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Prediction of the prognosis of advanced hepatocellular carcinoma by TERT promoter mutations in circulating tumor DNA

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Key words
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Abstract

Background and Aim: Human telomerase reverse transcriptase (TERT) promoter mutations were the most prevalent mutations in patients with hepatocellular carcinoma (HCC). We tried to detect the mutations with plasma circulating tumor DNA (ctDNA) in patients with advanced HCC and elucidated their clinical utility.

Methods: Circulating tumor DNA in plasma was extracted from 130 patients with advanced HCC who were treated with systemic chemotherapy (n = 86) or transcatheter arterial chemoembolization (n = 44), and TERT promoter mutations were examined with digital droplet polymerase chain reaction. The correlations between these mutations and the clinical outcome of patients were analyzed.

Results: Of the 130 patients examined, 71 patients (54.6%) were positive for TERT promoter mutations in ctDNA, of which 64 patients were −124bp G > A and 10 were −146bp G > A. The presence of TERT promoter mutations was correlated with large intrahepatic tumor size (P = 0.05) and high des-gamma carboxyprothrombin (P = 0.005). Overall survival of the patients with the mutations was significantly shorter than those without them (P < 0.001), and the patients with high (≥ 1%) fractional abundance of the mutant alleles showed shorter survival than those with low (< 1%) fractional abundance. Multivariate analysis revealed that TERT promoter mutation (hazard ratio [HR]: 1.94; 95% confidence interval [CI], 1.18–3.24; P < 0.01), systemic chemotherapy (HR: 2.38; 95% CI, 1.29–4.57; P < 0.01), and vascular invasion (HR: 2.16; 95% CI, 1.22–3.76; P < 0.01) were significant factors for poor overall survival.

Conclusions: TERT promoter mutations in ctDNA were associated with short survival and could be a valuable biomarker for predicting the prognosis of patients with advanced HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and deadliest cancers worldwide.1 Approximately 70–90% of HCC develop on a background of chronic liver disease caused by hepatitis B virus (HBV), hepatitis C virus (HCV), or alcohol intake.2 Disease that is diagnosed at an advanced stage or with progression after locoregional therapy has a dismal prognosis.3 Systemic treatment is recommended in patients with well-preserved liver function (Child–Pugh class A or B) at an advanced stage and at an intermediate stage that is unsuitable for locoregional treatment or transcatheter arterial chemoembolization (TACE).4 The introduction of new targeted agents such as sorafenib, lenvatinib, and regorafenib has drastically changed the systemic treatment of HCC.5 However, as the treatment options increase, there are many clinical questions, such as the timing of treatment changes and prognostic factors.

Hepatocarcinogenesis is a complex multistep process in which many signaling cascades are altered, leading to a heterogeneous molecular profile.6 The main mutations include those in TP53, CTNNB1, and human telomerase reverse transcriptase (TERT). Mutations of the TERT promoter are found in approximately 50% of HCC,7,8 which are the most frequent somatic genetic alterations of HCC and are involved in its early stages. These mutations create a potential binding site for E-twenty six/ternary complex factor transcription factors and increase promoter activity and TERT transcription.9 Several clinical studies have revealed that the presence of TERT promoter mutations was closely correlated with poor prognosis in solid tumors such as lung and breast cancer.10 However, the association between these mutations and clinicopathological features in patients with HCC has not been well elucidated.

Recently, circulating tumor DNA (ctDNA) in plasma has been applied as a non-invasive marker for cancer diagnosis.11 ctDNA is single-stranded or double-stranded DNA released by the tumor cells into the blood and thus harbors the mutations of the original tumor.12 Tissue biopsy is the standard diagnostic procedure for...
cancers and also provides a material for genotyping, which can assist in decisions regarding treatment strategies. However, it is often difficult to obtain tissue clinically from HCC because most cases are hypervascular and the patients often show bleeding tendencies. Moreover, there are other problems with tissue biopsy including seeding and tumor heterogeneity. On these backgrounds, liquid biopsy, which is a minimally invasive procedure, is useful in the clinical practice of HCC.

This study was designed to estimate the clinical utility of plasma ctDNA for detecting TERT promoter mutations in patients with advanced HCC and to reveal the correlation between these mutations and prognosis.

**Methods**

**Patients.** One hundred and thirty consecutive patients with advanced HCC who were treated with systemic chemotherapy (so-rafenib or lenvatinib) or TACE at Okayama University Hospital between September 2013 and September 2018 were enrolled in this study. All patients provided written informed consent for the use of their plasma in this study. We investigated the detection rate of TERT promoter mutations in plasma. Clinical data were taken from an electronic database. This study was approved by our institutional review board, registered at UMIN in 2013 (UMIN000011814), and conducted according to the Helsinki Declaration.

**DNA isolation from plasma.** In all 130 patients, plasma samples were obtained from their blood prior to the treatments. ctDNA was extracted from 1-mL plasma samples with QIAamp Circulating Nucleic Acid Kit (QIAGEN, Hilden, Germany) with the QIAvac 24 Plus vacuum manifold in accordance with the manufacturer’s instructions and was stored at −30°C until analysis. DNA concentration was evaluated with the NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

**Droplet digital polymerase chain reaction.** TERT promoter mutations (−124bp G > A and −146bp G > A) were analyzed by droplet digital polymerase chain reaction (ddPCR; Bio-Rad Laboratories, Hercules, CA, USA).

For detection of −124bp G > A, we used a TERT expert design assay (Bio-Rad Laboratories, Hercules, CA, USA), which was designed to detect TERT promoter mutation at −124bp G > A. Five microliters of DNA from plasma was added to 10-μL droplet PCR supermix (Bio-Rad Laboratories), 1-μL primer/probe mixture (The TERT C228T...88 Assay, Bio-Rad Laboratories), 3.5-μL sterile DNase-free and RNase-free water, 2 μL 5-M betaine, 0.25-μL 80-mM ethylenediaminetetraacetic acid, and 0.25-μL CviQI enzyme. A total of 22 μL mixture was added to 70-μL droplet generator oil (Bio-Rad Laboratories) to generate droplets. The emulsion was thermal cycled, starting with enzyme activation for 10 min at 95°C, followed by 40 cycles of 30 s at 94°C and 1 min at 60°C. The cycling then finished with 10 min at 98°C for enzyme deactivation and holding at 4°C. The rate of temperature rise was set at 2.5°C/s.

For the detection of −146bp G > A, we designed the primer probe set. The primer and probe sequences for each TaqMan assay were as follows: TERT forward primer, 5′-GGCCGCGGAAGTGGAG-3′; TERT reverse primer, 5′-CCCCCTACCTCCAGCTTC-3′; TERT FAM probe(G), 5′-CCCG+G+A+AGG+GG-3′; and TERT HEX probe(A), 5′-CCCG+G+A+AGGG. Two microliters of DNA from plasma was added to 10-μL droplet PCR supermix (Bio-Rad Laboratories), 1-μL primer/probe mixture for FAM, 1-μL primer/probe mixture for HEX, and 6-μL sterile DNase-free and RNase-free water. A total of 20-μL mixture was added to 70-μL droplet generator oil. The emulsion was thermal cycled, starting with enzyme activation for 10 min at 95°C, followed by 40 cycles of 30 s at 94°C and 1 min at 53°C. The cycling then finished with 10 min at 98°C for enzyme deactivation and holding at 4°C. The rate of temperature rise was set at 2.5°C/s.

When the cycling was complete, the fluorescence signal of each droplet was measured by QX200 Droplet Reader (Bio-Rad Laboratories) using QUANTASOFT (Bio-Rad Laboratories). All samples were analyzed in duplicate.

**Statistical methods**

All statistical analyses were performed using JMP Pro 12.0.1 (SAS Institute, Cary, NC, USA). Baseline characteristics were summarized with medians and ranges. Categorical data were analyzed with χ² test or Fisher’s exact test. Overall survival (OS) was calculated according to the Kaplan–Meier method, and Wilcoxon signed-rank test was used to compare OS among patient subgroups.

The following parameters were used to analyze the factors for OS: age, sex, viral markers (HBV surface antigen and HCV antibody), Child–Pugh class, number of tumors, intrahepatic tumor size, vascular invasion, UICC stage, total bilirubin, albumin, aspartate aminotransferase, alanine aminotransferase, platelets, prothrombin activity, α-fetoprotein, des-gamma carboxyprothrombin (DCP), and the presence of TERT promoter mutations in plasma. Variables associated with OS were assessed by Cox proportional hazard model. Variables with P values of < 0.05 in univariate comparison were subjected to multivariate analysis. P < 0.05 was considered statistically significant.

**Results**

**Demographic and clinical characteristics of patients.** Median age was 72 years old, and 89 (68.5%) patients were men. As for HCC treatments, 86 (66.2%) and 44 patients (33.8%) received systemic chemotherapy (sorafenib and/or lenvatinib) and TACE, respectively. HCV antibody and Hepatitis B surface (HBs) antigen were positive in 61 patients (46.9%) and 18 patients (13.8%), respectively. Median intrahepatic tumor size was 30 mm (range, 0–195 mm), and the clinical stages of 27, 25, 35, and 41 patients were II, III, IVa, and IVb, respectively. The median follow-up period was 12.9 months (interquartile range, 7.1–24.0 months) (Table 1).

**Determination of cut-off of amplitude to detect TERT promoter mutation.** The results of ddPCR were displayed as a two-dimensional histogram showing wild-type and mutational types. First, we defined the threshold of
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Table 1  Clinical characteristics of the patients included in the present study

| Variable                          | Value          |
|-----------------------------------|----------------|
| Patients, n                       | 130            |
| Sex, n (%)                        |                |
| Male                              | 89 (68.5)      |
| Female                            | 41 (31.5)      |
| Age, year                         | 72 (42–88)     |
| Treatment, n (%)                  |                |
| Systemic chemotherapy             | 86 (66.2)      |
| TACE                              | 44 (33.8)      |
| Etiology, n (%)                   |                |
| HBV                               | 18 (13.8)      |
| HCV                               | 61 (46.9)      |
| Alcohol                           | 18 (13.8)      |
| Others or unknown                 | 33 (25.4)      |
| Total bilirubin, mg/dL            | 0.8 (0.3–3.0)  |
| Albumin, g/dL                     | 3.5 (2.3–4.5)  |
| Aspartate aminotransferase, IU/L  | 37 (16–510)    |
| Alanine aminotransferase, IU/L    | 27.5 (6–142)   |
| Platelet, ×10^3/μm^3              | 120 (21–414)   |
| Prothrombin activity, %           | 91.5 (23–127)  |
| Tumor number, n (%)               |                |
| 0–1                               | 41 (31.5)      |
| ≥ 2                               | 89 (68.5)      |
| Intrahepatic tumor size, mm       | 30 (0–195)     |
| Child–Pugh class, n (%)           |                |
| A                                 | 100 (76.9)     |
| B                                 | 30 (23.1)      |
| Tumor marker                      |                |
| AFP, ng/mL                        | 29.8 (1.2–415 825) |
| Des-gamma carboxyprothrombin, mAU/mL | 240 (10–926 400) |
| Tumor stage, n (%)                |                |
| II                                | 27 (21.1)      |
| III                               | 25 (19.5)      |
| IVa                               | 35 (27.3)      |
| IVb                               | 41 (32.0)      |
| Vp, n (%)                         |                |
| 0                                 | 101 (77.7)     |
| 1                                 | 9 (6.9)        |
| 2                                 | 12 (9.2)       |
| 3                                 | 5 (3.8)        |
| 4                                 | 3 (2.3)        |
| Follow-up duration, day           | 385.5 (31–2124) |
| hTERT promoter mutation (ctDNA), n (%) | 71 (54.6) |
| −124bp G > A                      | 64 (49.2)      |
| −146bp G > A                      | 10 (7.8)       |

Values are indicated as median (range) unless otherwise noted.

Two cases had no data available.

AFP, α-fetoprotein; ctDNA, circulating tumor DNA; HBV, hepatitis B virus; HCV, hepatitis C virus; TACE, transcatheter arterial chemoembolization.

TERT promoter mutations in plasma. Of the 130 patients examined, 71 patients (54.6%) were positive for TERT promoter mutations in ctDNA, of which 64 patients were −124bp G > A and 10 patients were −146bp G > A. Clinicopathological characteristics according to mutational status are shown in Table 2. The presence of TERT promoter mutations was correlated with large intrahepatic tumor size (P = 0.05) and high DCP (P = 0.005).

Relationship between TERT promoter mutations and overall survival. In this study, we analyzed the relationship between TERT promoter mutations and OS after initiation of therapy by systemic chemotherapy (sorafenib or lenvatinib) or TACE. We calculated and plotted Kaplan–Meier survival curves. The OS of the patients with these mutations was significantly shorter than those without them (P < 0.001; Fig. 1a). Median survival time was 12.8 and 27.0 months in patients with and without TERT promoter mutations in plasma, respectively. The OS of the patients with the mutation of −124bp G > A was significantly shorter than those without it, but not for the mutation of −146bp G > A (Fig. S1).

The same relationship was observed when we divided the patients by treatments. OS of the HCC patients with TERT promoter mutations treated with systemic chemotherapy (median, 11.2 months) was significantly shorter than those without them (median, 22.2 months; P = 0.002; Fig. 1b). Similarly, the OS of the HCC patients with TERT promoter mutations treated with TACE (median, 27.6 months) was significantly shorter than those without them (median, 54.7 months; P = 0.02; Fig. 1c).

We also evaluated the fractional abundance of mutant alleles of TERT promoter in ctDNA among those samples with TERT promoter mutations. The OS of the patients who had high (≥ 1%) fractional abundance was significantly shorter than the patients who had low (< 1%) fractional abundance (Fig. 2).

In univariate analysis with Cox proportional hazards model, TERT promoter mutations (hazard ratio [HR]: 2.19; 95% confidence interval [CI]: 1.36–3.61; P = 0.001), systemic therapy (HR: 3.30; 95% CI: 1.87–6.14; P < 0.001), high α-fetoprotein (HR: 1.85; 95% CI: 1.16–2.97; P = 0.01), high DCP (HR: 2.01; 95% CI: 1.25–3.25; P = 0.003), metastasis (HR: 1.78; 95% CI: 1.07–2.92; P = 0.03), and vascular invasion (HR: 3.31; 95% CI: 1.96–5.48; P = 0.001) were significant factors for poor OS. Multivariate analysis revealed that TERT promoter mutations (HR: 1.94; 95% CI: 1.18–3.24; P = 0.009), systemic chemotherapy (HR: 2.38; 95% CI: 1.29–4.57; P = 0.006), and vascular invasion (HR: 2.16; 95% CI: 1.22–3.76; P = 0.009) were significant factors for poor OS (Table 3).

We further evaluated the effect of HCV and HBV infection on the relationship between TERT promoter mutations in plasma ctDNA and the prognosis of patients. The OS of the HCC patients with HCV and the mutations was significantly shorter than those without the mutations (P < 0.001; Fig. 3a). Median survival time was 12.8 and 34.7 months, respectively. However, no difference in OS was observed in HCC patients with and without TERT promoter mutations in cases of HBV infection (P = 0.77; Fig. 3b). The OS of the HCC patients with non-HBV and non-HCV (NBNC) and the mutations was significantly shorter than those without the mutations (P = 0.02; Fig. S2).

fluorescence amplitude using positive and negative control samples from the plasmid. According to the results, we defined an amplitude greater than 4500 as positive for mutational types and greater than 2500 as positive for wild type (data not shown).
Somatic mutations in the transcriptional regulatory region of the TERT gene have been reported in a range of cancers, including HCC. In this study, we analyzed the ctDNA of 130 HCC patients who were treated with systemic chemotherapy (sorafenib or lenvatinib) or TACE. We detected TERT promoter mutations in 71 cases of the 130 cases analyzed (54.6%) by ddPCR, which was similar to the previously reported prevalence (44.4–65%).7,8,14 The prevalence of the mutations located 124bp and 146bp upstream of the ATG (codon) start site were 49.2% and 7.8%, respectively. Furthermore, we demonstrated that the TERT promoter mutations in ctDNA were closely correlated with a poor prognosis in OS, especially in patients with HCV infection.

**Table 2** Demographic clinical variables in 130 patients with HCC by TERT promoter mutations in circulating tumor DNA

| Variable                        | TERT promoter mutated | TERT promoter non-mutated | P value |
|---------------------------------|-----------------------|---------------------------|---------|
| Patients, n (%)                 | 71 (54.6)             | 59 (45.4)                 |         |
| Gender, n (%)                   |                       |                           | 0.54    |
| Male                            | 47 (66.2)             | 42 (71.2)                 |         |
| Female                          | 24 (33.8)             | 17 (28.8)                 |         |
| Treatment, n (%)                |                       |                           | 0.99    |
| Systemic chemotherapy           | 47 (66.2)             | 39 (66.1)                 |         |
| TACE                            | 24 (33.8)             | 20 (33.9)                 |         |
| Median age (range), year        | 73 (36–88)            | 71 (42–88)                | 0.29    |
| PS, n (%)                       |                       |                           | 0.25    |
| 0                               | 50 (70.4)             | 48 (81.4)                 |         |
| 1                               | 19 (26.8)             | 9 (15.3)                  |         |
| 2                               | 2 (3.4)               | 2 (2.8)                   |         |
| Etiology, n (%)                 |                       |                           | 0.08    |
| HBV                             | 9 (12.7)              | 9 (15.3)                  | 0.67    |
| HCV                             | 35 (49.3)             | 26 (44.1)                 | 0.55    |
| Alcohol                         | 13 (18.3)             | 5 (8.5)                   | 0.11    |
| Others or unknown               | 14 (19.7)             | 19 (32.2)                 | 0.34    |
| Median total bilirubin (range), mg/dL | 0.9 (0.31–2.99) | 0.74 (0.31–1.82) | 0.09    |
| Median albumin (range), g/dL    | 3.5 (2.5–4.5)         | 3.6 (2.3–4.4)             | 0.49    |
| Median aspartate aminotransferase (range), IU/L | 41 (17–510) | 35 (16–213) | 0.22    |
| Median alanine aminotransferase (range), IU/L | 28 (9–121) | 26 (6–142) | 0.29    |
| Median platelet (range), ×10^9/mm$^3$ | 115 (35–414) | 126 (21–297) | 0.95    |
| Median prothrombin activity (range), (%) | 92 (23–127) | 91 (23–124) | 0.56    |
| Tumor number, n (%)             |                       |                           | 0.10    |
| 0–1                             | 18 (25.4)             | 23 (39.0)                 |         |
| ≥ 2                             | 53 (74.7)             | 36 (61.0)                 |         |
| Median intrahepatic tumor size (range), mm | 33 (0–128) | 23 (0–195) | 0.05    |
| Child–Pugh class, n (%)         |                       |                           | 0.80    |
| A                               | 54 (76.1)             | 46 (78.0)                 |         |
| B                               | 17 (22.9)             | 13 (22.0)                 |         |
| Tumor marker                    |                       |                           |         |
| Median AFP (range), ng/mL       | 75.7 (1.4–415,825)    | 17.1 (1.2–17,859)         | 0.07    |
| Median des-gamma carboxyprothrombin (range), mAU/mL | 417 (10–650,210) | 163 (10–926,400) | < 0.01  |
| Tumor stage, n (%)              |                       |                           | 0.63    |
| II                              | 10 (14.1)             | 17 (29.8)                 |         |
| III                             | 18 (25.4)             | 7 (12.3)                  |         |
| Iva                             | 23 (32.4)             | 12 (21.1)                 |         |
| Ivb                             | 20 (28.2)             | 21 (36.8)                 |         |
| Vp, n (%)                       |                       |                           | 0.34    |
| 0                               | 52 (73.2)             | 49 (83.1)                 |         |
| 1                               | 6 (8.5)               | 3 (5.1)                   |         |
| 2                               | 8 (11.3)              | 4 (6.8)                   |         |
| 3                               | 4 (5.6)               | 1 (1.7)                   |         |
| 4                               | 1 (1.4)               | 2 (3.4)                   |         |

Values are indicated as median (range) unless otherwise noted.

AFP, α-fetoprotein; HBV, hepatitis B virus; HCV, hepatitis C virus; PS, Performance Status; TACE, transcatheter arterial chemoembolization.

**Discussion**

Somatic mutations in the transcriptional regulatory region of the TERT gene have been reported in a range of cancers, including HCC. In this study, we analyzed the ctDNA of 130 HCC patients who were treated with systemic chemotherapy (sorafenib or lenvatinib) or TACE. We detected TERT promoter mutations in 71 cases of the 130 cases analyzed (54.6%) by ddPCR, which was similar to the previously reported prevalence (44.4–65%).7,8,14 The prevalence of the mutations located 124bp and 146bp upstream of the ATG (codon) start site were 49.2% and 7.8%, respectively. Furthermore, we demonstrated that the TERT promoter mutations in ctDNA were closely correlated with a poor prognosis in OS, especially in patients with HCV infection.

Telomeres are responsible for the maintenance of chromosomal integrity and genome stability. Telomerase activity is
inactivated during gestation and thereafter is reactivated in 90% of human cancer cells, including HCC. There are two mechanisms of reactivation of TERT activity: (i) through epigenetic regulation and (ii) through somatic mutations in the TERT promoter, which has been recently discovered in other solid tumors. Knowing that telomeres are necessary for cellular self-renewal, the mechanisms responsible for telomere maintenance have a crucial role in cancer development and might be important oncological biomarkers. Wang et al. reported that overexpression of TERT was associated with poor survival in human solid tumors, such as lung cancer, glioblastoma, and breast cancer, and our results showed a similar trend in HCC.

TERT promoter mutations have two hot spots: /C0124bp G > A and /C0146bp G > A. In this study, we demonstrated that the OS of the patients with the mutation of /C0124bp G > A was significantly shorter than those without it, but not for the mutation of /C0146bp G > A. However, we speculated that /C0146bp G > A has the same effect as /C0124bp G > A because previous research showed that both mutations are functionally active. However, this result may be due to the small number of mutated samples with /C0146bp G > A.

Figure 1 Overall survival (OS) curves according to TERT promoter mutational status evaluated by the Kaplan–Meier method. OS of hepatocellular carcinoma patients with TERT promoter mutations was significantly shorter than the patients without the mutations ($P < 0.01$) (a). The same relationship was observed in patients treated with systemic chemotherapy ($P < 0.01$) (b) and transcatheter arterial chemoembolization ($P = 0.02$) (c).

Figure 2 Effect of fractional abundance of mutant alleles of TERT promoter. Overall survival was significantly shorter in patients with high allele frequency than those with low allele frequency ($P < 0.01$). Low allele frequency (< 1%); High allele frequency (≥1%).
The detection of TERT promoter mutations in plasma may reflect an abundance of immortal and rapidly proliferating cells in HCC, which might result in poor prognosis. It is difficult to evaluate the whole tumor information by tissue biopsy, because the amount of tissue biopsy is limited despite the presence of tumor heterogeneity. Differing from tissue biopsy, liquid biopsy might have another potential: ctDNA can be a representative of the genome at the poorest differentiation site in the tumor. We have already clarified the usefulness of liquid biopsy in patients with pancreatic, gastric, and gallbladder cancer, not only for prediction of prognosis but also for detection of the cancers.18–20 These results support the results of the current study with HCC.

The frequency of the mutations was higher in HCV-positive cases (35/61; 57%) than those in HBV-positive cases (9/18; 50%). This is almost the same result as previous reports,21 although there was no statistically significant difference in our study.

**Table 3** Predictors of overall survival for patients with advanced hepatocellular carcinoma

| Variable | Univariate analysis | Multivariate analysis |
|----------|---------------------|----------------------|
|          | HR (95% CI)         | P value | HR (95% CI) | P value |
| With hTERT promoter mutation (−124bp G > A or −146bp G > A) | 2.19 (1.36–3.61) | < 0.01 | 1.94 (1.18–3.24) | < 0.01 |
| With hTERT promoter mutation (−124bp G > A) | 2.55 (1.59–4.15) | < 0.01 |           |           |
| With hTERT promoter mutation (−146bp G > A) | 0.87 (0.30–1.96) | 0.76  |           |           |
| Age (≥ 72 years) | 1.30 (0.81–2.08) | 0.28  |           |           |
| Male | 0.87 (0.54–1.44) | 0.58  |           |           |
| Systemic chemotherapy (versus TACE) | 3.30 (1.87–6.14) | < 0.01 | 2.38 (1.29–4.57) | < 0.01 |
| PS (≥ 1) | 0.69 (0.37–1.19) | 0.18  |           |           |
| HBV (positive) | 1.96 (0.99–3.55) | 0.05  |           |           |
| HCV (positive) | 0.81 (0.50–1.28) | 0.36  |           |           |
| Total bilirubin (≥ 0.8 mg/dL) | 1.48 (0.93–2.38) | 0.1   |           |           |
| Albumin (< 3.5 g/dL) | 1.58 (0.99–2.52) | 0.05  |           |           |
| Aspartate aminotransferase (≥ 37 IU/L) | 1.34 (0.84–2.15) | 0.22  |           |           |
| Alanine aminotransferase (≥ 27.5 IU/L) | 0.85 (0.53–1.37) | 0.51  |           |           |
| Platelet (≥ 120 × 10^3/mm^3) | 1.50 (0.94–2.41) | 0.09  |           |           |
| Prothrombin activity (≥ 91.5%) | 1.0 (0.62–1.59) | 0.99  |           |           |
| Number of tumors ≥ 2 (vs 0–1) | 1.51 (0.91–2.58) | 0.11  |           |           |
| Intrahepatic tumor size (≥ 30 mm) | 1.10 (0.69–1.78) | 0.68  |           |           |
| Child–Pugh class B (versus A) | 1.33 (0.75–2.15) | 0.33  |           |           |
| AFP (≥ 29.75) | 1.85 (1.16–2.97) | 0.01  | 1.20 (0.71–2.03) | 0.49 |
| Des-gamma carboxyprothrombin (≥ 240) | 2.01 (1.25–3.25) | 0.003 | 1.62 (0.98–2.71) | 0.06 |
| With metastasis | 1.78 (1.07–2.92) | 0.03  | 1.34 (0.77–2.33) | 0.30 |
| Vascular invasion (positive) | 3.31 (1.96–5.48) | < 0.01 | 2.16 (1.22–3.76) | < 0.01 |

AFP, α-fetoprotein; CI, confidence interval; HBV, hepatitis B virus; HCV, hepatitis C virus; HR, hazard ratio; PS, Performance Status; TACE, transcatheter arterial chemoembolization.

**Figure 3** Effect of TERT promoter mutational status in hepatocellular carcinoma (HCC) patients with different viral infections. TERT promoter mutations were associated with poor prognosis in (a) hepatitis C virus-related HCC patients (P < 0.01) but not in (b) hepatitis B virus-related patients (P = 0.77). ———, Mutations(−); ———, Mutations(+).
Moreover, we demonstrated that TERT promoter mutations were associated with poor prognosis in HCV-related HCC but not HBV-related HCC. The most likely explanation is that HBV-associated HCCs involve other mechanisms of maintaining telomere integrity.\textsuperscript{22} It has been reported that the TERT promoter is one of the most frequent integration sites for the HBV genome in HCC. Inserting a viral genome causes the juxtaposition of viral enhancers to be near the TERT gene and the potential activation of TERT expression.\textsuperscript{23} Kawai-Kitahata et al.\textsuperscript{8} reported that HBV integration into the TERT locus was found in 47% HBV-related HCC cases who were negative for TERT promoter mutations and most (89%) HBV integrants were in the HBx region, which was known to have effects on the transcriptional levels of many genes.

We also showed in this study that the patients who had a higher fractional abundance of TERT promoters in ctDNA had a poorer prognosis. The rapid turnover of cancer cells may have caused this result. This appears to further emphasize that TERT mutations are associated with prognosis. However, there are few reports describing the fractional abundance of TERT promoter mutations, and further research is needed.

This study has some limitations. First, this is a retrospective cohort and a single-center study. Prospective and multiple-center studies are warranted for further verification in other patient populations. Second, we only focused on TERT promoter mutations. Combined analysis of other mutated genes such as TP53 and CTNNB1 might result in precise prognosis prediction. Another limitation is the difficulties in determining the origin of the TERT promoter mutations in plasma. This is a universal weak point of liquid biopsy. We performed a previous study to elucidate the positivity between tissue and plasma or serum of HCC patients who had surgical resection, but more strong evidence might be made, if sampling tissue from patients who had systemic therapy prior to the treatment.

In conclusion, TERT promoter mutations in ctDNA were associated with poor prognosis and could be a good target for liquid biopsy. Further research is needed to confirm the clinical usefulness of TERT promoter mutations and of other mutations that lead to more accurate prediction of prognosis and aid in the selection of treatment strategies.

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Supporting information

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

Figure S1. The overall survival curves according to the telomerase reverse transcriptase promoter mutation status of -124bp G>A (A) and -146bp G>A (B).

Figure S2. The overall survival of the patients with NBNC-HCC according to the telomerase reverse transcriptase promoter mutation status.