



Circulating tumor DNA predicts neoadjuvant immunotherapy efficacy and recurrence-free survival in surgical non-small cell lung cancer patients

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Background: There is currently a lack of effective biomarkers to evaluate efficacy of neoadjuvant therapy (NAT) for resectable non-small cell lung cancer (NSCLC) patients. Circulating tumor DNA (ctDNA) has been investigated as a non-invasive tool for the assessment of tumor burden and minimal residual disease (MRD). The utility of ctDNA profiling in reflecting NAT efficacy, however, has not been confirmed. This study explored the association of ctDNA change with treatment response to NAT and recurrence-free survival (RFS) after surgery.

Methods: Eligible patients with stage IB–IIIA NSCLC were retrospectively included if they had received neoadjuvant immunotherapy combined with chemotherapy (IO+Chemo), dual immunotherapy (IO+IO), or chemotherapy alone (Chemo). We conducted ctDNA profiling before and after NAT, after surgery, and during follow-ups using an ultra-deep lung cancer-specific MRD (LC-MRD) sequencing panel.

Results: A total of 22 patients who received NAT followed by surgery between August 2018 and July 2019 were included in this study. The major pathological response (MPR) rates were 58.33% (7/12) in the IO+Chemo group, 25.00% (1/4) in the IO+IO group, and 16.67% (1/6) in the Chemo group. The ctDNA dynamics during NAT were highly concordant with pathologic response, demonstrating 100% sensitivity and 83.33% specificity, for an overall accuracy of 91.67%. Pre-surgery detectable ctDNA (after NAT) trended to correlate with inferior RFS [hazard ratio (HR), 7.41; 95% confidence interval (CI): 0.91–60.22, log-rank $P=0.03$]. At 3–8 days after surgery, ctDNA was detectable in 31.8% of patients and was an independent risk factor for recurrence (HR, 5.37; 95% CI: 1.27–22.67; log-rank $P=0.01$). The presence of ctDNA at 3 months after surgery showed 83% sensitivity and 90% specificity for predicting relapse (C-index, 0.79; 95% CI: 0.62–0.95). During disease monitoring after surgery, molecular recurrence by means of ctDNA preceded radiographic relapse, with a median time of 6.83 months.

Conclusions: This study investigated the potential of ctDNA in evaluating NAT efficacy in NSCLC, implying the high concordance between ctDNA and pathological response. We also set out the prognostic value of perioperative ctDNA in predicting recurrence.

Keywords: Non-small cell lung cancer (NSCLC); circulating tumor DNA (ctDNA); neoadjuvant therapy (NAT); recurrence

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Introduction

Lung cancer is the leading cause of cancer death worldwide (1). For resectable non-small cell lung cancer (NSCLC) patients, radical surgery is the standard of care as recommended by the National Comprehensive Cancer Network (NCCN) guidelines. Yet, a considerable proportion of patients (ranging from 50% for IA disease to 80% for IIIA disease) eventually succumb to recurrence within 5 years after resection (2-4). Several trials have suggested that neoadjuvant and adjuvant systemic therapy and/or radiotherapy, collectively defined as perioperative therapy, can improve clinical outcomes with a benefit of 5% on overall survival (OS), based on which perioperative therapies are routinely applied (3,5). Recent studies have revealed a promising efficacy and safety profile of neoadjuvant immunotherapy in NSCLC. In the phase 2 randomized NEOSTAR trial for operable NSCLC, the major pathological response (MPR) rates induced by nivolumab and nivolumab + ipilimumab were 24% (5/21) and 50% (8/16), respectively. Pathological complete response (pCR) rate was higher in patients administered dual immunotherapy (IO+IO) than in those treated with nivolumab alone (38% *vs.* 10%) (6). More recently, the NADIM clinical trial, which evaluated the combination of chemotherapy and immunotherapy has reported an unprecedented pCR of 63% (7) and 36-month progression-free survival (PFS) and OS of 81.1% and 91.0%, respectively, among patients with resectable, stage IIIA NSCLC (8) shifting the paradigm of stage III NSCLC from that of a lethal disease to a curable one. Similarly, in CheckMate 816, neoadjuvant nivolumab + chemotherapy increased pCR rate to 24% in NSCLC, higher than the 2.2% induced by chemotherapy alone (Chemo) (9). Currently, imaging modalities, such as computed tomography (CT) and positron emission tomography (PET), are utilized to evaluate response to treatment and

detect postoperative relapse during surveillance. The discordance between radiologic and pathological response underpins the need to develop effective biomarkers that can accurately predict therapeutic efficacy (10,11). In addition, the suboptimal detection limit of radiographic imaging makes minimal lesions unmeasurable when the long axis of the lesion is smaller than 10 mm. Therefore, a biomarker that can detect molecular/minimal residual disease (MRD) prior to radiologic recurrence is also urgently needed to guide timely treatment when the tumor/cancer burden is minimal. Collectively, effective biomarkers that can evaluate treatment response and expedite the identification of patients destined to relapse are unmet needs during the perioperative period.

Tumor immune microenvironment (TIME) makers such as PD-L1 expression, abundance of tumor-infiltrating lymphocytes (TILs), CD8⁺ T cells, and CD56^{dim} natural killer cells, and density of M1/M2-tumor-associated macrophages (12-15) were reported to be associated with pathological response in primary tumors subjected to immunotherapy; however, testing TIME markers relies on tumor tissue samples, which needs an invasive access. Circulating tumor DNA (ctDNA), a component of cell-free DNA (cfDNA), can reflect inter- and intra-tumoral heterogeneity. The ctDNA could noninvasively track dynamics of tumor burden and has been proposed as a real-time biomarker that can provide both predictive and prognostic values. In several cancers, including lung cancer, bladder cancer, and colorectal cancer, ctDNA-based profiling following surgery has shown encouraging performance in revealing MRD (16-19). In addition, ctDNA clearance has been shown to pose correlations with better treatment efficacy in advanced cancers (20-23), based on which its' effects in evaluating the efficacy of neoadjuvant therapy (NAT) have also been exploited. In bladder cancers, ctDNA has been found to be correlated with response to NAT (24). As for lung cancer, no data have been revealed

except for a few preliminary results of ongoing trials, including CheckMate 816 (NCT02998528) and NADIM trial (NCT03081689) released at the American Association for Cancer Research (AACR) annual meeting 2021 (9) and IASLC 2021 World Conference on Lung Cancer 2021, respectively (25). The former reported that ctDNA clearance was associated with pathologic response and the latter that pre-treatment ctDNA levels as well as post-treatment ctDNA detection identified patients at high risk of progression and death and outperformed radiological response assessed according to RECIST criteria v 1.1 in the prediction of survival. Our study primarily aimed to evaluate the utility of ctDNA profiling using next-generation sequencing (NGS)-based lung cancer-specific MRD (LC-MRD) panel to assess response to NAT. The association of pre-surgery and post-surgery ctDNA with postoperative relapse was also explored. We present the following article in accordance with the STARD reporting checklist (available at <https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-106/rc>).

Methods

Participants and study design

This was a cohort study investigating the feasibility of ctDNA to reflect treatment response and predict recurrence in surgical NSCLC patients. Patients with NSCLC who received neoadjuvant immunotherapy combined with chemotherapy (IO+Chemo), IO+IO, or Chemo between August 2018 and July 2019 at the Tianjin Medical University Cancer Institute and Hospital were retrospectively analyzed. Patients were included if they had a stage IB–IIIA resectable NSCLC, at least 1 radiologically measurable target lesion, and an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–1. Patients were excluded if they had distant organ metastasis, secondary primary tumors, *EGFR*, *ALK*, and other actionable driver mutations, previous exposure to anti-cancer therapy, including chemotherapy, radiotherapy, and immunotherapy, and previous exposure to immunosuppressive drugs within 3 weeks before NAT. The study protocol, standard operating procedure (SOP) of data collection, and case report form (CRF) were prospectively designed before the initiation of the study to guarantee the quality of data. Blood samples were procured from patients at 3–8 days before NAT, within 1 week before surgery, at 3–8 days after surgery, and every 2–3 months thereafter

until recurrence. All plasma samples were compared against their paired white blood cell (WBC) control and subjected to ultra-deep sequencing with LC-MRD panel at 3D Medicines, Inc., a College of American Pathologists (CAP)-accredited and Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory. Parallel CT surveillance was conducted to monitor disease. Patients who had at least 2 postoperative follow-ups were included. Patients were identified according to their electronic medical records. All procedures performed involving human participants were conducted in accordance with Declaration of Helsinki (as revised in 2013). This study was approved by the ethics committee of Tianjin Medical University Cancer Institute and Hospital (No. E2020444A), and written informed consents were provided by all the patients.

Outcomes and assessment

This study aimed to investigate the utility of ctDNA profiling using the LC-MRD panel to evaluate the therapeutic efficacy and predict recurrence. Radiologic imaging was performed on primary tumor lesions using CT scans before, after NAT, and at follow-ups postoperatively as per Response Evaluation Criteria in Solid Tumors version (RECIST) 1.1 (26). Recurrence-free survival (RFS) was defined as the time between the date of surgery and the date when local recurrence or distant relapses were diagnosed. ORR was defined as the frequency of patients who have had achieved complete response (CR) or partial response (PR) at two consecutive CT assessment at least 4 weeks apart. Hematoxylin and eosin (H&E) staining of surgical resection was applied for assessing histopathologic responses. The definition of MPR involved showing no more than 10% residual viable tumor cells, while a pCR referred to no residual tumor cells. All the radiologic and pathological data were reviewed by at least 2 independent radiologists or pathologists. The data were last edited on 16 April 2021.

LC-MRD-panel development

The MRD was detected by an NGS panel that identifies somatic single nucleotide variants (SNVs), insertion-deletions, and fusions in 194 genes (Table S1, Appendix 1: Supplementary Methods), of which at least 1 mutation would occur in >90% of NSCLC tumors. To build the panel, we first included exons covering recurrent mutations in driver genes of NSCLC from The Catalogue of Somatic Mutations in Cancer (COSMIC; <https://cancer.sanger>.

ac.uk/cosmic). Next, we developed an iterative algorithm to maximize the coverage of patients with minimum panel size using sequencing data of 1,577 NSCLC samples in the 3DMED database (<https://www.3dmedcare.com/technology/data.htm>). Mutations with top recurrence index were selected into the design. Last, we added important actionable mutations and Chinese NSCLC patient-specific mutations into the panel, with the final size of 153 kb.

LC-MRD-panel technical validation

For determination of SNV/Indel limit of detection (LoD) of the LC-MRD ctDNA assay, ctDNA reference standards of known mutations (including *EGFR* p.L858R, *EGFR* p.E746_A750del) were diluted with wild-type cfDNA reference standard to create variant allele frequency (VAF) titration series, including 0.3%, 0.2%, 0.1%, and 0.05%. As for the determination of fusion LoD, genomic DNA of the tumor cell line NCI-H2228 with fusion of *ALK-EML4* was diluted in the cell line without *ALK-EML4* fusion to generate the titration series of 1%, 0.5%, 0.25%, and 0.125%, with at least 3 replications for each titration point. These DNA pools were subsequently analyzed by the LC-MRD panel sequencing at the coverage of 30,000 \times and detected by digital droplet polymerase chain reaction (ddPCR) as reference. The LoD was defined as the lowest VAF at which 95% of replicates are reliably detected for the variant type. Accordingly, the LoD of SNV/Indel and fusion were determined to be 0.1% and 0.25%, respectively (Figure S1).

Intra-assay precision was assessed for repeatability. We prepared 4 cfDNA mixture pools with known VAF of 2%, 1%, 0.5%, and 0.2% for each variant using ctDNA reference standards or variant overexpressing cell lines and ran them in triplicate under the same operating conditions. Every 3 replications formed a combination, and the rate of consistent detection of a known mutation in each combination was calculated for repeatability. Inter-assay precision was evaluated for reproducibility. The same sample pools in the repeatability study were prepared and subjected to a subsequent run. Every 2 different batch replications formed a combination, and the rate of consistent detection in each combination was calculated for reproducibility. The repeatability and reproducibility of the LC-MRD panel assay turned out to be 100% (Table S2).

Blood collection and processing

We collected 20 mL blood samples in a Streck tube from

each patient and centrifuged them at 1,600 g at 4 °C for 10 min within 2 h of collection. The plasma supernatant was collected and transferred to a new microfuge tube, followed by centrifugation at 16,000 g at 4 °C for 10 min. The supernatant and cell pellets were collected separately and stored at -80 °C until DNA extraction.

NGS and sequencing data analysis

The NGS and sequencing data analyses were performed at 3D Medicines, Inc. (27,28). Briefly, cfDNA and genomic DNA from the paired WBCs were extracted using QiAmp Circulating Nucleic Acid Kit (QIAGEN, Hilden, Germany) and QIAamp DNA Mini Kit (QIAGEN), respectively. DNA extracts (30–200 ng) were sheared to 250 bp fragments using an S220 focused-ultrasonicator (Covaris, Woodbrun, MA, USA). The median cfDNA input was 47.13 ng (range, 14.98 to 60 ng). The cfDNA and genomic DNA libraries were constructed using the xGen Prism DNA Library Prep Kit (Integrated DNA Technologies, Inc., Coralville, IA, USA). They were individually barcoded with unique molecular identifiers (UMI), followed by probe-based hybridization. The captured libraries were subsequently loaded onto a NovaSeq 6000 platform (Illumina, San Diego, CA, USA) for paired-end sequencing with a mean sequencing depth of 50,000 \times . Sequencing data were mapped to the human genome hg19. Single-strand and duplex consensus sequences were generated to remove errors yielded from sequencing and PCR. Background noise was further polished by a self-developed database with cell-free DNA sequencing data from a healthy Chinese population. Germline and clonal hematopoiesis-derived mutations were filtered out based on data obtained by NGS profiling of paired WBCs. An in-house model for detecting loci-specific variants was applied to improve specificity (27,28).

Statistical analysis

Categorical variables were presented as absolute and relative frequencies, and numerical variables were denoted as mean \pm standard deviation (SD), median, and range. The pCR, MPR, and ctDNA response rates were compared using the Fisher's exact test. To evaluate the effects of ctDNA change on predicting pathological response, relative delta mean variant allele fraction ($R\Delta$ mean VAF) was calculated to depict dynamic changes of ctDNA upon NAT among pathological responders (MPR) and non-responders (non-MPR). Relative delta mean VAF = (mean VAF on treatment

– mean VAF at baseline)/mean VAF at baseline (29,30). A receiver operator characteristic (ROC) curve was generated to analyze the sensitivity and specificity of $R\Delta$ mean VAF predicting pathological response. The cutoff for defining responders was determined at -0.98 as the threshold where the maximum sum of specificity and sensitivity arrived. A 2-sided Fisher's exact test was conducted to compare ctDNA change between pathologic responders and non-responders. As the LoD of the panel assay used was 0.25% for fusions and 0.1% for SNVs/insertions and deletions (Indels), only variants of AF $>0.3\%$ detected at baseline were analyzed to increase the accuracy of prediction. A Kaplan-Meier curve was generated using a log-rank test to analyze RFS. Cox proportional hazards model was employed to compute hazard ratios (HR) and 95% confidence intervals (CI). A multivariate Cox regression model was established to identify independent risk factors for recurrence. The C-index (31) and landmark (32) analyses were performed to analyze the association of ctDNA with recurrence. The correlations between serum tumor biomarkers and pathological response to NAT were analyzed by using the bivariate linear regression model. The levels of tumor biomarkers during neoadjuvant treatment were recorded. A two sided P value less than 0.05 was considered statistically significant. Statistical analysis was performed using SPSS software (version 25).

Results

Patient characteristics and baseline plasma sample analysis

A total of 22 patients who received NAT and surgery were enrolled between 7 August 2018 and 26 July 2019 (Figure S2). The median age of the cohort (n=22) was 62.50 years (range, 53 to 72 years) and the majority of patients were male (77.27%, 17/22). Approximately half of the patients had stage III disease, and the majority had an ECOG performance score of 0 (95.45%, 21/22). Sixty-four percent of patients were classified as lung squamous cell carcinomas (LUSC). A high percentage of the cohort had a smoking history (81.82%, 18/22). In the neoadjuvant setting, 12 cases were treated with IO+Chemo, 4 with IO+IO, and 6 with Chemo. The clinicopathological characteristics of cases are summarized in Table 1. We performed NGS on baseline plasma and paired WBC samples, which identified 71 mutations from the entire cohort (n=22). A summary of mutation frequency is shown in Table S3. Baseline *RBM10* mutation was associated with

inferior RFS (data not shown). Until 22 January 2021, the median follow-up time after surgery was 17.67 months (range, 2.83 to 23.41 months).

The correlation between ctDNA and the efficacy of NAT

The pCR and MPR rates were 41.67% (5/12) and 58.33% (7/12) in the IO+Chemo group, 25% (1/4) and 25% (1/4) in the IO+IO group, and 0 (0/6) and 16.67% (1/6) in the Chemo group, respectively. Surgical histopathology displayed a median tumor regression of 68% (range, 5% to 100%). It was found that MPR occurred in 9 cases, which were therefore regarded as pathological responders (40.9%, 9/22) (Figure 1A). The remaining 13 cases had a tumor regression of only 5–80% and were defined as non-responders. According to CT assessment, 17 (77.2%, 17/22) cases achieved CR or PR, and 5 (22.7%, 5/22) had stable disease (SD) (Table 2) upon NAT. A total of 10 cases had discordant pathologic and radiologic response. According to CT imaging, 1 case had SD, but was turned out to have an MPR, and 9 CR/PR cases were found to be non-MPR, showing 88.89% sensitivity, 30.77% specificity, and an overall accuracy of 54.55%. Serendipitously, ctDNA demonstrated better association with pathologic response compared with CT (Table 2). The ctDNA dynamics, defined as a relative delta mean VAF ($R\Delta$ mean VAF) upon NAT, yielded a sensitivity of 100% (95% CI: 54.07% to 100%), a specificity of 83.33% (95% CI: 35.88% to 99.58%), and an overall accuracy of 91.67% (95% CI: 61.52% to 99.79%) when the $R\Delta$ mean VAF cutoff was set to -0.98 (Figure 1B, Table 2).

We also sought to analyze the routinely used tumor biomarkers and found that serum carbohydrate antigen 19-9 (CA19-9), carcinoembryonic antigen (CEA), or neuron-specific enolase (NSE) was not associated with pathologic response (Figure S3). These results were consistent with previous findings that the predictive or prognostic value of the currently used serum tumor protein biomarkers are limited (33,34).

When cases were stratified based upon the treatment regimens, those who had received IO+Chemo obtained a numerically better response than the cases treated with IO+IO or Chemo alone (pCRs: 42% vs. 25% vs. 0%). Consistently, the IO+Chemo group had a numerically higher rate of ctDNA response than the other 2 groups (Figure S4). Patients in the IO+Chemo group showed a significant better ORR than those in the Chemo alone according to CT assessment. These data suggest that ctDNA dynamics, defined as the relative $R\Delta$ mean VAF, had

Table 1 Characteristics of patients with NSCLC at baseline

Characteristic	All (n=22)	Patients with MPR (n=9)	Patients without MPR (n=13)
Sex, n (%)			
Male	17 (77.27)	9 (100.0)	8 (61.54)
Female	5 (22.73)	0 (0)	5 (38.46)
Age, years			
Mean \pm SD	61.36 \pm 5.76	60.78 \pm 5.76	61.77 \pm 5.96
Median (range)	62.50 (53.00–72.00)	63.00 (53.00–68.00)	62.00 (53.00–72.00)
Stage, n (%)			
I	5 (22.73)	3 (33.33)	2 (15.38)
II	4 (18.18)	1 (11.11)	3 (23.08)
III	13 (59.09)	5 (55.56)	8 (61.54)
Pathology, n (%)			
Sq	14 (63.64)	8 (88.89)	6 (46.15)
Non-sq	8 (36.36)	1 (11.11)	7 (53.85)
NAT, n (%)			
IO+IO	4 (18.18)	1 (11.11)	3 (23.08)
IO+Chemo	12 (54.55)	7 (77.78)	5 (38.46)
Chemo	6 (27.27)	1 (11.11)	5 (38.46)
Smoking, n (%)			
Yes	18 (81.82)	9 (100.0)	9 (69.23)
No	4 (18.18)	0 (0)	4 (30.77)
ECOG score, n (%)			
0	21 (95.45)	9 (100.0)	12 (92.31)
1	1 (4.55)	0 (0)	1 (7.69)

NSCLC, non-small cell lung cancer; MPR, major pathological response, defined as 10% or less of viable tumor cells in the resected lesions after neoadjuvant therapy; SD, standard deviation; Sq, lung squamous cell carcinomas; NAT, neoadjuvant therapy; IO, immunotherapy; Chemo, chemotherapy; ECOG score, Eastern Cooperative Oncology Group performance score.

a better association with neoadjuvant efficacy.

CtDNA detection for identifying MRD and relapse

We then investigated the dynamic changes in somatic mutation profiles of blood samples from our cohorts at treatment milestones and various postoperative follow-up time points to understand their feasibility in reflecting tumor burden or MRD. A total of 19 cases (86%, 19/22) had mutation detected from plasma samples at baseline, and the detection rate of ctDNA decreased to 59.1% and 31.8%, respectively, after neoadjuvant treatment and at the

first follow-up (3 to 8 days after surgery) (*Figure 2*).

Until 22 January 2021, a total of 8 cases had radiologically confirmed recurrence, ranging from 2.53 to 12.66 months after surgery. In 5 of 8 (62.5%) participants with CT-confirmed relapse, ctDNA was detectable in the blood sample at 3 to 8 days after surgery, suggesting the reliable reflection of MRD. All of the 8 cases that relapsed had ctDNA detected at any follow-up, occurring between 0.16 and 8.36 months after surgery (*Figure 3A*). In participants without detectable ctDNA at any time point post-operatively, the recurrence rate was 0% (0/8). Additionally, in the 8 cases with CT-confirmed relapse, the

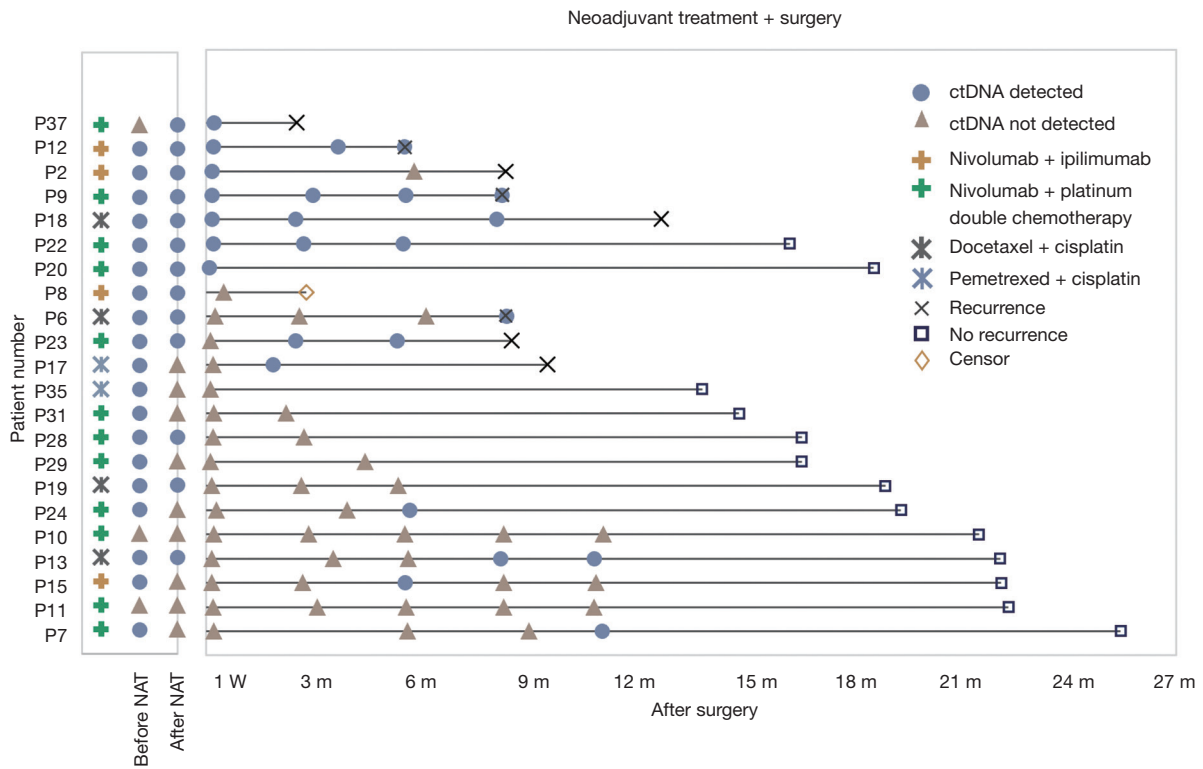


Figure 2 ctDNA monitoring at treatment milestones and during follow-ups. Blue circles denote the presence of ctDNA; while yellow triangles denote the absence of ctDNA. Neoadjuvant treatment regimens were represented by the indicated signs. Radiologic recurrence was denoted as multiplication sign. ctDNA, circulating tumor DNA.

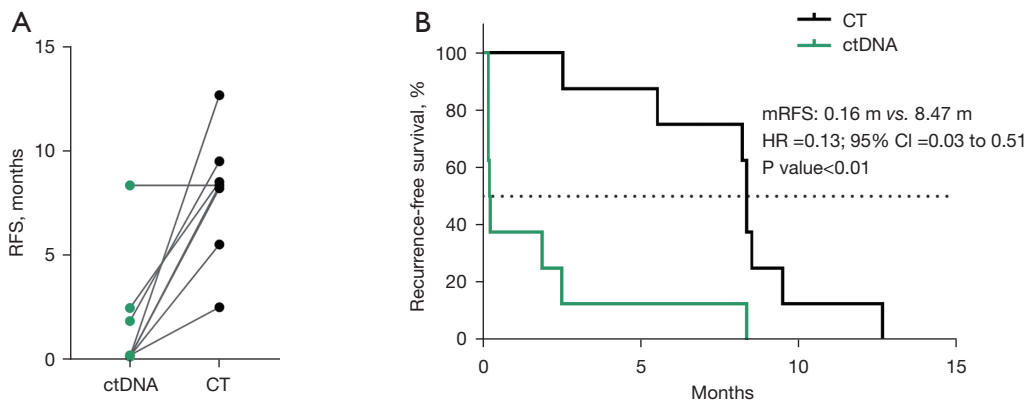


Figure 3 Monitoring of post-operative ctDNA predicts recurrence prior to radiologic imaging. (A) RFS determined by molecular or radiologic recurrence. The 8 patients with CT confirmed relapse were analyzed herein. Molecularly predicted recurrence was defined as the first presence of ctDNA at any time point after surgery. The presence of ctDNA was defined as having had any mutation detected from the used panel. Gray straight line denotes each patient's recurrence predicted by molecular profiling and radiologic imaging. (B) Kaplan-Meier survival curves of RFS determined by ctDNA profiling and CT imaging. Paired CT scan and ctDNA profiling were conducted at 3–8 days after surgery and every 2–3 months during the follow-up. ctDNA, circulating tumor DNA; RFS, recurrence-free survival; CT, computed tomography.

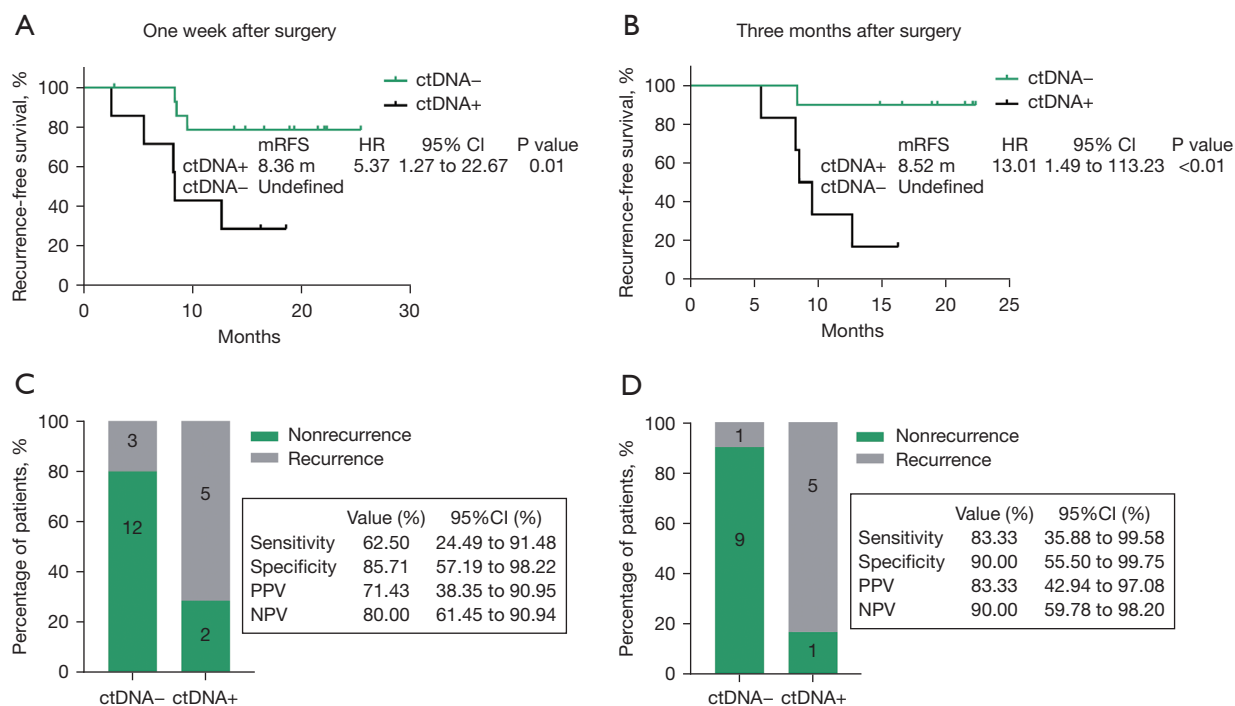


Figure 4 Absence of ctDNA after surgery correlates with better RFS. Kaplan-Meier survival curves depicting RFS of patients having had ctDNA detection at 1 week (n=22) (A) and 3 months (n=16) (B) after surgery. The performance of ctDNA profiling at 1 week (C) and 3 months (D) postoperatively in predicting recurrence in reference to radiologic imaging. ctDNA was defined as any mutation detected from the used panel. Gray color indicates relapse cases and green color denotes relapse-free events confirmed by radiologic imaging. The characters in the columns represent the number of patients with the corresponding status. ctDNA, circulating tumor DNA; RFS, recurrence-free survival; mRFS, median RFS; PPV, positive predictive value; NPV, negative predictive value.

RFS indicated by ctDNA was significantly shorter than that by CT (Figure 3B) (ctDNA vs. CT, 0.16 vs. 8.47; log-rank P<0.01). The ctDNA-determined recurrence was defined as the first detection of any mutation from the used panel after surgery. Post-surgery ctDNA presence preceded radiologic relapse with a median leading time of 6.83 months (0–12.50 months).

Perioperative ctDNA detection for prognosis

We also explored the prognostic and predictive value of ctDNA during the perioperative period to predict RFS in neoadjuvant immunotherapy and/or chemotherapy-treated NSCLC. First, cases who had detectable ctDNA after NAT (at 1–8 days before surgery) trended towards an inferior RFS than those with no mutation detected (ctDNA+ vs. ctDNA-, 12.66 months vs. NA, HR, 7.41; 95% CI: 0.91 to 60.22; log-rank P=0.03, Figure S5). Postoperative ctDNA was found to be prognostic for

RFS. Patients who had detectable ctDNA at 3–8 days after surgery were associated with high risk for recurrence (ctDNA+ vs. ctDNA-, 8.36 months vs. NR; HR 5.37; 95% CI: 1.27 to 22.67; P=0.01). Additionally, cases that had detectable ctDNA at 3 months postoperatively correlated with remarkably inferior RFS (ctDNA+ vs. ctDNA-, 8.52 months vs. NR; HR, 13.01; 95% CI: 1.49 to 113.23; log-rank P<0.01) (Figure 4). Orthogonal analysis of CT-confirmed relapse revealed the performance of post-surgery ctDNA in predicting recurrence. The ctDNA+ at 3–8 days after surgery showed 62.50% sensitivity, 85.71% specificity, 71.43% positive predictive value (PPV), and 80% negative predictive value (NPV) for identifying relapse, while ctDNA+ at 3 months after surgery displayed 83% sensitivity, 90% specificity, 83% PPV, and 90% NPV. Considering ctDNA as a time-varying covariate, we have also tried the C-index and landmark approaches to analyze the association of ctDNA with recurrence. Both the C-index or landmark analysis confirmed that ctDNA presence at

one week [C-index, 0.72, 95% CI: 0.57–0.87; landmark, 0–12 months, $P=0.0101$, 12 months later ($P=0.0555$)] and three months after surgery (C-index, 0.79, 95% CI: 0.62–0.95; landmark, 0–12 months: $P=0.003$, 12 months later $P=0.0339$) was associated with recurrence. Of note, results obtained using these three approaches were consistent that ctDNA presence at three months after surgery was better associated with recurrence than ctDNA at one week.

We further looked into the predictive value of ctDNA after surgery within 3–8 days among patients treated with IO+Chemo. The ctDNA-negative participants showed a trend for longer RFS (ctDNA+ *vs.* ctDNA–, 13.4 months *vs.* NR; $P=0.12$) than the ctDNA-positive cases. The predictive value of ctDNA at 3 months after surgery was of more interest, which showed that ctDNA-negative patients were more highly associated with better RFS than their counterparts (ctDNA+ *vs.* ctDNA–, 8.52 months *vs.* NR; log-rank $P=0.03$), indicating post-surgery ctDNA was a prognostic biomarker for RFS in neoadjuvant immunotherapy-treated NSCLC (Figure S6).

Depending on data availability, univariable and multivariable analyses were conducted in participants who had ctDNA analyzed at 3 to 8 days after surgery to identify predictors of RFS. Smoking (HR 0.19; 95% CI: 0.05 to 0.83; log-rank $P=0.01$) and ctDNA presence at 3 to 8 days after surgery (HR 5.37; 95% CI: 1.27 to 22.67; log-rank $P=0.02$) were significantly correlated with inferior RFS (Table S4). In the multivariate analysis, among smoking and ctDNA presence at 3 to 8 days after surgery, ctDNA presence was the only independent risk factor that associated with inferior RFS in neoadjuvant immunotherapy and/or chemotherapy-treated NSCLC (HR 4.59; 95% CI: 1.04 to 20.33; $P=0.045$).

Discussion

Overall, our study revealed that lung cancer-specific ctDNA profiling during the perioperative period could evaluate efficacy of neoadjuvant immunotherapy and/or chemotherapy, reflect post-surgery MRD, and predict recurrence in surgical NSCLC patients. Several findings are presented: (I) ctDNA change during NAT was highly concordant with pathologic response, showing 100% sensitivity and 83.33% specificity, for an overall accuracy of 91.67%; (II) post-surgical ctDNA presence predicted recurrence, preceding radiographic relapse with a median leading time of 6.83 months; (III) both pre-surgery and post-surgery ctDNA predicted RFS; (IV) ctDNA presence

at 3 months after surgery showed 83% sensitivity and 90% specificity for relapse prediction.

In the neoadjuvant setting, no effective biomarkers are available for evaluating therapeutic efficacy. Biomarkers reflecting treatment response may facilitate the pre-surgery identification of non-responders, thus for whom other optimal treatment regimens can be explored and offered. The CT imaging has been clinically utilized for response assessment but with limited concordance with pathologic response (10,11). Change of ctDNA was shown to be predictive for treatment efficacy in advanced cancers (20–23). In locally advanced, unresectable, NSCLC ctDNA has been shown to predict survival outcomes (35). Similarly, its use to evaluate response to NAT has also been investigated. In this regard, ctDNA could predict pathological response of neoadjuvant chemoradiotherapy in patients with advanced rectal cancer (36). Similar results have been observed in bladder cancers (24). With respect to lung cancer, little is known about the effects of ctDNA changes on predicting NAT therapy. This study found that the ctDNA dynamics during NAT were highly associated with pathologic response (sensitivity, 100%; specificity, 83%). Perioperative ctDNA dynamics could predict neoadjuvant efficacy in NSCLC treated with neoadjuvant immunotherapy and/or chemotherapy. Our finding agrees with the preliminary results of CheckMate 816 (NCT02998528) and NADIM trial (NCT03081689) presented at the AACR 2021 annual meeting (9) and IASLC 2021 World Conference on Lung Cancer 2021 (25), in which ctDNA clearance was reported to be associated with pathologic response and survival, respectively. Most recently, data of a single-arm trial (NCT02927301) investigating ctDNA association with pathologic response and outcomes were presented on the ESMO Immuno-Oncology Congress 2021, showing that ctDNA reductions post-atezolizumab correlated with pathologic response, and 2-year disease-free survival (DFS) was better in NSCLC patients who were ctDNA– post-surgery (37).

The optimal neoadjuvant regimen for resectable NSCLC patients remains unclear. A meta-analysis of 13 randomized control trials has shown that neoadjuvant chemotherapy significantly improved the OS of NSCLC patients compared with surgery alone (5). Moreover, immune checkpoint inhibitors (ICIs), which have demonstrated encouraging antitumor activities and few toxicities in advanced NSCLC patients, have attracted increasing attention in the perioperative setting. In a phase 2 trial, neoadjuvant nivolumab induced an MPR

of 45%, heralding an era of neoadjuvant immunotherapy for NSCLC patients (10). Finally, according to NADIM and CheckMate 816 data, it is becoming clear that the combination of chemotherapy and immunotherapy can significantly improve survival outcomes and multiple trials are therefore currently underway to confirm these findings (NCT02998528, NCT04941417, NCT04459611, NCT04197076, NCT03838159). Accordingly, in our study, the response elicited by nivolumab plus chemotherapy was numerically better than that by either the combinational nivolumab plus ipilimumab or single chemotherapeutic drug corresponding to the increase in pCR, MPR, or ctDNA clearance rates. Since the primary tumor can serve as an antigen source activating tumor-specific T cells, we, therefore propose that immunotherapy may enhance chemotherapeutic efficacy, and the combinatory therapy might be a better neoadjuvant treatment option for resectable early-stage NSCLC patients (38-40). Given the small sample size and the retrospectively design, these data could not deliver a solid conclusion, and the analysis power was compromised. Further studies are required to confirm the results.

The presence of ctDNA after surgery could reflect the residual tumor burden and indicate recurrence prior to radiographic relapse (16-19). In our cohort, among patients without detectable ctDNA at any time point post-operatively, no recurrence occurred (0/8). All of the 8 cases that had CT-confirmed recurrence had detectable ctDNA at any follow-up after surgery, occurring between 0 and 12.50 months (median 6.83 months) ahead of radiologic recurrence. Consistently, despite the various definitions of ctDNA recurrence and different NGS panels employed, a leading time of ctDNA-determined relapse prior to radiologic relapse has been reported in multiple cancers, including lung cancer, bladder cancer, and colorectal cancers (16-19). That leading time in detecting molecular recurrence may open a window for earlier intervention. From our observation, patients who were ctDNA positive at 3 months after surgery exhibited a significantly inferior RFS than ctDNA negative cases, suggesting patients who are ctDNA-positive after surgery have a high risk of relapse and may need adjuvant therapy or maintenance therapy. This hypothesis was favored by the data revealed most recently in bladder cancers (41). Multiple ongoing trials exploring the ctDNA-determined MRD in lung cancer will reveal more data regarding its guiding value in the perioperative setting and during surveillance (ClinicalTrials.gov No. NCT04585477, NCT04385368, NCT04642469,

NCT04585490, NCT04367311, NCT04267237, NCT0425699, NCT04611776).

For ctDNA detection, our study employed a well-designed LC-MRD ultra-deep sequencing panel, which was developed to maximize the coverage of patients with minimum panel size using sequencing data of 1,577 Chinese NSCLC cases in the 3DMed database. The presence of ctDNA at 3 months after surgery identified recurrence with 83% sensitivity and 90% specificity, comparable with the 92% and 90% in Signatera, and inferior to the 93% and 97% in CAPP-Seq. Still, this panel has several advantages over the customized NGS assays. As reported, Signatera and CAPP-seq, which represent tumor-informed and tumor-uniformed strategy, respectively, were applied for ctDNA analysis in early-stage NSCLC (16,19). Signatera was developed with a tumor-informed algorithm, aiming to detect patient-specific somatic variants; therefore, it was able to obtain a good accuracy with high sensitivity and specificity. Of note, the first evaluation time point of Signatera was at 30 days after surgery (16), which may not be sufficiently timely as a few patients were reported to have relapsed within 3 months postoperatively (3,4,42). Similarly, in the CAPP-Seq study, the first time point of postoperative ctDNA detection was within 1 month after surgery; whereas in our observation, the presence of ctDNA within 3-8 days after surgery could also independently predict recurrence. Additionally, ctDNA based profiling can minimize the tumor heterogeneity. It is well known that clonal hematopoiesis-derived mutations constitute an important source of false positive rates (43). This study utilized an ultra-deep ctDNA sequencing with a paired WBC control, which could offset clonal evolution to some extent. Moreover, except for the variants selected by an iterative algorithm, this panel included other actionable mutations and Chinese NSCLC patient-specific mutations to improve clinical applicability, thus potentially facilitating the guiding of subsequent treatments.

This work was limited by its retrospective nature. The potential use of combinational agents during the operation and follow-ups may affect data interpretations. A small sample size and a lack of clinical validation are other significant limitations, for which the power of data was compromised to some extent, particularly in the subgroup analysis. Nevertheless, this study has shown that ctDNA dynamics upon NAT demonstrate high sensitivity and specificity for predicting NAT efficacy in resectable NSCLC patients. Monitoring of ctDNA presence and dynamic changes can be used as a predictor of benefit

from neoadjuvant immunotherapy and/or chemotherapy in patients with NSCLC, which may potentially assist therapeutic decision-making and personalized treatment. Also, both pre-surgery and post-surgery ctDNA were found to be highly associated with RFS. Collectively, we envision the potential of ctDNA monitoring in guiding patients' management in the perioperative setting to define treatment response to NAT, identify MRD after surgery, and pre-emptively predict relapse. Prospective studies with larger sample sizes are warranted to validate our findings.

This is one of the first studies to reveal the potential of ctDNA in evaluating NAT efficacy in surgical NSCLC, demonstrating the high concordance between ctDNA and pathologic response. We also set out the prognostic value of perioperative ctDNA in predicting recurrence.

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Footnote

Reporting Checklist: The authors have completed the STARD reporting checklist. Available at <https://tclr.amegroups.com/article/view/10.21037/tclr-22-106/rc>

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://tclr.amegroups.com/article/view/10.21037/tclr-22-106/coif>). LC, YG, TB, XZ, Bei Z, and YB declare that they are employees of 3D Medicines Inc. Atocha Romero reports personal fees from AstraZeneca, outside the submitted work. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed involving human participants were conducted

in accordance with Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics committee of Tianjin Medical University Cancer Institute and Hospital (No. E2020444A). Written informed consent was provided by individual participants.

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