Received: 17 September 2024 | Accepted: 27 December 2024

DOI: 10.1097/HC9.0000000000000652

ORIGINAL ARTICLE

OPEN

Multiple features of cell-free mtDNA for predicting transarterial chemoembolization response in hepatocellular carcinoma

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Abstract

Background: Transarterial chemoembolization (TACE) is the primary treatment modality for advanced HCC, yet its efficacy assessment and prognosis prediction largely depend on imaging and serological markers that possess inherent limitations in terms of real-time capability, sensitivity, and specificity. Here, we explored whether multiple features of cell-free mitochondrial DNA (cf-mtDNA), including copy number, mutations, and fragmentomics, could be used to predict the response and prognosis of patients with HCC undergoing TACE treatment.

Methods: A total of 60 plasma cell-free DNA samples were collected from 30 patients with HCC before and after the first TACE treatment and then subjected to capture-based mtDNA sequencing and whole-genome sequencing.

Results: Comprehensive analyses revealed a clear association between cf-mtDNA multiple features and tumor characteristics. Based on cf-mtDNA multiple features, we also developed HCC death and progression risk prediction models. Kaplan-Meier curve analyses revealed that the high-death risk or high-progression–risk group had significantly shorter median overall survival (OS) and progression-free survival than the low-death risk or low-progression-risk group (all $p < 0.05$). Moreover, the change in cf-mtDNA multiple features before and after TACE treatment exhibited an exceptional ability to predict the risk of

Abbreviations: AASLD, American Association for the Study of Liver Diseases; cf-mtDNA, cell-free mitochondrial DNA; cf-DNA, cell-free DNA; CN, copy number; CNV, copy number variant; HC, healthy control; HDR, HCC death risk; HPP, HCC prognosis prediction; HPR, HCC progression risk; MAF, minor allele frequency; mRECIST, Modified Response Evaluation Criteria in Solid Tumors; mtDNA, mitochondrial DNA; mtDNA Cap-seq, capture-based mtDNA sequencing; OS, overall survival; PD, progressive disease; PFS, progression-free survival; PR, partial response; SD, stable disease; TACE, transarterial chemoembolization; WGS, whole-genome sequencing.

Dang Miao, Wang Siyuan, and Peng Fan contributed equally.

Supplemental Digital Content is available for this article. Direct URL citations are provided in the HTML and PDF versions of this article on the journal's website, www.hepcommjournal.com.

death and progression in patients with HCC (log-rank test, all $p < 0.01$; HRs: 0.36 and 0.33, respectively). Furthermore, we observed the consistency of change between the cf-mtDNA multiple features and copy number variant burden before and after TACE treatment in 40.00% (12/30) patients with HCC. **Conclusions:** Altogether, we developed a novel strategy based on profiling of cf-mtDNA multiple features for prognosis prediction and efficacy evaluation in patients with HCC undergoing TACE treatment.

Keywords: cell-free mitochondrial DNA, HCC, multiple features, prognosis, transarterial chemoembolization

INTRODUCTION

HCC, a primary liver malignancy, poses a substantial global health burden owing to its high incidence, aggressive nature, and limited therapeutic options, especially in advanced stages. Among various therapeutic strategies, transarterial chemoembolization (TACE) remains the cornerstone treatment for advanced HCC, offering a palliative yet effective means of controlling tumor growth. The Modified Response Evaluation Criteria in Solid Tumors (mRECIST) assessment is recommended for evaluating the efficacy of TACE treatment based on the reduction of the arterially hyperenhancing viable tumor diameter by CT or MRI. However, the image-based assessment method is easily interfered with by numerous factors (eg, tumor location, lipiodol deposition, coagulative hemorrhage, and necrosis) and greatly relies on the experience of radiologists, which makes it relatively subjective. In addition, the image-based assessment method is less time-sensitive. Therefore, response to TACE varies widely among patients, necessitating the identification of predictive factors to guide personalized treatment decisions.

Liquid biopsy, as a noninvasive detection method, plays a significant role in predicting the efficacy of TACE treatment.[1–3] Previous studies have shown that single-nucleotide variants and copy number variants (CNVs) identified from cell-free DNA (cfDNA) are highly consistent with the genetic profiles of HCC tissues and can reflect tumor burden in real-time.[4,5] Dong et al[6] have demonstrated that CNVs in cfDNA could serve as a strategy for evaluating TACE treatment outcomes in patients with HCC. Sefrioui et al[7] have suggested that single-nucleotide variants in cfDNA may predict disease progression after TACE treatment. Previous studies have mainly focused on cell-free nuclear DNA (cf-nDNA) but ignored the existence of mitochondrial DNA (mtDNA) in plasma.

The mtDNA, with a length of 16,569 bp, residing within the mitochondria, plays a crucial role in energy production, cellular metabolism, and apoptosis. Its unique features, including a high copy number (CN) per cell and high mutation rate because of the lack of a DNA repair system and histone protection, make mtDNA an attractive target for biomarker development. Previously, we have developed a versatile and cost-effective capture-based sequencing approach for the comprehensive detection of cell-free mitochondrial DNA (cf-mtDNA) with multiple features, including mtDNA CN, mtDNA mutations, and mtDNA fragmentomics.[8,9] Moreover, our study has demonstrated that tumor-derived mtDNA mutations are present in the plasma.[9] We have also demonstrated that mtDNA CN and their fragmentomic features are sensitive and cost-effective biomarkers for cancer detection (under publication). However, whether cf-mtDNA multiple features can be used to predict the efficacy has not been reported.

To address this issue, we analyzed cf-mtDNA multiple features in 30 patients with HCC before and after the first TACE treatment using capture-based ccf-mtDNA sequencing data. Then, we comprehensively evaluated the clinical value of cf-mtDNA multiple features in predicting the efficacy of TACE treatment by comparing it with the CNV burden.

METHODS

Patient enrollment

A total of 30 patients with HCC undergoing TACE were enrolled from the Tangdu Hospital of Fourth Military Medical University (May 2019 to August 2019). Patients with HCC were diagnosed based on the American Association for the Study of Liver Diseases (AASLD) guidelines.[10] All patients received TACE treatment after being diagnosed with HCC and were followed up every 3 months, with a final follow-up period of February 2024. All patients died of HCC during follow-up, with a median follow-up duration of 10.5 months (range: 3–54 mo). Overall survival (OS) was defined as the

time from diagnosis to death, and progression-free survival (PFS) was defined as the time from diagnosis to cancer progression or death, whichever occurred first. The clinical characteristics of patients are shown in Figure 1B. The tumor stage was determined according to the Barcelona Clinic Liver Cancer (BCLC) staging system. This study was conducted in accordance with the Declarations of Helsinki and Istanbul, ensuring that all research involving human participants adhered to the highest ethical standards. The study was approved by

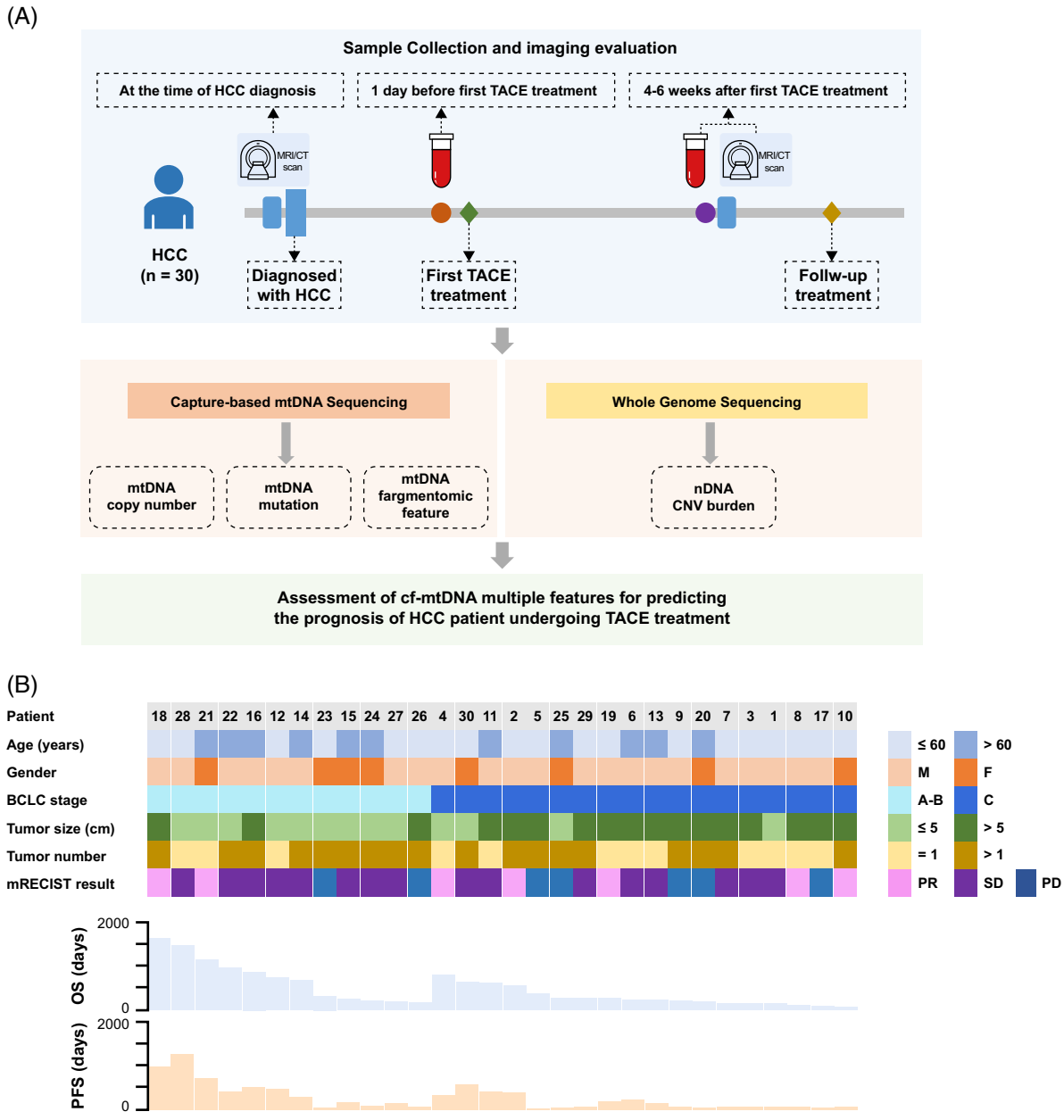


FIGURE 1 Study design and characteristics of patients. (A) Study design. Blood samples were collected from patients with HCC in 2 distinct phases: the first sample was collected from patients with HCC 1 day before the first TACE treatment, and a second sample was collected concurrently with the imaging evaluation performed at 4–6 weeks after the first TACE treatment. Each sample was subjected to both capture-based mtDNA sequencing and whole-genome sequencing. Subsequently, capture-based mtDNA sequencing was used to analyze the mtDNA copy number, mtDNA mutations, and the cf-mtDNA fragmentomic features. Meanwhile, whole-genome sequencing was employed to analyze CNV burden. Finally, we conducted an assessment of cf-mtDNA multiple features to predict the prognosis of patients with HCC undergoing TACE treatment. (B) Patient characteristics. Age, gender, BCLC stage, tumor size, tumor number and mRECIST result of each patient were shown in each column. In gender, M represents Male, while F represents Female. According to mRECIST, patients with HCC were classified into 4 categories: complete response (CR), partial response (PR), progressive disease (PD), and stable disease (SD). OS and PFS of patients were shown in days. Abbreviations: BCLC, Barcelona Clinic Liver Cancer; cf-mtDNA, cell-free mtDNA; DNA, nuclear DNA; mRECIST, Modified Response Evaluation Criteria in Solid Tumors; mtDNA, mitochondrial DNA; OS, overall survival; PD, progressive disease; PFS, progression-free survival; PR, partial response; SD, stable disease; TACE, transarterial chemoembolization.

the Institutional Review Board of Tangdu Hospital, Fourth Military Medical University (permission number: KY20183331-1; Date issued: 2018-03-08). Written informed consent was obtained from all participants.

mRECIST evaluation

mRECIST evaluation was carried out by at least 2 experienced radiologists based on postoperative MRI or CT images within 4–6 weeks. TACE responses in patients with HCC were classified into 4 categories: complete response, disappearance of all target lesions; partial response (PR), at least a 30% decrease in the sum of diameters of viable target lesions; progressive disease (PD), minimum 20% increase in the sum of the diameters of viable target lesions; and stable disease (SD), between PR and PD.

Sample collection, DNA extraction, library construction, and sequencing

Peripheral blood (5 mL/patient) was collected into EDTA tubes 1 day before and 4–6 weeks after the first TACE treatment, with plasma isolated within 2 hours and stored at −80°C for no >3 months until DNA extraction. cfDNA was extracted using the QIAamp Circulating Nucleic Acid Kit (Qiagen) and quantified a Qubit 4.0 (Thermo Fisher). The whole-genome sequencing (WGS) libraries were constructed for plasma cfDNA (10–20 ng/sample) using an NEB Ultra v2 kit (NEB).[11,12] As previously described, capture-based mtDNA sequencing (mtDNA Cap-seq) was performed using homemade biotinylated probes.[8,9] Both the WGS and captured mtDNA libraries were sequenced on an Illumina HiSeq XTen platform using paired-end runs with 2×150 cycles (PE 150).

Sequencing data analysis

Illumina adapters and low-quality bases (Q < 30) were trimmed from raw sequencing data using the Fastp software (v.0.20.0).[13] WGS data were mapped and aligned to the human genome reference (hg19) using the BWA software (v.0.7.1521). And mtDNA Cap-seq data were aligned to both revised Cambridge reference sequence of the human mitochondrial DNA and hg19 using BWA software (v.0.7.1521) to diminish contaminations from nuclear sequences of mitochondrial origin, retaining only those mapped to revised Cambridge reference sequence with high mapping quality (MAPQ ≥ 20). All Reads were sorted and de-duplicated using Picard Tools (v.1.119) and Picard Mark Duplicates (v.1.81). Finally, local realignment was performed using Genome Analysis Toolkit 4 (v.3.2-2).

Calculation of mtDNA CN

Based on the method established by our group,[8] the average sequencing depth of mtDNA and the nuclear reference gene was used to calculate the relative mtDNA CN.[14] The following formula was used to calculate the mtDNA CN: 2 × average depth of mtDNA/average depth of the nuclear reference gene.

MtDNA mutation calling

First, we counted the read numbers of the major and minor alleles for each site of the mitochondrial genome and calculated the site-specific minor allele frequency (MAF). Then, the mtDNA mutation calling was carried out according to the following filter conditions: (1) at least 3 reads on each strand for the mutation site; (2) minimum MAF cutoff of 1%; (3) to remove heterogeneity sites in revised Cambridge reference sequence repeat regions (66–71, 303–311, 514–523, 12418–12425, 16184–16193); (4) to remove C > A / G > T mutations with low MAF (<10%), which is known to arise from artificial guanine oxidation during sequencing library preparation; (5) to remove mtDNA mutations if the mutant rate and mutant base quality do not pass binominal test ($p > 0.001$).[15] Finally, mutations with an MAF < 50% were used for further analysis.

Analysis of cf-mtDNA fragmentomic features

The fragmentomic features, including fragment size, fragmentation profile, 5′ end base preference, and 5′ end motif diversity score, were extracted from the mtDNA Cap-seq data as described in our previous study (under publish). First, fragment size was determined using Picard tools, categorizing fragments as short or long based on the median size per sample. Next, we computed fragment counts spanning mtDNA sites (1–16569), defined fragment lengths by median size, and assessed the fragment size distribution (FSD) score by comparing long-to-short fragment coverage adjusted for GC content via LOESS. The FSD scores were standardized as z-scores, enabling the depiction of fragmentation profiles across all mtDNA sites in each cf-mtDNA sample. Spearman rank correlation coefficient was calculated between the ccf-mtDNA fragmentation profile of each sample and the median healthy control (HC) profile, which was generated in a previous study. The peak on the fragmentation profile was defined by setting the peak width ≥5 bp. Compared to the peaks in the median HC profile, new peaks were defined as those with a distance of more than 20 bp, and the mitochondrial genome was equally divided into 255 windows. The areas of the positive and

negative peaks were calculated for each window at a baseline FSD score of zero. Then, the Euclidean distance from each fragmentation profile to the median HC profile was calculated within each window using the Scipy software (v.1.6.2).

Additionally, paired-end sequencing reads were analyzed to determine 5′ end base preferences and the proportion of 5' end 4-mer motifs for both the heavy and light strands of cf-mtDNA. In brief, the proportion of 5′ end base was normalized based on the base composition of the mitochondria reference genome, and the 5′ end base preference was finally defined. To reflect the frequency distribution of 5′ end 4-mer motifs, we adopted the normalized Shannon entropy as a mathematical approach to calculate the 5′ end motif diversity score.

In our previous study, based on the cf-mtDNA fragmentomic features, we developed a HCC prognosis prediction (HPP) model using the LASSO-Cox regression analysis and calculated the HPP score,which was significantly associated with death risk. Therefore, in the present study, we calculated the HPP score for each sample of 30 patients with HCC based on previous HPP model.

Calculation of CNV burden

The CNV burden was calculated as described.[14] Briefly, a stability score (S-score) was defined to reflect the extent of CNV deviation from the diploid genome and was calculated using the following formula:

$$CNV\ burden = \sqrt{\frac{\sum_i^n (Xi - 0)^2}{n}}$$

where Xi is the $\log_2$ ratio of the bin-specific value calculated using the ichorCNA algorithm, 0 represents the desirable $\log_2$ ratio of the diploid genome, and n is the number of bins (n = 2475).

Construction of the HCC death and progression risk prediction models

Cox proportional hazards analysis was employed to evaluate the influence of varying combinations of cf-mtDNA multiple features (including cf-mtDNA CN, mutation, and fragmentomics) and CNV burden on patients' survival outcomes, resulting in the calculation of coefficients for each feature within each combination (Supplemental Table S1, http://links.lww.com/HC9/B901). For each patient, we computed the risk score by adding the weighted values of each feature, where the weight of each value was determined by its corresponding coefficient obtained from the Cox proportional hazards model. We named the model that incorporates 3 cf-mtDNA features for predicting the OS and PFS of patients as the HCC death risk (HDR) prediction model and the HCC progression risk (HPR) prediction model, respectively. Subsequently, the HDR and HPR scores were obtained. This was done for the data of all plasma samples collected before and after TACE treatment. Leave-one-out cross-validation was used to evaluate model performance.

Statistical analysis

Statistical analyses were performed using GraphPad Prism v.9.5.1 (GraphPad Software). The Mann-Whitney *U* test was used to compare differences between 2 groups with continuous variables. Spearman rank correlation coefficient was calculated to assess the association between cf-mtDNA multiple features and the clinical characteristics of patients with HCC. Kaplan-Meier analysis with a log-rank test was used to compare PFS and OS among different groups of patients, and associations were expressed as HR with 95% CI. All reported *p* values were two-tailed, with a significance level of 0.05.

RESULTS

Study design and characteristics of patients

To investigate the clinical value of cf-mtDNA multiple features in patients with HCC undergoing TACE, a total of 60 plasma samples were collected from 30 patients with HCC 1 day before TACE and 4–6 weeks after the first TACE and then subjected to WGS and mtDNA cap-seq. The detailed study design is shown in Figure 1A. The clinical characteristics before TACE treatment for 30 patients with HCC were presented in Figure 1B. The median diameter of tumors among them was 5.40 cm (range, 2.10–13.00 cm), 60.0% (18/30) of them had multiple tumors, and 60.00% (18/30) of them were classified as BCLC stage C. After the first TACE treatment, according to the mRECIST criteria, seven patients (23.3%) achieved PR, 16 patients (53.3%) had SD, and 7 patients (23.3%) showed PD.

The analysis of cf-mtDNA multiple features in patients with HCC before TACE treatment

Tumor stage, number, and size are essential indicators of HCC severity and prognosis. Therefore, we first analyzed the relationship between cf-mtDNA multiple features, including mtDNA CN, number of mtDNA mutations, and mtDNA fragmentomics with the HPP score as the quantification index and the tumor

characteristics of patients with HCC before TACE treatment. As shown in Figure 2A, our data revealed that patients at BCLC stage C exhibited significantly higher mtDNA CN, number of mtDNA mutations, and HPP score than patients at BCLC stage A–B. Furthermore, significant increases in mtDNA CN, number of mtDNA mutations, and HPP score were observed in patients with HCC with bigger tumor size (>5 cm) with no <2 tumor sites (>1) (all $p<0.05$, Figure 2B, C). However, no significant differences in cf-mtDNA multiple features were observed among the groups with different tumor numbers (all $p>0.05$). In addition, positive correlations were observed between cf-mtDNA features and tumor sizes, AFP, or AST levels in 30 patients with HCC before TACE (Supplemental Figure S1, http://links.lww.com/HC9/B902).

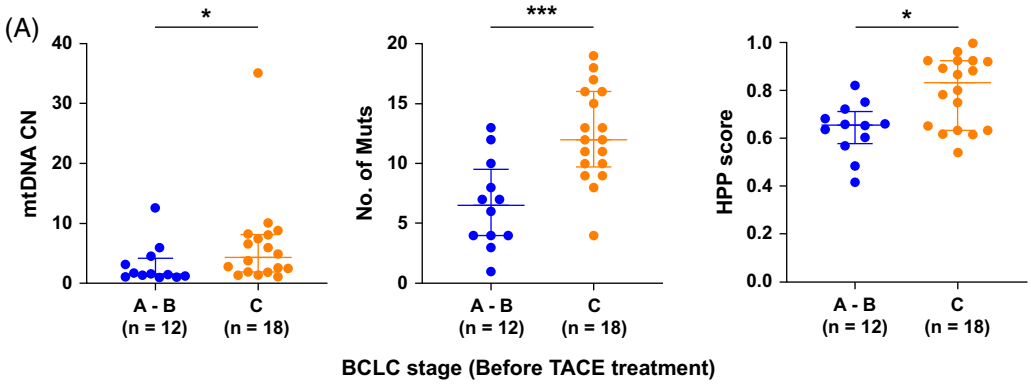
To further investigate whether pre-TACE cf-mtDNA multiple features could provide a prognostically relevant classification of patients, we employed Cox proportional hazards analysis to construct HCC prognosis prediction models based on different combination of mtDNA CN, number of mtDNA mutations, and HPP score. As shown in Supplemental Table S2, http://links.lww.com/HC9/B901 and Supplemental Figure S2, http://links.lww.com/HC9/B902, and Figure 2D, E, the combination of mtDNA CN, number of mtDNA mutations, and HPP score showed optimum performance for predicting HCC prognosis compared to any other combination or one of these features alone. The patients with HCC were stratified into high-risk and low-risk groups based on the median HDR score of 4.56 from the death risk prediction model and the median HPR score of 5.31 from a progression risk prediction model, both of which were constructed using 3 cf-mtDNA multiple features. Subsequently, Kaplan-Meier curve analyses revealed a significantly shorter median OS and PFS in the high-risk group compared to the low-risk group (log-rank test, all $p<0.01$; HR: 0.30 and 0.37, respectively). Moreover, we examined the relationship between HDR or HPR scores and the variables of HCC progression. Notably, as shown in Supplemental Figure S3, http://links.lww.com/HC9/B902, both HDR and HPR scores were significantly correlated with tumor characteristics. These results suggest that pre-TACE cf-mtDNA multiple features may serve as effective prognostic biomarkers, reflecting the tumor burden in HCC.

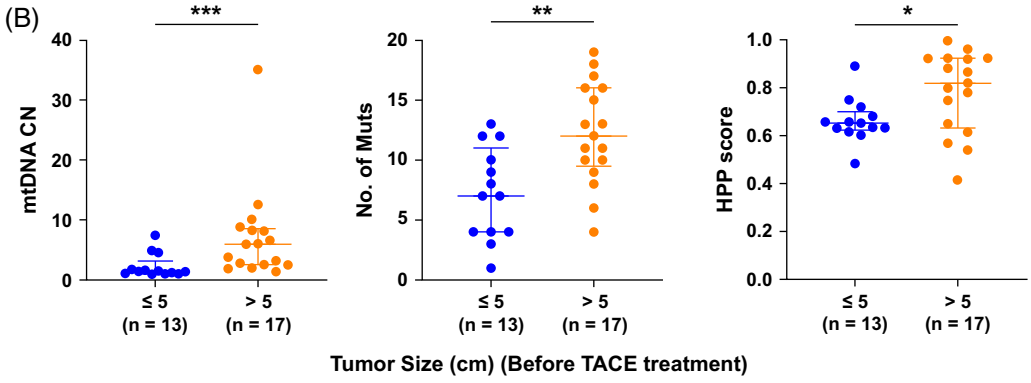
The clinical value of post-TACE cf-mtDNA multiple features in patients with HCC

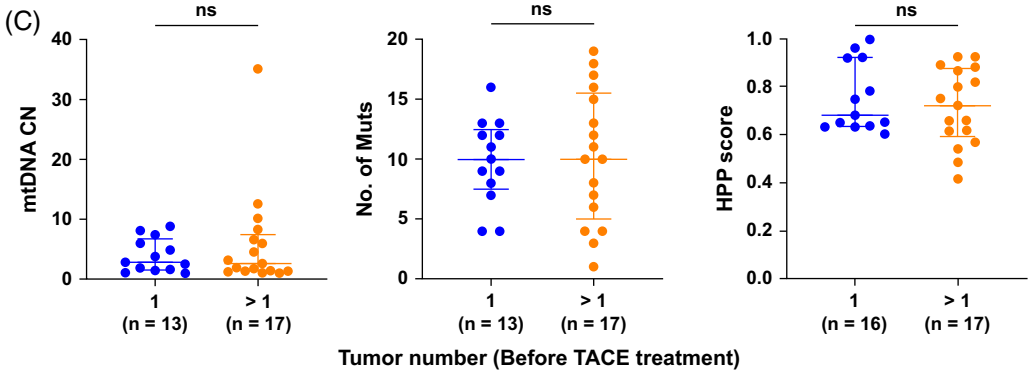
To evaluate whether cf-mtDNA multiple features could reflect tumor burden after TACE treatment, we analyzed the relationship between cf-mtDNA multiple features in post-TACE plasma samples and post-TACE tumor characteristics. As shown in Figure 3A, patients with HCC of different tumor sizes exhibited significant differences in cf-mtDNA multiple features. Specifically, larger tumors were associated with higher mtDNA CN, number of mtDNA mutations, and HPP scores. Furthermore, positive correlations were observed between cf-mtDNA multiple features and tumor sizes, AFP, or AST levels (Supplemental Figure S4, http://links.lww.com/HC9/B902). Next, we evaluated the clinical value of post-TACE cf-mtDNA multiple features for prognosis prediction. As shown in Supplemental Table S3, http://links.lww.com/HC9/B901, the combination of mtDNA CN, the number of mtDNA mutations and the HPP score exhibited optimum performance for predicting HCC prognosis when compared with any other combination or one of these features alone. Patients with HCC were stratified into high-risk and low-risk groups based on a median HDR score of 1.99 from a death risk prediction model and a median HPR score of 3.81 from a progression risk prediction model, both constructed using post-TACE cf-mtDNA multiple features. Subsequently, Kaplan-Meier curve analyses revealed a significantly shorter median OS and PFS in the high-risk group than in the low-risk group (log-rank test, all $p<0.01$; HR: 0.33 and 0.28, respectively). We found that the post-TACE HDR and HPR scores were closely related to tumor characteristics (Supplemental Figure S5, http://links.lww.com/HC9/B902). These results suggest that post-TACE cf-mtDNA multiple features may be used to predict HCC prognosis.

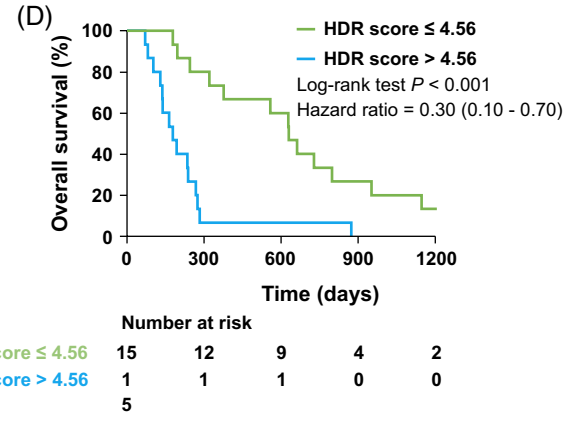
Efficiency assessment of TACE treatment using cf-mtDNA multiple features

It is widely known that mRECIST, based on image diagnosis, serves as a benchmark for evaluating the efficacy of TACE treatment. Here, according to the mRECIST criteria, among 30 patients with HCC undergoing the first TACE treatment, 7 (23.3%) patients achieved PR, 16 (53.3%) patients had SD, and 7 (23.3%) patients showed PD. To investigate whether the dynamic change in cf-mtDNA multiple features could reflect therapeutic efficacy, we calculated the decrease in cf-mtDNA multiple features between the pre-TACE and post-TACE groups. As shown in Figure 4A–C, when the threshold for absolute value change of all 3 cf-mtDNA features was set to 0, among the 30 patients with HCC, 14 (46.7%) patients showed a decrease in mtDNA CN, 23 (76.7%) patients showed a decrease in the number of mutations, and 20 (67.7%) patients showed a decrease in HPP score. As shown in Figure 4A, the PR and SD groups in the mRECIST criteria significantly overlapped with the decreased mtDNA CN group, and the PD group was significantly enriched in the group with increased mtDNA CN. Similar results were observed for the number of mutations and HPP


(A)
mtDNA CN
No. of Muts
HPP score
*
***
*
A - B (n = 12)
C (n = 18)
BCLC stage (Before TACE treatment)


(B)
mtDNA CN
No. of Muts
HPP score
***
**
*
≤ 5 (n = 13)
> 5 (n = 17)
Tumor Size (cm) (Before TACE treatment)


(C)
mtDNA CN
No. of Muts
HPP score
ns
1 (n = 13)
> 1 (n = 17)
1 (n = 16)
> 1 (n = 17)
Tumor number (Before TACE treatment)


(D)
Overall survival (%)
HDR score ≤ 4.56
HDR score > 4.56
Log-rank test P < 0.001
Hazard ratio = 0.30 (0.10 - 0.70)
Time (days)
Number at risk
HDR score ≤ 4.56 15 12 9 4 2
HDR score > 4.56 15 1 1 1 0 0

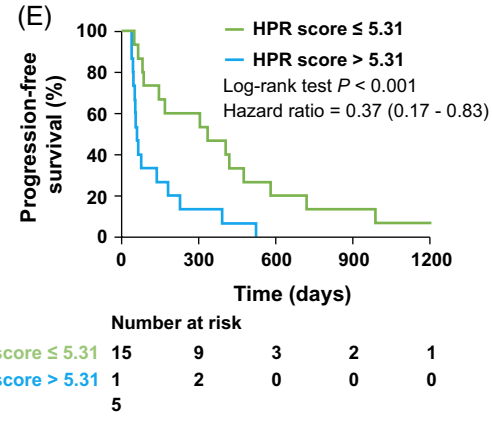

(E)
Progression-free survival (%)
HPR score ≤ 5.31
HPR score > 5.31
Log-rank test P < 0.001
Hazard ratio = 0.37 (0.17 - 0.83)
Time (days)
Number at risk
HPR score ≤ 5.31 15 9 3 2 1
HPR score > 5.31 15 2 0 0 0

FIGURE 2 The analysis of ccf-mtDNA multifeatures in patients with HCC before TACE treatment. Comparison of mtDNA CN, number of Muts, and HPP score between (A) BCLC stages A-B and stage C groups, (B) different tumor size groups, and (C) different tumor number groups. (D) The Kaplan-Meier survival curve was used to compare the overall survival in patients with HCC with an HDR score ≤ 4.56 (green) with those with an HDR score > 4.56 (blue) based on the HCC death risk prediction model. (E) The Kaplan-Meier survival curve was used to compare the progression-free survival in patients with HCC with an HPR score ≤ 5.31 (green) with those with an HPR score > 5.31 (blue) based on the HCC progression risk prediction model. HPP scores were calculated from a developed HCC prognosis prediction model based on cf-mtDNA fragmentomic features in a previous study. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. In A–C, the center line indicates the median and the lower and upper hinges represent the 25th and 75th percentiles, respectively. Abbreviations: BCLC, Barcelona Clinic Liver Cancer; CN, copy number; HDR, HCC death risk; HPP, HCC prognosis prediction; HPR, HCC progression risk; mtDNA, mitochondrial DNA; No. of Muts, number of mtDNA mutations; TACE, transarterial chemoembolization.

scores (Figure 4B, C). Furthermore, post-TACE cf-mtDNA multiple features were significantly different between patients achieving PR/SD and PD (all $p < 0.05$, Figure 4D–F). These results indicate that the assessment of TACE efficacy using alterations in cf-mtDNA multiple features is in good agreement with the mRECIST evaluations.

cf-mtDNA multiple features-based strategy markedly outperforms CNV burden for HCC prognosis prediction

Previous studies have demonstrated that CNV burden reflects tumor burden in real time.[6] Similar to the results of cf-mtDNA multiple features, the PR groups in the

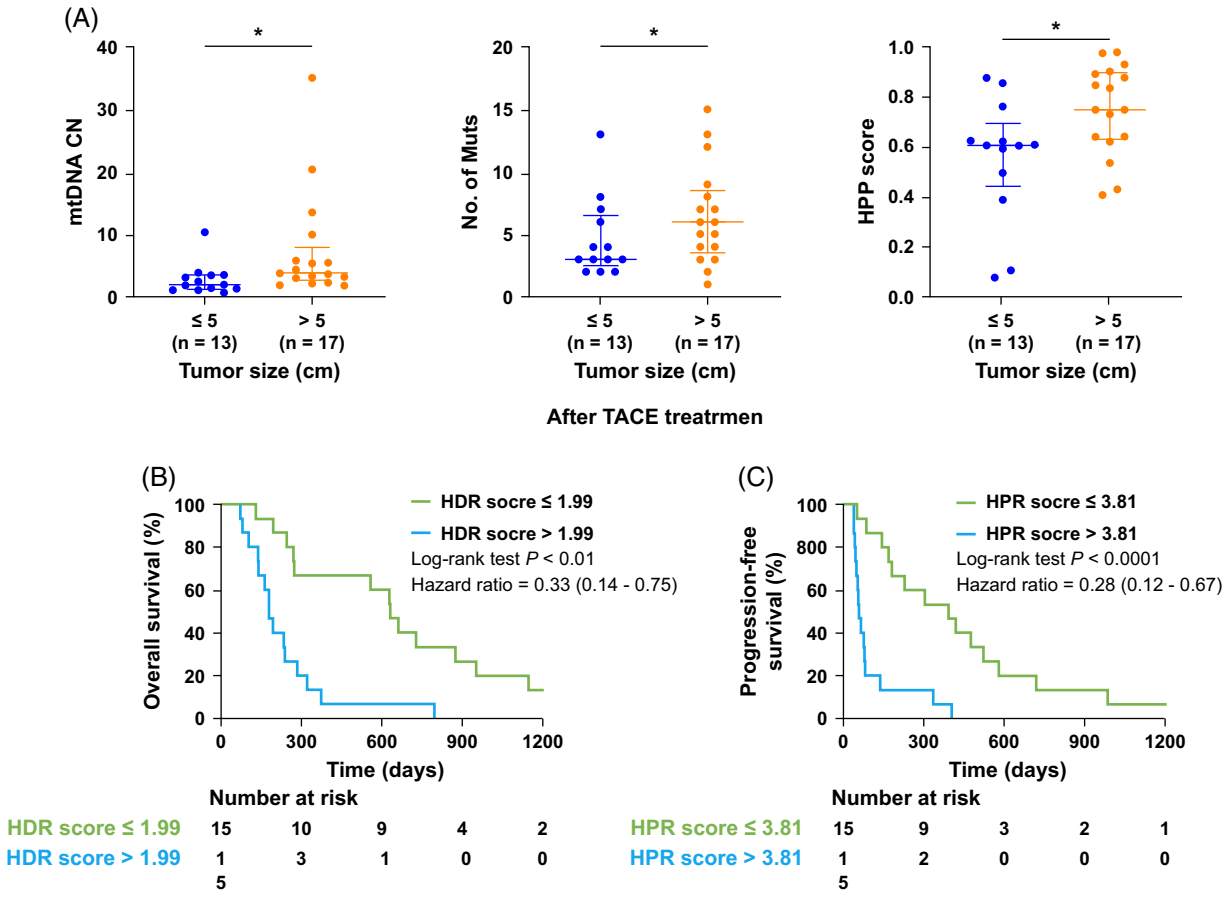


FIGURE 3 The analysis of ccf-mtDNA multifeatures in patients with HCC after TACE treatment. (A) Comparison of mtDNA CN, No. of Muts, and HPP score between different tumor size groups after TACE treatment. (B) The Kaplan-Meier survival curve was used to compare the overall survival in patients with HCC with an HDR score ≤ 2.53 (green) with those with an HDR score > 2.53 (blue) based on the HCC death risk prediction model. (C) The Kaplan-Meier survival curve was used to compare the progression-free survival in patients with HCC with an HPR score ≤ 1.67 (green) with those with an HDR score > 1.67 (blue) based on the HCC progression risk prediction model. Tumor size of patients with HCC was obtained after TACE treatment. *$p < 0.05$. In A–C, center line indicates the median, the lower and upper hinges represent the 25th and 75th percentiles, respectively. Abbreviations: CN, copy number; HPP, HCC prognosis prediction; HDR, HCC death risk; HPR, HCC progression risk; mtDNA, mitochondrial DNA; No. of Muts, number of mtDNA mutations; TACE, transarterial chemoembolization.

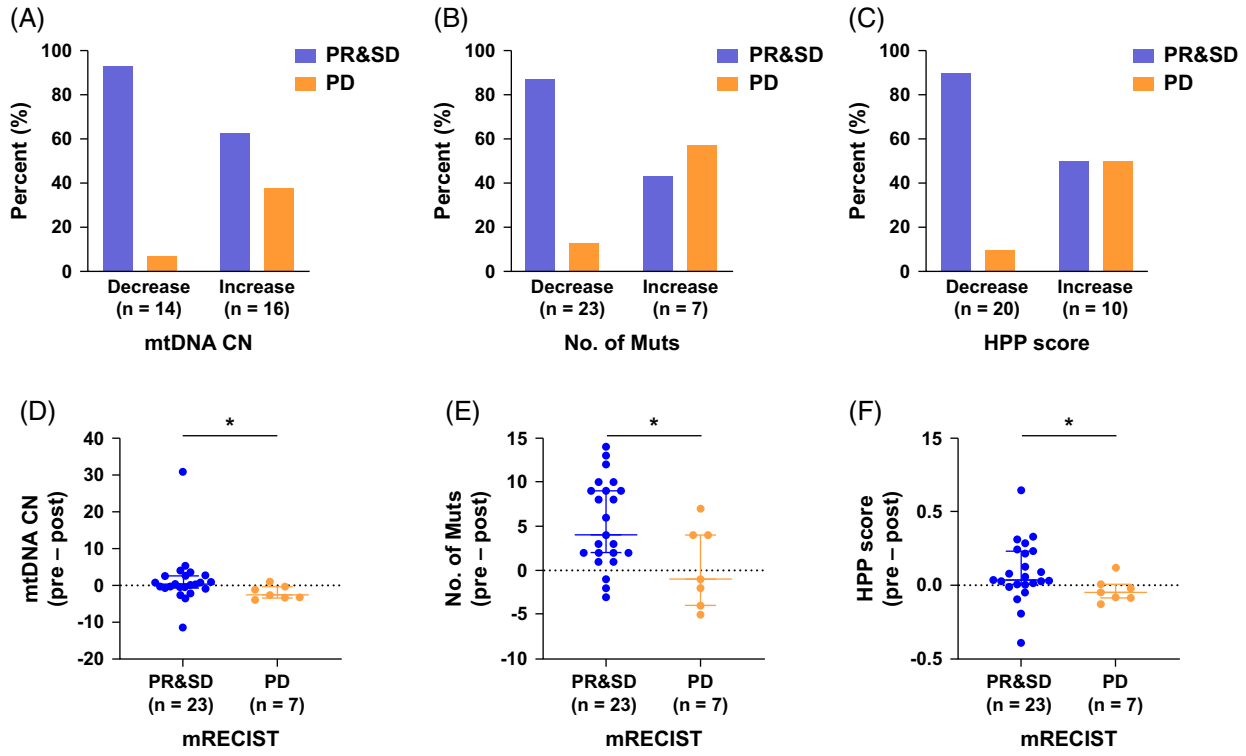


FIGURE 4 Therapeutic efficiency assessment of TACE treatment using cf-mtDNA multiple features. The percentage of patients achieved PR and SD or PD within the (A) mtDNA CN decrease and increase groups, (B) No. of Muts decrease and increase groups, and (C) HPP score decrease and increase groups. (D–F) mtDNA CN, No. of Muts, and HPP score change in the PR and SD, and PD groups. For example, in graph A, based on the change of mtDNA CN before and after TACE treatment, 30 patients were divided into 2 groups: the mtDNA CN decrease group (pre-post $\geq$ 0, n = 14) and the mtDNA CN increase group (pre-post < 0, n = 16). In the mtDNA CN decrease group, 15 (92.90%) patients achieved PR or SD, and 1 (7.10%) patients achieved PD. In the mtDNA CN increase group, 10 (62.50%) patients achieved PR or SD, and 6 (37.50%) patients achieved PD. Pre-post means subtracting the value obtained after TACE treatment from the value obtained before TACE treatment. *$p < 0.05$. In D–F, the center line indicates the median and the lower and upper hinges represent the 25th and 75th percentiles, respectively. Abbreviations: CN, copy number; HPP, HCC prognosis prediction; mRECIST, Modified Response Evaluation Criteria in Solid Tumors; No. of Muts, number of mtDNA mutations; PD, progressive disease; PR, partial response; SD, stable disease; TACE, transarterial chemoembolization.

mRECIST criteria significantly overlapped with the increased CNV burden group, and the change in CNV burden after TACE treatment exhibited significant differences between patients achieving PR/SD and PD (all $p < 0.01$; Supplemental Figure S6, http://links.lww.com/HC9/B902). We then compared the performance of the cf-mtDNA multiple features-based strategy with that of the CNV burden-based strategy, where the CNV burden was calculated based on WGS data. Patients with HCC were first stratified into high-risk and low-risk groups based on changes in cf-mtDNA multiple features before and after TACE treatment. Specifically, if the values of any 2 of the 3 features (mtDNA CN, the number of mtDNA mutations, and HPP score) increased after TACE treatment, patients with HCC were classified into the high-risk group; otherwise, they were classified into the low-risk group. As shown in Figure 5A, B, the Kaplan-Meier curve analyses revealed a significantly shorter median OS and PFS in the high-risk group than in the low-risk group (log-rank test, all $p < 0.01$; HR: 0.36 and 0.33, respectively). Furthermore, the patients with decreased CNV burden had a median OS of 284 days and a median PFS of 228 days, compared to 236 days and 53 days in patients with increased CNV burden (OS: $p = 0.20$, HR: 0.63; PFS: $p < 0.01$, HR: 0.38; Figure 5C, D). Additionally, the combination of cf-mtDNA multiple features and CNV burden did not significantly improve the prediction of HCC prognosis compared to cf-mtDNA multiple features alone (Figure 5E, F). As shown in Supplemental Tables S2 and 3, http://links.lww.com/HC9/B901, pre- and post-TACE cf-mtDNA features showed better performance in predicting HCC prognosis compared to CNV burden.

Consistency analysis between alterations of cf-mtDNA multiple features and changes in CNV burden before and after TACE treatment

We then observed the correlation between alterations of cf-mtDNA multiple features and changes in CNV burden before and after TACE treatment. As shown in Figure 6, among the 30 patients with HCC, 12 showed

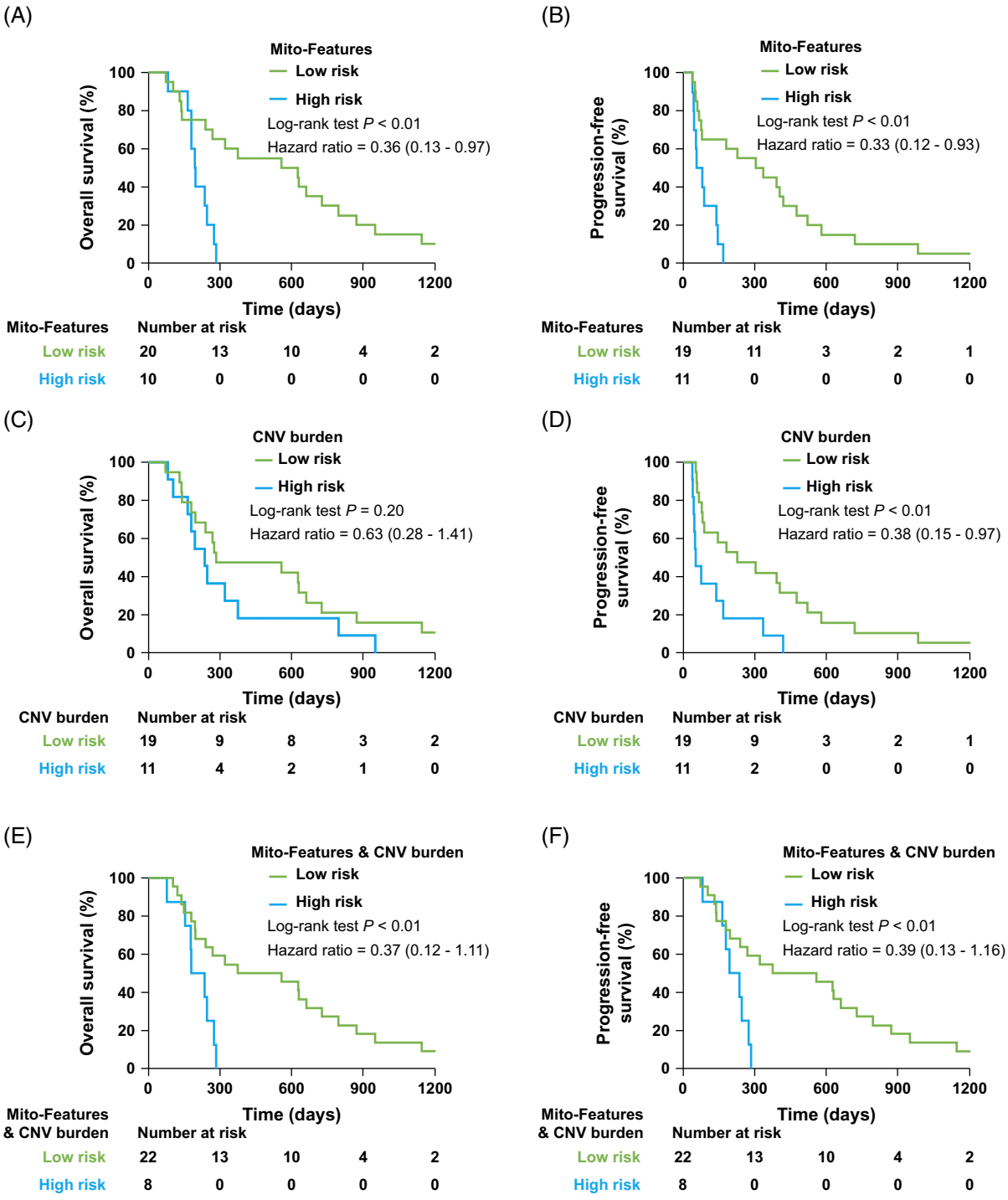


FIGURE 5 cf-mtDNA multiple features-based strategy markedly outperforms CNV burden for HCC prognosis prediction. The Kaplan-Meier survival curves were used to compare (A) OS or (B) PFS in patients with HCC with low risk (green) with those with high risk (blue) based on the Mito features. Specially, if the values of any 2 of the 3 features (mtDNA copy number, number of mtDNA mutations, and HPP score) increase after TACE treatment, the patients with HCC would be classified into the high-risk group; otherwise, they would be classified into the low-risk group. The Kaplan-Meier survival curves were used to compare (C) OS or (D) the PFS in patients with HCC with low risk (green) with those with high risk (blue) based on the CNV burden. Patients with CNV burden decrease after TACE treatment were categorized as low-risk group, whereas those with CNV burden increase after TACE treatment were categorized as high-risk group. The Kaplan-Meier survival curves were used to compare (E) OS or (F) PFS in patients with HCC with low risk (green) with those with high risk (blue) based on the combination of Mito features and CNV burden. Specially if the values of any 3 of the 4 features (mtDNA copy number, number of mtDNA mutations, HPP score, and CNV burden) increase after TACE treatment, the patients with HCC would be classified into the high-risk group; otherwise, they would be classified into the low-risk group. Abbreviations: CNV, copy number variation; HPP, HCC prognosis prediction; Mito-features, cf-mtDNA multiple features including mtDNA copy number, number of mtDNA mutations, and HPP score; OS, overall survival; PFS, progression-free survival.

consistent trends in the changes in mtDNA CN, number of mtDNA mutations, HPP score, and CNV burden before and after TACE treatment. Of these, 10 patients exhibited a significant decrease in all features, and 2 patients exhibited a significant increase in all features. Interestingly, these 10 patients achieved PR or SD after the first TACE treatment, and another 2 patients achieved PD.

DISCUSSION

HCC is a highly aggressive and heterogeneous malignancy that frequently presents at an advanced stage, rendering surgical resection infeasible in many patients. By targeting tumor-feeding arteries with chemotherapeutic and embolizing agents, TACE aims to restrict tumor growth and prolong survival. However, as a class of highly heterogeneous tumors, patients with HCC often show varied responses to TACE treatment, and there is an urgent need to develop an effective predictive strategy to guide clinical decisions regarding TACE treatment. Currently, the assessment of patient responses to TACE primarily relies on the interpretation of radiological images by radiologists, which is highly dependent on the expertise and experience of radiologists. This can lead to variations in assessments among radiologists. Furthermore, subtle changes in imaging may be difficult to quantify accurately, especially in cases where the tumor boundaries are indistinct or vascular invasion is complex. These limitations have prompted the exploration of nonimaging-based approaches to assist or supplement the current strategies.

In this study, we found that calculating cf-mtDNA multiple features (CN, mutation, and fragmentomics) before or after the first TACE treatment based on mtDNA cap-seq could provide an accurate and cost-efficient strategy for predicting prognosis in patients with HCC. We further found that changes in cf-mtDNA multiple features between pre-TACE and post-TACE treatment could predict the OS and PFS of patients with HCC.

In the previous study, Zhou et al[16] have found that cf-mtDNA CN is a potential noninvasive prognostic biomarker in patients with HCC receiving TACE treatment. Previous studies from our and other groups have also demonstrated that increased cf-mtDNA CN and aberrant cf-mtDNA fragmentomic features were observed in cancer patients when compared with HC, indicating that cf-mtDNA is a potential diagnosis biomarker.[17] In the present study, we observed a close

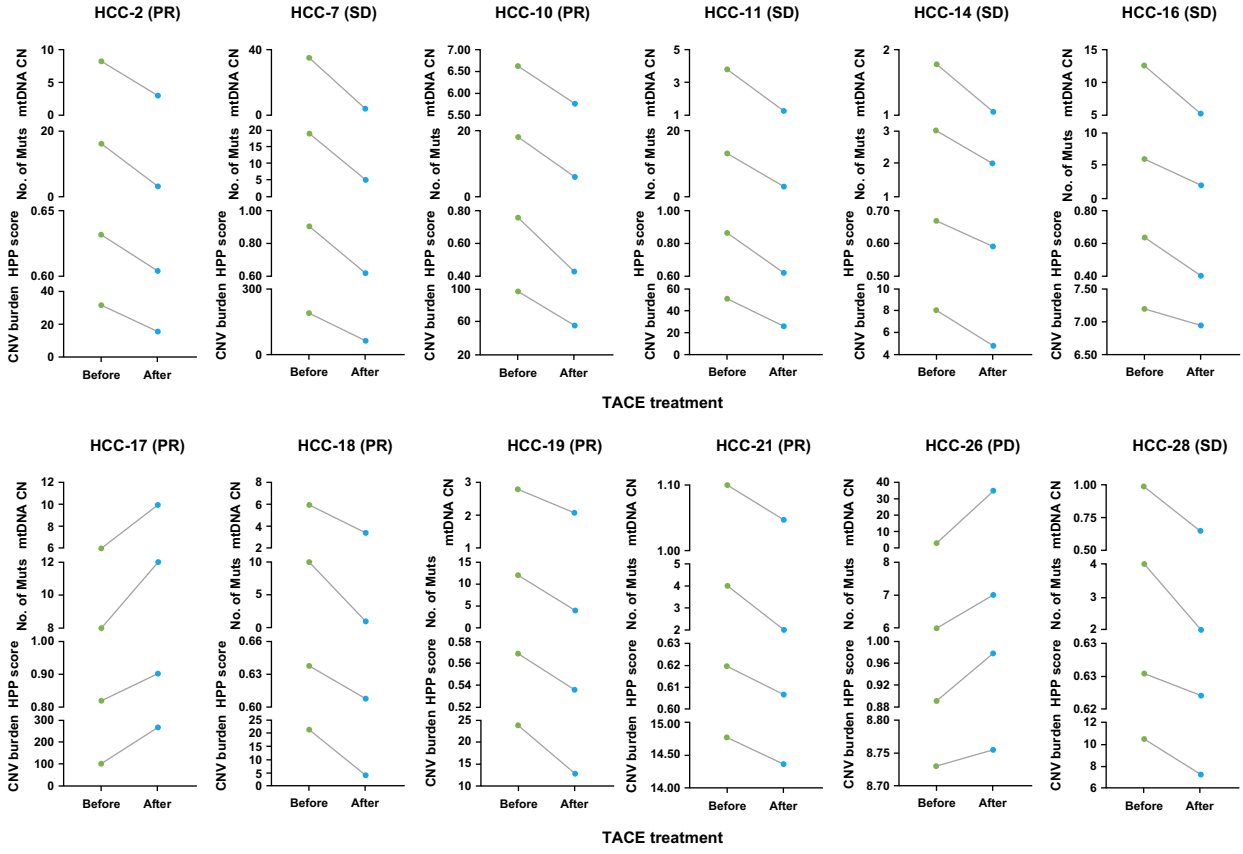


FIGURE 6 Consistency analysis of cf-mtDNA multiple features changes and CNV burden changes before and after TACE treatment. Abbreviations: CNV, copy number variation; HPP, HCC prognosis prediction; mtDNA, mitochondrial DNA; mtDNA CN, mtDNA copy number; No. of Muts, number of mtDNA mutations; PD, progressive disease; PR, partial response; SD, stable disease; TACE, transarterial chemoembolization.

relationship between cf-mtDNA multiple features and pre-TACE tumor characteristics (BCLC stage, tumor number, and tumor size). We also found that multiple features of cf-mtDNA positively correlated with AFP and AST levels. One possible explanation for this phenomenon is that the accumulation of mtDNA CN may be a pivotal factor in eliciting persistent mitochondrial-deficient activities, eventually contributing to cancer pathogenesis and progression.[18] Sun et al[19] have also demonstrated that an increased mtDNA CN significantly facilitates cell proliferation and inhibits apoptosis of tumor cells. Furthermore, a previous study has shown that mtDNA mutations likely cause mitochondrial dysfunction and modify HCC progression.[20] Another possible explanation is that there are high levels of oxidative stress inside tumor cells, which can lead to mtDNA damage and mutations that further affect mitochondrial function, forming a vicious cycle and promoting tumor progression. Moreover, the disease status can lead to changes in mtDNA fragmentomic features, which may be related to tumor progression; the mechanisms underlying cf-mtDNA multiple features and tumor progression need to be further explored in future studies.

Previous studies have demonstrated that the CNV burden of cf-nDNA calculated based on low-depth WGS data can serve as biomarkers for patients' tumor burden and could monitor HCC progression in real-time, providing a potential alternative strategy for evaluating the prognosis and monitoring the therapeutic efficacy of TACE treatment.[6] Remarkably, we obtained more information about genomic variation using mtDNA cap-seq than using low-depth WGS. Our results indicate that cf-mtDNA multiple features can be used to predict the prognosis of patients with HCC and outperform CNV burden. Possible explanation is that CNV burden reflect limited tumor information and are not as comprehensive as mtDNA multiple features. Our study emphasizes the value of moving beyond single-biomarker analysis and advocates the exploration and integration of cf-mtDNA multiple features to pursue more accurate and comprehensive prognostic models. Ignatiadis M and colleagues have also emphasized the necessity of integrative multiomics analyses.[21] Future studies should focus on elucidating the functional implications of these cf-mtDNA multiple features, refining predictive algorithms, and validating their clinical utility in larger and more diverse patient cohorts. The TACE procedure is based on cytotoxic and ischemic combined effects, which may have facilitated the release of cfDNA within a few days of treatment. An increased release of cfDNA after cytotoxic treatment has already been reported and may reflect the apoptosis process occurring in tumor cells a few hours after drug administration.[22,23] However, the release of cfDNA 1 month after TACE treatment did not reflect the apoptosis process occurring in tumor cells. Sefrioui et al[24] enrolled consecutive patients treated with TACE, with samples collected at baseline (D-1), day 2 (D-2), and 1 month (M-1), and also observed cfDNA significantly increased from (D-1) to D-2, decreased from D-2 to M-1.

The mRECIST criteria can reflect a tumor's response to treatment and disease progression, which is commonly used as a standard method to assess the treatment efficacy of solid tumors. In the present study, we found that the changes in cf-mtDNA multiple features and CNV burden after TACE treatment were significantly different between patients with PR/SD and those with PD. Similarly, a recent study has found that the change in CNV burden after TACE was related to the results of mRECIST evaluation and showed a striking association with PFS.[6] Concurrently, the combination of cf-mtDNA multiple features clearly enhanced the predictive performance for HCC prognosis compared to the CNV burden. Furthermore, we observed that the degree of abnormality determined by cf-mtDNA multiple features closely matched the CNV burden. Interestingly, patients with a decrease in all 4 indicators (cf-mtDNA CN, cf-mtDNA mutation, cf-mtDNA fragmentomics, and CNV burden) after TACE treatment showed PR or SD, whereas patients with an increase in all 4 indicators showed PD. This further highlights the importance of cf-mtDNA multiple features for predicting TACE efficacy. To our knowledge, this study is the first to explore the application of cf-mtDNA multiple features in predicting TACE efficacy and the prognosis of HCC patients undergoing TACE treatment. Moreover, further exploration of the biological mechanisms underlying the association of cf-mtDNA multiple features with HCC prognosis is warranted.

Although our results are promising, it is important to acknowledge the limitations of this study. The relatively small sample size, lack of external validation, and the absence of multicenter data limit the generalizability of our findings. Future studies with larger cohorts, external validation, and multicenter collaborations are warranted to confirm and extend our observations. Additionally, further investigation into the specific mechanisms underlying the changes in cf-mtDNA multiple features in response to TACE may provide deeper insights into the biology of HCC and inform the development of more targeted and effective therapeutic strategies. Also, developing a method to ascertain the molecular origin of cf-mtDNA and exploring more tumor-specific markers and techniques are crucial to predict TACE response.

In conclusion, our study demonstrated that the combination of cf-mtDNA multiple features could serve as a robust and cost-effective strategy for evaluating TACE treatment outcomes in patients with HCC. Our preliminary findings represent an important step forward in the search for improved HCC biomarkers and precision medicine.

DATA AVAILABILITY STATEMENT

The raw sequencing data underlying this article are available in BIG Data Center, Beijing Institute of Genomics (BIG) with access number PRJCA030058.

AUTHOR CONTRIBUTIONS

Liu Yang and Guo Xu conceived and designed the project. Dang Miao, Wang Siyuan, Peng Fan, Zhang Runjiao, Jiao Huanmin, Zhang Huanqin, Zhang Hongxin, Dong Haiying, Xing Jinliang, Guo Xu, and Liu Yang performed the selection of patients, collection of samples, and clinical data and mtDNA sequencing. Dang Miao, Wang Siyuan, Peng Fan, Jiao Huanmin, and Liu Yang analyzed the sequencing data. Dang Miao, Wang Siyuan, Peng Fan, Xing Jinliang, Guo Xu, and Liu Yang prepared the manuscript. All co-authors reviewed and approved the final draft of the manuscript.

FUNDING INFORMATION

This work was supported by the National Natural Science Foundation of China (grant no. 82330073 and 82402731), the Science & Technology Co-ordination and Innovation Project of Shaanxi Province, China (grant no. 2023-ZDLSF-46), Science and Technology Research and Development Program of Shaanxi Province, China (grant no. 2019SF-037).

CONFLICTS OF INTEREST

The authors have no conflicts to report.

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How to cite this article: Dang M, Wang S, Peng F, Zhang R, Jiao H, Zhang H, et al. Multiple features of cell free mtDNA for predicting transarterial chemoembolization response in hepatocellular carcinoma. Hepatol Commun. 2025;9: e0652. https://doi.org/10.1097/HC9.0000000000000652