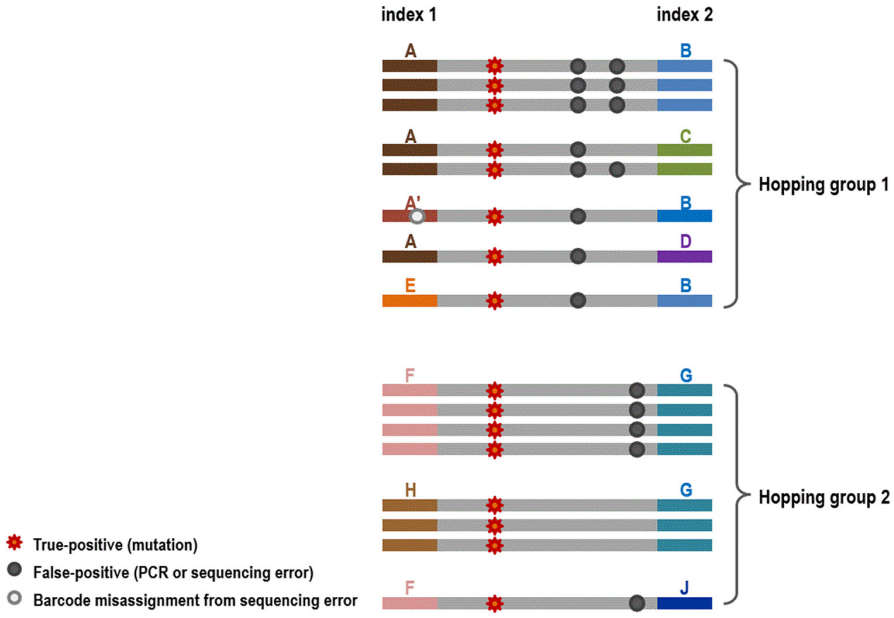

FIGURE 1 Schematic concepts underlying the condensation of hopped indexes (Chi-Seq) algorithm. Hopping groups were constructed based on the assumption of swapped unique molecular indexes, such that unique molecular indexes on one side that matched two different unique molecular indexes on the other side within the same genomic region were considered swapped; the swapped unique molecular indexes were merged into and regarded as a single group of unique molecular indexes. Variants present in all reads within a specific unique molecular index group were considered true positives, whereas variants present in a small proportion of reads were considered false positives that had resulted from PCR errors or sequencing errors.

categorical variables between two groups were performed using the Wilcoxon test, while comparisons of categorical variables among ≥3 groups were performed using the Kruskal–Wallis test. Proportions were compared between two and ≥3 groups using Fisher's exact test and the chi-squared test respectively. Receiver-operating characteristic curve analysis and the DeLong test were performed using the pROC package in R software. A p value of <0.05 was considered statistically significant.

3 | RESULTS

3.1 | Clinical characteristics of the study population

The study included 143 patients (median follow-up period, 19.1 months): 102 had HCC, 7 had a benign liver tumour (BLT) and 34 had chronic liver disease (CLD). The BLT group included two patients with angiomyolipoma, four patients with focal nodular hyperplasia and one patient with hepatic adenoma. The CLD group included 20 patients with autoimmune hepatitis, 9 patients with liver cirrhosis and 4 patients with viral hepatitis. Mean ages at diagnosis in the HCC, BLT and CLD groups were 60, 42 and 64 years respectively. Men were predominant in the HCC group, while there were more women in the CLD group (26/34, 76.5%) (Table 1). When categorized according to aetiology, 71, 6 and 6 HCC patients had a history of HBV-positive hepatitis, HCV-positive hepatitis and heavy alcohol consumption respectively. The remaining 18 patients had negative HBV and HCV serology findings (non-B non-C, NBNC). Cirrhosis was present in 60 (58.8%) patients with HCC and 22 (64.7%) patients with CLD (Table 1).

3.2 | Limit of detection and accuracy

Limit of detection and accuracy were evaluated using commercial reference materials from Horizon and SeraCare that harboured different mutations in various genes including *TP53*, *CTNNB1*, *KRAS* and *PIK3CA*. Custom controls were constructed from cell lines that contained mutations in *CTNNB1*, *KRAS*, *PIK3CA*, *PTEN*, *TERT* and *TP53* (Table S1). In repeat experiments with a mean coverage of 2309× UMIs per region, we were able to detect all mutations with MAFs >0.5% in the Horizon and Seraseq reference materials and in custom controls, except for mutations in the *TERT* promoter, which had low sequencing coverage. However, additional amplicon sequencing of the *TERT* promoter revealed the presence of low-frequency mutations. Reduced detection rates were observed in diluted samples with MAFs <0.5%, indicating that the LoD of the assay was approximately 0.5%.

Excellent linearity was observed between MAFs calculated by our assay and MAFs that were theoretically predicted, based on the dilution ratio (r^2 = 0.997 and 0.920 for the reference materials and custom controls respectively; Figure S1).

3.3 | Cell-free DNA concentrations

The concentrations of cfDNA in HCC and other liver diseases ranged from 0.150 to 761.9 ng/ml plasma; there were no significant differences according to age or sex. The cfDNA concentration tended to be higher in the HCC group than in the BLT or CLD group, particularly in advanced BCLC stages, although this difference was not statistically significant (Figure S2).

TABLE 1 Baseline characteristics of patients in this study

Parameter	HCC (n = 102)	HCC with matched tissue (n = 27)
Age (years)		
<50	10 (9.8)	5 (18.5)
50–59	39 (38.2)	13 (48.1)
60–69	32 (31.4)	5 (18.5)
≥70	21 (20.6)	4 (14.8)
Sex		
Female	15 (14.7)	2 (7.4)
Male	87 (85.3)	25 (95.6)
Cause		
HBV	71 (69.6)	19 (70.4)
HCV	6 (5.9)	1 (3.7)
Alcohol	9 (8.8)	3 (11.1)
NBNC	16 (15.7)	4 (14.8)
Other/unknown	0 (0.0)	0 (0.0)
Cirrhosis		
Yes	60 (58.8)	14 (51.9)
No	42 (41.2)	13 (48.1)
BCLC stage		
A (Early)	43 (42.2)	4 (14.8)
B (Intermediate)	15 (14.7)	6 (22.2)
C (Advanced)	44 (43.1)	17 (63.0)
AFP (ng/ml)		
≥10	74 (72.5)	24 (88.9)
<10	28 (27.5)	3 (11.1)
PIVKA-II (mAU/ml)		
≥40	71 (70.3)	25 (92.6)
<40	30 (29.7)	2 (7.4)
Treatment modalities		
Operation or RFA	20 (16.7)	
TACE	34 (33.3)	
Concurrent chemoradiotherapy	21 (20.6)	
Systemic chemotherapy	15 (14.7)	

Abbreviations: AFP, α-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NBNC, non-B non-C; PIVKA-II, prothrombin induced by vitamin K absence-II; RFA, radiofrequency ablation; TACE, transarterial chemoembolization.

3.4 | Circulating tumour DNA mutation profiles

Among the 102 HCC cases, 49 (48.0%) had tier 1/2 or oncogenic mutations in at least one gene. *TERT* was most frequently mutated (31 cases, 30%), followed by *TP53* (16 cases, 16%), *CTNNB1* (14 cases, 14%), *ARID2* (5 cases, 5%), *ARID1A* (4 cases, 4%), *NFE2L2* (4 cases, 4%), *AXIN1* (3 cases, 3%) and *KRAS* (1 case, 1%). Seven cases had three concurrent mutations in three different genes, while 15 cases had two concurrent mutations. No oncogenic mutations were detected in the BLT and CLD groups, suggesting that our assay exhibited high specificity (Figure 2). *TP53* mutations were distributed across different locations, whereas *CTNNB1* mutations were clustered around well-known hotspot sites in codons 32–45 (Figure 3A,B). *TP53* mutations had the highest mean MAF, presumably because they were associated with advanced stages (Figure 3C). There were no significant differences in mutation frequencies according to age or sex. *TERT* mutations were frequently observed in HCV-related HCCs (5/6, 83.3%; $p = 0.031$). Enrichment analyses showed significant associations between *TP53* mutation and HBV infection ($p = 0.002$) and between *CTNNB1* mutation and NBNC aetiology ($p = 0.033$; Figure 3D). The mutation frequency increased as the BCLC stage increased. The mean mutation frequencies were 25.6%, 33.3% and 75.0% for BCLC stages A, B and C respectively ($p < 0.001$, Table 2).

Circulating tumour DNA mutations were detected at a high frequency in cases with AFP ≥10 ng/ml (55.4%) and PIVKA-II ≥40 mAU/ml (57.7%). However, ctDNA mutations were detectable in a substantial proportion of cases with AFP <10 ng/ml (28.6%) and PIVKA-II <40 mAU/ml (26.7%), suggesting an additional role for ctDNA testing in HCC diagnosis (Table 2). Consistent with these findings and with the high specificity (~100%) of ctDNA testing, receiver-operating characteristic curve analyses defined the confirmatory diagnosis by pathological examination as an outcome in which a combination of ctDNA with AFP and/or PIVKA-II had superior diagnostic performance compared to protein markers alone. The areas under the curve for AFP and PIVKA-II were 0.726 and 0.822 respectively. In contrast, the areas under the curve for of AFP + ctDNA, PIVKA-II + ctDNA and AFP + PIVKA-II + ctDNA were 0.813, 0.863 and 0.865 respectively. These values were all significantly greater than the area under the curve of AFP alone ($p = 0.002$, 0.014 and 0.002 respectively) (Figure 4).

3.5 | Mutation profiles of tumour tissues

Matched tissue biopsy samples were available for 27 cases. Nearly all mutations in the target genes could be detected in both tissue and plasma except for *TERT* promoter mutations; such mutations had a low MAF (<25%) in tissue samples and were missed in matched plasma samples (three cases). Matched tissue samples were analysed via targeted sequencing of 200 HCC-related genes. According to the mutation profiles of tumour tissues, *TP53* was most frequently mutated (12 cases, 44.4%), followed by *CTNNB1* (7 cases, 25.9%), *AXIN1* (3 cases, 11.1%), *ARID2*, *ARID1A*, *ALB*, *PDE4DIP*, *PIK3CA*, *RB1*, *RYR2* (2 cases, 7.4%) and others (1 case, 3.7%) (Figure S3). *TP53* mutations were associated with stage C. Except for TERT, 15 of the

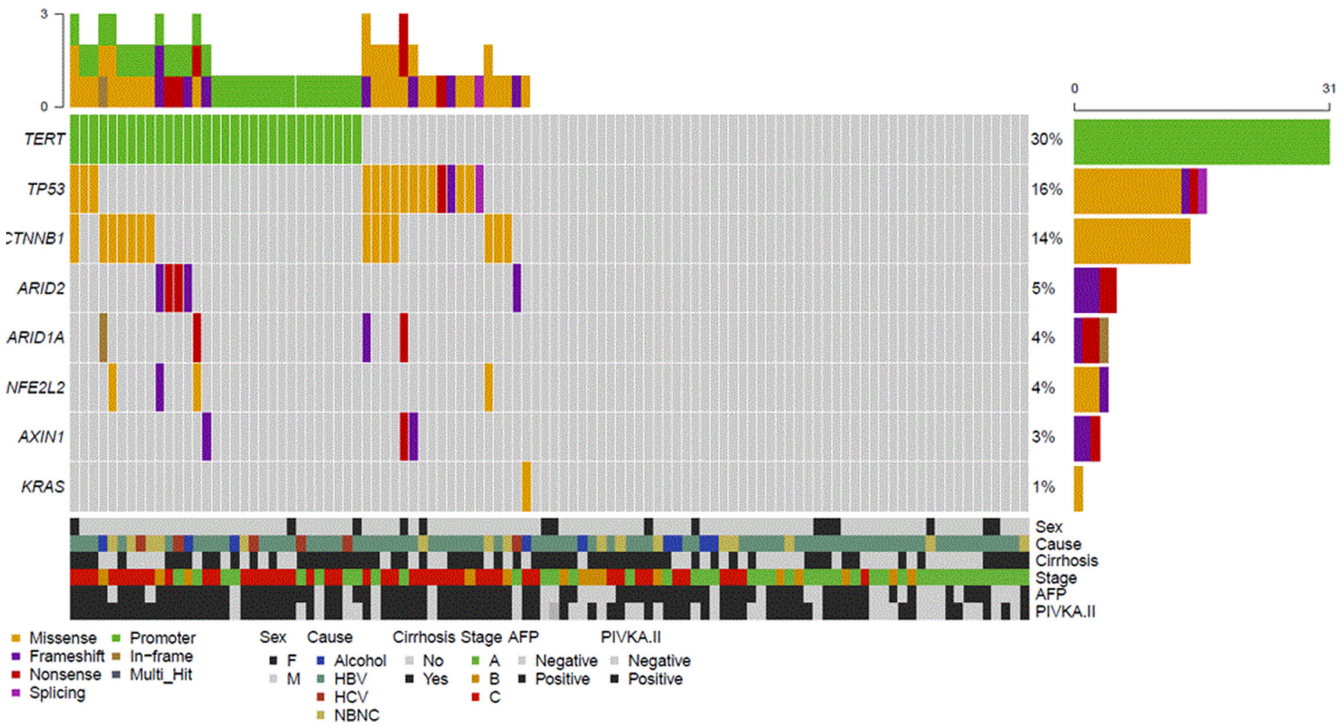

FIGURE 2 ctDNA mutation profiles in 102 hepatocellular carcinoma patients and their associations with demographic and clinical parameters. ctDNA, circulating tumour DNA.

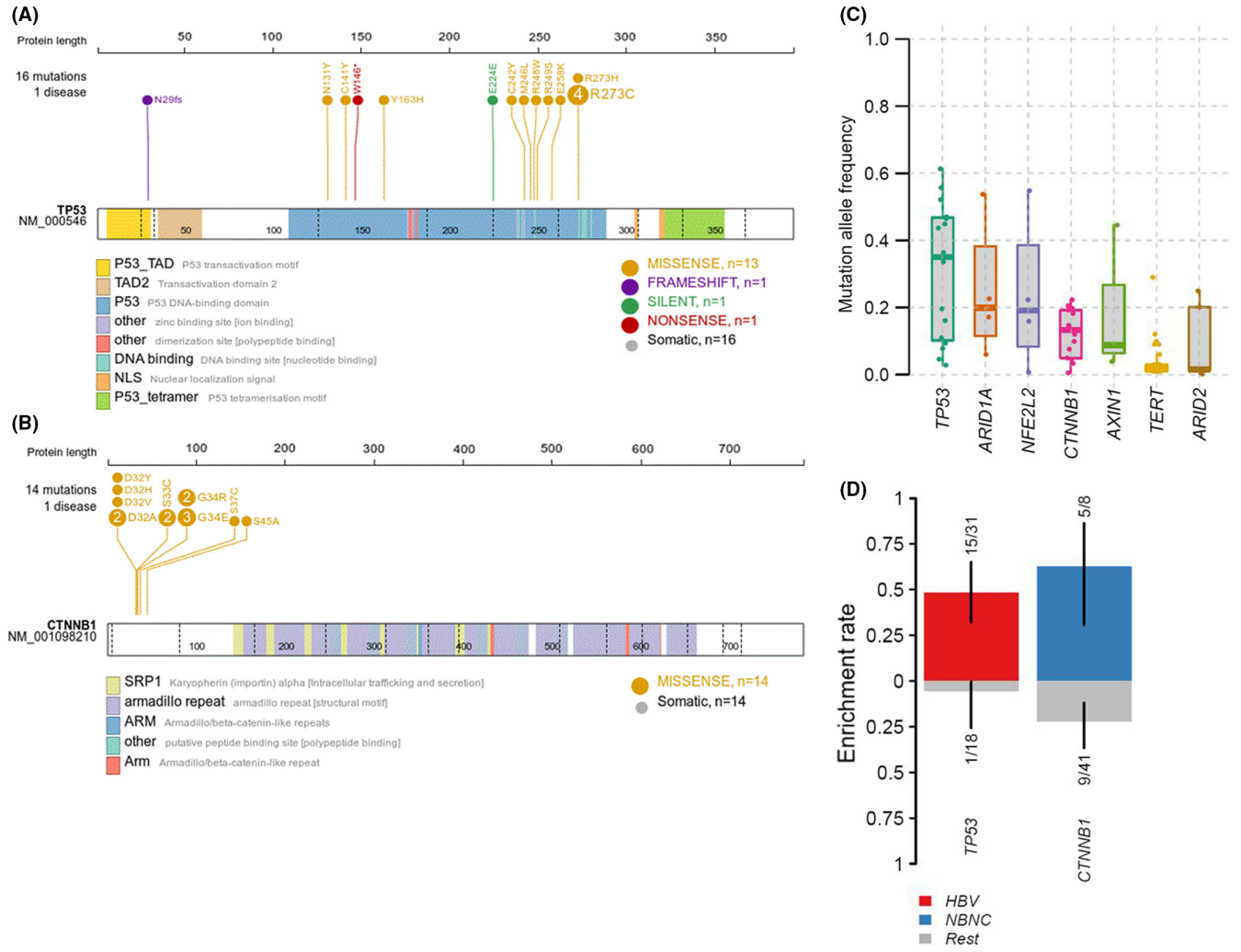

FIGURE 3 Characteristics of ctDNA mutations. Locations of ctDNA mutations in *TP53* (A) and *CTNNB1* (B) genes, mutant allele frequencies according to genes (C) and enrichment rates according to clinical parameters (D). ctDNA, circulating tumour DNA.

18 (83.3%) ctDNA mutations were also detected in tumour tissue samples (Table S4).

3.6 | Clinical outcomes according to circulating tumour DNA mutations

In terms of survival, 30 (29.4%) HCC patients died, whereas all BLT and CLD patients survived. Survival analysis using the Kaplan–Meier method showed that patients with ctDNA mutations tended to have worse survival than did patients without these mutations, although the difference was not statistically significant ($p = 0.063$). Patients with *TP53* mutations exhibited significantly worse survival than did patients without these mutations ($p = 0.007$), whereas survival remained unaffected in patients with *CTNNB1* or *TERT* mutations (Figure 5A–D). In patients with BCLC stage A ($n = 43$) and BCLC stage C ($n = 44$), overall survival did not significantly differ according to the *TP53* mutation status (BCLC stage A: $p = 0.706$; BCLC stage C: $p = 0.528$; Figure S4A,C). The difference was statistically significant among patients with BCLC stage B ($n = 15$, $p < 0.001$), but this was presumably because the death occurred in the only patient who had *TP53* mutation (Figure S4B). Multivariate analysis showed no significant associations of survival with *TP53*, *TERT* and *CTNNB1* mutations, except BCLC stage (Table S5).

4 | DISCUSSION

In this study, we found that ctDNA mutation profiles were closely associated with tissue mutation profiles in Korean HCC patients. We confirmed high frequencies of *TP53*, *TERT* and *CTNNB1* mutations in these patients. Among 102 HCC patients, 48% had tier 1/2 or oncogenic mutations in at least one gene. *TERT* was the most frequently mutated gene, followed by *TP53* and *CTNNB*. Patients with *TP53* mutations had significantly worse survival rates than did patients without these mutations. Other mutations did not affect survival.

In cancer research, liquid biopsies are currently used to investigate circulating tumour cells, tumour-derived extracellular vesicles

TABLE 2 Mutation profiles of HCC patients according to clinical parameters

	n	ctDNA mutation (%)	*p*	*TP53* mutated (%)	*p*	*CTNNB1* mutated (%)	*p*	*TERT* mutated (%)	*p*
Age (years)									
<50	10	8 (80.0)	0.154	3 (30.0)	0.541	1 (10.0)	0.359	5 (50.0)	0.391
50–59	39	16 (41.0)		6 (15.4)		3 (7.7)		12 (30.8)	
60–69	32	14 (43.8)		5 (15.6)		5 (15.6)		7 (21.9)	
≥70	21	11 (52.4)		2 (9.5)		5 (23.8)		7 (33.3)	
Sex									
Female	15	5 (33.3)	0.270	3 (20.0)	0.701	1 (6.7)	0.687	3 (20.0)	0.544
Male	87	44 (50.6)		13 (14.9)		13 (14.9)		28 (32.2)	
Cause									
HBV	71	31 (43.7)	0.069	15 (21.1)	0.141	7 (9.9)	0.162	18 (25.4)	0.031
HCV	6	6 (100.0)		0 (0.0)		1 (16.7)		5 (83.3)	
Alcohol	9	4 (44.4)		0 (0.0)		1 (11.1)		3 (33.3)	
NBNC	16	8 (50.0)		1 (6.3)		5 (31.3)		5 (31.3)	
Cirrhosis									
Yes	60	30 (50.0)	0.690	12 (20.0)	0.178	6 (10.0)	0.246	19 (31.7)	0.828
No	42	19 (45.2)		4 (9.5)		8 (19.0)		12 (28.6)	
BCLC stage									
A	43	11 (25.6)	<0.001	2 (4.7)	0.004	1 (2.3)	0.009	8 (18.6)	0.016
B	15	5 (33.3)		1 (6.7)		2 (13.3)		3 (20.0)	
C	44	33 (75.0)		13 (29.5)		11 (25.0)		20 (45.5)	
AFP (ng/ml)									
≥10	74	41 (55.4)	0.025	14 (18.9)	0.223	11 (14.9)	0.753	26 (35.1)	0.099
<10	28	8 (28.6)		2 (7.1)		3 (10.7)		5 (17.9)	
PIVKA-II (mAU/ml)									
≥40	71	41 (57.7)	0.005	14 (19.7)	0.139	13 (18.3)	0.059	26 (36.6)	0.060
<40	30	8 (26.7)		2 (6.7)		1 (3.3)		5 (16.7)	

Abbreviations: AFP, α-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; ctDNA, circulating tumour DNA; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NBNC, non-B non-C; PIVKA-II, prothrombin induced by vitamin K absence-II.

and ctDNA.[33] Selected cancer-derived circulating tumour cells can be used for the genomic characterization of a pure cancer cell population. Extracellular vesicles, such as microvesicles and exosomes, also contain tumour-specific, noncoding, small and long regulatory RNAs and proteins.[34] ctDNA can be examined for DNA quantity, integrity, methylation and gene mutations. While it is easy to measure cfDNA quantity or integrity, it is difficult to distinguish an increase in ctDNA from non-specific increases in cfDNA caused by benign conditions (e.g. inflammation).

Various cancers have been investigated for the presence of somatic ctDNA mutations.[11–13] However, there are limited data regarding ctDNA mutations in HCCs.[35] A notable challenge concerning the detection of ctDNA mutations in HCCs is that such mutations are highly variable, with few hotspots of frequent mutation.[36] An exception is the *TERT* promoter mutation c.1-124C > T (also known as C228T), which is predominant in HCCs; this mutation was also found in our patients. Accordingly, previous studies have investigated *TERT* mutations using target-specific PCR techniques such as ddPCR.[37,38] *TP53* R249S mutations, which are frequently associated with aflatoxin exposure and HBV infections, occur predominantly in South Asia and sub-Saharan Africa. Therefore, some researchers have attempted to detect R249S mutations using ddPCR.[39,40] In our Korean cohort, however, *TP53* mutations were highly variable across different locations, while R249S was observed in only one case; these findings suggest that *TP53* mutations should be examined using more sophisticated technologies such as NGS. *TP53* mutations are generally associated with poor outcomes in HCC patients. In this study, the presence of ctDNA with *TP53* mutations was associated with the best survival, followed by ctDNA without *TP53* mutations, and then the absence of ctDNA ($p = 0.022$).

Several previous studies have used multi-gene NGS for the detection of ctDNA mutations in HCCs.[22,41–47] Although these studies employed different assay methods and had cohort sizes that were insufficient for definitive conclusions, ctDNA testing was presumed to be highly specific, with few positives detected

in patients with benign liver diseases. Instead, the sensitivity of ctDNA mutation testing tended to be low in early stage HCCs.[35,48] This may be partly attributable to the presence of minimal ctDNA (from small tumours) in blood samples.[49] We also observed low detection rates in early stage cancers, despite applying the use of more advanced and sensitive NGS techniques that involved ultra-high depth and UMI barcoding.[34,50] Nonetheless, our study showed that ctDNA mutation testing may be beneficial in a substantial proportion of patients, especially patients with negative protein markers. A combination of ctDNA and protein markers demonstrated enhanced diagnostic performance. ctDNA mutation testing appeared to complement other diagnostic modalities. A previous study in HBV-positive individuals suggested that combined detection of ctDNA alterations and protein markers could identify early stage HCCs.[47] Many studies have shown that genetic ctDNA variations could reflect the mutational landscape of corresponding tumour tissues. The specificity of detection methods can approach 100%, but the sensitivity is generally low and depends on the type of DNA alteration. Because of the extremely small amount of ctDNA, highly sensitive and advanced molecular detection technologies are required along with cfDNA-specific isolation methods.

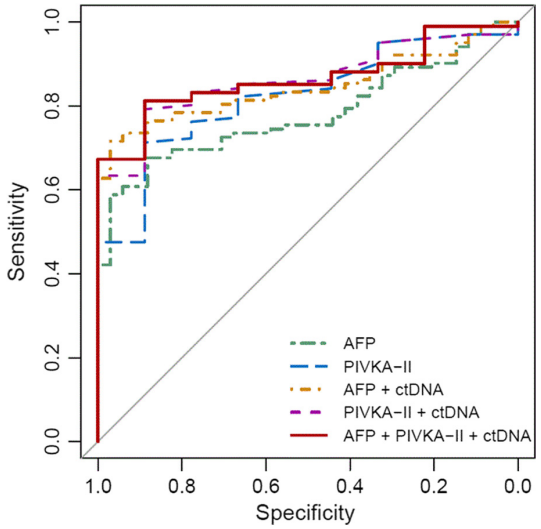

FIGURE 4 Area under the curve analysis of the protein markers, AFP and PIVKA-II, in combination with ctDNA mutations. AFP, α-fetoprotein; ctDNA, circulating tumour DNA; PIVKA-II, prothrombin induced by vitamin K absence-II.

Our results also showed that ctDNA mutation profiles were closely associated with cancer tissue mutation profiles. Consistent with previously established characteristics of HCCs,[51] we observed high frequencies of *TP53*, *TERT* and *CTNNB1* mutations. Most *TERT* mutations were c.1-124C>T (29/31, 93.5%); *CTNNB1* mutations

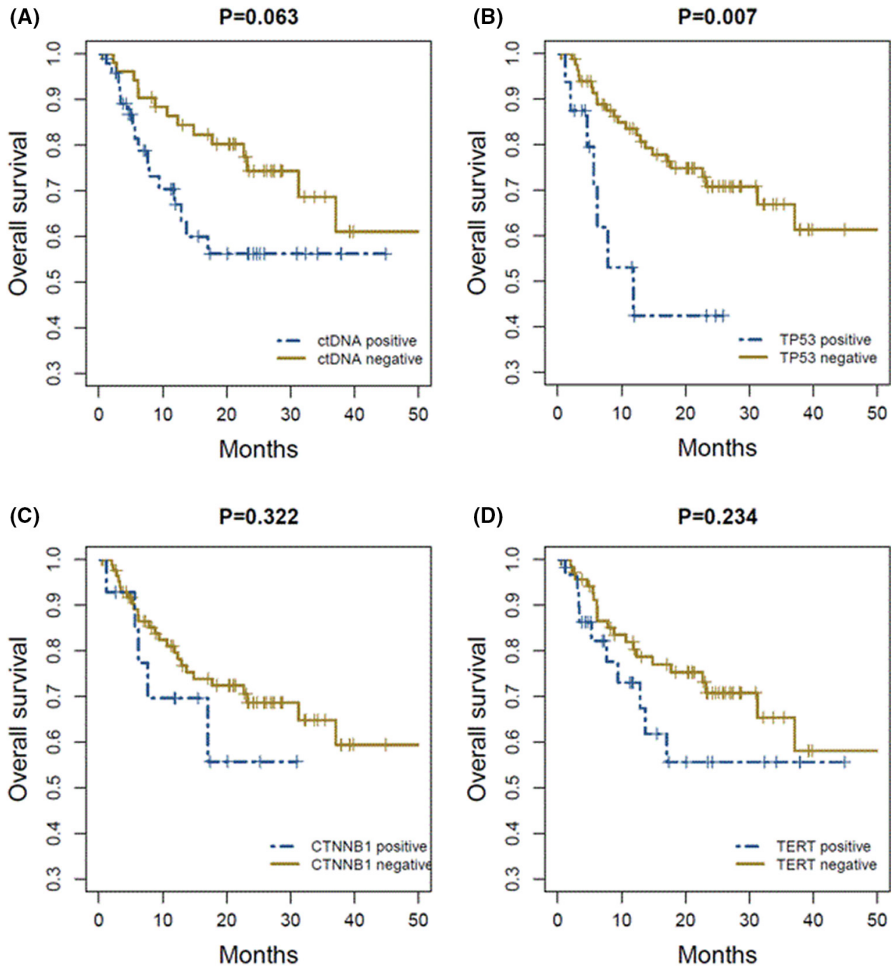

FIGURE 5 Survival analysis according to all ctDNA mutations (A) and *TP53* (B), *CTNNB1* and *TERT* (C) mutations. ctDNA, circulating tumour DNA.

were clustered in N-terminal regions, whereas *TP53* mutations were scattered without a hotspot. Therefore, our data suggested that ctDNA mutation testing could be a complementary or alternative option for genomic testing using tissue samples.

A limitation of this study was the lower rate of detection for *TERT* mutations, compared with previous studies.[37,38] We examined mutations via enrichment sequencing (instead of ddPCR) because the *TERT* promoter region is difficult to amplify due to its high GC content. Despite this limitation, enrichment sequencing retains advantages over ddPCR in terms of discovering unknown mutations and analysing multiple target genes. Another limitation of this study was the inclusion of an insufficient number of matched tumour tissue biopsies. The tumour tissue samples from 27 of the 102 HCC patients were not representative of the entire sample; this lack of representativeness could have led to bias.

In summary, we used advanced NGS techniques to investigate ctDNA mutation profiles in HCC patients. Our results confirmed that ctDNA testing offers dynamic and personalized information concerning tumour biology, which can facilitate clinical diagnosis and management. ctDNA testing also has the potential as a minimally invasive approach for prognostic stratification and post-therapeutic monitoring. Further studies of HCC patients are needed to confirm the potential applications of ctDNA in predicting treatment responses to various tyrosine kinase inhibitors, prognostic outcomes before and after immune checkpoint administration and tumour recurrence.

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CONFLICT OF INTEREST

The authors do not have any disclosures to report.

ETHICS APPROVAL STATEMENT

The study protocol was approved by the Severance Hospital Institutional Review Board (IRB No. 4-2015-0184).

PATIENTS CONSENT

All patients' consent was achieved for this study.

PERMISSION TO REPRODUCE MATERIAL FROM ANOTHER SOURCE

NA.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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