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Short-term dynamics of circulating tumor DNA predicting efficacy of sintilimab plus docetaxel in second-line treatment of advanced NSCLC: biomarker analysis from a single-arm, phase 2 trial

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ABSTRACT

Objective Robust biomarker predicting efficacy of immunotherapy is limited. Circulating tumor DNA (ctDNA) sought to effectively monitor therapeutic response as well as disease progression. This study aims to investigate predictive role of ctDNA short-term dynamic change (6 weeks postimmunotherapy) in a single-arm, phase 2 trial of sintilimab plus docetaxel for previously treated advanced non-small cell lung cancer (NSCLC) patients.

Methods A total of 33 patients with advanced NSCLC with disease progression during or after any first-line treatment were prospectively enrolled between 2019 and 2020. Patients received sintilimab (200 mg, day 1, every 3 weeks) plus docetaxel (75 mg/m^2, day 3, every 3 weeks) for 4–6 cycles, followed by maintenance therapy with sintilimab (200 mg, day 1, every 3 weeks) until disease progression or unacceptable toxic effects. Blood samples were prospectively collected at baseline, and after 2 cycles of treatment (6 weeks post-treatment). All samples were subjected to targeted next-generation sequencing with a panel of 448 cancer-related genes. The landscape of high-frequency genomic profile of baseline and 6th week was described. Major molecular characteristics in preselected genes of interest associated with response to second-line chemoimmunotherapy were analyzed. The curative effects and prognosis of patients were evaluated.

Results Patients with ctDNA clearance at 6th week had decreased tumor volume, while most patients with positive ctDNA at 6th-week experienced an increase in tumor volume. Positive 6th-week ctDNA was associated with significantly shorter progression-free survival (PFS) (91 vs NR days; p<0.0001) and overall survival (47 vs 467 days; p=0.0039). Clearance of clonal mutations and none new clonal formation at 6th week were associated with longer PFS (mPFS 89 vs 266 days, p=0.003). ctDNA clearance at 6th week was an independent risk factor for progression or death (HR=100 (95% CI 4.10 to 2503.00), p=0.005).

Conclusion ctDNA status and ctDNA mutation clearance putatively serve as predictive biomarkers for sintilimab combined with docetaxel chemotherapy in pretreated advanced NSCLC patients.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ PD-L1 expression is the only clinically validated and Food and Drug Administration-approved biomarker to identify putative responder to immune checkpoint inhibitor (ICIs) in both treatment naïve and pretreated advanced non-small cell lung cancer (NSCLC) patients without driver mutations. Analysis of circulating tumor DNA (ctDNA) has been shown to have prognostic value in a variety of cancers, including lung cancer, as it enables detection of residual proliferative disease in the therapeutic setting and estimation of tumor burden in the metastatic setting.

WHAT THIS STUDY ADDS

⇒ In addition to PD-L1 Tumor Proportion Score, short-term ctDNA kinetics (ctDNA status, clonal clearance at 6th week) putatively indicate prognosis and therapeutic efficacies for patients with advanced NSCLC receiving second-line chemo/ICI-combined therapy.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ ctDNA kinetics could impact second-line clinical practice of advanced NSCLC, especially identifying putative long-term and short-term responders for chemoimmunotherapy. For putative short-term responders for chemoimmunotherapy indicated by uncleared ctDNA at 6th week, future clinical trials should be designed to investigate better therapeutic approaches.

INTRODUCTION

Immune checkpoint inhibitors (ICIs) dramatically changed the treatment landscape for advanced non-small cell lung cancer (NSCLC) patients without actionable driver mutations.[1 2] Phase III studies including CheckMate 057,[3] KEYNOTE 010,[4] and OAK,[5] demonstrated that programmed death 1 (PD-1) inhibitors nivolumab and pembrolizumab, and the programmed death ligand 1 (PD-L1) inhibitor atezolizumab, provided more survival benefit than chemotherapy, resulting the approval of these three ICIs as second-line treatments in advanced or metastatic NSCLC by the US Food and Drug Administration (FDA). Currently, combination of ICI-based immunotherapy with chemotherapy, such as sintilimab (anti-PD-1 antibody) plus platinum and gemcitabine in the treatment of advanced or metastatic squamous NSCLC, and other pivotal clinical trials,[6 7] appears to be a viable strategy which has demonstrated encouraging results. However, clinical trials involving chemoimmunotherapy approaches in NSCLC patients almost in the first-line setting, and it remains unclear whether the combined therapy can further improve patients' progression-free survival (PFS) compared with immunotherapy alone. Recently, a retrospective study[8] and a phase 2 randomized clinical trial[9] displayed that PD-1/PD-L1 inhibitor in combination with chemotherapy was well tolerated and substantially improved PFS and objective response rate (ORR) in patients with advanced NSCLC who had previous progression after platinum-based chemotherapy. However, another retrospective study revealed that addition of chemotherapy to PD-1 inhibitors did not improve overall survival (OS) or PFS compared with PD-1/PD-L1 inhibitor alone for NSCLC patients as second-line and later therapy.[10] These inconsistent results highlight the need to further investigate the potential role of this combination of treatments in the second-line setting, and warrants the use of more appropriate biomarkers to select potential benefit patients.

PD-L1 expression, detected by immunohistochemistry, is the only clinically validated and FDA-approved biomarker to identify putative responder to ICIs in both treatment naïve and pretreated advanced NSCLC patients without driver mutations.[11 12] Patients with low PD-L1 expression (Tumor Proportion Score, TPS, 1%–49%) with concurrent chemotherapy plus pembrolizumab and those with high PD-L1 expression (TPS ≥50%) with pembrolizumab alone or with concurrent chemotherapy.[13 14] However, a subset of patients with PD-L1 1%–49% can respond to immunotherapy alone, for instance, Keynote-024 trial,[11] so that PD-L1 expression is a less than perfect predictor of response.[15] In addition, other ICI agents such as nivolumab,[3] atezolizumab,[5] and durvalumab[16] have marked clinical benefits in the treatment of NSCLC without requirement for specific PD-L1 levels. Therefore, PD-L1 expression alone is not sufficiently accurate to identify all potential responders to PD-1/PD-L1 blockade-based ICI in NSCLC, and such imperfect also prompted the investigation of other markers of response.

In recent years, tissue tumor mutational burden (TMB) has become an alternative biomarker, as well as microsatellite instability, mismatch repair gene deficiency, special gene mutations, tumor immune microenvironment, gene expression profiles, and antigen presentation defects, have also been investigated as possible predictive markers for the efficacy of ICIs.[17] All these aforementioned markers require an invasive biopsy, however ideally, routine clinical biomarkers should be assessed in a minimally invasive manner. Therefore, whole blood, serum or stool-based markers, such as blood-based TMB (bTMB),[18] inflammatory cytokines,[19] and intestinal micro-organism[20] are an attractive option given the ease of obtaining such measures. Particularly, circulating tumor DNA (ctDNA) has been proposed as a noninvasive biomarker to provide information for determining a patient's disease state and capturing dynamic changes during treatment.[21] Cabel *et al* demonstrated that patients who with low or undetected ctDNA level at the beginning of and during therapy often have better responses to anti-PD-1 immunotherapy.[22] According to Goldberg *et al*, patients with ≥50% decrease compared with ctDNA baseline levels showed superior PFS and OS than those by <50%.[23] In the latter study, Nabet *et al* concluded that adding on-treatment measurement of ctDNA dynamics and peripheral immune features to pretreatment ctDNA could improve the prediction for those who will benefit from the given ICI therapy.[24] However, more work is needed to examine baseline ctDNA level, and ctDNA level on and post-treatment, could be prognostic and predictive factors in patients receiving ICIs.

Recently, we have designed and conducted a single-arm, phase 2 study to evaluate the clinical efficacy and safety of sintilimab combined with docetaxel in the second-line treatment for patients with advanced NSCLC with disease progression during or after platinum-based chemotherapy or intolerance/failure of any first line tyrosine kinase inhibitor (TKI) (ChiCTR1900027634). We reported here the potential biomarkers that can predict immunotherapeutic response and long-term survival results in this phase 2 trial, especially the predictive value of differences in ctDNA levels before and after treatment.

MATERIALS AND METHODS

Patients

A total of 33 patients with advanced NSCLC with disease progression during or after platinum-based chemotherapy or intolerance/failure of any first-line TKI between 2019 and 2020 from a prospective cohort study (ChiCTR1900027634) were enrolled. Detailed patient recruitment methods are provided in online supplemental material.

Treatment and samples collection

Patients received sintilimab (200 mg, day 1, every 3 weeks) plus docetaxel (75 mg/m^2, day 3, every 3 weeks) for 4–6 cycles, followed by maintenance therapy with sintilimab

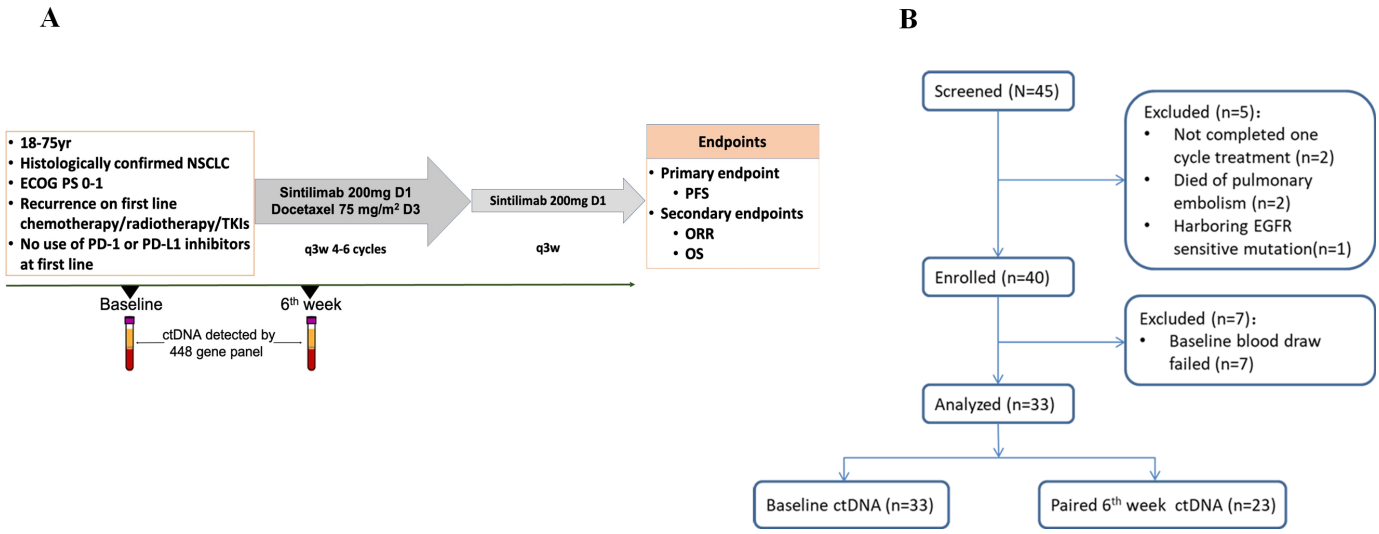

Figure 1 Study design and time point of ctDNA collection (A), and flow chart of patient selection in this study (B). ctDNA, circulating tumor DNA; ECOG, Eastern Cooperative Oncology Group; NSCLC, circulating tumor DNA; ORR, objective response rate; OS, overall survival; PFS, progression-free survival.

(200 mg, day 1, every 3 weeks) until disease progression or unacceptable toxic effects. Clinical response was evaluated by the investigators according to the RECIST V.1.1. Patients were characterized as having either durable clinical benefit (DCB) or no durable benefit (NDB) in addition to complete/partial response (CR/PR), stable disease (SD) or progressive disease (PD) as best overall response (BOR). DCB was defined as CR or PR or SD for at least 6 months, whereas NDB was defined as PD within 6 months from start of treatment. Patient blood samples were collected presecond-line treatment (at baseline), and after two cycles of treatment or before the start of the third cycle (at 6th week). Blood sample from each patient (10 mL/time, two times) was collected in Streck tube. The study design and time point of blood collection were shown in figure 1A.

Sample preparation, DNA extraction, library construction, and targeted sequencing

Blood sample was processed for plasma isolation with ctDNA extraction using a QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. AmoyDx Blood and Leukocyte DNA Kit (AmoyDx, Xiamen, China) was used to extract genomic DNA from peripheral blood lymphocytes as the normal control for mutation calling from ctDNA. DNA concentration was quantified with the QuantFluor dsDNA System on a Quantus Fluorometer (Promega, Madison, Wisconsin, USA) according to manufacturer's instructions. The library fragment size distribution was analyzed in a Bioanalyzer 2100 using the High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, California, USA). All samples were subjected to targeted next-generation sequencing (NGS) with a panel of 448 cancer-related genes (AmoyDx, Xiamen, China) performed on the Illumina NovaSeq 6000 platform (San Diego, California, USA) according to the manufacturer's recommendations at an average depth of 5600×to 17,500×.

Base calling and sequence alignment

Paired-end sequenced reads were separated according to sample-specific barcodes. Sequencing data were stored as FASTQ files using ADXTMB448V2-pbMut (V.0.1.5, AmoyDx, Xiamen, China, inhouse). After removing adaptor and low-quality reads, clean FASTQ data were aligned to the human reference genome (hg19) using the BWA-MEM aligner with default parameters (http://bio-bwa.sourceforge.net/). FormatFastq (V.2.4.0, AmoyDx, Xiamen, China, inhouse) was used to remove duplicated reads for tumor and germline genomic DNA. For ctDNA, duplicated reads were identified by unique molecular identifiers and the position of template fragments to eliminate errors introduced by PCR or sequencing using SSBC (V.1.4.0, AmoyDx, Xiamen, China, inhouse). The aligned bam file was sorted by coordination with samtools_V.1.9 (https://sourceforge.net/projects/samtools/).

Variants calling, annotation and filtration

Single-nucleotide variants (SNVs) were identified and recorded by SSBC-VarScan (V.1.3.0; AmoyDx, Xiamen, China, inhouse). Insertions/deletions (Indels) were called by IndelCaller (V.0.2.1; AmoyDx, Xiamen, China, inhouse). All variants identified were annotated with Annotator (V.0.3.6; AmoyDx, Xiamen, China, inhouse). Variants classification and criteria for filtering SNVs, Indels and fusion were provided in online supplemental material.

Blood tumor mutation burden

For the calculation of the bTMB, three criteria for competent mutations were applied: (1) somatic but not germline mutation; (2) located in coding region, and (3)

nonsynonymous SNVs/Indels. The bTMB was calculated as the number of competent mutations divided by the length of the panel-covered genomic region (1.16 Mb), thus when there was only one mutation, bTMB was 1/1.16=0.86 mut/Mb.

Analysis of clonal and subclonal mutations

All SNV gene alterations were subjected to clonal and subclonal mutation analysis. According to the previous study,[25] a cancer fraction was calculated as the allele frequency of the mutation relative to the maximum somatic variant allele frequency present in the sample. Analyses based on categorical assessment of clonality, mutations with cancer fraction ≥0.5 were considered clonal mutation, while mutations with cancer fractions <0.5 were considered subclone.

Statistical analysis

ctDNA values were dichotomized as positive (≥2 mutations detectable) and negative (≤1 mutation detectable). Demographic characteristic and mutational landscape of patients were analyzed using descriptive statistic. χ^2 or Fisher's exact probability tests were performed when rate or percentage was compared for significance. Non-parametric Wilcoxon rank sum tests were used for the comparison of medians between two datasets. Kaplan-Meier survival analysis was used to evaluate the association between bTMB/ctDNA and PFS. Survival curves were compared by using the log-rank test. Univariate and multivariate Cox regression analysis was performed to figure out the potential risk factors. All statistical analyses were performed with the R software (https://www.r-project.org/) or SPSS V.22.0 (IBM). A p<0.05 was considered statistically significant.

Table 1 The demographic characteristics of the 33 patients

Features	No (n)	%
Gender		
Male	26	78.79
Female	7	21.21
Age 56 (31–71)		
≥60 years	8	24.24
<60 years	25	75.76
Smoking history		
Yes	18	54.55
No	15	45.45
ECOG PS		
0	4	12.12
1	29	87.88
Histology		
Adenocarcinoma	29	87.88
Squamous	4	12.12
Stage		
IIIc	7	21.21
IV	26	78.79
Metastasis		
None	3	9.09
Single	9	27.27
Multiple	21	63.64
PD-L1 staining (TPS)		
<1%	5	15.15
10%–20%	1	3.03
>50%	3	9.09
NA	24	72.73

ECOG PS, Eastern Cooperative Oncology Group Performance Status; NA, not available; TPS, Tumor Proportion Score.

RESULTS

Patients' characteristics and genomic profiling of advanced NSCLC from baseline and 6th-week ctDNA

Blood samples from 33 Chinese patients with advanced NSCLC (7 female and 26 male) were subjected to ctDNA extraction and targeted NGS. The median age was 56 (range 31–71) years. The flow chart (figure 1B) describes selection of the 33 patients analyzed in this study starting from the total population (n=45). Table 1 shows the demographic characteristics of the patients.

The mutational spectrum of ctDNA was established using the baseline blood samples. Mutations were detected in ctDNA of 32 patients (figure 2A) while not detected in one patient. A total of 236 somatic mutations were identified with an average of 7.38 per sample (ranging from 1 to 27), including 220 SNVs, 15 Indels, and 1 fusion. *TP53* (56%), *EGFR* (22%), *LRP1B* (16%), *KRAS* (12%), *PTPRD* (12%), and *DNMT3A* (12%) exhibited the highest mutational frequencies (figure 2A). Mutations in other canonical lung cancer driver genes such as *RET*, *PIK3CA*, *KEAP1*, *CDKN2A*, and *PTEN* were also observed in this cohort. After two cycles of treatment with sintilimab plus docetaxel, paired blood ctDNA samples from 23 patients were sequenced using the same gene panel and same sequencing platform. As shown in figure 2B, among the 30 genes with the highest mutation frequency, 6 patients developed 8 new gene mutations (including *KRAS*, *ERBB2*, *DNMT3A*, *MED12*, and *CBL*) or new mutation sites (including *EGFR* and *LRP1B*) after two cycles of treatment compared with the baseline; And a total of 25 gene mutations disappeared in 11 patients, among which *TP53* mutation disappeared in 5 patients, *PTPRD* mutation disappeared in 3 patients, *CBL*, *MED12*, *EP300*, *IRF4*, and *PXDNL* disappeared in 2 patients, respectively, and *EGFR*, *DNMT3A*, *LRP1B* and *ERBB2* disappeared in 1 patient, respectively; In addition, a total of 37 gene mutations were reappeared in 11 patients, among which *TP53* mutation reappeared in 6 patients, *EGFR* mutation reappeared in 5 patients (and new mutation sites were detected in one of them), *LRP1B*, *DNMT3A*, *TAF1*, and *MAGI2* reappeared in 2 patients, respectively (one of them detected two new *LRP1B* mutation sites).

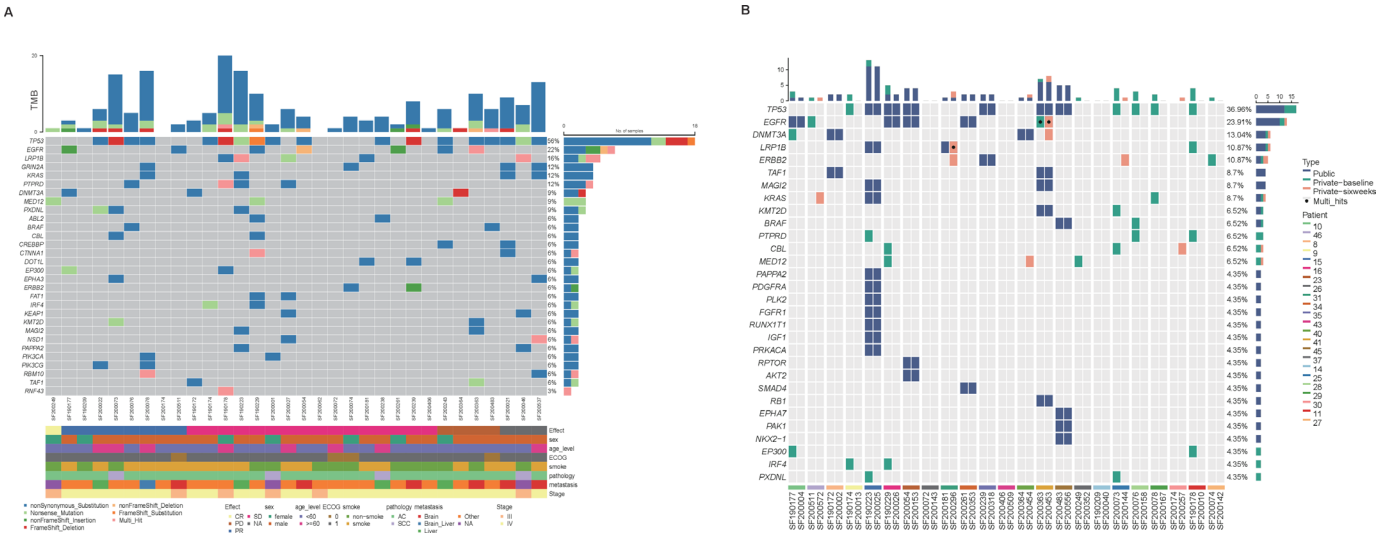

Figure 2 The landscape of high-frequency genomic alterations. Genes with somatic mutations were listed on the x-axis, and samples were shown on the y-axis, mutation frequencies of each gene were shown on the right. (A) Somatic mutation profile of baseline ctDNA sequencing of 448 tumor genes in 32 patients. (B) Somatic mutations of 23 paired baseline and 6th-week ctDNA sequencing of 448 tumor genes. ctDNA, circulating tumor DNA; TMB, tumor mutational burden.

Association of major genetic alterations with therapeutic response to the second-line chemo/ICI combined therapy and prognosis

Primary and adaptive resistance to immunotherapy has been associated with specific genetic alterations.[26] Thus, the association of alterations in preselected genes of interest of this patient cohort with response to chemoimmunotherapy therapy were assessed. Clinical data of 27 patients with available RECIST evaluation were collected and included in the analysis. Among these patients, 8 achieved DCB and 19 showed NDB. The compared frequencies of mutations between the two groups were shown in figure 3A. We identified mutations that were previously associated with response to ICIs in our patient cohort, namely *PIK3CG*,[27] two DCB patients harbored mutant *PIK3CG*, and two DCB patients harbored mutant *GRIN2A*, while none of the NDB patients harbored mutations of these two genes. However, the difference did not reach statistical significance, and whether mutant *PIK3CG* or *GRIN2A* was a protective factor needs to be illustrated in the future. Consistent with previous reports,[28] we found patients with *EGFR* mutations rarely derive benefit from treatment with ICI, in addition, *DNMT3A* and *ABL2* mutations were also exclusively found in NDB group.

The association of genetic alterations in these patients with BOR to the therapy were also assessed. Eight patients achieved CR or PR, and 19 were SD or PD. The compared frequencies of mutations between these two groups were shown in figure 3B. Two of the eight CR/PR patients harbored mutant *PIK3CG*, and two CR/PR patients harbored mutant *RBM10*, while none of the SD/PD patients harbored *PIK3CG* or *RBM10* mutations, also, no significance difference was observed between the two groups. Previous reports have found that *LRP1B* mutations were associated with improved outcomes to ICIs across multiple cancer types,[29 30] however, inconsistent with these findings, *LRP1B* mutations were exclusively found in SD/PD group in our cohort.

Univariate analysis was performed to figure out the significant mutant genes associated with PFS and found that *EPHA7* (p=0.0103) and *MAGI2* (p=0.0122) were associated with disease progression. Multivariate Cox regression analysis adjusted by age, gender, smoking history, Eastern Cooperative Oncology Group (ECOG) and stage was further performed. And as shown in figure 3C, after adjusting, *MAGI2* mutant might be an independent risk factor associated with disease progression, as only 2 of 29 patients were *MAGI2* mutated, which warrants validation in larger patient cohorts.

Association of bTMB and ctDNA status with patients' overall response

In order to further search for biomarkers that can effectively predict the efficacy of second-line immunotherapy combined with chemotherapy, the association of bTMB and ctDNA status with patients' therapeutic effect as analyzed. A significant difference was identified in the mean tumor volume change between patients with and without detectable ctDNA at 6th week. Overall, patients with ctDNA-negative had decreased tumor volume, while most patients with positive ctDNA had increased tumor volume (figure 4A). We further explored the potential predicting efficacy of ctDNA status on therapeutic response, and the result showed that patients with ctDNA clearance were more likely to achieve clinical efficacy (PR/CR) (figure 4B). The bTMB change degree and change percentage (bTMB_change% = [6th-week bTMB - baseline bTMB]/baseline bTMB×100%) of patients reaching DCB/BOR were also compared. The bTMB change degree and change percentage of patients reaching DCB (durable of response (DOR) ≥6 months) was significantly different to that of patients with NCB (DOR<6 months), and patients

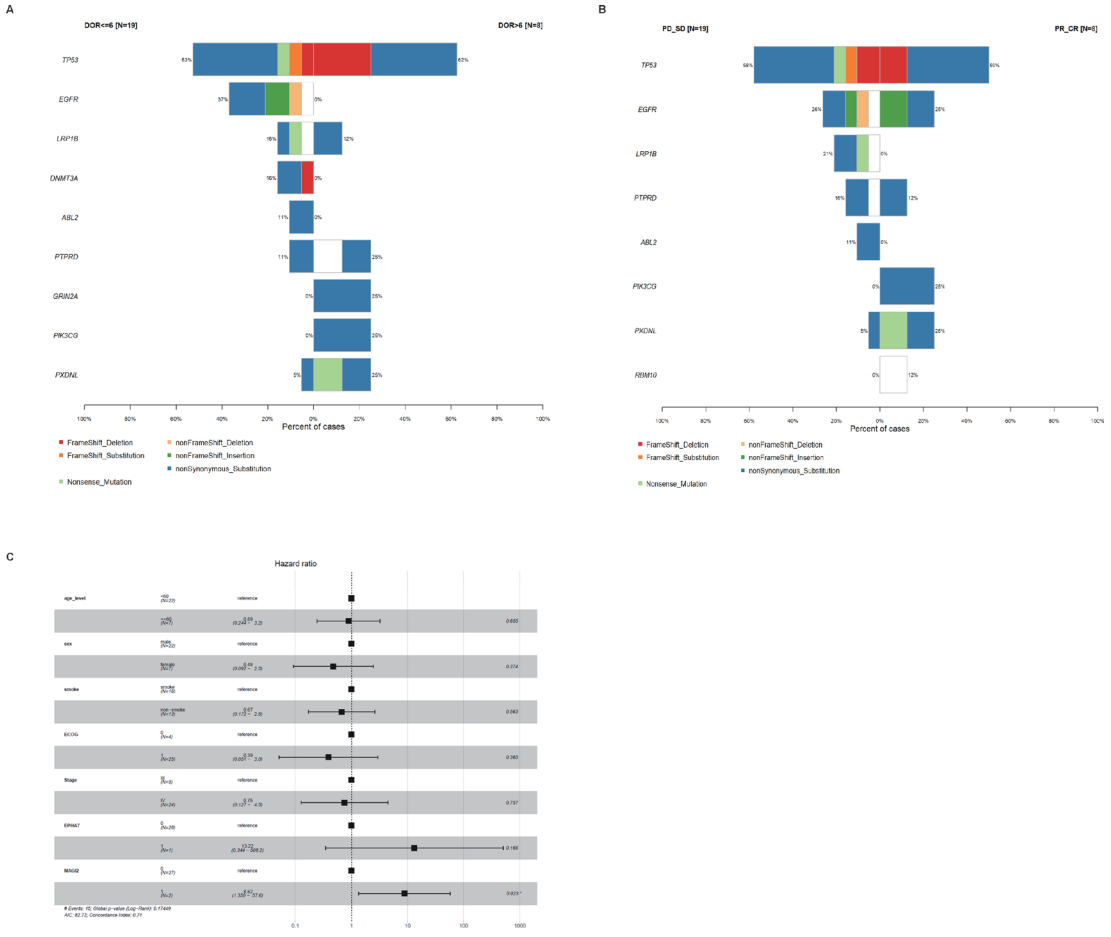

Figure 3 Major molecular characteristics in preselected genes of interest associated with response to second-line chemoimmunotherapy. (A) Gene alterations associated with DCB and NCB. (B) Gene alterations associated with BOR. (C) Mutated gene significantly associated with PFS was analyzed by a multivariate Cox regression model. BOR, best overall response; DCB, durable clinical benefit; NCB, no clinical benefit; PFS, progression-free survival.

with significantly decreased bTMB had a higher probability to obtain DCB (p>0.05), though bTMB at 6th weeks in the response group (patients reaching PR/CR) was significantly lower than that in the no response group (patients reaching SD/PD) (p < 0.05) (online supplemental figure 1A). However, the degree and percentage of change in bTMB were not significantly associated with BOR ($P > 0.05$) (online supplemental figure 1B). Among 23 patients with paired ctDNA results, 7 patients had eligible PD-L1 TPS score ((online supplemental figure 2A). For the 3 PD-L1-positive patients, the dynamic changes of ctDNA levels and PD-L1 expression consistently associated to overall response and duration of response (online supplemental figure 2B). However, for four PD-L1 negative patients, two patients with ctDNA clearance were benefited from second-line chemo/ICI-combined therapy. Thus, patients with ctDNA clearance and a significant decrease in bTMB were more likely to benefit from ICI-based immunotherapy combined with chemotherapy.

Association of bTMB and ctDNA status with patients' prognosis

For the association of ctDNA status at 6th weeks and patients' prognosis, patient with positive ctDNA was associated with significantly shorter PFS and OS compared with patients with negative ctDNA (PFS: 91 vs NR days; HR 8.423; 95% CI, 2.695 to 26.33; p<0.0001; OS: 147 vs 467 days; HR 7.21; 95% CI 1.325 to 39.24; p=0.0039; figure 4C). The association of baseline ctDNA status and the patients' prognosis was also evaluated, and the results showed that the baseline ctDNA positive was associated with shorter PFS (147 vs NR days; HR 2.828; 95% CI 1.14 to 6.99; p=0.074; online supplemental figure 3A) and shorter OS (378 vs NR days; HR,5.007; 95% CI 1.519 to 16.5; p=0.08; online supplemental figure 3B), although this difference was not statistically significant. Furthermore, the association of baseline bTMB and patients' prognosis after treatment of combined ICIs and chemotherapy was assessed in this study. Interesting but non-statistically significant differences between higher and lower baseline bTMB was observed. When the cut-off value of baseline bTMB to discriminate DCB by recipient operating characteristic (ROC) curve was 16 Mut/Mb, higher baseline bTMB was associated with longer PFS (147 vs NR days; HR 3.174; 95% CI 0.02 to 26.33; p=0.23; online supplemental figure 3C); the same as the OS (378 vs NR days; HR, NA; p=0.22; online supplemental figure

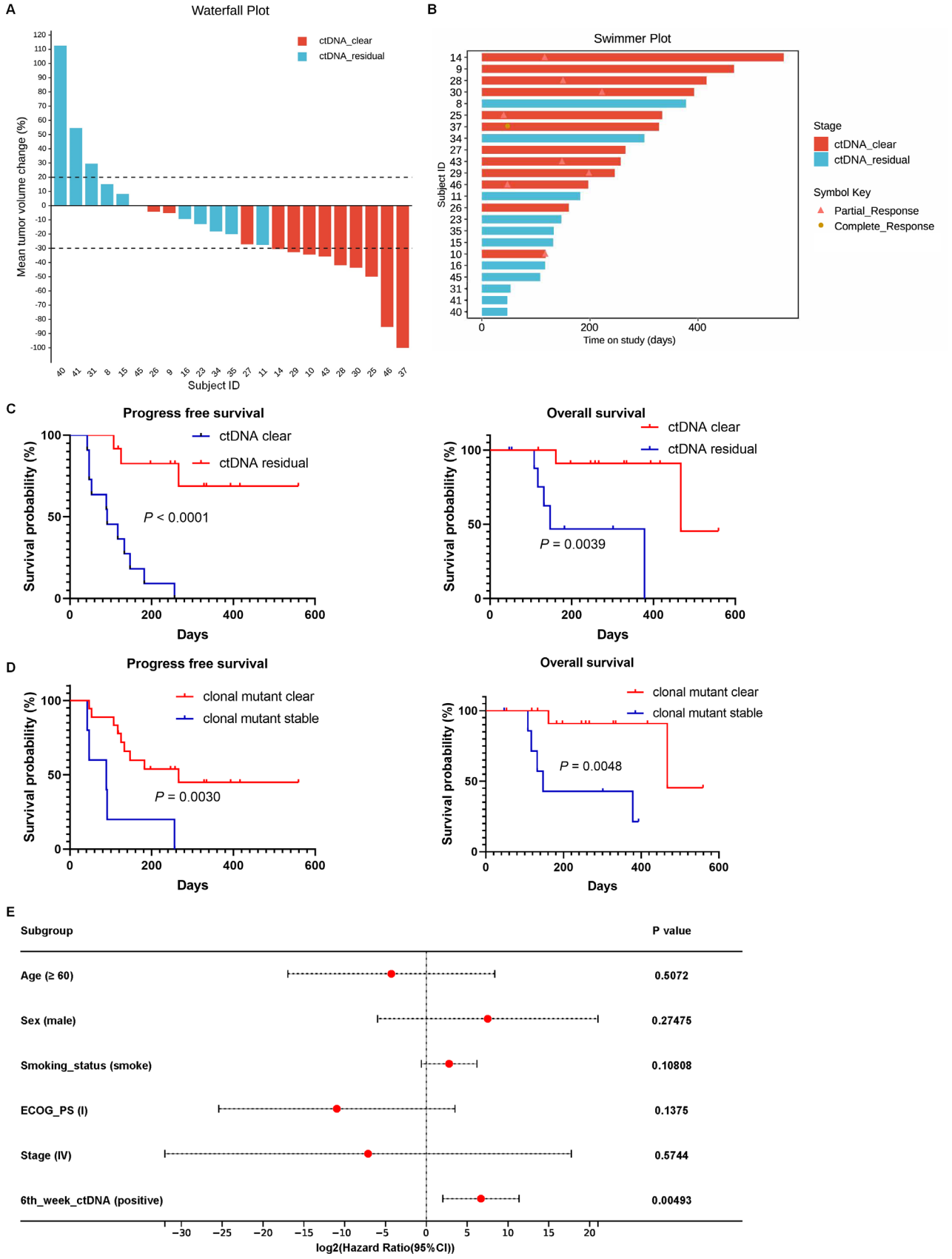

Figure 4 Association of 6th-week ctDNA status with patient prognosis and overall response. (A) Association of ctDNA status at 6th-week and tumor volume. (B) Association of ctDNA status at 6th-week and overall response. (C) Association of ctDNA status at 6th weeks and patient prognosis. (D) Association of primary clonal mutations clearance and longer OS. (E) ctDNA residual at 6th weeks was an independent risk factor for progression or death. ctDNA, circulating tumor DNA; ECOG-PS, Eastern Cooperative Oncology Group Performance Status; OS, overall survival.

Table 2 The ctDNA clearance of the 23 patients after two cycles of treatment

Features	No (n)	%	ctDNA clear (%)	ctDNA residual
Gender				
Male	18	78.26	9	9
Female	5	21.74	3	2
Age 56 (31–71)				
≥60 years	4	17.39	3	1
<60 years	19	82.61	9	10
Smoking history				
Yes	13	95.65	6	7
No	10	4.35	6	4
ECOG PS				
0	4	17.39	3	1
1	19	82.61	9	10
Histology				
Adenocarcinoma	22	95.65	11	11
Squamous	1	4.35	1	0
Stage				
IIIc	3	13.04	2	1
IV	20	86.96	10	10
Metastasis				
None	1	4.35		
Single	9	39.13	5	4
Multiple	13	56.52	6	7
NA	0	0	0	0

ctDNA, circulating tumor DNA; ECOG PS, Eastern Cooperative Oncology Group Performance Status; NA, not available.

3D). However, when the cut-off value of baseline bTMB to predict death/recurrence by ROC was 2.155 Mut/Mb, higher baseline bTMB was associated with shorter PFS (147 vs NR days; HR 3.106; 95% CI 1.28 to 7.56; p=0.051; online supplemental figure 3E). While the cut-off value of baseline bTMB to discriminate death by ROC was 3.875 Mut/Mb, higher baseline bTMB was significantly associated with shorter OS (176 vs 490 days; HR 4.492; 95% CI 1.50 to 13.50; p=0.0087; online supplemental figure 3F). Furthermore, multivariate Cox regression analysis adjusted by age, gender, smoking history, ECOG and stage was performed. And as shown in figure 4E, after adjusting, ctDNA residual at 6th week was an independent risk factor for progression or death (HR=100 (95% CI 4.10 to 2503.00), p=0.005). Since somatic mutations detected in ctDNA from plasma can be an indicator of disease progression, response to therapy, and clonality of primary and metastatic lesions. The ctDNA clearance of the 23 patients after 2 cycles of treatment were analyzed and showed in table 2. Kaplan-Meier survival analysis was used to evaluate the association between clearance of clonal mutations and patients' prognosis at 6th week. The results showed that clearance of clonal mutations was associated with longer OS (mOS 147 vs 467 days, HR=7.01 (95% CI 1.30 TO 37.69), p=0.0048) (figure 4D). In addition, clearance of clonal mutations and no clonal formation at 6th week were associated with longer PFS (mPFS 89 vs 266 days, HR=4.40 (95% CI 0.84 to 23.16), p=0.003 (figure 4D). For the association between clearance of primary clonal mutations and patients' prognosis, the results showed that the ctDNA clear (primary clone clearance) showed numerically better OS than that with ctDNA residual (primary clone residue) (255 vs 467 days; HR 4.445; 95% CI 0.5401 to 36.58; p=0.0296; online supplemental figure 3G).

Representative patients monitored with variant allele frequency and radiology

Among the 23 patients, 7 patients who agreed to monitor were selected. All selected patients had been diagnosed with advanced NSCLC with disease progression during or after platinum-based chemotherapy or intolerance/failure of any first-line TKI, and all patients received sintilimab (200 mg, day 1, every 3 weeks) plus docetaxel (75 mg/m2, day 3, every 3 weeks) for 4–6 cycles, followed by maintenance therapy with sintilimab (200 mg, day 1, every 3 weeks) until disease progression or unacceptable toxic effects. Clinical response including CTs and variant allele frequency (online supplemental tables) was evaluated by the investigators.

Patient eight was a male in his early 40 s, smoker, diagnosed with advanced NSCLC. A total of four genetic mutations were found at baseline, among which the mutation frequencies of three genes, *DNMT3CA*, *TAF1*, and *SMARCA4*, tended to increase at 6th week, while only the mutation frequency of *SDHB* tended to decrease at 6th week. The corresponding CT images were shown in figure 5, which demonstrated that his efficacy evaluation of ICIs combined with chemotherapy was PD according to RECIST V.1.1.

Patient 10 was male smoker, diagnosed with advanced NSCLC in his early 50 s. A total of three genetic mutations including *EP300*, *EGFR*, *DNMT3A* were found at baseline, and the variant allele frequencies tended to decrease overall at 6th week. The corresponding CT images were shown in figure 5, which demonstrated that his efficacy evaluation of ICIs combined with chemotherapy was PR according to RECIST V.1.1.

Patient 23 was a male smoker who was diagnosed with advanced NSCLC in his late 50s, and had mutations in four genes of *RPTOR*, *AKT2*, *EGFR*, and *TP53* (splicing and R273L) at baseline. The variant allele frequencies of almost all genes were decreased, except for *TP53* (R273L), which was slightly upregulated. The corresponding CT images were shown in figure 5, which demonstrated that his efficacy evaluation of ICIs combined with chemotherapy was SD according to RECIST V.1.1.

Patients 25 was a male, non-smoker, diagnosed with advanced NSCLC in his late 60s. He had mutations in 16 genes including *NFE2L2*, *EPHA3*, *EIF4A2*, *IL7R*, *ROS1*, *PXDNL*, *BIRC3*, *ATM*, *CBL*, *KMT2D*, *GLI1*, *TP53*, *NCOR1*,

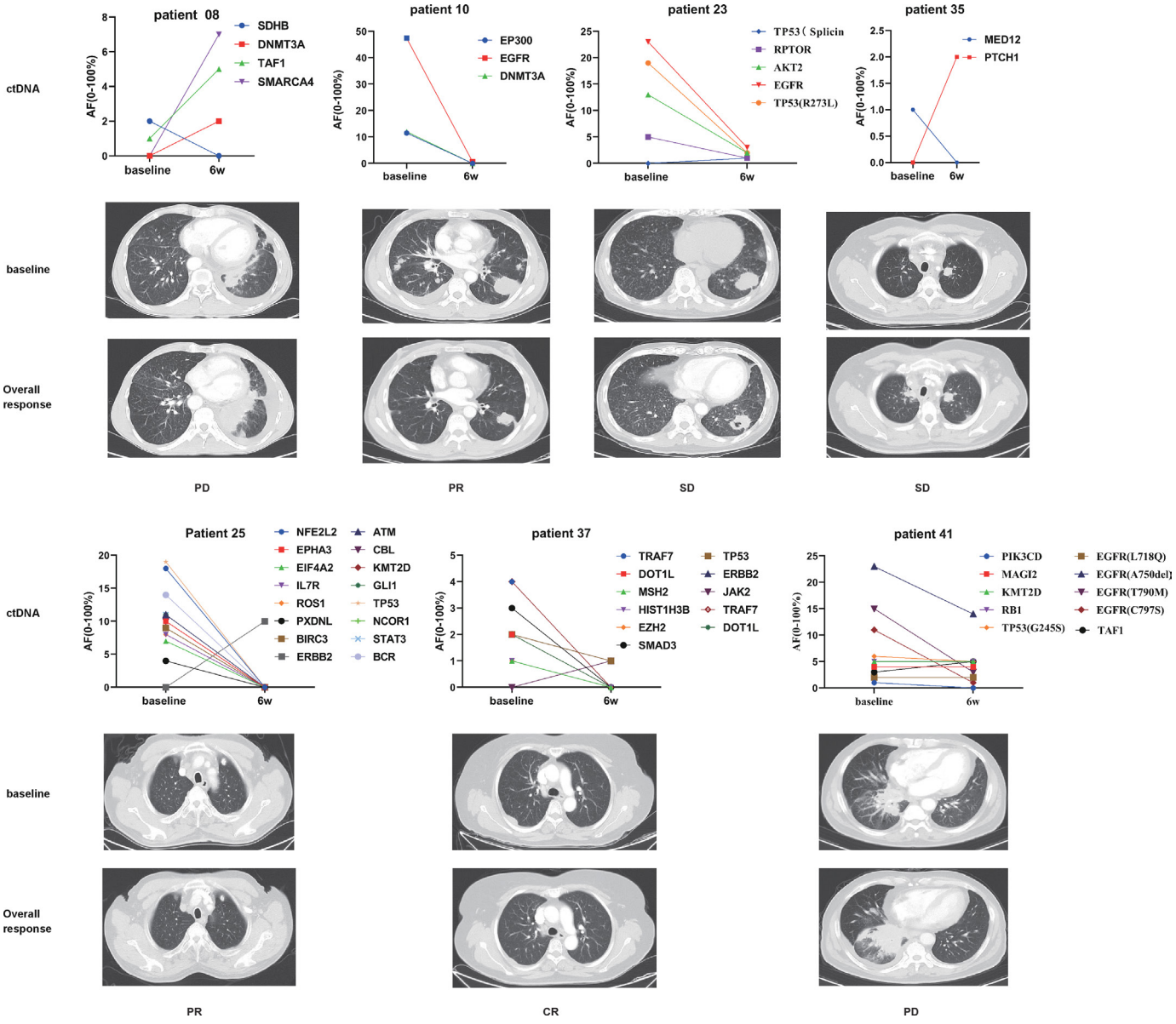

Figure 5 Variant allele frequency of ctDNA and CT images of seven patients who agreed to monitor. CR, complete response; ctDNA, circulating tumor DNA; PD, progressive disease; PR, complete response.

STAT3, *BCR* and *ERBB2* at baseline. The variant allele frequencies of 15 genes tended to decrease to 0 at 6th week, while only the variant allele frequency of *ERBB2* tended to increase at 6th week. The corresponding CT images were shown in figure 5, which demonstrated that his efficacy evaluation of ICIs combined with chemotherapy was SD according to RECIST V.1.1.

Patient 35 was a male and non-smoker, diagnosed with advanced NSCLC in his early 30s. A total of two genes were found to have mutations at baseline, the mutation frequency of *MED12* decrease to 0 at 6th week, while the mutation frequency of *PTCH1* tended to increase at 6th week. The corresponding CT images were shown in figure 5, which demonstrated that his efficacy evaluation of ICIs combined with chemotherapy was SD according to RECIST V.1.1.

Patient 37 was a female, non-smoker, diagnosed with advanced NSCLC in her late 50s. A total of nine genes were found to have mutations at baseline. The variant allele frequencies of *TRAF7*, *DOT1L*, *MSH2*, *HIST1H3B*, *EZH2* and *SMAD3* tended to decrease to 0 at 6th week, while the variant allele frequencies of *TP53* (exon 5), *ERBB2* and *JAK2* tended to slightly increase at 6th week. The corresponding CT images were shown in figure 5, which demonstrated that her efficacy evaluation of ICIs combined with chemotherapy was CR according to RECIST V.1.1.

Patient 41 was a male in his early 50s, non-smoker, diagnosed with advanced NSCLC. A total of seven genes were found to have mutations at baseline. The variant allele frequencies of *PIK3CD*, *MAGI2*, *KMT2D*, *RB1*, *TP53* (exon7), *TAF1* and *EGFR* (exon18, exon19, exon20,

exon20) tended to decrease at 6th week. The corresponding CT images were shown in figure 5, which demonstrated that his efficacy evaluation of ICIs combined with chemotherapy was PD according to RECIST V.1.1.

DISCUSSION

Here, we reported a single arm, phase 2 study of 33 patients to explore the potential biomarkers that can predict therapeutic effect and prognosis, and the results showed that major molecular characteristics in preselected genes of interest were associated with response to second-line chemoimmunotherapy. Besides, Cox regression demonstrated that ctDNA status after two cycles of treatment could be an independent risk factor for disease progression, and either ctDNA clearance or primary clonal mutation clearance was significantly associated with PFS and OS.

Non-invasive blood-based liquid biopsy and circulating cell-free DNA analysis has become a routine approach to elucidate patient's genomic landscape and actionable information to help for identifying therapeutic targets and combined treatment strategies.[31] The spectrum and frequency of NSCLC gene mutations described in tissue and their concordance with plasma ctDNA has been well published.[21] According to the previous study, primary and adaptive resistance to immunotherapy has been associated with specific genetic alterations.[26]

In our research, blood samples collected from 33 advanced NSCLC patients were used to analyze the genomic profiling between baseline and 6th week, and the results demonstrated that the landscape of gene mutations was consistent with the previous study,[32] for instance, *TP53* and *EGFR* were the most frequent gene in NSCLC. *TP53*, *EGFR*, *LRP1B*, *KRAS*, *PTPRD*, and *DNMT3A* exhibited the highest mutational frequencies in the baseline, while several patients developed new gene mutations such as *KRAS*, *ERBB2*, *DNMT3A*, *MED12*, and *CBL* or new mutation sites including *EGFR* and *LRP1B* after two cycles of treatment.

Immunotherapy with anti-PD-1 inhibitors is revolutionizing the treatment of NSCLC. To maximize the efficacy of immunotherapy in patients with lung cancer, it is critical to identify which patients may benefit from different treatments. Recently, ctDNA has been proved can reflect the burden of tumors and carry the original tumor mutations.[33] Prior research has associated ctDNA level with OS in various advanced solid tumors, including brain, lung, breast, and gastrointestinal, independent of intervening method.[34] And the correlation of ctDNA detection with the emergence of recurrent or PD has been shown in early-stage NSCLC patients and in advanced NSCLC patients receiving targeted therapies.[35 36] It also has been reported that ctDNA levels and bTMB have a significant impact on the prognosis of tumor patients.[37] In our study, the association of baseline and 6th-week ctDNA status with patients' prognosis was evaluated, and the results showed that ctDNA status after two cycles of treatment (6th week) could be an independent prognostic biomarker, the ctDNA clearance was significantly associated with better PFS as well as overall survival (OS).

Some mutant subclones were considered probably responsible for drug resistance and disease progression.[38] For example, the variant allele frequencies of *EGFR* subclone resistance mutations in NSCLC patients were associated with patients' PFS and OS.[39 40] Our study also supports this conclusion as the results showed that the clearance of clonal mutations was associated with longer prognosis. Further, the variant allele frequencies of patient 10 tended to decrease overall at 6th week and his efficacy evaluation of ICIs combined with chemotherapy was PR according to RECIST V.1.1. In addition, the efficacy evaluation of ICIs combined with chemotherapy of patient 37 was CR according to RECIST V.1.1, as the variant allele frequencies of 11 genes detected at baseline were all tended to decrease significantly at 6th week. On the contrary, the variant allele frequencies of the patient 8 tended to increase overall, so that his efficacy evaluation of ICIs combined with chemotherapy was PD according to RECIST V.1.1. Further, it should be noticed that these radio-genomic results were concordant to previously reported studies,[41–43] confirming that the complementary and synergistic combination of the liquid biopsy and imaging can provide an attractive choice in the personalized treatment of advanced lung cancers.

It was previously reported that higher TMB resulted stronger tumor immunogenicity due to trigger stronger T cell response and the antitumor response by production of higher load of new antigens.[44] Our research results showed that bTMB could not be used as an independent prognostic factor. Patients with lower baseline bTMB showed the trend to have benefit from immunotherapy. That was consistent with the previously reported studies.[45 46] Rather than being associated with neoantigen loads, bTMB might be more closely associated with tumor burden,[35] amount of ctDNA,[44] as well as with the increased intra-tumor heterogeneity especially induced by prior treatment.[47]

Further, a recent meta-analysis revealed that the levels and the clearance of ctDNA can be used as independent prognostic factors for immunotherapy, while the prognostic impact of bTMB in cancer patients undergoing immunotherapy is worth further discussion and exploration.[48] However, for monitoring response to treatment with bTMB in patients with advanced NSCLC receiving ICIs, our results showed that the degree or percentage of change in bTMB was significantly associated with therapeutic effect of DCB/BOR, which indicated that changes in bTMB could possibly predict therapeutic response, even though there was no significant relationship between bTMB change and the long-term survival.

There are several limitations in this study. First, as a single-arm, phase 2 clinical trial, the estimated sample size might have been limited. Second, though ctDNA kinetics and change in bTMB could indicate the response of chemo-immunotherapy, no chemotherapy arm could

be used as control in this study to infer whether these findings were predictive specifically for immunotherapy. Third, PD-L1 staining was not amendatory in current study due to re-biopsy was not eligible to all NSCLC patients progressed from prior treatment. While, in 23 patients with paired blood ctDNA samples, PD-L1 results were available in seven patients. Kinetics of ctDNA was more relevant to the therapeutic efficacy than PD-L1 TPS score (1% as cut-off) (online supplemental figure 2). Similar phenomena have also been reported in a previous study.[49] Finally, longer follow-up is required to fully assess the effect of ctDNA kinetics in predicting prognosis. Thus, larger prospective trials of anti-PD-1 plus chemotherapy vs chemo-monotherapy in first/second-line treatment of advanced NSCLC with PD-L1 TPS score will be necessary and warranted to validate these findings and determine ctDNA kinetics could be used as predictive marker.

In conclusion, findings from this phase 2 trial demonstrated the feasibility that negative ctDNA status, as well as the clearance of primary clonal mutation after treatment could be a potential biomarker to predict therapeutic response and long-term survival. However, the results of this study need to be further verified. In addition, the clinical value of bTMB level in immunotherapy of NSCLC needs further explored.

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