Usefulness of Cell-Free Human Telomerase Reverse Transcriptase Mutant DNA Quantification in Blood for Predicting Hepatocellular Carcinoma Treatment Efficacy

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Although the usefulness of liquid biopsy as a biomarker in the treatment of hepatocellular carcinoma (HCC) has been suggested, its usefulness in transcatheter arterial chemoembolization (TACE) or tyrosine kinase inhibitor (TKI) therapies has not been reported in detail. In this study, we investigated the clinical value of a cell-free (cf)DNA quantification system targeting the human telomerase reverse transcriptase (*hTERT*) promoter mutation in advanced HCC treatment. Plasma from 67 patients with advanced HCC, treated with TACE and TKI, was used for extraction of cfDNA. We defined cfDNA with the *hTERT* promoter C228T mutation as circulating mutant DNA (mutant DNA) and without the mutation as circulating wild-type DNA (wild-type DNA). We analyzed the changes in mutant and wild-type DNA levels during HCC treatment and examined the relationship between changes in the cfDNA level and the clinical course. Mutant DNA was detected in 73.1% (49/67) of the patients during HCC treatment. In univariate analysis, factors associated with detection of mutant DNA before treatment were the intrahepatic maximum tumor diameter ($P = 0.015$) and protein induced by vitamin K absence (PIVKAII) ($P = 0.006$). The degree of mutant DNA change after TACE was significantly correlated with tumor volume ($P < 0.001$), reflecting the treated tumor volume. Responders with peak cfDNA levels within 1 week of TKI initiation had significantly better progression-free survival than nonresponders ($P = 0.004$). *Conclusion:* Changes in blood *hTERT* promoter mutant DNA levels during TACE or TKI treatment indirectly reflect the amount of HCCs and are useful for predicting long-term treatment responses. (*Hepatology Communications* 2021;5:1927-1938).

Hepatocellular carcinoma (HCC) is the sixth most common malignancy in the world and one of the most common causes of cancer-related deaths.[1] Many tyrosine kinase inhibitors (TKIs) have been developed as the main chemotherapeutic agents for the treatment of advanced HCC, and currently lenvatinib (LEN) and sorafenib (SOR) are used as first-line treatment while regorafenib and

Abbreviations: AST, aspartate aminotransferase; cfDNA, cell-free DNA; CT, computed tomography; ctDNA, circulating tumor DNA; HCC, hepatocellular carcinoma; hTERT, human telomerase reverse transcriptase; LEN, lenvatinib; OR, odds ratio; OS, overall survival; PFS, progression-free survival; PIVKAII, protein induced by vitamin K absence; SOR, sorafenib; TACE, transcatheter arterial chemoembolization; TKI, tyrosine kinase inhibitor.

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cabozantinib are used as second-line treatment.[2,3] Nonetheless, their effect remains limited, and the development of appropriate biomarkers enabling accurate response prediction is awaited.

Recent advances in sequencing (next-generation sequencing [NGS]) technology have gradually revealed the profile of genetic abnormalities in HCC tissues.[4-6] As a noninvasive test in cancer treatment, attempts to detect genetic abnormalities in HCC using cell-free DNA (cfDNA) in blood through NGS technology have been actively pursued. Fragmented cfDNA appears in blood following cell apoptosis,[7,8] and patients with cancer have more cfDNA than healthy individuals, including circulating tumor DNA (ctDNA). It has been suggested that analysis of cfDNA in blood has potential applications for the early detection of cancer, monitoring of posttreatment recurrence, and identification and evaluation of therapeutic targets.[9-11]

Cancer-related genetic mutations can be used as markers of disease progression when abnormalities are concentrated in limited hotspots in specific genes that are correlated with the appearance of malignant tumors, such as estrogen receptor 1 (*ESR1*) mutations in metastatic cases of breast cancer,[12] epidermal growth factor receptor (*EGFR*) mutations in lung cancer,[13] and v-Ki-ras2 Kirsten rat sarcoma viral oncogene (*KRAS*) mutations in colon cancer.[14] HCC is a tumor that progresses in multiple stages from precancerous lesions to advanced cancer; during this process, genetic abnormalities involving multiple signaling pathways are observed. A large-scale study of HCC mutations has identified genes involved in human telomerase reverse transcriptase (*hTERT*), tumor protein P53 (*TP53*), Wnt-β-catenin signaling, and chromatin remodeling.[6,15] Among them, *hTERT* promoter mutations are the most frequently observed in HCC and are concentrated in specific hotspots, suggesting a potential role as a clinical biomarker.[16,17]

In our previous study using resected HCCs, the *hTERT* promoter mutation C228T was observed most frequently among other genes, even at the earliest histologic grade, suggesting that the *hTERT* promoter mutation C228T could be an appropriate target gene for ctDNA analysis.[18] Several studies have observed changes in the amount of ctDNA in the blood after cancer treatment.[14,19,20] However, it is not clear how changes in the amount of ctDNA, in particular, the changes caused by treatment, reflect the tumor tissue; how ctDNA changes quantitatively in a short period of time during treatment; and how the dynamic changes vary with different treatments.

In this study, we focused on the short-term dynamics in cfDNA levels after transcatheter arterial chemoembolization (TACE) and TKI treatment for advanced HCC, with particular attention to detection of the *hTERT* promoter mutation in blood, and investigated the relationship between the dynamics of cfDNA levels and clinical factors, including treatment response.

Patients and Methods

PATIENTS

Among patients with advanced HCCs at the University of Yamanashi Hospital from April 2018

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to April 2020, 239 patients were treated with TACE and 41 patients were treated with TKI. TACE was performed with epirubicin or cisplatin, according to institutional protocol. Thirty-two consecutive patients treated with TACE and 35 consecutive patients treated with TKI, with adequate available plasma, were included in the study. Of the patients treated with TKI, 6 were treated with SOR and 29 with LEN. The criteria for TKI induction were Child-Pugh A and advanced HCC ineligible for TACE, but 1 patient of Child-Pugh B and 1 patient of Child-Pugh C were treated after adequate informed consent.

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki by the Human Ethics Review Committee of the University of Yamanashi. Informed consent in writing was obtained from each patient.

SAMPLE COLLECTION AND DNA EXTRACTION

Blood samples were collected 3 times in patients treated with TACE (before treatment, the day after treatment, and 3-5 days after treatment) and 6 times in patients treated with TKI (before treatment, the day after treatment, and 3, 5, 7, and 14 days after treatment). We collected 8 mL of blood using BD Vacutainer cell preparation tubes (Becton-Dickinson, NJ) and centrifuged the samples within a few hours. The plasma fraction was collected and stored at −20℃ until use. Plasma DNA was extracted using QIAamp Circulating Nucleic Acid kits (Qiagen, Milan, Italy), according to the manufacturer's instructions.

DEFINITION OF MUTANT DNA AND WILD-TYPE DNA

Because there is no classification of cfDNA focusing on tumor-associated mutations, we defined circulating wild-type DNA without tumor-associated mutations as wild-type DNA and circulating mutant DNA with tumor-associated mutations as mutant DNA (Supporting Fig. S1). In this study, the *hTERT* promoter C228T mutation was the targeted hotspot for mutant DNA and wild-type DNAs. In the 2 patients where biopsy tissues were obtained before the start of LEN, mutant DNA was detected in both blood and tissue (Supporting Table S1).

DETECTION OF *hTERT* PROMOTER MUTATION USING DIGITAL POLYMERASE CHAIN REACTION

The *hTERT* promoter mutation was detected using the QuantStudio 3-Dimension Digital Polymerase Chain Reaction (dPCR) system (Thermo Fisher, Waltham, MA). The details are given in the Supporting Materials and Methods.

EVALUATION OF TUMOR VOLUME

Tumor sizes and numbers were assessed by computed tomography (CT) and magnetic resonance imaging before treatment and CT during hepatic arteriography and during arterioportography during treatment. TACE was performed according to the institution's protocol. In the evaluation of tumor volume, we used the longest tumor diameter as a surrogate of tumor volume because the Response Evaluation Criteria in Solid Tumors uses longest diameters as a measure of treatment response[21] and because previous studies also reported a correlation between tumor volume with longest diameter.[22] In addition, cubes of tumor diameters were also used as an indicator of tumor volume in the treated liver. Using the number of tumors (n) and tumor diameter (r_n [cm]), the tumor volume index (eTV in cm^3) is expressed as $eTV = \Sigma\ [k = 1, n]\ r_k^3$.

CHANGES IN MUTANT AND WILD-TYPE DNA LEVELS AFTER TREATMENT

The amounts of mutant DNA and wild-type DNA detected in the plasma are expressed as the copy number per 1 mL of plasma. If mutant DNA was not detected during treatment, it was not possible to distinguish mutant DNA-negative cases from those with a low level of detection. Therefore, in the analysis of the factors enabling detection of mutant DNA before treatment, those cases without detection of mutant DNA during treatment were excluded from the analysis.

For patients treated with TACE, changes in mutant and wild-type DNA were analyzed by pretreatment values, 1-day posttreatment values, and final observation values. Patients treated with TKI were analyzed by pretreatment values, peak values in the first week of the posttreatment period, and final observation

values. In patients treated with TACE, the relationship between mutant or wild-type DNA levels and tumor volume or liver damage was examined. In patients treated with TKI, the pattern of change in mutant DNA was used to define the short-term treatment response. As shown in the results, we defined responders as those whose mutant DNA increased more than 1.5-fold from baseline and peaked within 1 week of TKI treatment and nonresponders as those whose mutant DNA did not increase in this fashion.

The association between short-term mutant DNA response and long-term treatment response was assessed using the progression-free survival (PFS) rate and overall survival (OS) rate.

STATISTICAL ANALYSIS

Continuous variables are presented as median (range) and were compared using the Mann-Whitney U test or Wilcoxon signed-rank test. Categorical variables were evaluated using Fisher's exact test. Bonferroni correction was used for multiple comparisons in multiple groups. Trends in the prevalence of patients with mutant DNA before treatment were analyzed using the Cochran-Armitage test. Correlation and linear regression analysis were performed between the longest diameter or tumor volume index and mutant/wild-type DNA and between the aspartate aminotransferase (AST) value and mutant/wild-type DNA. Statistical significance was defined as $P < 0.05$. The difference in OS or PFS in patients with and without mutant DNA before treatment or with and without a mutant DNA response to treatment were assessed by visualizing Kaplan-Meier survival curves and tested by the difference between the survival curves, using the log-rank test. Analyses were conducted in R version 3.5.1 and Microsoft Excel.

Results

BACKGROUND CHARACTERISTICS OF PATIENTS

Background characteristics of the 67 patients (32 TACE, 35 TKI) studied are shown in Table 1. Patients treated with TKI had more extrahepatic metastases ($P < 0.001$) and more were at an advanced stage ($P = 0.002$) than patients treated with TACE.

MUTANT AND WILD-TYPE DNA LEVELS BEFORE AND AFTER HCC TREATMENT

Mutant DNA was found in the plasma of 22 of 67 patients (32.8%) before treatment and in 49 of 67 patients (73.1%) after treatment, demonstrating that mutant DNA was detectable more frequently ($P = 0.001$) after treatment (Fig. 1). The prevalence of patients positive for mutant DNA tended to be lower in patients infected with hepatitis B virus (HBV) (44.4%) than with hepatitis C (82.6%) ($P = 0.075$, data not shown). This result is consistent with previous reports that *hTERT* promoter mutations are positively associated with age and hepatitis C infection but negatively associated with HBV infection.[23,24] In contrast, wild-type DNA was found in the plasma of all patients before treatment, but the quantity was significantly increased after treatment (Fig. 1).

MUTANT DNA DETECTION BEFORE HCC TREATMENT

In the 49 patients in whom mutant DNA was detectable during treatment, mutant DNA was detected more frequently before treatment in advanced HCC ($P = 0.012$; Supporting Fig. S4). According to univariate analysis, the factors associated with the detection of mutant DNA before treatment were maximum tumor diameter (odds ratio [OR] 1.07; 95% confidence interval [CI], 1.01-1.12; $P = 0.015$) and protein induced by vitamin K absence (PIVKAII) (OR 6.55; 95% CI, 1.73-24.69; $P = 0.006$) (Table 2). Patients positive for mutant DNA before treatment had a significantly poorer prognosis (Fig. 2). In the analysis of the prognosis, we further classified patients according to treatment modality, and a significant difference of prognosis was observed between mutant DNA-positive and mutant DNA-negative cases in those treated with TKI, while this difference was not evident in those treated with TACE (Supporting Fig. S5).

CHANGES IN MUTANT AND WILD-TYPE DNA DURING TACE

The cfDNA of patients treated with TACE was measured at 3 time points as follows: pretreatment,

TABLE 1. DEMOGRAPHIC CHARACTERISTICS OF 67 PATIENTS TREATED WITH TACE AND TKI

	All (N = 67)	TACE (n = 32)	TKI (n = 35)	*P*
Age (years)	71.5 (47-88)	73.5 (47-88)	69.5 (52-86)	0.200
Sex				
Male/female	59/8	26/6	33/2	0.139
Treatment				
TACE/LEN/SOR	32/29/6	—	—	
Etiology				
HBV/HCV (after SVR)/HBV+HCV/others	9/35 (13)/2/21	5/17 (5)/0/10	4/18 (8)/2/11	0.754
Maximum diameter of intrahepatic tumors (mm)	24.0 (6-180)†	29.6 (8-180)†	21.0 (6-180)†	0.245
Numbers of intrahepatic tumors	4 (0-28)†	4 (1-28)†	4 (0-19)†	0.927
Tumor volume index (cm^3)	26.0 (0.2-5,832.0)	35.3 (0.5-351.2)	20.0 (0.2-5,832.0)	0.337
Extrahepatic metastasis				
n (%)	11 (16.4)	0 (0)	11 (31.4)	<0.001
Stage				
I/II/III/IVa/IVb	3/18/26/6/11	3/11/15/3/0	0/9/12/3/11	0.002
Child-Pugh				
A/B/C	57/9/1	25/7/0	33/1/1	0.023
ALBI score	–2.34 (–3.58-0.74)	–2.21 (–3.10-1.34)	–2.36 (–3.58-0.74)	0.192
Alb (g/dL)	3.6 (2.3-5.0)	3.5 (2.5-4.3)	3.7 (2.3-5.0)	0.326
AST (U/L)	37 (17-152)	37 (20-76)	36 (17-152)	0.551
ALT (U/L)	27 (10-143)	26 (13-90)	28 (10-143)	0.980
TBil (mg/dL)	0.7 (0.3-4.1)	0.8 (0.3-4.1)	0.7 (0.3-4.1)	0.280
PT%	87.4 (47.6-116.0)	87.4 (47.6-116.0)	91.0 (63.6-125.0)	0.291
AFP (ng/mL)	194.8 (1.0-1,466,703.0)	14.6 (1.0-13,711.0)	211.3 (1.8-1,466,703.0)	0.121
PIVKAII (mAU/mL)	433.0 (11.0-501,890.0)	291.0 (11.0-40,434.0)	445.5 (11.0-501,890.0)	0.311

*Three patients in the TKI group had no intrahepatic tumor, and 1 patient in the TKI group had unmeasurable tumor.
†Three patients in the TACE group and 5 patients in the TKI group had diffuse tumors.
Abbreviations: AFP, alpha-fetoprotein; Alb, albumin; ALBI, albumin-bilirubin; ALT, alanine aminotransferase; PT, prothrombin time; SVR, sustained virological response; TBil, total bilirubin.

1 day after treatment, and 3-5 days after treatment. Changes in mutant DNA, wild-type DNA, and AST levels in 32 patients treated with TACE are demonstrated in Fig. 3. All those values were elevated at 1 day after treatment and had decreased by 3-5 days after treatment.

hTERT promoter mutant DNA was detected in 24 of the 32 patients (75.0%) during TACE therapy. The change in mutant DNA after TACE was significantly correlated with the longest diameter of tumors (R^2 = 0.372, P = 0.002; Supporting Fig. S6) and tumor volume index (R^2 = 0.449, P < 0.001; Fig. 4), and the change in wild-type DNA was significantly correlated with the change in AST levels (R^2 = 0.483, P < 0.001; Fig. 4). These results suggest that the cfDNA changes after TACE originated from the treated tumor and background normal liver.

CHANGES IN MUTANT AND WILD-TYPE DNA DURING TKI

The cfDNA from patients treated with TKI was measured at baseline and 1, 3, 5, 7, and 14 days after treatment; the data from 3 time points (pretreatment, peak-value during treatment, and last value obtained) were used for the analysis. Mutant DNA and wild-type DNA levels peaked within 1 week of treatment (Fig. 5). Based on this result, we defined responders as those whose mutant DNA increased more than 1.5-fold from baseline and reached a peak within 1 week of TKI treatment and nonresponders as those whose values did not increase in this fashion. Of the responders, 11 were patients treated with LEN and 2 were patients treated with SOR; in the nonresponders, 8 were patients treated with LEN and 4 were patients

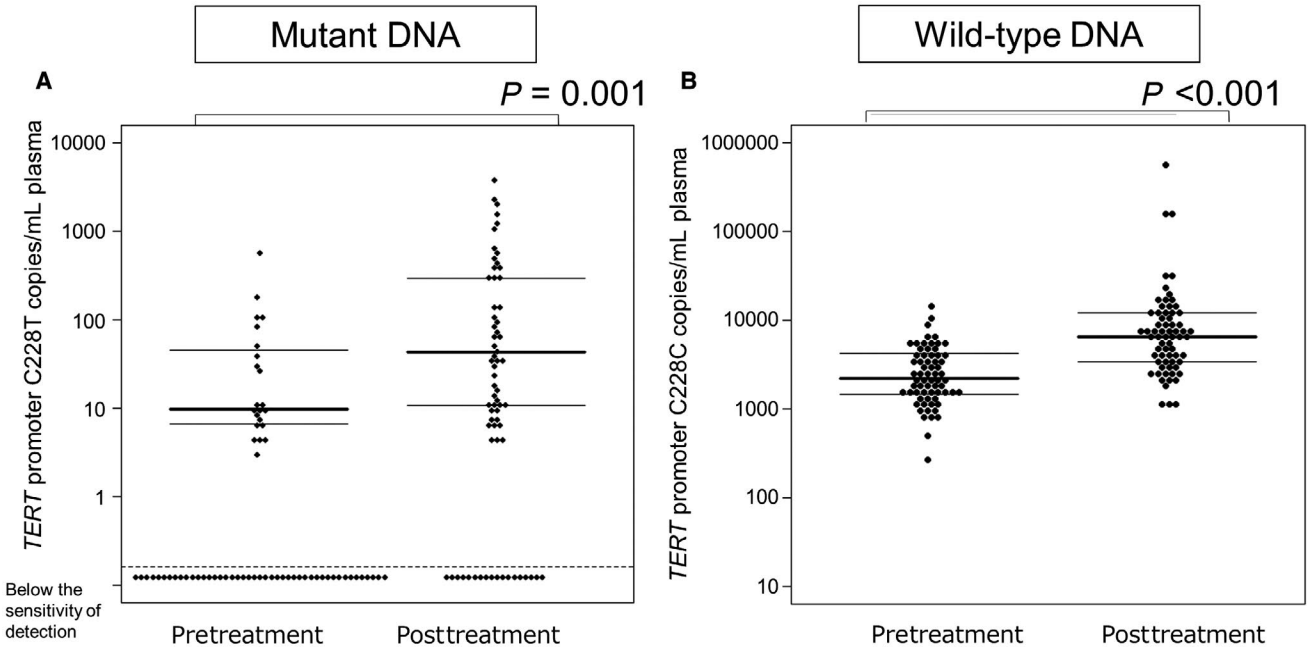

FIG. 1. Mutant and wild-type DNA detected in the plasma of patients treated with TACE or TKI. (A) Mutant DNA. (B) Wild-type DNA. Before treatment, mutant DNA was found in plasma from 22 of 67 patients (32.8%); after treatment, mutant DNA was found in 49 of 67 patients (73.1%). Posttreatment mutant DNA and wild-type DNA levels were each higher than the pretreatment levels.

TABLE 2. CLINICAL FACTORS RELATED TO THE DETECTION OF MUTANT DNA BEFORE TREATMENT

	Mutant DNA-Positive Cases (n = 49)			
	Univariate		Multivariate	
Covariate	OR (95% CI)	*P*	OR (95% CI)	*P*
Extrahepatic metastasis	1.78 (0.35-8.96)	0.486		
Vascular invasion	3.73 (0.83-16.71)	0.085	1.57 (0.2-12.27)	0.666
Numbers of intrahepatic tumors >10	2.51 (0.68-9.25)	0.165		
Maximum diameter of intrahepatic tumors (mm)	1.07 (1.01-1.12)	0.015	1.05 (0.99-1.1)	0.105
Age	0.99 (0.94-1.05)	0.842		
Sex	4.77 (0.51-44.33)	0.169		
AFP >10 ng/mL	3.09 (0.82-11.67)	0.096	2.39 (0.42-13.54)	0.327
PIVKAII >100 mAU/mL	6.55 (1.73-24.69)	0.006	4.17 (0.84-20.83)	0.081

Multivariable logistic regression analyses, including factors with significance or trend for significant association ($P < 0.10$) in univariable logistic regression analyses.
Abbreviation: AFP, alpha-fetoprotein.

treated with SOR (Supporting Fig. S7; Supporting Table S2). There was no difference in clinical and tumor factors between responders (n = 13) and nonresponders (n = 12) (Supporting Table S2). The change of mutant DNA was significantly correlated with the longest diameter and tumor volume index in the responders but not the nonresponders (Supporting Figs. S8 and S9), and there was also a tendency that changes in mutant DNA correlated with the AST changes induced by TKI (Supporting Fig. S10). Such a correlation was not found in the nonresponders (Supporting Figs. S8-S10).

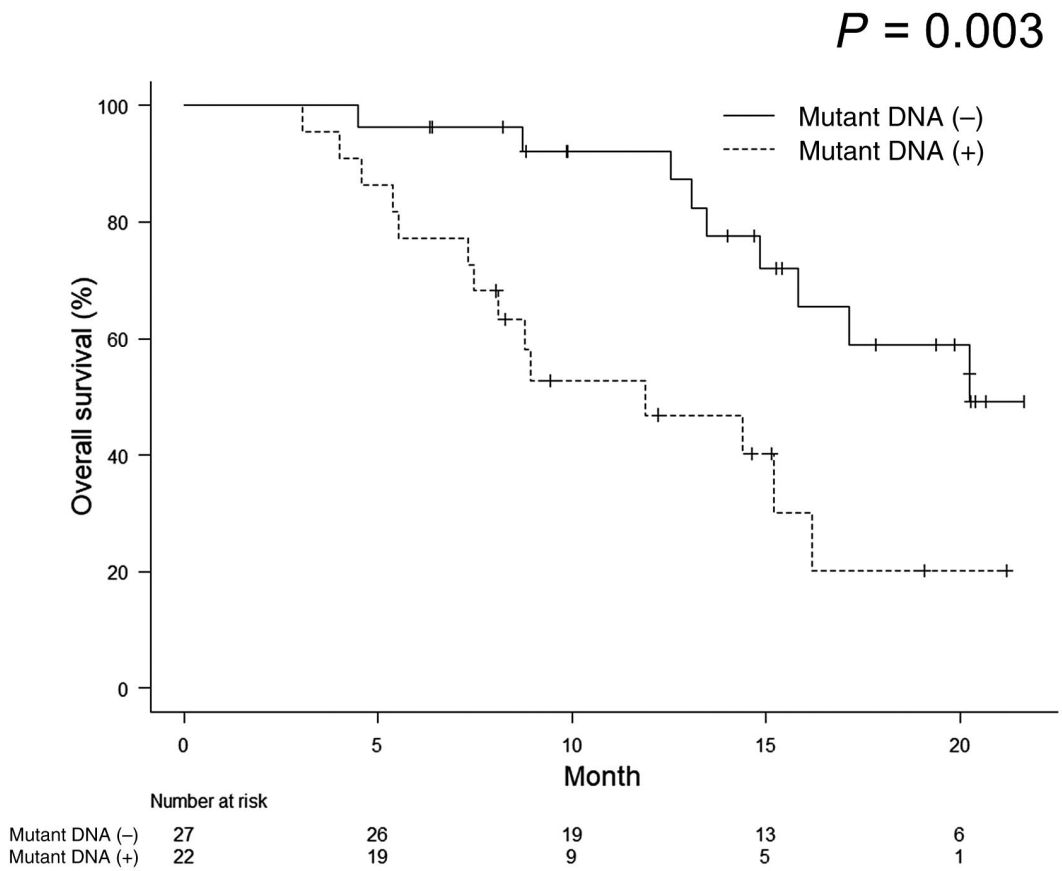

FIG. 2. OS of patients treated with TACE and TKI classified by mutant DNA detection before therapy. Patients positive for mutant DNA before treatment had significantly poorer prognoses than patients negative for mutant DNA. Median survival times were 11.9 months in patients positive for mutant DNA before treatment and 20.2 months in patients negative for mutant DNA. [+]Censored.

PROGNOSTIC PREDICTION OF RESPONSE IN TKI-TREATED PATIENTS, ACCORDING TO SHORT-TERM MUTANT DNA CHANGES

Of the 25 patients treated with TKI with *hTERT* promoter mutant DNA, 8 were discontinued due to adverse events (AEs) before the first treatment outcome was determined. Based on logistic regression analysis, the factor associated with AE discontinuation of TKI was the albumin-bilirubin score[23] (OR, 17.17; 95% CI, 1.26-234.17; $P = 0.033$) (Supporting Table S3).

Excluding patients with AE discontinuation, the initial treatment response was partial response (PR) in 5, stable disease (SD) in 4, and no progressive disease (PD) among the TKI responders (n = 9) and PR in 1, SD in 3, and PD in 4 among the nonresponders (n = 8), showing that responders had better initial treatment response ($P = 0.045$). Although there was no evident difference in OS between responders and nonresponders (Supporting Fig. S11), the PFS of the responders was superior to that of the nonresponders ($P = 0.004$; Fig. 6).

Discussion

In order to clarify the role of quantification of serum *hTERT* promoter mutations during HCC therapy, we defined *hTERT* promoter mutation-positive cfDNA as mutant DNA and mutation-negative cfDNA as wild-type DNA. We also used dPCR to analyze the relationship between the amount of cfDNA and clinical factors during HCC treatment, focusing on TACE and TKI therapies, including changes over time.

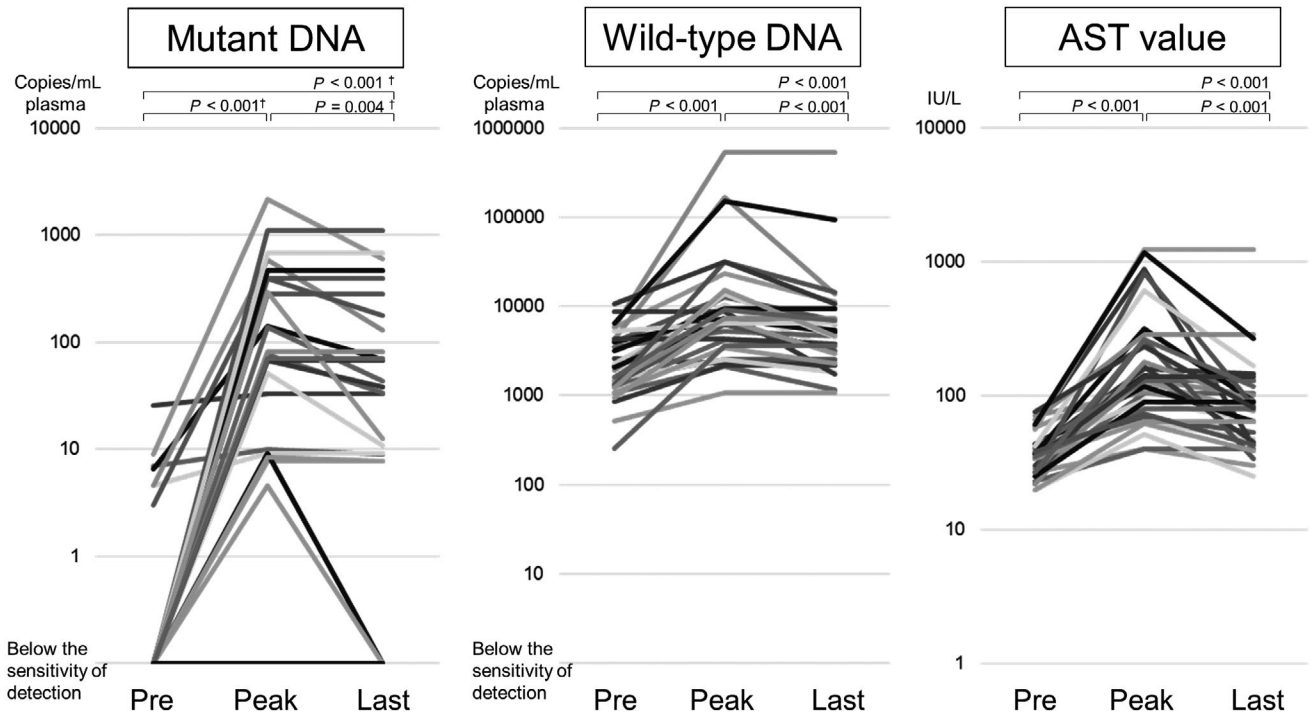

FIG. 3. Changes in mutant DNA, wild-type DNA, and AST levels in 32 patients treated with TACE. The mean amounts of mutant DNA per 1 mL of plasma at pretreatment (prevalue), posttreatment (peak value), and last observation (last value) were 2.5, 296.9, and 179.7 copies, respectively (prevalue vs. peak value, $P < 0.001$; prevalue vs. last value, $P < 0.001$; peak value vs. last value, $P = 0.004$). The mean amounts of wild-type DNA per 1 mL of plasma were 2,957.2 copies at prevalue, 34,771.8 copies at peak value, and 24,452.4 copies at last value (prevalue vs. peak value, $P < 0.001$; prevalue vs. last value, $P < 0.001$; peak value vs. last value, $P < 0.001$). The mean serum AST levels reflecting liver injury were 39.0 IU/L at prevalue, 260.6 IU/L at peak value, and 128.3 IU/L at last value (prevalue vs. peak value, $P < 0.001$; prevalue vs. last value, $P < 0.001$; peak value vs. last value, $P < 0.001$). †Analyzed in 24 patients positive for mutant DNA.

First, we analyzed cfDNA extracted from the plasma of patients treated with TACE or TKI and showed that mutant DNA was detected in 73.1% of the samples during the course of TACE or TKI therapy. The detection rate of mutant DNA during treatment was close to the reported positive rate of *hTERT* promoter mutations in HCC tissues,[25] suggesting that the prevalence of genetic mutations in cfDNA reflects the prevalence of mutations in tissues; when tumors were positive for *hTERT* promoter mutations, the cfDNA became positive for the mutations during treatment in most cases. *hTERT* promoter mutations are frequently observed in early to advanced HCC[16,18] and have been recognized as one of the trunk mutations of intratumor and intertumor heterogeneity.[26,27] Based on these previous findings, it is suggested that the presence of *hTERT* promoter mutation indicates the presence of HCC and the amount of mutant cfDNA could be a surrogate marker of tumor volume, although the amount of mutant cfDNA may also be partially influenced by tumor vascular invasion.[28] At the same time, because *hTERT* promoter mutation might reflect tumor volume, *hTERT* promoter mutant DNA could also be used as an early HCC diagnostic marker if a highly sensitive detection system is introduced.[29,30] We hypothesized that changes in mutant DNA levels reflect the amount of tumor necrosis caused by treatment and focused on changes in mutant DNA levels and treatment effects in advanced cancer. In a cross-sectional analysis of factors associated with the detection of *hTERT* promoter mutant DNA in the blood before HCC treatment, tumor diameter was extracted as a factor. We found that a larger tumor diameter was more likely to be detected.

As to the change in cfDNA after TACE, it was shown that changes in mutant DNA correlated with tumor volume while changes in wild-type DNA were

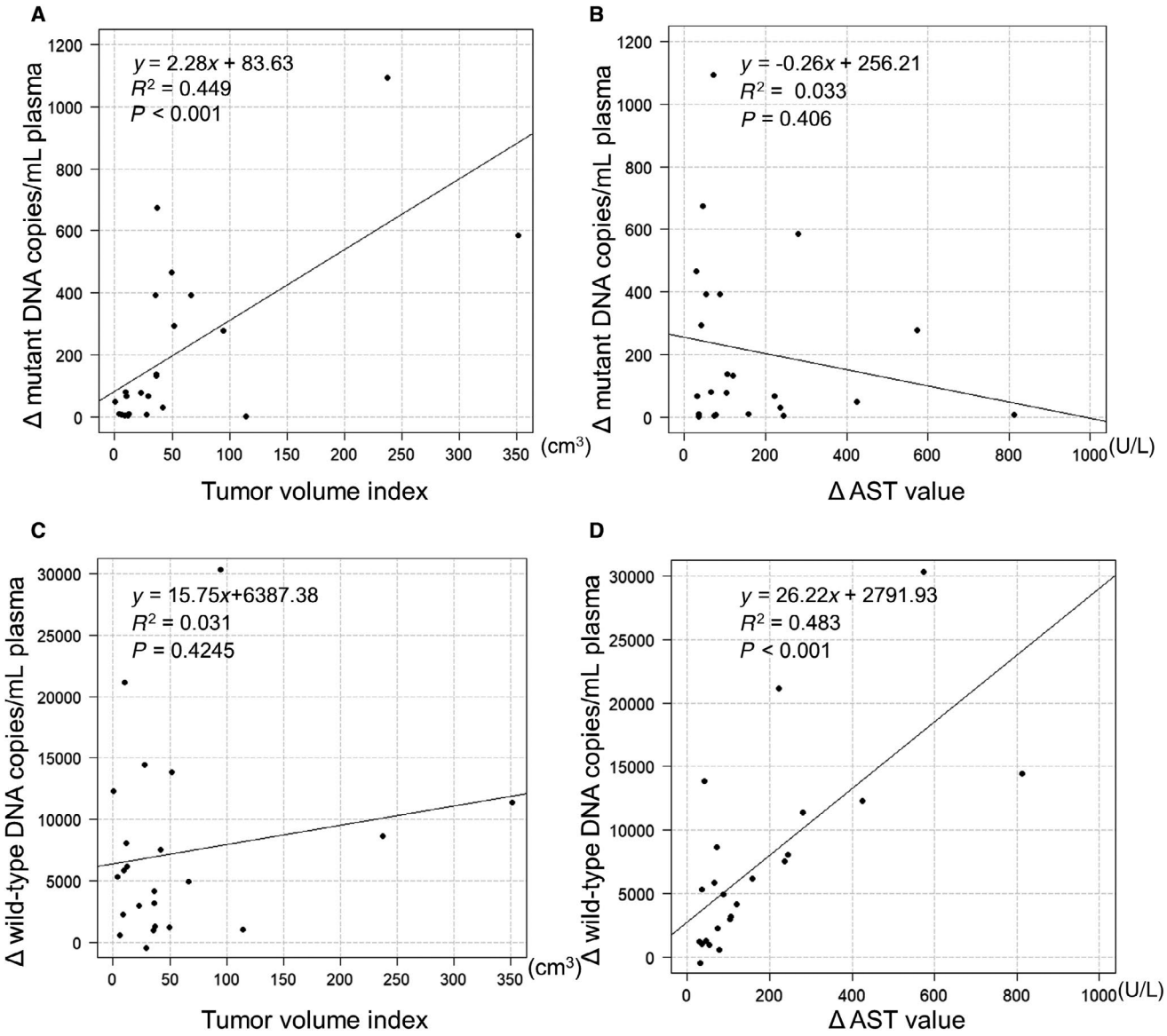

FIG. 4. Correlation between cell-free DNA changes and tumor volume or serum AST levels in 24 patients of *hTERT* promoter mutants treated with TACE. (A,B) Mutant DNA was significantly correlated with tumor volume index but not with serum AST levels. (C,D) Wild-type DNA was significantly correlated with serum AST levels but not with the tumor volume index.

associated with changes in serum AST levels. The correlation between mutant DNA level and tumor volume after TACE indicates that the mutant DNA level reflects the amount of tumor necrosis caused by treatment; the association between wild-type DNA level and serum AST level after TACE indicates that the wild-type DNA level reflects the volume of liver damaged during TACE. If the change in mutant DNA level after HCC treatment reflects the amount of tumor necrosis, then that change can be used to monitor the effect of tumor treatment not only in TACE but also in TKI treatment. Ono et al.[28] also reported that short-term changes were found in mutant DNA levels after TACE and that the peak increase in mutant DNA levels occurred within a few days of treatment, as in the present study. Because the half-life of cfDNA is considered to be only a few hours,[31] quantification of mutant DNA in blood may be a sensitive method to detect changes in cfDNA levels resulting from tumor necrosis.

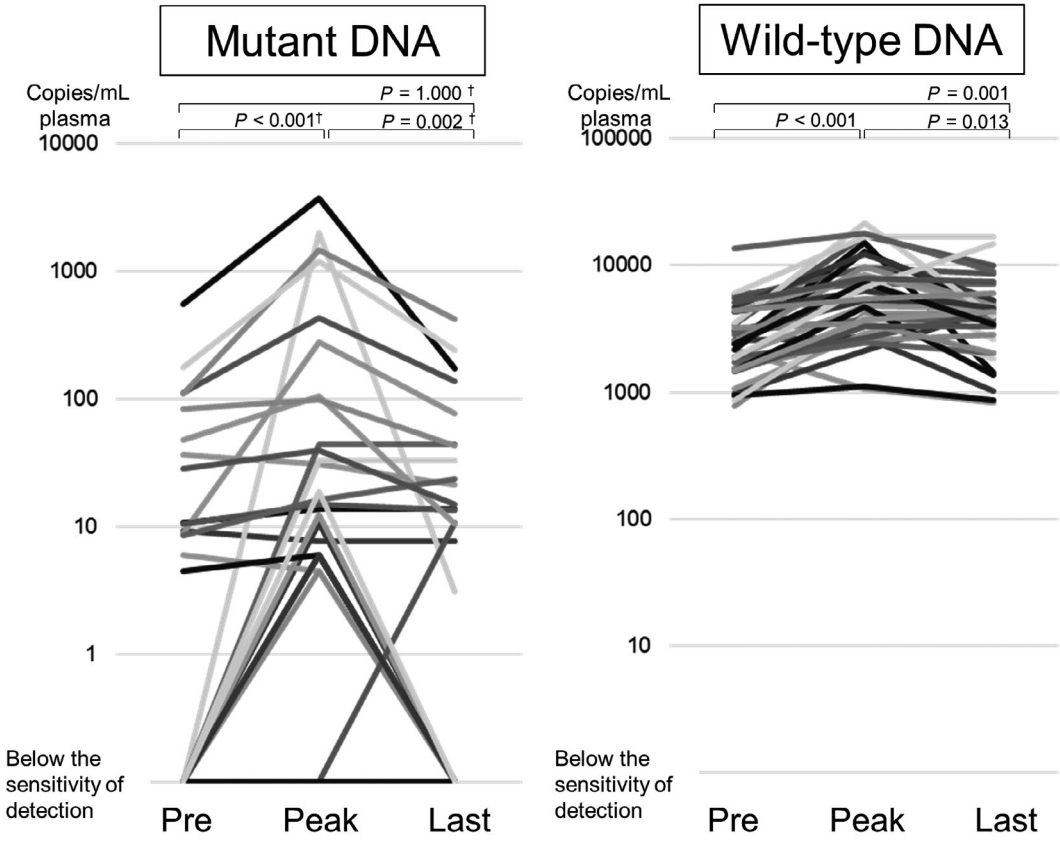

FIG. 5. Mutant DNA and wild-type DNA changes in 35 patients treated with TKI. The average amounts of mutant DNA per 1 mL of plasma were 48.2 copies at prevalue, 385.2 copies at peak value, and 51.7 copies at last value (prevalue vs. peak value, 0.001; prevalue vs. last value, $P = 1.000$; peak value vs. last value, $P = 0.002$). Average amounts of wild-type DNA per 1 mL of plasma were 3,089.2 copies at prevalue, 7,141.3 copies at peak value, and 4,923.5 copies at last value (prevalue vs. peak-value, $P < 0.001$; prevalue vs. last value, $P = 0.001$; peak value vs. last value, $P = 0.013$). †Analyzed in 25 patients positive for mutant DNA.

We measured the amount of cfDNA and its dynamics after TKI and showed that it reached a peak within 1 week after the start of treatment by analyzing cfDNA at 5 or 6 time points (baseline and days 1, 3, 5, 7, and 14). To our knowledge, this study is the first to show short-term dynamic changes in cfDNA levels by obtaining multiple time-point data after TKI treatment of HCC with statistical analysis. Interestingly, continued TKI treatment did not result in a sustained increase in mutant DNA. This finding has also been observed in changes in cfDNA quantity after TKI treatment for lung cancer. Riediger et al.[13] reported a case in which EGFR-targeted mutant DNA reached a peak 1 day after the start of TKI but became undetectable after 1 week. The changes in mutant DNA levels in that report and our study suggest that tumors that respond to TKIs become necrotic within a few days of treatment. Unfortunately, undetectable mutant DNA after TKI initiation does not indicate necrosis of all tumors. In a previous study, the variant allele frequency of *hTERT* promoter mutations in HCC tissues was reported to be approximately 30% and DNA positive for *hTERT* promoter mutations was reported to not reflect all tumor cells.[18] In the future, analysis of DNA levels targeting multiple tumor-specific mutations will be needed, reflecting the information from all tumors, even though it is currently difficult to selectively extract only tumor-derived DNA from blood.

This study also showed that the changes in mutant DNA levels within 1 week of TKI initiation predicted PFS and that the change in mutant DNA levels could be a predictor of long-term treatment response. On the other hand, a favorable short-term response after TKI initiation did not contribute to prolongation of OS. It is not surprising that the effect of initial response to

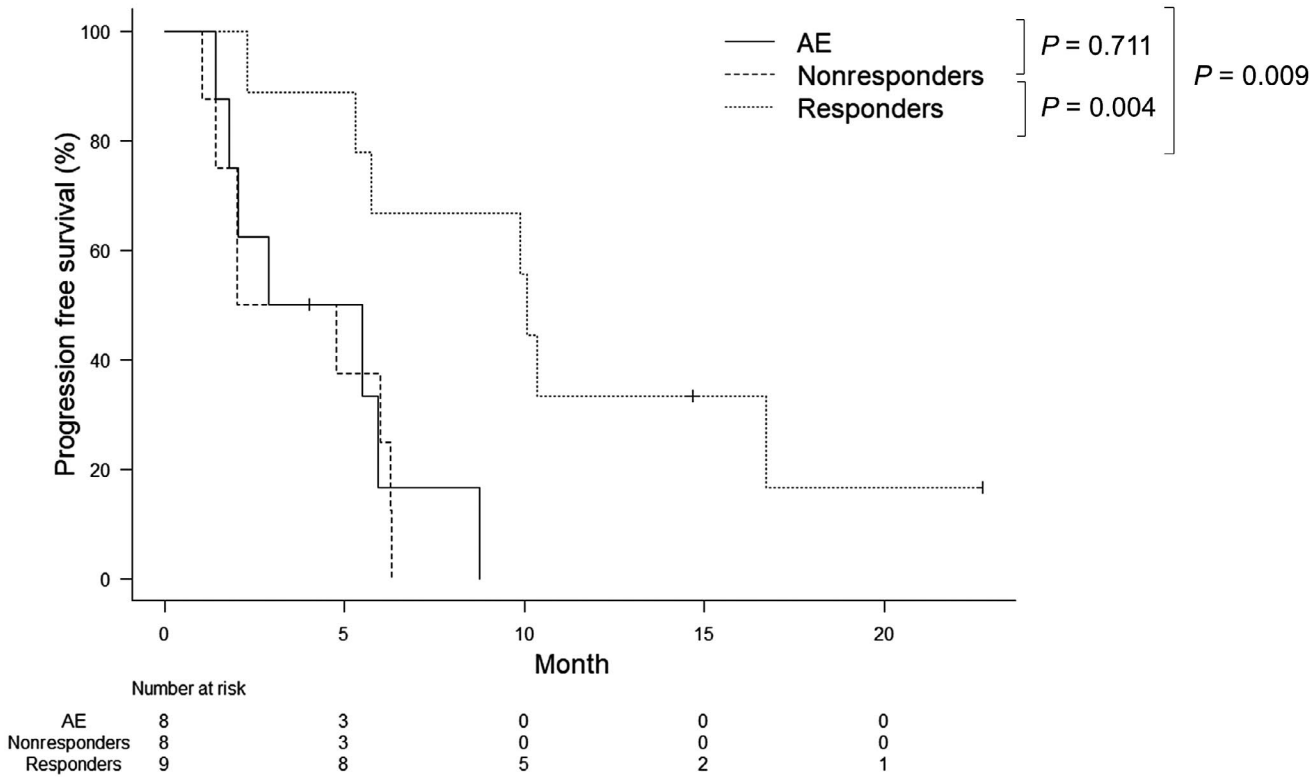

FIG. 6. PFS of patients classified by short-term mutant DNA response in TKI therapy. Responding patients had significantly better PFS than patients with AEs or nonresponders. The median period of PFS was 4.2 months for those with AE, 3.4 months for nonresponders, and 10.1 months for responders. [+]Censored.

TKI is not directly linked to improved survival because the choice of treatment after first-line treatment failure of TKI varies from patient to patient. However, as the number of patients analyzed increases, we suspect that differences may reach significance, although further analysis is required. On the other hand, it is also undeniable that HCCs, which initially responded well to TKIs, became more biologically malignant as soon as they became resistant to TKIs; as a result, the prognosis of patients did not improve. Because recent studies suggest that TKI resistance may be induced by a variety of factors[32,33] and current *hTERT*-targeted cfDNA tests alone cannot distinguish between resistant and sensitive tumors, sensitive detection of small changes that indicate resistance may be key to changing treatment strategies in the future.

There are limitations to this study. First, we analyzed cfDNA targeting only the *hTERT* promoter mutation, which is the most frequent genetic mutation in HCC; however, 30% of tumors are negative for this mutation. Target genes complementary to the *hTERT* promoter mutation should be searched for in tissue- and plasma-derived DNA. Second, the number of analyzed patients was small, especially for patients treated with TKI, many of whom discontinued TKIs due to AEs. These patients were classified as the AE group and were excluded from analysis for the role of mutant and wild-type DNA. Third, we focused on short-term changes in cfDNA levels, and analysis of long-term changes in cfDNA levels also is required.

In conclusion, we demonstrated that the changes in *hTERT* promoter mutant DNA levels in the blood indirectly reflect the amount of tumor necrosis during TACE and TKI therapy. The changes in *hTERT* promoter mutant DNA levels could be a predictor of long-term treatment response, although further studies are needed.

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Supporting Information

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