

Circulating tumor DNA serial monitoring of relapse and responses to tislelizumab immunotherapy as second-line monotherapy for metastatic esophageal squamous cell carcinoma: A prospective study

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Abstract. Anti-programmed cell death 1 immuno-monotherapy has become the second-line standard treatment for advanced esophageal squamous cell carcinoma (ESCC) after the failure of first-line chemotherapy. However, new biomarkers are still needed to identify patients at risk of tumor progression and to select patients with advanced ESCC who are likely to benefit from immunotherapy. A total of 12 patients with advanced ESCC treated with tislelizumab were prospectively enrolled and endoscopic biopsy samples were collected. Plasma was obtained prior to and after every 2-3 treatment cycles with tislelizumab and when disease progression occurred. Targeted sequencing of 425 genes from plasma cell-free DNA, DNA from leukocytes and fixed esophageal tumor biopsies was performed. The patients underwent imaging analyses every 6-8 weeks until disease progression. The association between status of circulating tumor DNA (ctDNA) or changes in ctDNA following tislelizumab immunotherapy and response, tumor progression and survival was determined. All patients had evaluable next-generation sequencing results at the time of analysis. The results showed that patients with ESCC with liver metastasis had a significantly shorter median progression-free survival (mPFS: 1.4 vs. 11.7 months; $P=0.037$). TSC complex subunit 2 [11.7 months vs. not reached (NR); $P=0.004$] and zinc finger protein 217 (11.7 months vs. NR; $P=0.022$) gene mutations were the independent and negative prognostic factors for median overall survival (OS), respectively. Of note,

ctDNA dynamic changes expressed as Δ mutant molecules per milliliter of plasma (Δ MMPM; MMPM detected at the first monitoring time-point after the first infusion of tislelizumab as baseline MMPM) predicted progression-free survival (PFS) and OS more accurately compared to the ctDNA change of an individual gene. Δ MMPM $<20\%$ was an independent predictor of PFS (2.8 vs. 14.6 months; $P=0.029$), although there was no significant difference for OS (16.7 vs. 17.6 months; $P=0.830$). In conclusion, changes in ctDNA levels were associated with anti-tumor effects, progression and disease-specific survival. ctDNA sequencing is promising for predicting response and progression after tislelizumab immunotherapy as second-line monotherapy for advanced ESCC [the present study was part of the RATIONALE-302 study (ClinicalTrials.gov identifier no. NCT03430843; 29.01.2018)].

Introduction

Esophageal squamous cell carcinoma (ESCC) is the predominant (90%) histological type of Esophageal cancer. The prognosis of advanced ESCC is poor, with a median overall survival (OS) of ~1 year, highlighting the great need for new therapies (1,2). Recent clinical results support the use of new immune checkpoint blockers, such as anti-programmed cell death 1 (PD-1) and PD-1 ligand 1 (PD-L1) antibodies, as part of the antitumor treatments in several cancer types, including ESCC. These inhibitors are recommended to be used either as second-line monotherapy or in combination with platinum doublet chemotherapy in patients with tumors expressing PD-L1 at any level in ESCC (3-8). Such treatments would be facilitated by the selection of biomarkers that can sensitively and noninvasively detect early responses and predict prognoses. The reality is that conventional clinical assessment and imaging, such as endoscopy, endoscopic ultrasound and computed tomography (CT), are not sufficiently robust methods of gauging treatment response to anti-PD-1/PD-L1 therapy, such as pseudoprogression, which manifests in delayed clinical responses in certain patients, consisting of an initial increase in tumor size, which is subsequently followed by tumor shrinkage due to tumor immune cell infiltration (9,10). These results demonstrate that clinical assessment and imaging cannot

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always reliably predict clinical benefits and survival in patients who received immunotherapy. Accordingly, there is an unmet clinical need to identify biomarkers that can sensitively detect early progression and predict prognosis in patients with ESCC receiving anti-PD-1 immunotherapy. Circulating tumor DNA (ctDNA) is a new biomarker that has been mostly investigated as a tool for liquid biopsy, as it harbors mutations similar to those in DNA extracted from tumor biopsies (11-13). Of note, ctDNA detection techniques are quantitative, and changes in ctDNA levels during chemotherapy and targeted therapy have been associated with tumor response or progression in several tumor types; ctDNA levels may predict outcomes for patients undergoing esophagectomy for ESCC (14-16). However, to the best of our knowledge, no previous study has applied ultrasensitive next-generation sequencing (NGS) methods to detect ctDNA changes in patients with ESCC receiving anti-PD-1 monotherapy as a second-line therapy. The present study reported on the deep sequencing of 425 cancer-related genes in 43 ctDNA samples collected before treatment and at different time-points during treatment with tislelizumab (an anti-PD-1 drug) in 12 patients with ESCC. It was demonstrated that ctDNA sequencing is promising for predicting the response and prognosis after treatment with tislelizumab as a second-line monotherapy for advanced ESCC.

Patients and methods

Subjects and study procedure. Our research center is one of the sub-centers of the RATIONALE-302 (17) study (ClinicalTrials.gov identifier no. NCT03430843), of which 20 subjects were screened and 15 were enrolled, of which 12 patients were eligible for this exploratory study at Zhejiang Cancer Hospital (Hangzhou, China) between March 2018 and September 2020, whose tumor tissue or plasma was available for NGS. Eligible patients were aged 18-73 years and previously treated with systematic chemotherapy for stage IV ESCC, who were being treated with tislelizumab immuno-monotherapy 200 mg intravenous drip every 3 weeks until disease progression. Specific inclusion and exclusion criteria were those of the RATIONALE-302 study (17). This study was approved by the Institutional Review Board of Zhejiang Cancer Hospital (Hangzhou, China) and all patients provided written informed consent. The study was conducted in accordance with the established ethical standards of the institutional and/or national research committee, as outlined in the Declaration of Helsinki.

Radiographic data and clinical outcomes. Patients underwent CT imaging at a follow-up visit at every 6-8 weeks during immunotherapy and chest and abdominal CT scans were evaluated according to Response Evaluation Criteria in Solid Tumours (RECIST 1.1) (18), by a radiologist blinded to circulating cell-free DNA (cfDNA) data. Progression-free survival (PFS) was defined as the interval between treatment initiation and the date of disease progression or death from any cause, whichever occurred earlier. Overall survival (OS) was defined as the time interval from treatment initiation to death from any cause. A censor date of June 30, 2021 was applied if no endpoint was met.

Tumor and blood sample collection. Fresh ESCC tumor samples from patients undergoing esophagogastroscope were obtained from 12 patients before immunotherapy. A total of 35 blood samples for ctDNA were obtained at baseline, when patients achieved a status of partial response (PR) or stable disease (SD), or after progressive disease (PD) from March 2018 through to September 2020. Tumor samples were subjected to pathological diagnosis, graded according to the World Health Organization system (19) and then prepared into formalin-fixed and paraffin-embedded (FFPE) blocks. Peripheral blood (8-10 ml) was collected from each patient at each time-point in EDTA-coated tubes (BD Biosciences), and plasma was separated within 2 h after peripheral blood collection. All samples were sent to the centralized testing center of Nanjing Geneseeq Technology, Inc. (Nanjing, China) for targeted NGS.

DNA extraction and library construction. Genomic DNA from FFPE tissues was extracted using a QIAamp DNA FFPE Tissue Kit (Qiagen GmbH). DNA from white blood cells (WBC) was extracted with the DNeasy Blood & Tissue Kit (Qiagen GmbH) and sequenced as normal controls to distinguish germline variations. Plasma-derived cfDNA was extracted using the QIAamp Circulating Nucleic Acid Kit (Qiagen GmbH). DNA was quantified with a Qubit 3.0 (Thermo Fisher Scientific, Inc.) and the quality was assessed using a Nanodrop 2000 (Thermo Fisher Scientific, Inc.). FFPE and WBC-derived DNA was sheared into 300-350 bp fragments using a Covaris M220 instrument (Covaris, Inc.). cfDNA or fragmented genomic DNA was prepared using a KAPA Hyper Prep Kit (KAPA Biosystems) as previously described (20). In brief, the DNA was subjected to end repair, A-tailing and adaptor ligation. DNA was then amplified and purified. A customized NGS panel containing 425 cancer-related genes was used for library enrichment. The captured libraries were amplified, purified and quantified. All the experimental procedures were performed at Nanjing Geneseeq Technology, Inc.

Library sequencing and bioinformatics analysis. The prepared library was sequenced using a HiSeq4000 platform (Illumina, Inc.). The mean coverage depth was 150x μ m for WBC controls and 800x μ m for tissue samples. For cfDNA samples, the mean coverage sequencing depth was 5,000x. Base calling was performed on a bcl2fastq v2.16.0.10 (Illumina, Inc.) to generate sequence reads in the FASTQ format (Illumina 1.8+ encoding). Quality control was performed using the Trimmomatic software version 0.40 (<https://github.com/usadellab/Trimmomatic>). High-quality reads were aligned to the human genome (hg19; Genome Reference Consortium Human ref. 37) using Burrows-Wheeler Aligner 0.7.12 (<https://github.com/lh3/bwa/tree/master/bwakit>). The data were further processed using Picard 1.119 (<https://github.com/broadinstitute/picard/releases/latest>) and the Genome Analysis Toolkit (GATK; version 3.4.0; <https://software.broadinstitute.org/gatk>).

Single nucleotide variants (SNVs) and short insertions/deletions (indels) were identified using VarScan2 and HaplotypeCaller/UnifiedGenotyper in GATK, with a minimum mutant allele frequency cut-off of 0.5% for tissue, 0.3% for cfDNA and a minimum of three unique mutant reads

with good quality scores. All SNVs/indels were annotated using ANNOVAR (default version; <https://bioweb.pasteur.fr/packages/pack@annovar@24.10.2019>) and manually checked using the Integrative Genomics Viewer version 2.10.3 (<https://igv.org>). Gene fusions/rearrangements were identified using FACTERA version 1.4.4 (<https://factera.stanford.edu/download.php>) and copy number gain or loss was analyzed using ADTEX version 2.0 (<https://sourceforge.net/projects/adtex>). The log₂ ratio cutoff defined for copy number gain was 2.0 for tissue and 1.6 for cfDNA. A log₂ ratio cutoff defined for copy number loss was 0.6 for all sample types. Tissue tumor mutation burden (tTMB) was calculated according to Fang's study (21). High-level tTMB (tTMB-H) was defined as ≥10 mutations/Mb (mut/Mb) and <10 mut/Mb was defined as tTMB-L.

ctDNA monitoring and MMPM analysis. A longitudinal ctDNA analysis was performed and the mutant molecules per milliliter of plasma (MMPM) were measured, which quantifies ctDNA for all variants. The overall MMPM for each sample was defined as the mean MMPM (mMMPM) across SNVs and indels, which was calculated as previously described (22,23). In brief, the mMMPM was calculated from the mean allele fraction (mAF) of variants by multiplying by the extracted mass (ng), dividing by the plasma volume (ml) and adjusting by a factor of 330 haploid human genome equivalents per ng:

$$mMMPM = \frac{mAF \times \text{extracted mass (ng)} \times 330}{\text{Plasma volume (ml)}}$$

ΔmMMPM was defined as the percentage change of mMMPM in plasma at the first monitoring time-point after the first infusion of tislelizumab (ranging from cycles 2 to 6) since baseline for each patient:

$$\Delta mMMPM = \frac{mMMPM \text{ at first monitoring} - mMMPM \text{ at baseline}}{mMMPM \text{ at baseline}} \times 100\%$$

Statistical analysis. Quantitative data are presented as the median (range) or n (%). Proportion comparisons between the groups were performed using Fisher's exact test. Kaplan-Meier curves were used to perform the survival analysis. Genes with mutations that were significantly associated with PFS and OS were screened using univariate Cox models. A two-sided P<0.05 was considered statistically significant for all tests unless indicated otherwise. All analyses were performed using R software version 3.6.3 (<https://cran.r-project.org/bin/windows/base/old/3.6.3/>).

Results

Patient characteristics. Between March 2018 and September 2020, 15 patients who received tislelizumab as a second-line treatment for advanced ESCC in the RATIONALE-302 study (17) were recruited for the present study. Among them, 12 patients had endoscopic biopsy samples taken before tislelizumab immunotherapy, as well as plasma samples before and after every 2-3 (6-9 weeks) treatment cycle and disease progression. These 12 patients were included in the subsequent analysis. All 12 patients had interpretable data at the pretreatment time-point and 10 patients (83.3%) had interpretable data of NGS at the dynamic time-points and were used for analyses.

The median age of the patients was 62.5 years (range, 46-73 years). There were 11 men (91.7%), nine current or former heavy drinkers (75.0%) with >1 l of liquor per day. The Eastern Cooperative Oncology Group performance status (24) of all the 12 patients was 0-1. Among these 12 patients, a total of four patients (33.3%) had undergone surgery and nine patients (75.0%) had received local radiotherapy, one of them had undergone both surgery and radiotherapy. Of the 12 patients, nine (75%) had evaluable lymph node metastases, six (50%) had lung metastases and two (16.7%) had liver metastases. All patients received tislelizumab as the second-line treatment. A total of six patients (50.0%) received ≥12 cycles of tislelizumab. The overall response rate was 33.3%. After a median follow-up of 19.3 months, eight patients (66.7%) exhibited confirmed tumor progression, of whom six (50.0%) died (Table I). The median PFS of the 12 patients treated with tislelizumab was 7.9 months (Fig. 1A) and the median OS was 17.6 months (Fig. 1B). Tumor evaluation was performed after every 2 injections of tislelizumab at 6 weeks of treatment for all patients, except for one patient with severe immune-related thrombocytopenia, who died quickly after only one injection of tislelizumab. This patient only underwent endoscopic biopsy and plasma samples were taken before treatment and were included in the following ctDNA analyses. Of the 12 patients with ESCC in the present study, almost all clinical characteristics (such as age, sex, treatments, cycles of treatment and number of metastatic organs) were not significantly associated with clinical response and outcome (data not shown), except for liver metastasis. The present results showed that patients with liver metastasis had a significantly shorter mPFS (1.4 vs. 11.7 months; P=0.037) (Fig. 1C) and a tendency of shorter OS (13.2 vs. 17.6 months; P=0.560) (Fig. 1D). Table I describes the population characteristics and clinical response to tislelizumab immunotherapy of the patients of this study.

Genomic profiling of ESCC from pre-treatment tumor DNA and ctDNA detection. Tumor tissues were available from all 12 patients and were subjected to targeted sequencing of 425 cancer-associated genes. Tumor mutation profiles included high-frequency alterations in tumor protein (TP)53 and cell cycle genes, such as fibroblast growth factor (FGF)19 and cyclin (CCN)D1 (Fig. 2A). According to the Kaplan-Meier survival analysis, the median OS for TSC complex subunit 2 (TSC2) and zinc finger protein 217 (ZNF217) gene mutation was significantly shorter than that for TSC2 [11.7 months vs. not reached (NR), P=0.004, Fig. 2B] or ZNF217 wild type (11.7 months vs. NR, P=0.022, Fig. 2C), while this trend was not significant for PFS. Furthermore, patients with FGF19 gene amplification had a slightly longer PFS (Fig. S1A) and OS (Figs. 2D and S1B) than patients with FGF19 gene wild-type, but the differences were not significant. However, when patients were stratified by the tTMB, patients with tTMB-H had longer PFS and OS than patients with tTMB-L, although no significant differences were obtained (PFS: 10.8 vs. 7.2 months, P=0.750; OS: 17.0 vs. 13.2 months, P=0.680) (Fig. S1C and D).

Subsequently, pretreatment ctDNA levels were analyzed in 12 patients for whom baseline plasma samples were available. Table SI shows the ctDNA results for all 35 plasma samples collected by NGS for each patient. ctDNA was detected in 7/12 (58.3%) patients at baseline. Patients with undetectable

Table I. Clinical characteristics of patients receiving tislelizumab therapy and their tumor response in the present study (n=12).

Characteristic	Value
Age, years	62.5 (46-73)
≤65	6 (50.0)
>65	6 (50.0)
Sex	
Male	11(91.7)
Female	1 (8.3)
ECOG PS	
0	0 (0)
1	12 (100)
Stage	
I-III	0 (0)
IV	12 (100)
Previous therapies	
Surgery	4 (33.3)
Radiotherapy	9 (75.0)
Systemic anticancer therapy	12 (100)
Cycles of treatment	12 (1-28)
<12	6 (50.0)
≥12	6 (50.0)
Number of metastasis organs	
≤3	9 (75.0)
>3	3 (25.0)
Site of metastases	
Lymph node	9 (75.0)
Liver	2 (16.7)
Lung	6 (50.0)
Best overall response	
Complete response	0 (0)
Partial response	4 (33.3)
Stable disease	4 (33.3)
Progressive disease	4 (33.3)
Response	
Confirmed objective response	4 (33.3)
Confirmed disease control	8 (66.7)

Values are expressed as median (range) or n (%). ECOG PS, Eastern Cooperative Oncology Group performance status.

ctDNA at baseline showed prolonged OS compared to those with detected ctDNA (NR vs.13.2 months; $P=0.017$) and had a better tendency in PFS (18.2 vs. 5.5 months; $P=0.078$) (Fig. 3A and B). The mMMPM in these 10 patients at baseline was 91.68 (range: 0-462.72). ctDNA remained undetectable in two patients during the entire serial monitoring.

ctDNA dynamics and treatment efficacy. Three patterns of ctDNA molecular status were observed in the 10 patients who had plasma samples taken after tislelizumab treatment.

The first pattern, seen in two of the ctDNAs undetectable in the whole serial monitoring (Patients 11 and 12), was consistent with radiologic PR or SD for >1 year up to the end of follow-up time (Fig. 3C and D). In these two cases, tumor-specific variants were undetectable during serial monitoring of tislelizumab immunotherapy. The follow-up duration for these two patients were 12 and 17 months, respectively, and no disease progression had occurred at the last follow-up.

In the second pattern, ctDNA levels displayed a relatively obvious rise (defined as $\Delta\text{mMMPN} \geq 20\%$) 2-6 cycles after therapeutic initiation. However, two patients with $\Delta\text{mMMPN} \geq 20\%$ showed no clinical benefit. One patient exhibited PD and another patient showed SD with a PFS of <4 months. As a representative case, ctDNA levels in patient 04 continued to rise from the time of initiation of tislelizumab immunotherapy, which is consistent with radiographically confirmed PD (Fig. 4A).

The last pattern, among the patients with $\Delta\text{mMMPM} < 20\%$ (n=8), showed a marked reduction or no relatively obvious rise in ctDNA on average at 3 cycles from treatment initiation. All patients showed clinical benefit with at least PR or SD, with a PFS of >6 months, except for one patient who had assessed PD. For instance, for patient 05, ctDNA-based molecular analyses showed a marked reduction in molecular response at cycle 2, which coincided with a radiologic partial response, and at the time of acquired resistance at 60 cycles, while radiographic analysis showed disease progression according to RECIST 1.1 (18), consistent with rising ctDNA levels during tislelizumab immunotherapy (Fig. 4B).

Accordingly, ctDNA dynamic changes expressed as the ΔmMMPM more accurately predicted PFS and OS than tTMB (Fig. 3IC and D). Patients with $\Delta\text{mMMPN} < 20\%$ had significantly longer PFS than patients with $\Delta\text{mMMPN} \geq 20\%$ (14.6 vs. 2.8 months; $P=0.029$) (Fig. 4C), although no significant differences were observed in OS (17.6 vs. 16.7 months; $P=0.830$) (Fig. 4D). To a certain extent, $\Delta\text{mMMPN} < 20\%$ was an independent predictor of PFS in patients receiving second-line tislelizumab monotherapy for advanced and metastatic ESCC.

Discussion

Anti-PD-1 immunotherapy provides long-term survival benefits for patients with advanced ESCC. However, conventional imaging examinations cannot always reliably predict clinical benefits and survival in response to anti-PD-1/PD-L1 therapy due to tumor immune infiltration. Certain patients lose the opportunity to continue receiving immunotherapy due to pseudoprogression and some patients may develop hyperprogressive disease during immunotherapy (25,26). Discriminating patients who benefit from therapy from those who may progress before imaging examinations is a challenge faced by clinical oncologists. Previous studies have shown that ctDNA dynamic changes can sensitively monitor the efficacy of chemotherapy or targeted therapy in patients at an early stage, and ctDNA can better overcome tumor heterogeneity and represent the change in the tumor burden of patients (12-14).

Multiple clinical studies have confirmed that anti-PD-1 immunotherapy combined with chemotherapy is the standard first-line regimen for ESCC (27-30). Second-line anti-PD1

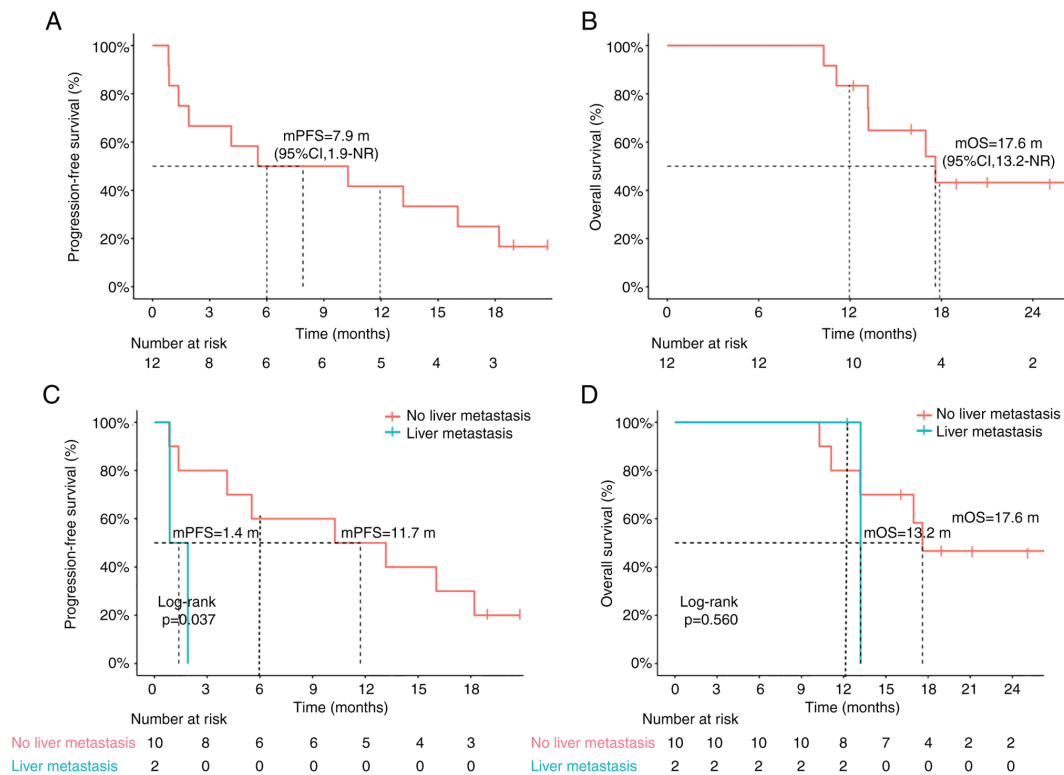


Figure 1. Survival benefit of 12 patients. (A) mPFS and (B) mOS of the 12 patients with tislelizumab. (C) Patients with liver metastasis had significantly shorter mPFS and (D) a tendency of shorter OS. mPFS, median progression-free survival; mOS, median overall survival; NR, not reached.

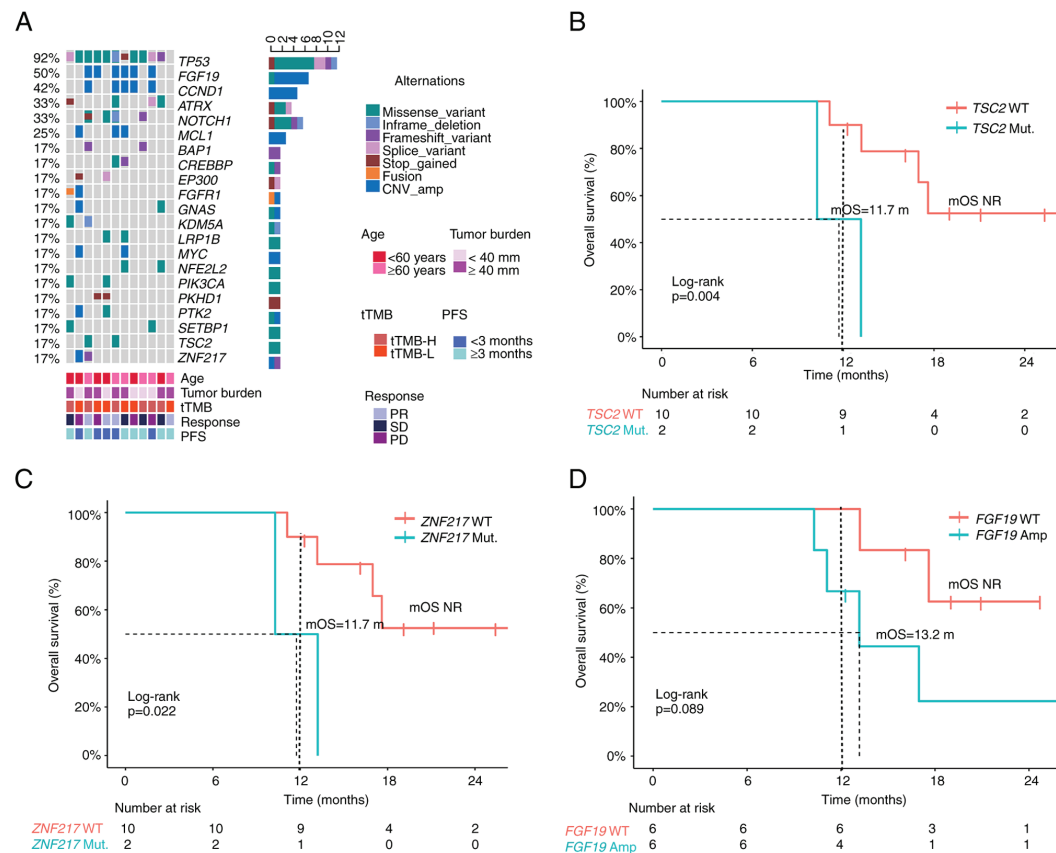


Figure 2. Results of 12 patient genetic variants and their association with survival. (A) Tumor mutation profiles in the 12 patients with tislelizumab therapy. (B and C) The mOS for (B) TSC2 and (C) ZNF217 gene mutation was significantly shorter than that for TSC2 or ZNF217 WT. (D) Patients with FGF19 gene Amp had slightly longer OS. mOS, median overall survival; PR, partial response; SD, stable disease; PD, progressive disease; WT, wild-type; Mut, mutant; NR/NA, not reached; Amp, amplification; m, months; y, years; tTMB-H, tissue tumor mutation burden high; ZNF217, zinc finger protein 217; TSC2, TSC complex subunit 2; FGF, fibroblast growth factor.

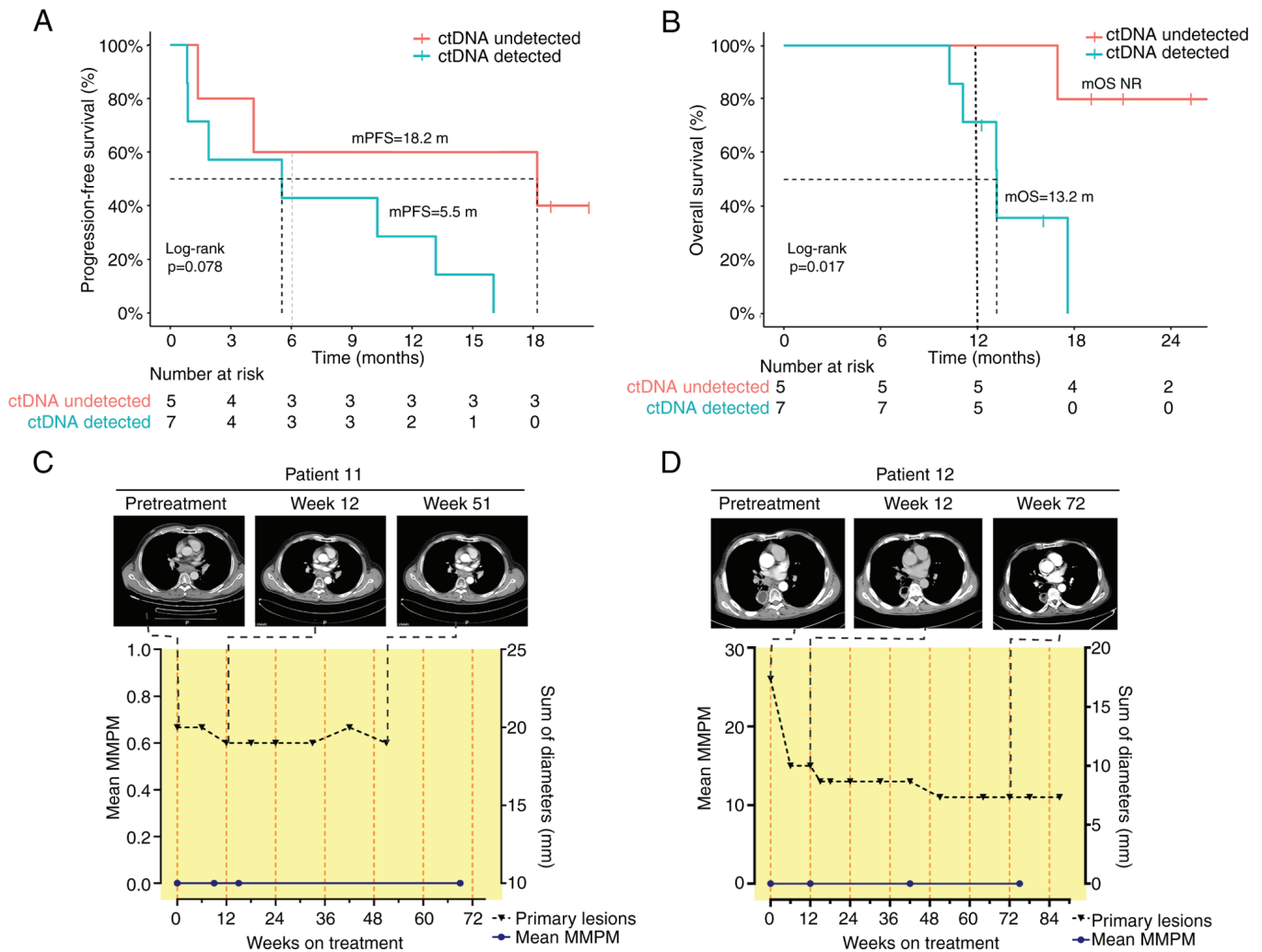


Figure 3. Relationship between ctDNA level with survival and examples. Patients with undetected ctDNA at baseline showed (A) prolonged OS in comparison to patients with detected ctDNA and (B) had a better tendency of PFS. A total of two patients had ctDNA undetectable in the whole serial monitoring, consistent with (C) radiologic stable disease (patient 11, male, 50 years) or (D) partial response (patient 12, male, 65 years). mPFS, median progression-free survival; mOS, median overall survival; ctDNA, circulating tumor DNA; NR, not reached; MMPM, mutant molecules per milliliter of plasma.

immunotherapy is superior to conventional chemotherapy in patients with ESCC who have received chemotherapy as first-line therapy (6,7,8,17). The present study found that the mPFS and mOS of patients with ESCC receiving second-line tislelizumab were 7.9 and 17.6 months, respectively. This is a partial result of the RATIONALE-302 trial and it is basically consistent with the published results of the whole RATIONALE-302 study (17). The survival time was higher than that reported in other studies, which may be due to the bias caused by the small sample size in the present study. Due to practical reasons, such as sample quality control and financial circumstances, patients from other centers were not included in the present study. With the current National Medical Products Administration of China approved indication for tislelizumab in second-line monotherapy in ESCC, real-world studies with larger sample sizes may be initiated in the future to obtain more evidence-based results. The present study also found that patients with liver metastasis were significantly less likely to benefit from anti-PD1 immunotherapy than patients without liver metastasis, which was consistent with the results of other studies (30,31), suggesting

that subjects with ESCC with liver metastasis are potentially a group of patients who are not sensitive to immune monotherapy.

In the present study, certain high-frequency mutated genes were identified in ESCC, such as *TP53* and genes involved in the cell cycle, such as *FGF19* and *CCND1*, which is consistent with previously reported results (32). *TSC2*, *ZFN217* mutation and *FGF19* amplification were negative predictors in patients with ESCC who received second-line tislelizumab. However, the mechanism of these two gene variants affecting PD-1/PD-L1 is not clear, which is also the study focus of our future work. The present study also found that patients with ESCC whose ctDNA was not detected during tislelizumab monotherapy had longer PFS and OS than those whose ctDNA was detected.

ctDNA was absent in the plasma during the whole therapy in two patients, and these two patients (patients 11 and 12) did not develop disease progression during >1 year of follow-up, suggesting that patients with ctDNA negativity may benefit from tislelizumab immunotherapy. Changes in mMMPM can predict the benefit of PFS. Patients with ESCC and

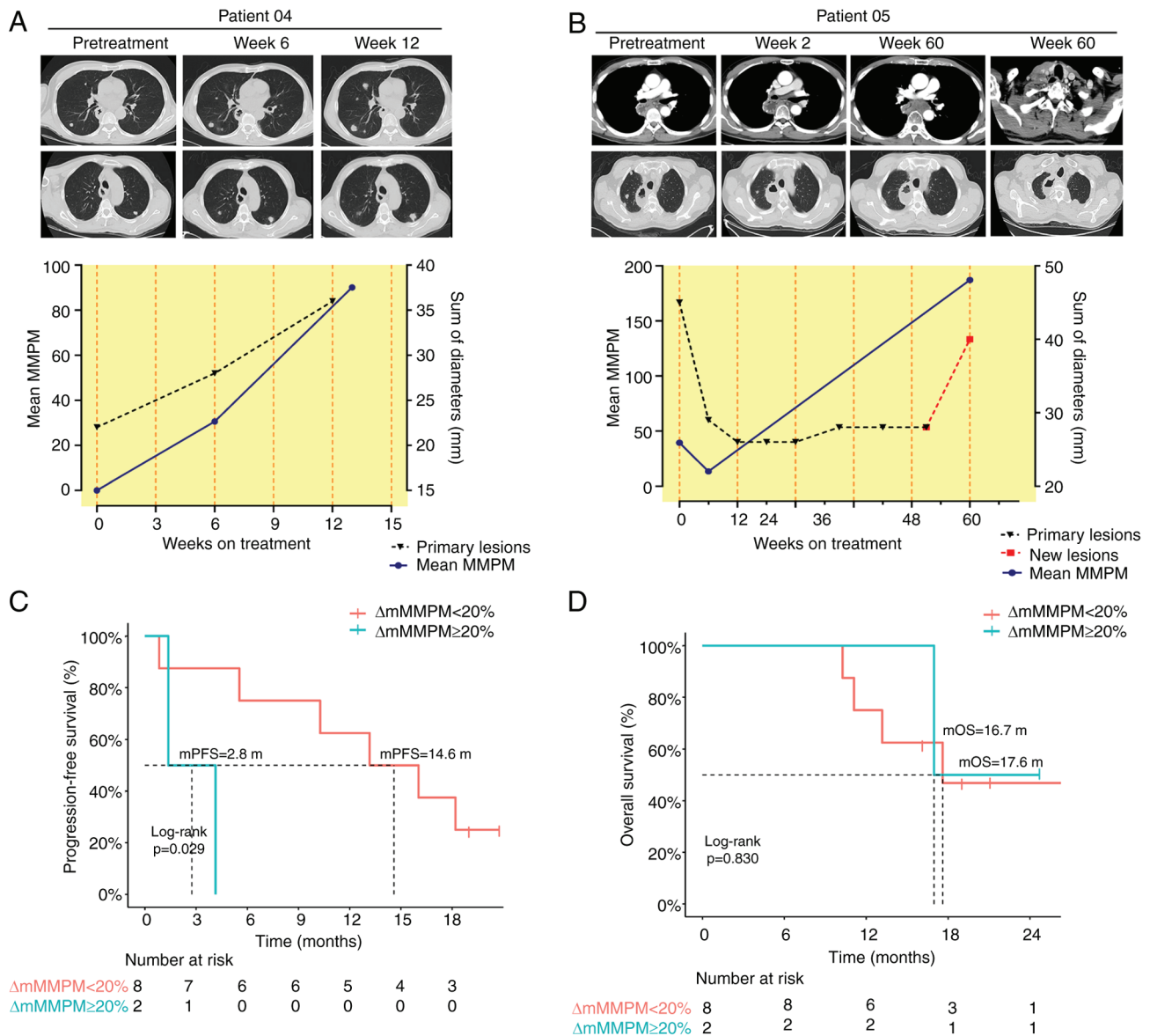


Figure 4. Relationship of ctDNA changes with response and survival with examples. (A) ctDNA levels in patient 04 (male, 59 years) continued to rise from the time of initiation of tislelizumab immunotherapy, consistent with radiographic confirmed disease progression. (B) For patient 05 (male, 56 years), ctDNA-based molecular analyses showed a markedly reduced molecular response at cycle 2, coinciding with a radiologic partial response, and at the time of acquired resistance at 60 cycles; however, radiography showed disease progression, consistent with rising ctDNA levels during the treatment with tislelizumab immunotherapy. (C) Patients with $\Delta mMMPN < 20\%$ had significantly longer PFS than patients with $\Delta mMMPN \geq 20\%$, although (D) no significant differences were observed in OS but an obvious tendency of longer OS was present. mPFS, median progression-free survival; mOS, median overall survival; ctDNA, circulating tumor DNA; $\Delta mMMPM$, mean percentual difference in mutant molecules per milliliter of plasma at first monitoring time-point vs. baseline.

$\Delta mMMPN < 20\%$ can significantly benefit from tislelizumab monotherapy, although there was no statistically significant change in OS. To our knowledge, this is the first study to systematically evaluate the efficacy and prognostic utility of ctDNA in a cohort of patients with ESCC treated with tislelizumab as a second-line monotherapy. Since the present analysis was exploratory, future studies will be needed to validate our findings, including the $\Delta mMMPM$ cutoff point. In addition, it would be interesting to combine ctDNA analysis with other potential clinical or molecular predictors of outcome in multiparameter models that may further improve outcome prediction.

However, the present study had certain limitations. It was a prospective study with a relatively small sample

size. Furthermore, owing to the lack of tumor tissue after immunotherapy, the present observation of putative emergent mutations in a subset of patients must be interpreted with caution, given that we cannot be entirely certain that these mutations originate from tumor cells. Future studies investigating emergent mutations should profile metastatic/recurrent tumor deposits in addition to pre- and post-immunotherapy plasma samples to conclusively establish the presence of these mutations in tumor cells.

In conclusion, in an analysis of cfDNA in plasma samples from patients who underwent tislelizumab immunotherapy for ESCC, the detection of ctDNA was associated with anti-tumor effects, progression and disease-specific

survival. It was demonstrated that ctDNA sequencing is promising for predicting response and relapse after tislelizumab immunotherapy as a second-line monotherapy for advanced ESCC.

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Availability of data and materials

The datasets supporting the conclusions of this study are included within the article and its additional files. All mutations reported in this study are provided in Table SI. The data presented in the study are deposited in the Genome Sequence Archive for Human (GSA-Human) repository (<https://bigd.big.ac.cn/gsa-human/>; accession no. HRA005982).

Authors' contributions

Conceptualization and design: QH, JY, MW, XY; methodology development: QH, XS, YJ; data acquisition: QH, XS, YJ, CG, XY; data analysis and interpretation (e.g., statistical analysis, bioinformatics and other computational analyses): QH, JY, MW; writing and/or revision of the manuscript: QH, JY, MW; administrative, technical or material support (e.g., reporting or organizing data, constructing databases): QH, XY. QH and XY checked and confirmed the authenticity of the raw data and supervised the study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of Zhejiang Cancer Hospital (Hangzhou, China) and all patients provided written informed consent. The study was conducted in accordance with the established ethical standards of the institutional and/or national research committee, as outlined in the Declaration of Helsinki.

Patient consent for publication

The authors confirm that they have obtained written consent from each patient to publish the manuscript.

Competing interests

JY and MW are employees of Nanjing Geneseeq Technology Inc. The remaining authors have no competing interests to declare.

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