



Original Articles

Unrevealing the therapeutic benefits of radiotherapy and consolidation immunotherapy using ctDNA-defined tumor clonality in unresectable locally advanced non-small cell lung cancer



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ABSTRACT

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Progression occurs in approximately two-thirds of patients with locally advanced non-small cell lung cancer (LA-NSCLC) receiving chemoradiation and consolidation immunotherapy. Molecular indicators for outcome prediction are under development. A novel metric, the ratio of mean to max variant allele frequency (mmVAF), was derived from 431 pre-treatment tissue biopsies from The Cancer Genome Atlas and evaluated in serial circulating tumor DNA (ctDNA) from 70 LA-NSCLC patients receiving definitive radiotherapy/chemoradiotherapy (RT/CRT) with/without immunotherapy. High mmVAFs in pre-treatment tissue biopsies, indicating clonal predominant tumors ($P < 0.01$), were associated with inferior overall survival [OS, hazard ratio (HR): 1.48, 95 % confidence interval (CI): 1.11–1.98]. Similar associations of mmVAF with clonality ($P < 0.01$) and OS (HR: 2.24, 95 % CI: 0.71–7.08) were observed in pre-treatment ctDNA. At 1-month post-RT, ctDNA mmVAF-high patients receiving consolidation immunotherapy exhibited improved progression-free survival (PFS) compared to those who did not (HR: 0.14, 95 % CI: 0.03–0.67). From the baseline to week 4 of RT and/or 1-month post-RT, survival benefits from consolidation immunotherapy were exclusively observed in ctDNA mmVAF-increased patients (PFS, HR: 0.39, 95 % CI: 0.14–1.15), especially in terms of distant metastasis (HR: 0.11, 95 % CI: 0.01–0.95). In summary, our longitudinal data demonstrated the applicability of ctDNA-defined clonality for prognostic stratification and immunotherapy benefit prediction in LA-NSCLC.

Abbreviations: bTMB, blood tumor mutation burden; CI, confidence interval; CRT, chemoradiotherapy; ctDNA, circulating tumor DNA; DMFS, distant metastasis-free survival; HR, hazard ratio; LA-NSCLC, locally advanced non-small cell lung cancer; mmVAF, ratio of mean to max variant allele frequency; No.s/m, proportion of subclones over all mutations; NR, not reached; OS, overall survival; PFS, progression-free survival; RT, radiotherapy; TCGA, The Cancer Genome Atlas; TP, time-point; VAF, variant allele frequency.

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1. Introduction

Locally advanced non-small cell lung cancer (LA-NSCLC), which accounts for approximately 30 % of global newly-diagnosed NSCLC [1], is associated with a relatively unfavorable prognosis [2–4]. Historically, definitive-dose radiotherapy with platinum-based doublet chemotherapy (CRT) has been the backbone treatment for patients with unresectable LA-NSCLC [5,6]. In 2018, based on the results of the PACIFIC trial, definitive CRT followed by durvalumab was established as the standard of care [7,8]. Despite the advances in treatment modalities, LA-NSCLC, which affects various patients with distinctive clinical and molecular characteristics, often recurs after definitive therapy. In addition to well-understood clinical factors, molecular features can indicate the prognosis of patients with LA-NSCLC and are under development to identify patients who would benefit from consolidation immunotherapy [9].

Nevertheless, collecting tumor tissue samples from patients with unresectable tumor treated with definitive radiotherapy (RT) or CRT, is difficult and poses a significant challenge for profiling the genomic characteristics of LA-NSCLC. In addition, predicting the efficacy of consolidation immunotherapy using treatment naïve tissue samples may not be ideal due to the influence of CRT on tumor genome and micro-environment, the knowledge regarding which is also limited. Liquid biopsy can overcome the limitations of tumor tissue biopsy because of its advantages including quicker turnaround, easier serial assessment, and relatively comprehensive heterogeneity evaluation. Circulating tumor DNA (ctDNA) released from the primary tumor into the bloodstream has been shown to present genetic mutations in primary tumor tissue with high sensitivity and specificity [10]. Thus, ctDNA surveillance has already been applied to investigating the efficacy or resistance mechanism of treatment, with the conception of “plasma first” being gradually accepted in postoperative or metastatic NSCLC [11].

Although ctDNA has been used as an alternative approach for clonal evolution investigation [12,13], early-detection of progression [14,15], and disease surveillance [16–18], the applicability of liquid biopsy to identify prognostic indicators for patients with LA-NSCLC treated with definitive RT/CRT is unknown. Additionally, there is a lack of longitudinal ctDNA surveillance data to identify potential biomarkers for predicting the benefits of immunotherapy at certain time points during treatment.

In this study, we aimed to reveal the prognosis-related genetic features of LA-NSCLC using pre-treatment tumor tissue samples from an external public database. Next, its prognostic value in liquid biopsies was validated in our study cohort, wherein serial peripheral plasma samples were collected at multiple time-points (TPs) during definitive RT/CRT and immunotherapy. Furthermore, the molecular feature dynamics under RT/CRT were investigated, which might be beneficial for identifying individuals with high disease progression risks or potential benefits from consolidation immunotherapy, with the purpose of optimizing treatment strategies.

2. Materials and methods

2.1. Study cohort and serial liquid biopsies

Patients diagnosed with LA-NSCLC at the National Cancer Center, Chinese Academy of Medical Sciences between May 2018 and March 2022 were enrolled in this prospective study. Patients fulfilling the following inclusion criteria were incorporated in this study: (1) ≥18 years of age; (2) initially diagnosed with LA-NSCLC, which was confirmed by pathology and radiography according to the 8th edition of the American Joint Committee on Cancer classification [19]; (3) those in whom tumors were or medically inoperable; (4) individuals who completed a total radiation dose of ≥50Gy in conventional fractions with intensity-modulated RT; (5) those who adhered to regular follow-up. Patients who subsequently underwent systemic therapy were

excluded. Demographic and clinical data were sourced from medical records, with the specifics of this study cohort outlined previously [20]. This study was approved by the Institutional Review Board of the National Cancer Center, Chinese Academy of Medical Sciences (No. 19/098–1883) and performed in accordance with the tenets of Declaration of Helsinki. All patients signed informed consent forms prior to enrollment and sample collection.

Serial plasma samples were obtained at five TPs. Pre-treatment plasma samples were procured before RT (TP0). Plasma samples were collected during definitive RT at week 4 (TP1). Participants were advised to maintain regular follow-up, approximately every three months after completing RT. Post-RT samples were acquired before consolidation immunotherapy (1 month post-RT, TP2), at three months post-RT (TP3), and at the point of radiological progression (TP4).

2.2. Exploratory The Cancer Genome Atlas (TCGA) cohort

Patients with unresectable LA-NSCLC who underwent definitive RT/CRT, the tumor tissue samples along with all the relevant data of whom were sourced from the TCGA database, were grouped into the TCGA cohort for analysis of prognostic molecular features. Tumor tissue samples were subjected to genomic profiling using whole genome or whole exome sequencing. The sequencing data, along with clinical details, treatment records, and overall survival (OS) data were downloaded from the TCGA data portal (portal.gdc.cancer.gov).

2.3. DNA extraction, library preparation, next-generation sequencing, and data processing

Peripheral blood (10 mL) was collected and centrifuged (at 1800×g, 10 min, room temperature) within 2 h to separate the plasma and leukocyte (normal control). The plasma fraction of blood samples was subjected to circulating free DNA extraction using the QIAGEN QIAamp Circulating Nucleic Acid Kit (QIAGEN, Dusseldorf, Germany), followed by purification, qualification (Nanodrop2000, Thermo Fisher Scientific, Waltham, MA, USA), and quantification (dsDNA HS Assay Kit, Life Technologies, Waltham, MA, USA). Sequencing libraries were prepared using the KAPA Hyper Prep kit (KAPA Biosystems, Wilmington, MA, USA) with an optimized manufacturer’s protocol. Customized xGen lockdown probes targeting 474 cancer- and RT-relevant genes (Radio-tron®, Nanjing Geneseq Technology Inc., Nanjing, China) were used for hybridization enrichment. Target-enriched libraries were then sequenced on Illumina sequencing platforms (Illumina, San Diego, CA, USA) followed by data analysis as described previously [21]. Single nucleotide variants and indels with a variant allele frequency (VAF) over 0.2 % and at least three unique mutant reads were retained. Copy number variations with a fold change ≥1.6 and ≤0.6 were identified as gain and loss, respectively.

2.4. Clonal and survival analysis

A genetic alteration was categorized as clonal if its VAF accounted for ≥25 % of the highest VAF in that specific sample, whereas one alteration with VAF <25 % was classified as subclonal [22]. Progression-free survival (PFS) was defined as the duration from the initiation of RT to disease progression, encompassing both local-regional recurrence and distant metastasis. OS was defined as the interval from RT initiation to death due to any cause. Distant metastasis-free survival (DMFS) and local-regional recurrence free survival (LRFS) were defined as the duration from the initiation of RT to distant metastasis and local-regional recurrence, respectively.

2.5. Statistical analysis

Fisher’s exact test and Mann-Whitney *U* test were performed to compare the frequencies and medians of the independent groups,

respectively. The median follow-up time was estimated using the reverse Kaplan-Meier method [23]. For survival data, Kaplan-Meier curves were generated, and log-rank tests were used to compare differences. Cox proportional hazards models were fitted to estimate hazard ratios (HR) with 95 % confidence intervals (CI), and the proportionality of hazards was assessed using log(-log) survival plots. Individuals with missing data were excluded from analysis. All quoted *P*-values were two-tailed, and *P*-values <0.05 were considered to be statistically significant. Data were analyzed using R software (version 4.0.3), and the *survival*, *prodlm*, and *epiR* packages.

3. Results

3.1. Clonal predominant tumor associated with inferior OS

The study commenced by examining the association between multiple genomic features and prognosis in the TCGA cohort including 431 patients with unresectable LA-NSCLC. Surprisingly, metrics such as max VAF, mean VAF, min VAF, and the number of detectable mutations showed no significant correlation with OS (Fig. 1A). Subsequently, we investigated the secondary molecular feature, mmVAF, which was defined as the ratio of mean VAF to max VAF in one sample. This was found to be inversely associated with the number of subclonals with VAFs $<25\%$ of max VAF ($P < 0.01$, Fig. 1B). We also observed a strong correlation between mmVAF and the proportion of subclonals among all mutations (No.s/m) ($P < 0.01$, $R = -0.86$, Fig. 1C). These findings indicated that mmVAF from tumor tissue could potentially reflect tumor clonality in unresectable LA-NSCLC, with high mmVAF indicating a clonal predominant tumor. Notably, patients with high mmVAF (above

the mean: >0.365) exhibited significantly worse OS than patients with low mmVAF (HR: 1.43, 95 % CI: 1.08–1.89, Fig. 1D). Additionally, when adjusted for clinical characteristics, including sex, age, histology, and disease stage, the association between mmVAF and OS remained statistically significant (HR: 1.48, 95 % CI: 1.11–1.98, Fig. 1E).

3.2. Applicability of liquid biopsies and ctDNA clonality

Considering the challenges of tissue sampling in unresectable LA-NSCLC, we assessed the applicability of liquid biopsies for molecular profiling. We enrolled 70 patients who underwent definitive RT/CRT at the National Cancer Center, Chinese Academy of Medical Sciences, between May 2018 and March 2022 (Fig. 2A). Baseline clinical characteristics of the patients and treatment regimens are summarized in Table 1. The median age of these patients was 64 years (range: 40–80 years), with the majority of them being males (85.7 %), current or former smokers (71.1 %), and patients with stage IIIB disease (55.7 %). Twenty-nine (41.4 %) and 37 (52.9 %) patients were diagnosed with adenocarcinoma and squamous cell carcinoma, respectively. A total of 55.7 % of the patients were treated with definitive RT/CRT followed by consolidation immunotherapy. By the end of follow-up (median follow-up: 17.0 months, interquartile range: 9.8–26.4 months), 52.9 % of the patients experienced recurrence and/or metastasis. The median PFS and median OS were 16.4 months (95 % CI: 12.4–26.5) and 25.4 months (95 % CI: 19.0–not reached [NR]), respectively. No significant differences were observed in terms of PFS (Supplementary Fig. 1A) or OS (Supplementary Fig. 1B) between patients with and without consolidation immunotherapy.

As designed, a total of 218 plasma samples were collected at five TPs,

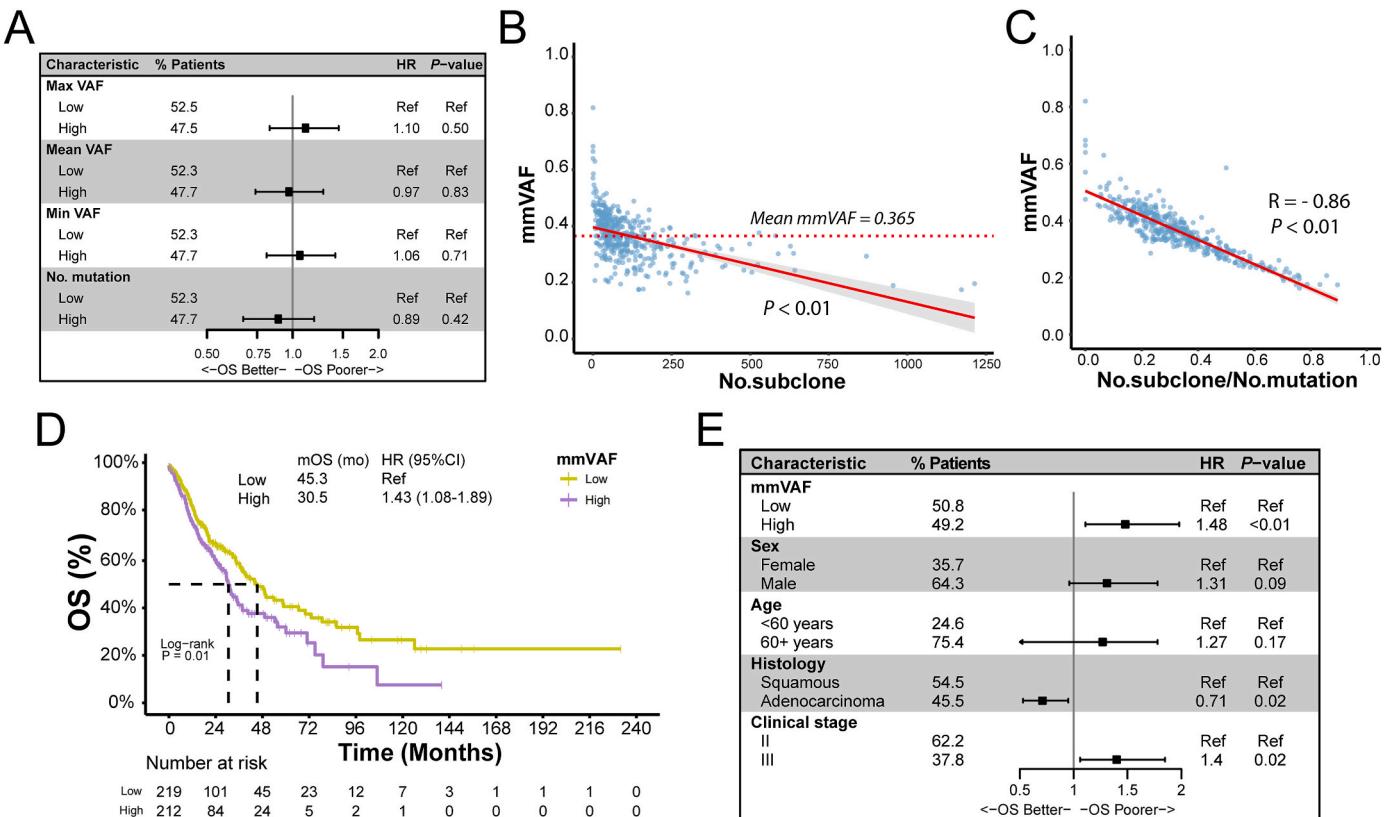


Fig. 1. Tissue-based mmVAF associated with tumor clonality and OS

(A) max VAF, mean VAF, min VAF, and number of detectable mutations were not associated with OS. (B) mmVAF negatively correlated with the number of subclonals. (C) mmVAF negatively correlated with the proportion of subclonals over total mutations (No.s/m). (D) Patients with high mmVAF (mmVAF >0.365) had worse OS than patients with low mmVAF (mmVAF ≤ 0.365). (E) The association between high mmVAF and worse OS remained statistically significant in the multivariable Cox regression model. (Abbreviations: VAF, variant allele frequency; mmVAF, the ratio of mean to max variant allele frequency; OS, overall survival; No.s/m: the proportion of subclonals over all mutations).

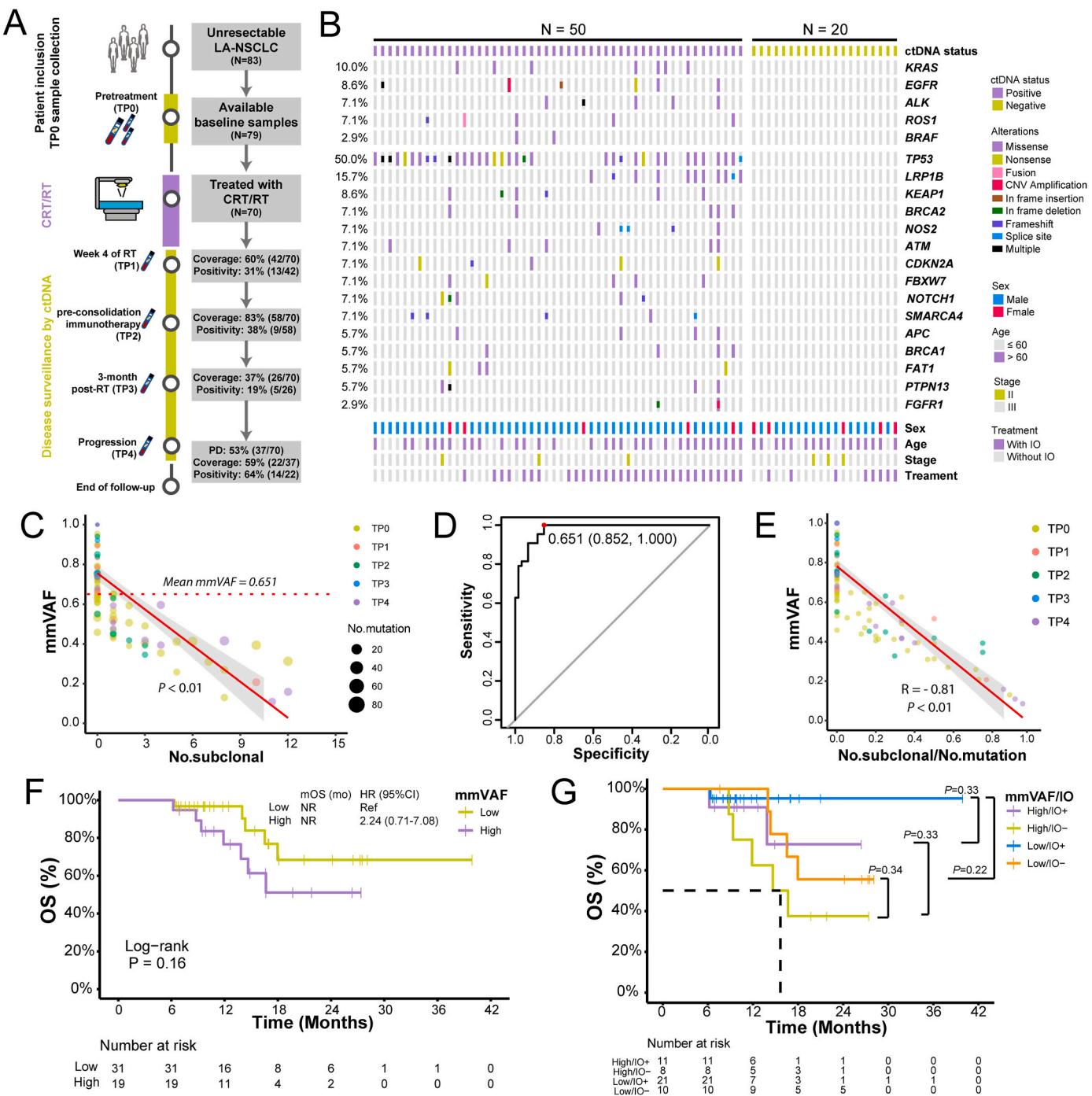


Fig. 2. Applicability of liquid biopsies and ctDNA

(A) Seventy patients with unresectable locally advanced non-small cell lung cancer receiving definitive RT/CRT with/without consolidation immunotherapy were enrolled, and serial plasma samples were collected at five time-points. (B) The mutation landscape using liquid biopsies at TP0, including 50 ctDNA-positive patients and 20 ctDNA-negative patients. (C) ctDNA-based mmVAF negatively correlated with the number of subclonals. (D) The mean of ctDNA-based mmVAF (0.651) was the best mmVAF cut-off to classify samples with and without subclonals. (E) ctDNA-based mmVAF negatively correlated with the proportion of subclonals over total mutations. (F) Patients with high ctDNA-based mmVAF (mmVAF>0.651) had potentially worse OS than patients with low mmVAF (mmVAF≤0.651). (G) Similar trends were observed in patients with and without consolidation immunotherapy. (Abbreviations: ctDNA, circulating tumor DNA; RT, radiotherapy; CRT, chemoradiotherapy; mmVAF, the ratio of mean to max variant allele frequency; OS, overall survival).

with an average sequencing depth of 1525X (range:93–5239). At baseline (TP0), 50 out of 70 (71.4 %) samples were ctDNA-positive (Fig. 2B). The most frequently altered gene was *TP53* (50.0 %), followed by *LRP1B* (15.7 %) and established lung cancer drivers, including *KRAS* (10.0 %), *EGFR* (8.6 %), *ALK* (7.1 %) and *ROS1* (7.1 %). TPO ctDNA positivity rates were comparable between patients who developed disease progression and those who did not (Supplementary Fig. 1C). Similar results were

obtained when comparing patients who had died by the end of follow-up with those who remained alive (Supplementary Fig. 1D). Additionally, there were no significant differences in PFS (Supplementary Fig. 1E) or OS (Supplementary Fig. 1F) between the TP0 ctDNA-positive and ctDNA-negative patients. Therefore, our data demonstrated that pre-treatment ctDNA positivity might not serve as a reliable prognostic biomarker.

Table 1
Baseline patient characteristics.

Characteristics	Patients (N = 70)
Age, median (range), y	64 (40–80)
Sex, No. (%)	
Female	10 (14.3)
Male	60 (85.7)
Smoking, No. (%)	
Current or ever	54 (77.1)
Never	16 (22.9)
Histology, No. (%)	
Adenocarcinoma	29 (41.4)
Squamous cell carcinoma	37 (52.9)
Other	4 (5.7)
T stage, No. (%)	
T1	13 (18.6)
T2	17 (24.2)
T3	22 (31.4)
T4	18 (25.7)
N stage, No. (%)	
N0	4 (5.7)
N1	10 (14.3)
N2	26 (37.1)
N3	30 (42.9)
Clinical stage, No. (%)	
IIA	1 (1.4)
IIB	5 (7.1)
IIIA	14 (20.0)
IIIB	39 (55.7)
IIIC	11 (15.7)
Consolidation immunotherapy, No. (%)	
Without	31 (44.3)
With	39 (55.7)
Chemotherapy regimen, No. (%)	
AP	26 (37.1)
EP	19 (27.1)
PC	15 (21.4)
RT alone	10 (14.3)

RT: radiotherapy, AP: pemetrexed plus cisplatin, EP: etoposide plus cisplatin, PC: paclitaxel plus carboplatin.

mmVAF negatively associated with the number of subclonals in 218 plasma samples in our study cohort ($P < 0.01$, Fig. 2C), which was similar to our findings in tissue samples from the TCGA cohort. Most pre-treatment ctDNA samples without detectable subclonals exhibited mmVAF values that exceeded the mean (0.651). Moreover, the mean mmVAF of 0.651 was identified as the optimal cut-off for classifying plasma samples with and without subclonals, demonstrating a specificity of 0.852 and sensitivity of 1.000 (Fig. 2D). A strong correlation was also observed between mmVAF and No.s/m ($P < 0.01$, $R = -0.81$, Fig. 2E), suggesting that liquid biopsy mmVAF could likewise reflect tumor clonality. Like the TCGA cohort, patients with relatively high TP0 mmVAF (>0.651) appeared to have poorer OS than other patients (HR: 2.24, 95 % CI: 0.71–7.08, Fig. 2F), irrespective of the fact that patients were treated with or without consolidation immunotherapy (Fig. 2G). When adjusted for chemotherapy regimens, immunotherapy usage, patient age, sex, NSCLC histology, and tumor stage, patients with high TP0 mmVAF patients exhibited a trend towards inferior OS compared to patients with negative TP0 ctDNA (HR: 4.44, 95 % CI: 0.93–21.2, Supplementary Fig. 2) and patients with low TP0 mmVAF (HR: 1.96, 95 % CI: 0.51–7.5, Supplementary Fig. 2), without strong influence from various chemotherapy regimens (Supplementary Fig. 2).

3.3. Immunotherapy benefits in clonal predominant tumor

We further explored the potential of pre-treatment mmVAF among 50 ctDNA-positive patients at TP0 in identifying patients who could benefit from consolidation immunotherapy. Among patients with elevated TP0 mmVAF, those receiving consolidation immunotherapy showed a trend towards improved PFS in comparison to those who did not receive the therapy (HR: 0.30, 95 % CI: 0.07–1.21, Fig. 3A).

However, there was minimal discrepancy in PFS of patients with low TP0 mmVAF between those receiving and not receiving consolidation immunotherapy (HR: 1.04, 0.38–2.87, Fig. 3A). Interestingly, high mmVAF potentially indicated better PFS in patients receiving consolidation immunotherapy, whereas it was likely to be associated with poorer PFS in patients not receiving consolidation immunotherapy (Fig. 3A), suggesting that the predictive value of mmVAF on PFS might depend on the specifics of the treatment regimen.

We also investigated the prognostic value of blood tumor mutation burden (bTMB) at TP0. No robust links between bTMB levels and consolidation immunotherapy benefits were discerned, regardless of whether bTMB-high subgroup was defined based on mmVAF being \geq the mean (Supplementary Figs. 3A and B), \geq the median (Supplementary Figs. 3C and D), or the top 25 % of bTMB levels (Supplementary Figs. 3E and F).

Given the strong correlation between mmVAF and No.s/m, the association between consolidation immunotherapy and PFS was assessed in patients with clonal predominant tumors, where TP0 No.s/m was lower than the mean. Although consolidation immunotherapy appeared to be associated with superior PFS, the difference was not statistically significant (HR: 0.50, 95 % CI: 0.18–1.37, Fig. 3B). Notably, No.s/m values for the 26 patients without detectable subclonals at TP0 were uniformly calculated to be zero, whereas these patients could be further stratified based on TP0 mmVAF (Fig. 3C). Among 18 patients categorized as mmVAF-high, those treated with consolidation immunotherapy had relatively better PFS than those treated without consolidation immunotherapy (HR: 0.33, 95 % CI: 0.08–1.31, Fig. 3D). This intriguing finding suggests that mmVAF might be instrumental in identifying patients who lacked detectable subclonals but stand to gain more benefits from consolidation immunotherapy.

Next, we investigated the prognostic implication of ctDNA after initiating definitive RT/CRT. Plasma samples were collected from 42 to 58 patients at TP1 and TP2, respectively. Compared with ctDNA-negative patients at TP1, ctDNA-positive patients displayed inferior PFS (HR: 2.40, 95 % CI: 1.04–5.50, Fig. 4A). Similarly, TP2 ctDNA positivity was associated with worse PFS (HR: 2.60, 95 % CI: 1.20–5.66, Fig. 4B). In the multivariable Cox regression model, after adjusting for consolidation immunotherapy, patient age, sex, histology, and clinical stage, TP2 ctDNA positivity remained significantly associated with diminished PFS (adjusted HR: 3.25, 95 % CI: 1.39–7.61, Fig. 4C). Additionally, consolidation immunotherapy was associated with enhanced PFS (adjusted HR: 0.33, 95 % CI: 0.14–0.78, Fig. 4C); however, this association was exclusively observed in TP2 ctDNA-positive patients (HR: 0.20, 95 % CI: 0.07–0.60, Fig. 4D). The TP2 ctDNA-positive patients were further categorized into four subgroups based on mmVAF values and consolidation immunotherapy. Among patients with clonal predominant tumor (mmVAF >0.651), those receiving consolidation immunotherapy had superior PFS compared to those who did not receive consolidation immunotherapy (HR: 0.14, 95 % CI: 0.03–0.67, Fig. 4E). Despite the limited sample size in each subgroup, a potential positive association between high TP2 mmVAF and improved PFS was noted in patients receiving consolidation immunotherapy, which was consistent with our findings at TP0.

3.4. Increased clonality correlated with poorer PFS but potentially benefits from consolidation immunotherapy

Following radiographic progression, TP4 samples exhibited a significant reduction in detected mutations compared to TP0 samples ($P < 0.01$, Fig. 5A), but the proportion of subclonals increased from 29.9 % to 62.5 % ($P < 0.01$, Fig. 5B). Additionally, during the treatment, mmVAFs at TP1 ($P < 0.01$, Fig. 5C) and TP2 ($P = 0.02$, Fig. 5C) were higher than those at TP0. As a result, patients with serial plasma samples at TP0 and TP1 were classified into three subgroups: mmVAF increase, mmVAF non-increase, and ctDNA remain-negative subgroups. In comparison to the mmVAF increase subgroup, patients with non-increased mmVAF

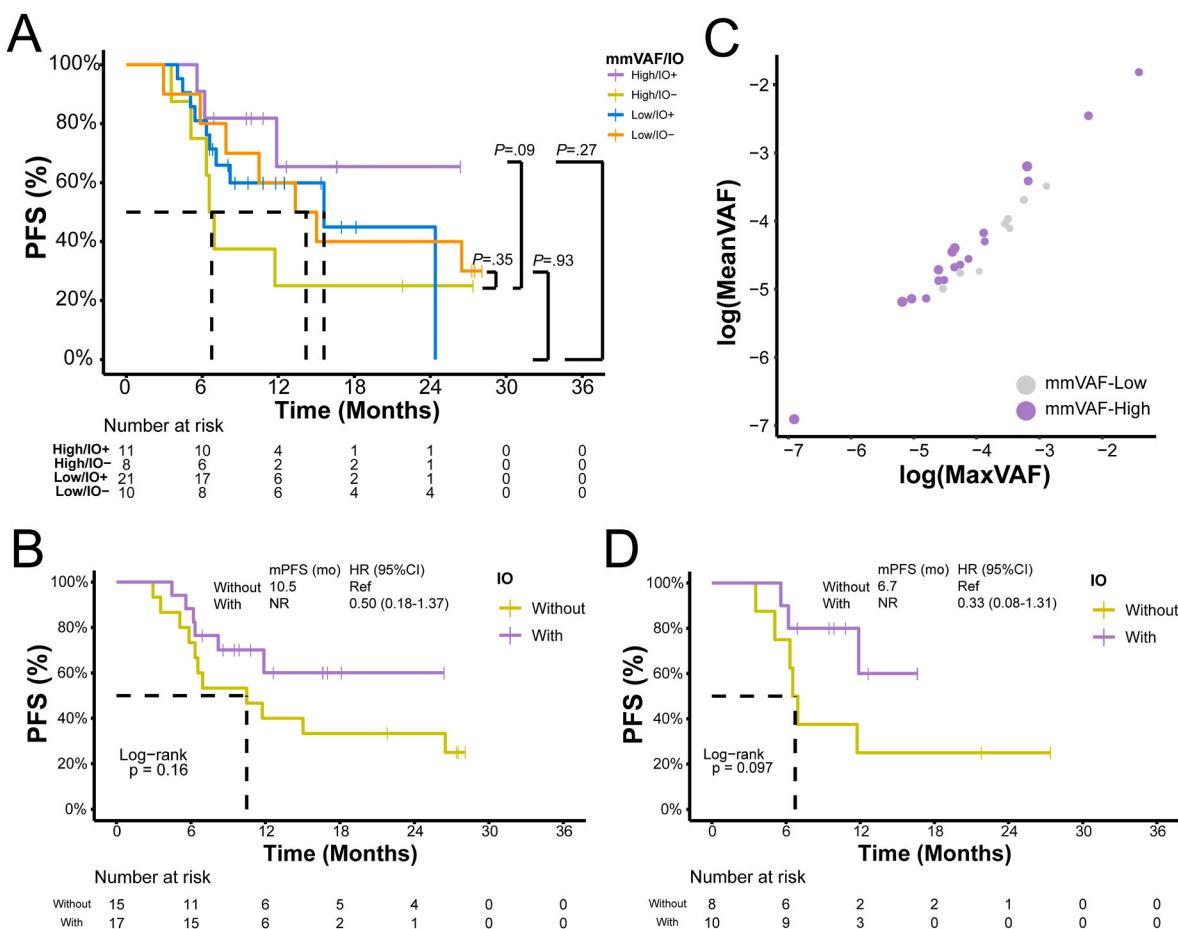


Fig. 3. Pre-treatment mmVAF and consolidation immunotherapy benefits

(A) Among patients with high TP0 mmVAF, consolidation immunotherapy was associated with potentially better PFS. (B) Among patients who had low No.s/m values at TP0, consolidation immunotherapy appeared to be associated with superior PFS; however, it was not statistically significant. (C) Twenty-six patients without detectable subclonal at TP0 could be further classified based on TP0 mmVAF. (D) Among 18 patients who did not have subclonal and were further categorized as mmVAF-high, patients treated with consolidation immunotherapy had relatively better PFS than patients who did not receive consolidation immunotherapy. (Abbreviations: mmVAF, the ratio of mean to max variant allele frequency; PFS, progression-free survival; No.s/m: the proportion of subclonal over all mutations).

(HR: 0.22, 95 % CI: 0.08–0.63, Fig. 5D) and undetectable ctDNA (HR: 0.31, 95 % CI: 0.11–0.87, Fig. 5D) displayed better PFS. Similar results were obtained between TP0 and TP2 (non-increase vs. increase, HR: 0.31, 95 % CI: 0.12–0.78, Fig. 5E). However, no statistically significant difference in PFS was observed between the mmVAF increase and ctDNA remain-negative subgroups (HR: 0.62, 95 % CI: 0.24–1.63, Fig. 5E).

We then explored the association between mmVAF dynamics and the benefits of consolidation immunotherapy. Due to the lack of plasma samples at TP3, patients with increased mmVAF from TP0 to TP1 and/or TP2 were grouped into the mmVAF increase subgroup (TP0-TP1-TP2 increase). Other patients with non-increased mmVAF or those who remained ctDNA-negative were grouped together for further analyses. Interestingly, all eight patients without consolidation immunotherapy in the increase subgroup developed radiographic progression, and the disease progression percentage was significantly lower in mmVAF-increased patients receiving consolidation immunotherapy (54.5 % vs. 100.0 %, $P = 0.05$). Survival analysis results also demonstrated that consolidation immunotherapy was potentially associated with better PFS in the increase subgroup (HR: 0.39, 95 % CI: 0.14–1.15, Fig. 5F). In contrast, patients whose ctDNA status remained undetectable or those who successfully achieved ctDNA clearance appeared to have favorable outcomes, with limited benefits from consolidation immunotherapy (HR: 1.04, 95 % CI: 0.42–2.57, Fig. 5F). Particularly, patients in the increase subgroup who received consolidation immunotherapy

exhibited significantly lower distant metastasis rates (14.2 % vs. 75.0 %, $P = 0.04$, Fig. 5G) and better DMFS (HR: 0.11, 95 % CI: 0.01–0.95, Fig. 5H) than those without consolidation immunotherapy.

4. Discussion

In this study, we demonstrated mmVAF as a potential prognostic molecular indicator based on the TCGA cohort and further assessed its value in liquid biopsies to overcome the drawbacks of tissue sampling for patients with unresectable LA-NSCLC receiving definitive RT/CRT. Our data also revealed the potential of ctDNA-defined mmVAF in identifying patients who would benefit from consolidation immunotherapy. Moreover, mmVAF dynamics determined by ctDNA surveillance during RT/CRT could aid in outcome prediction. Therefore, our findings could help identify LA-NSCLC patients with higher risks of progression and deepen the understanding of immunotherapy efficacy prediction.

Since tissue samples are traditionally considered the “gold standard” for clonality study, many tissue-based biomarkers have been developed as prognostic indicators [24,25]. For instance, the mutant-allele tumor heterogeneity (MATH) algorithm was applied to predicting treatment responses in patients with breast cancer and locally advanced rectal cancer [26–28]. However, tissue biopsy is risky and invasive in serial sample collection for patients during disease monitoring. Liquid biopsy,

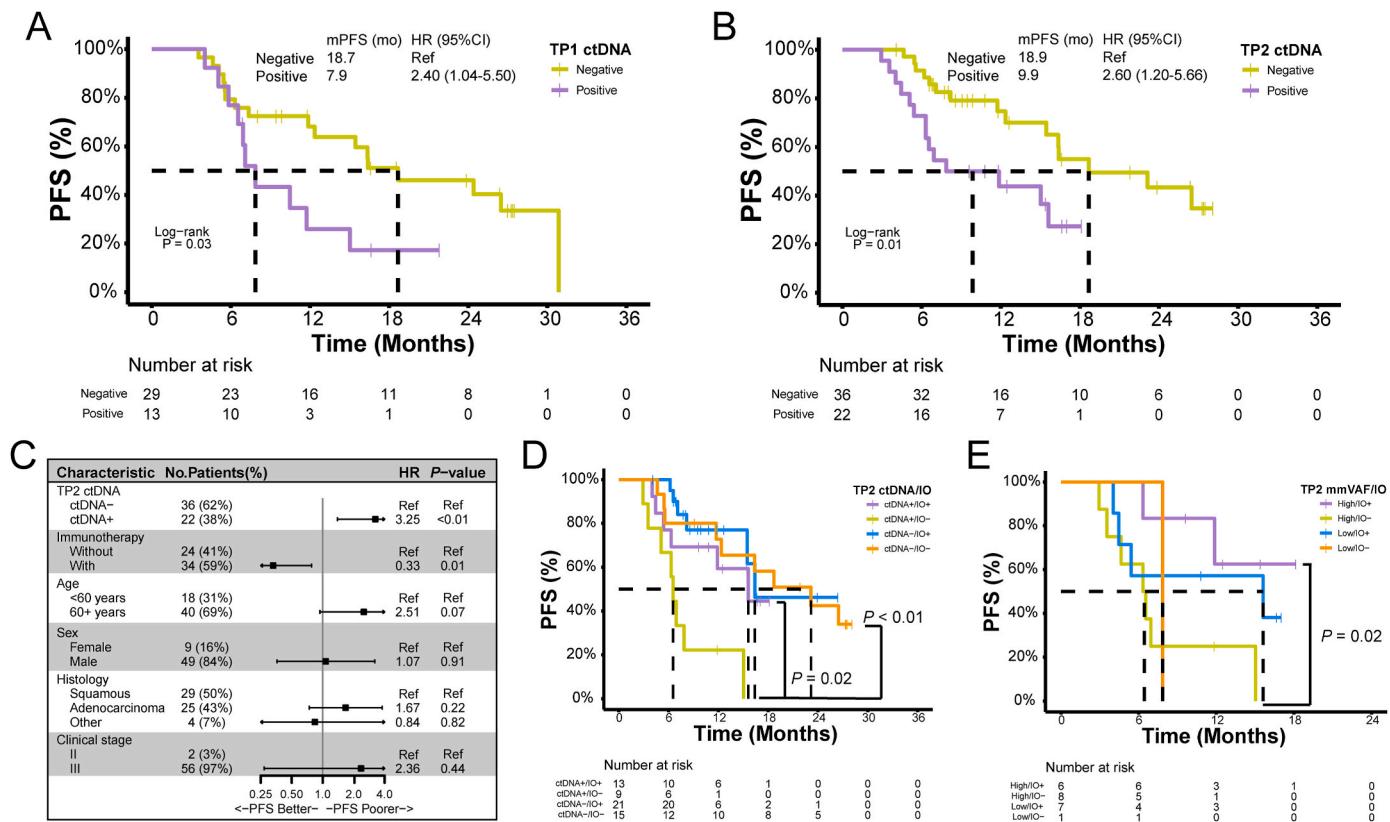


Fig. 4. ctDNA during/post-RT and prognosis prediction

(A) ctDNA-positive patients at TP1 had inferior PFS compared to ctDNA-negative patients. (B) ctDNA-positive patients at TP2 had inferior PFS than ctDNA-negative patients. (C) ctDNA positivity at TP2 remained significantly associated with poorer PFS, after adjusting for consolidation immunotherapy, patient age, histology, and disease stage. (D) The association of consolidation immunotherapy with superior PFS was exclusively observed in TP2 ctDNA-positive patients. (E) Among TP2 ctDNA-positive patients with mmVAF over the mean (0.651), those receiving consolidation immunotherapy had superior PFS compared to those who did not receive immunotherapy. (Abbreviations: ctDNA, circulating tumor DNA; RT, radiotherapy; PFS, progression-free survival; mmVAF, the ratio of mean to max variant allele frequency).

mainly blood, is an alternative approach for ascertaining diagnostic, prognostic, and predictive biomarkers [29]. Previous studies have demonstrated a high agreement in biomarker detection between tumor tissue and liquid biopsies in multiple cancer types. A previous study reported that the concordance of *EGFR* mutation status and subtypes between baseline NSCLC tumor tissue and plasma samples was approximately 95 % with sensitivity of over 65 % and specificity of over 99 % [30]. Another NSCLC study revealed a high *EGFR* mutation detection concordance of 88 % with sensitivity of 75 % and specificity of 96 % [31]. Multiple studies have also demonstrated comparable mutation detection rates and strongly correlated VAFs between ctDNA and tissue samples of NSCLC [32–35]. Furthermore, a breast cancer study showed that the overall gene-level concordance between tissue and ctDNA test ranged between 91.0 % and 94.2 % [36]. Similarly, ctDNA genomic profiling was reported to be reliable and practical for metastatic bladder cancer, with a concordance of 83.4 % compared to tumor tissue samples [37]. Liquid biopsies also demonstrated relatively good performance in detection of *KRAS*, *NRAS*, and *BRAF* in newly diagnosed colorectal cancer with liver metastases when compared to tissue based next-generation sequencing test, achieving an overall agreement of 81.94 %, and concordance of 96.15 % [38]. For metastatic castration-resistant prostate cancer, all somatic mutations in 72 clinically relevant genes observed in metastatic lesions in the bone or soft tissues were successfully identified in ctDNA [39]. Moreover, liquid biopsy may better capture resistance-related acquired alterations and achieve longitudinal surveillance [40–42]. In our study, tissue-based mmVAF was assessed using ctDNA data, with similar associations between clonality and prognosis in both sample types, suggesting the

generalizability of mmVAF and applicability of ctDNA. Considering the difficulty in tumor sample collection, ctDNA analysis may be useful for future research on clonal structure reconstruction and resistance mechanism in unresectable LA-NSCLC under definitive treatment. Thus, liquid biopsies might be involved in practical applications to assist in therapy decision making and prognosis evaluation, especially in situations where tumor tissue samples are not easily accessible.

Multiple studies have demonstrated associations between relapse risks after radical resection and ctDNA test results in various cancers, such as breast cancer, colorectal cancer and NSCLC [43–45]. A previous study by our team revealed that a positive ctDNA status at one month after RT/CRT could serve as an unfavorable prognostic indicator for LA-NSCLC [20]. Additionally, the detected ctDNA was able to indicate disease progression earlier than regular radiological assessment; thus, enhanced adjuvant therapy could be administered to control minimal residual disease [46,47]. In particularly, for patients with LA-NSCLC treated with definitive therapy, Moding et al. revealed that patients with detectable ctDNA after RT/CRT tended to have poor outcomes and could benefit from consolidation immunotherapy [9]. Similarly, in our study, TP2 ctDNA-positive patients treated with consolidation immunotherapy had significantly superior PFS compared to those who did not receive consolidation immunotherapy. Our data also revealed that mmVAF at pre-treatment could potentially distinguish patients with a potentially higher risk of death. Since the OS data are still immature, further follow-ups are required to confirm the association between mmVAF and long-term survival.

Tumor clonality profoundly influences the selection pressure of treatment and clinical outcome of patients [48]. Previous studies have

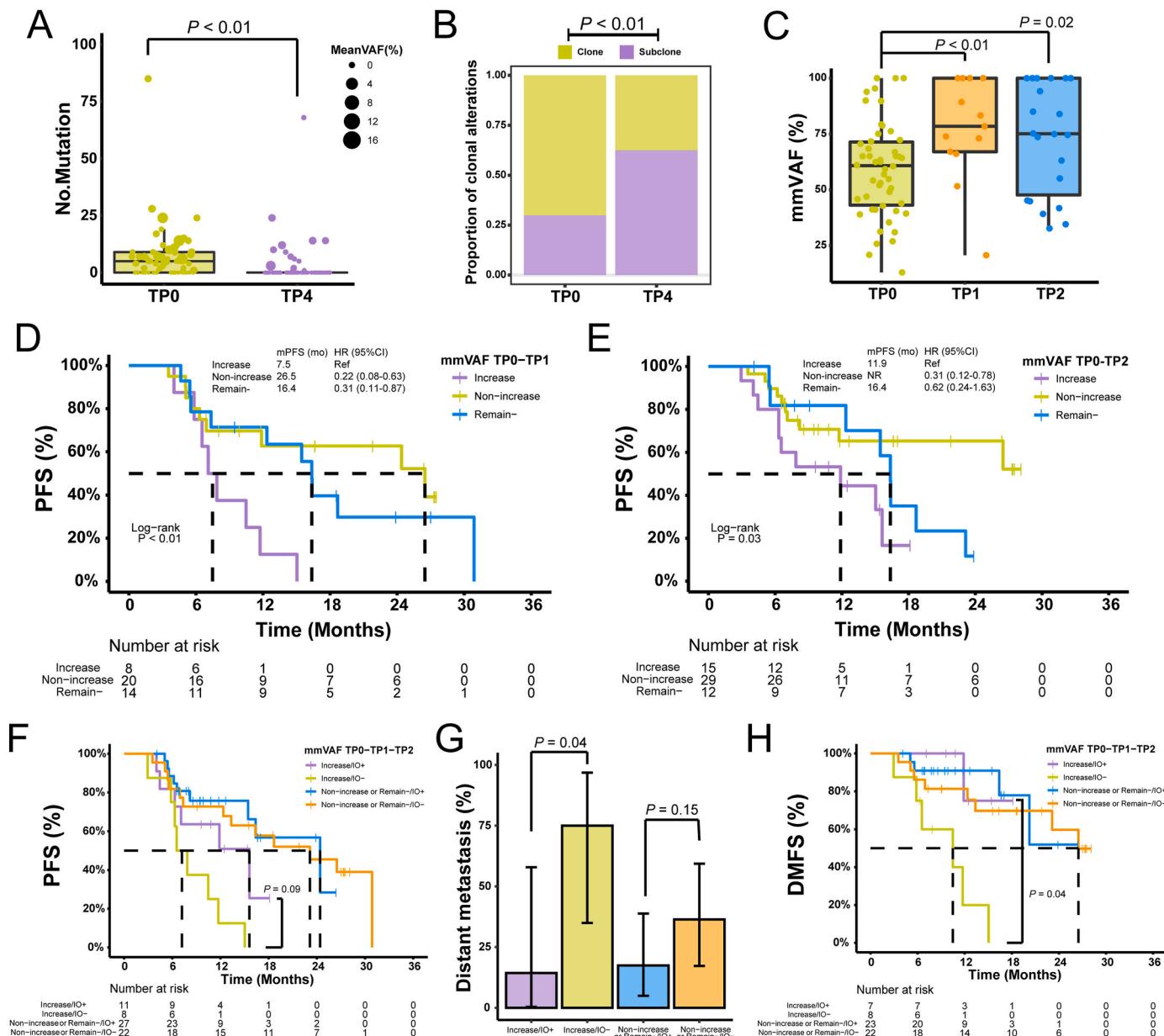


Fig. 5. ctDNA dynamic analysis and clonal evolution.

(A) Significantly fewer mutations were detected in TP4 samples than in TP0 samples. (B) The proportion of subclonals increased significantly from 29.9 % to 62.5 %. (C) mmVAFs at TP1 and TP2 were higher than in comparison to TP0. (D) Between TP0 and TP1, patients with increased mmVAF had worse PFS than patients with non-increase mmVAF. (E) Similar results were obtained between TP0 and TP2. (F) Consolidation immunotherapy was potentially associated with better PFS in the increase subgroup. (G) The distant metastasis rate was lower in mmVAF increased patients receiving consolidation immunotherapy than those who did not receive consolidation immunotherapy. (H) Consolidation immunotherapy was associated with better DMFS in the increase subgroup. (Abbreviations: ctDNA, circulating tumor DNA; mmVAF, the ratio of mean to max variant allele frequency; PFS, progression-free survival; DMFS, distant metastasis free survival).

mainly focused on patients with NSCLC undergoing resection or receiving systemic therapies [45,49]. However, our study is the first to delineate changes in clonality under definitive RT/CRT in LA-NSCLC. High mmVAF was associated with relatively poor prognosis in patients without consolidation immunotherapy after RT/CRT, suggesting that continuously expanding clones were likely to result in a higher possibility of RT/CRT resistance and shorter survival. In contrast, among patients receiving consolidation immunotherapy, those with high mmVAF were likely to benefit more. Consistent with our findings, several previous studies have revealed an association between tumor heterogeneity and immunotherapy efficacy. For instance, Wu et al. profiled NSCLC tissue samples using single-cell RNA sequencing and demonstrated a highly immunosuppressive environment and low

cancer-killing ability among NSCLC patients with high intratumor heterogeneity [50]. Another study suggested that the presence of a low ratio of VAF of a mutation to the max VAF defined by ctDNA could predict an inferior prognosis in patients with advanced NSCLC receiving immunotherapy [51]. Similarly, in breast cancer, tumors with high heterogeneity have been reported to be associated with lower immune cell infiltration and immune response [27]. Radiosensitivity is an intrinsic property of tumor clones, which takes both the highest cancer cell killing rates and minimal damage to normal tissues into consideration [52], and the cell clusters within a tumor exhibit different sensitivity. Aguilera et al. reported that mixing additional responsive breast cancer cells with unresponsive tumor cells could lead to better response to RT with different innate and adaptive immune components in the

matrix [53].

This study had a few limitations. First, the TCGA cohort consists of patients enrolled before the PACIFIC trial; thus, we could not validate the interaction between pre-treatment mmVAF and consolidation immunotherapy in tissue samples. Second, due to the modest sample size of the study cohort, no specific mutated genes or signaling pathways associated with prognosis were determined. Further research is warranted to identify the specific genetic alterations and their dynamics in serial liquid biopsies. Additionally, the mmVAF threshold defined in our study depended on VAFs of all detectable alterations, thus the size of sequencing panel and sequencing depth might influence the generalizability of our observations, which still need further verification. Finally, the path of cancer clonal evolution might be impacted not only by genetic variations, but also transcriptional adaptions and epigenetic modifications under RT/CRT [54]. Future integration of multi-omics data will provide new insights into mmVAF dynamics and clonality research.

5. Conclusions

Tissue or liquid biopsy-based mmVAF, a derivative molecular indicator correlated with tumor clonality, could help stratify the prognosis of patients with unresectable LA-NSCLC receiving definitive RT/CRT. ctDNA positivity, ctDNA-defined clonality before RT initiation, and mmVAF dynamics were also able to identify patients who could benefit from consolidation immunotherapy. Future studies with larger population and longer follow-up period are required for further validation.

Ethics approval and consent to participate

This study was approved by Institutional Review Boards of National Cancer Center, Chinese Academy of Medical Sciences (No. 19/098–1883). All patients signed informed consent forms prior to enrollment and sample collection.

Consent for publication

Not applicable.

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Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Yufan Yang: Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis. **Jianyang Wang:** Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation. **Jingbo Wang:** Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation. **Xiaotian Zhao:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Formal analysis. **Tao Zhang:** Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation. **Yin Yang:** Writing – review & editing, Resources, Investigation, Data curation. **Jiaohui Pang:** Writing – review & editing, Visualization, Software, Methodology, Formal analysis. **Qixiang Ou:** Writing – review & editing, Visualization, Project administration. **Linfang Wu:**

Writing – review & editing, Resources, Investigation, Data curation. **Xin Xu:** Writing – review & editing, Resources, Investigation, Data curation. **Kunpeng Xu:** Writing – review & editing, Resources, Investigation, Data curation. **Jingjing Zhao:** Writing – review & editing, Resources, Investigation, Data curation. **Na Bai:** Writing – review & editing, Resources, Investigation, Data curation. **Peng Yang:** Writing – review & editing, Investigation, Data curation. **Sha Wang:** Writing – review & editing, Software, Resources, Data curation. **Luhua Wang:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Nan Bi:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: X Zhao, J Pang, Q Ou, N Bai, P Yang, and S Wang are employees of Nanjing Geneseeq Technology Inc., China. The remaining authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.canlet.2023.216569>.

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