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# Correlation Between Circulating Tumor DNA Levels and Response to Tyrosine Kinase Inhibitors (TKI) Treatment in Non-Small Cell Lung Cancer

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Statistical Analysis C  
Data Interpretation D  
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**Background:** Clinical monitoring of EGFR-positive NSCLC patients is important to gauge treatment response. The current study addresses the usage of circulating tumor DNA (ctDNA) as a prognostic marker during treatment of first-generation TKIs.

**Material/Methods:** Serial samplings of peripheral blood from 200 EGFR-positive NSCLC patients were taken. Baseline ctDNA quantification was conducted by digital droplet PCR before TKI treatment was administered and compared to primary biopsies. Thereafter blood sampling at different treatment cycles were measured and assessed for its prognostic and predictive value.

**Results:** ctDNA was successfully detected in a number of patients and overall concordance rate was 84%. Importantly, we observed a strong correlation to ctDNA increase with disease progression using radiographic scans. In addition to survival analysis, we noted patients with the largest ctDNA variations had worst outcome. A significant number of EGFR patients during treatment developed a secondary mutation T790M and this cohort had worst survival outcome as well.

**Conclusions:** Our study demonstrated a highly associative relation of ctDNA to NSCLC patients during treatment that can be utilized to gauge treatment response. CtDNA is an attractive means compared with conventional core needle biopsies and presents new methods for accurately profiling NSCLC disease progression.

**MeSH Keywords:** **Biopsy • Carcinoma, Non-Small-Cell Lung • Genes, erbB-1 • Treatment Outcome**

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## Background

Non-small cell lung cancer (NSCLC) is a leading cause of mortality [1]. Treatment efforts have been focused on different molecular targets that exist within patient cohorts [2]. In advanced-stage NSCLC, there has been relatively good progress with a variety of therapy options [3–5]. For instance, EGFR-positive NSCLC patients respond well to EGFR tyrosine kinase inhibitors (TKIs) [4], while ALK- and ROS1-positive patients are shown to respond to crizotinib [5]. Despite the increase in treatment options, the disease is difficult to manage effectively, largely due to rapid changes at the molecular level in response to treatment [6].

Current practice in the pharmacological management of late-stage NSCLC patients relies on initial genetic profiling from resected tumors or biopsied samples [7]. Patients are rarely subjected to repeat biopsies during the course of treatment, which is critically needed to chart the dynamic and rapid changes that develop during the metastatic process or are induced by pharmacological treatment [8,9]. For instance, a significant number of EGFR-positive patients on first-generation TKIs developed a secondary T790M mutation during their course of treatment [2]. The presence of a bulkier methionine residue at position 790 abrogates the inhibitory role of the TKIs [10] and renders these patients resistant to therapy [11]. Even if repeat biopsy were to occur, it might be difficult for a single sampling to capture these events. Due to tumor heterogeneity, especially across multiple metastatic sites [12], it is challenging to retrieve tumor materials that contain these critical mutations.

The detection of circulating tumor DNA (ctDNA) in the blood stream as a result of necrotic or apoptotic tumor cells [13] is an attractive alternative to solid tumor extraction. Due to the relatively less invasive sample retrieval procedure [14], numerous specimens can be taken during the course of treatment [15]. Furthermore, as the circulating DNA is derived from different metastatic sites that shed tumor DNA, this might better represent the many molecular changes that occur during disease progression. The clinical utility of ctDNA has been studied in a number of cancers. For instance, it was shown that KRAS mutation in ctDNA could be a potential biomarker for pancreatic cancer and is highly associated with pancreatic adenocarcinoma [16,17]. In NSCLC, Chen et al. found that ctDNA can be reflective of cancer stage and tumor aggressiveness [18]. These studies show that ctDNA can be useful in the management of this disease.

In this study, our aim was to access the suitability of ctDNA detection and monitoring for EGFR-positive patients during their TKI treatment. Specifically, we targeted late-stage NSCLC patients who had not undergone previous chemotherapy and harbored an activating EGFR mutation. Blood sampling was

**Table 1.** Patient cohort characteristics at baseline.

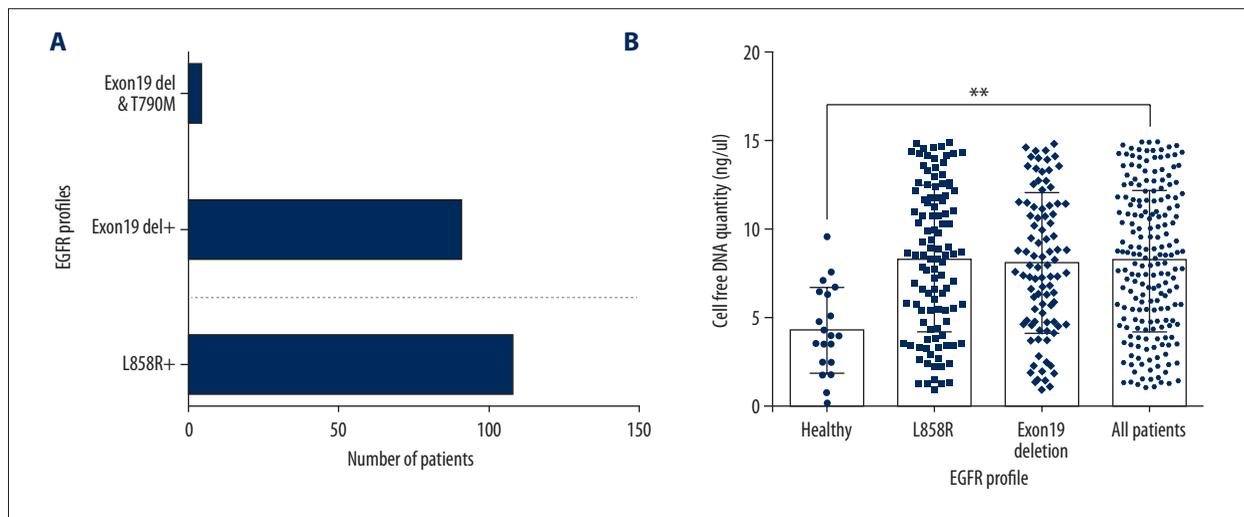
		N=200
Sex	Male	151
	Female	49
Median age (years)		58
ECOG performance status	0	39
	1	153
	Unknown	8
Smoker	Current	117
	Former	67
	Never	16
Disease stage	IIIB	38
	IV	162
Histology	Adenocarcinoma	200
EGFR genotype	Exon19del	90
	Exon19del + T790M	3
	L858R	107

performed before TKI was administered and at every cycle of treatment to gauge treatment efficacy. We hypothesize these measures have prognostic value, and we also selectively performed serial radiographic analysis. Our results showed a close correlation of ctDNA to the disease genotype. The quantity of measured ctDNA was also highly associated with disease progression and survival outcomes.

## Material and Methods

### Patient clinical characteristics and molecular profiles

A total of 200 patients who had activating EGFR mutations detected via routine tissue biopsies were recruited for this study. Patients were selected randomly and were treatment-naïve at the start of the investigation. A detailed summary of the patient characteristics is provided in Table 1. Patients who participated gave their informed consent and specimen extraction was performed using procedures approved by our Institutional Review Board (IRB). The median age of the patient cohort was 56 years old and most had adenocarcinoma on histology. As per guidelines, they were treated with EGFR TKIs erlotinib or gefitinib. Within this patient cohort, we noticed 3 patients with *de novo* T790M mutation. As a control for the study, we recruited 20 healthy volunteers who had been certified disease-free.



**Figure 1.** Baseline patient circulating DNA characteristics. (A) Distribution of NSCLC patients with different EGFR profiles. (B) Cell-free DNA quantity comparing different patient groups.

### Whole-blood extraction and cell-free DNA purification

We performed a series of blood specimen extractions from each patient at different time points. Peripheral blood drawn into 10-ml in ethylenediaminetetraacetic acid (EDTA) tubes were taken each time and processed immediately. For healthy volunteers, a single time point blood extraction was performed and plasma was extracted for ctDNA analysis. Blood plasma was obtained by centrifuging peripheral blood twice at 1000 g for 10 min at 4°C. The repeat centrifugation step was to remove any remaining contaminating cells from the supernatant from the first centrifugation. Cell-free DNA was purified using the Qiagen QIAamp Circulating Nucleic Acid kit (Qiagen Inc., USA) following the manufacturer's instructions. Approximately 5 ml of plasma was processed from each sample and stored at -20°C prior to molecular analysis. Quantification of DNA was done using a Nanodrop 2000 device (Thermo Scientific, USA).

### Detection of mutant EGFR via ddPCR

To ensure good sensitivity in mutant DNA detection, droplet digital PCR (ddPCR) was performed using the QX100 ddPCR system (BioRad, USA) on the cell-free DNA. Primers and probes for the EGFR mutations for L858R, Exon 19 deletions, and T790M were procured from BioRad PrimePCR™ ddPCR™ Mutation Assays (BioRad Lab Inc., USA). Assay validation runs were performed using control specimens of EGFR mutation containing plasmids. For molecular profiling, the procedures closely followed the manufacturer's recommendations. Briefly, each 20- $\mu$ l reaction was prepared and amplified using the following conditions. On the thermocycler (BioRad, USA), settings were programmed at 95°C for 10 min followed by 40 cycles of 94°C for 30 s and 55°C for 1 min, followed by an enzyme deactivation step at 98°C for 10 min. Samples were held at 4°C

thereafter. For analysis, each sample plate was analyzed using QuantaSoft software (Version 1.7, BioRad Lab Inc., USA).

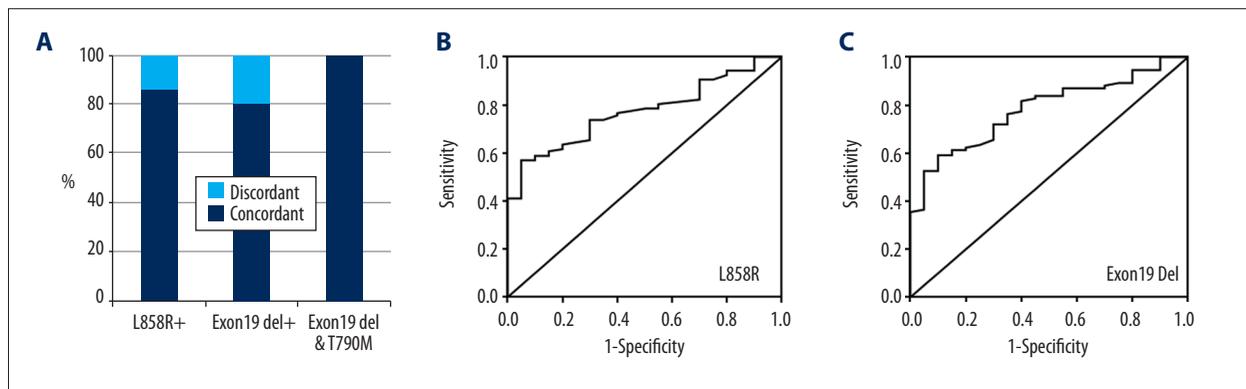
### Statistical analysis

We performed a series of analyses to determine the correlations of plasma DNA and tumor biopsies. Concordance rate was assessed by comparing the match between primary tissue profiling and ctDNA. Comparisons of healthy volunteers' cell-free DNA content were done using an unpaired *t* test. Comparisons of ctDNA concentrations at different time points for NSCLC patients used a paired *t* test. A receiver-operating curve (ROC) was established for healthy volunteers against patients with lung cancer to evaluate the suitability of cfDNA analysis as a detection assay. We measured the area under the curves (AUCs) to gauge accuracy. Survival analysis of the patient cohort was done using the Kaplan-Meier estimate with hazard ratios determined using the log-rank test. All statistical analyses were performed with Prism software (GraphPad Inc., USA).

## Results

### Study design and the importance of cell-free DNA in NSCLC patients

Our study addressed an important aspect of NSCLC treatment monitoring using circulating DNA in peripheral blood. A total of 200 patients were recruited as part of the study and these patients had advanced NSCLC. As the study aimed to follow through on patients who are EGFR-positive and treated with EGFR TKIs, patients in the trial were randomly selected but had either L858R or Exon 19 deletions at baseline; 3 patients in the cohort were discovered to have *de novo* T790M



**Figure 2.** Clinical correlation of circulating DNA to NSCLC. **(A)** Concordance rate comparing ctDNA with primary tumor tissue profiling at baseline measurement. **(B)** ROC analysis of healthy volunteers against EGFR-positive patients using cell-free DNA, showing good clinical prognostic value.

mutation. The proportion of patients with different molecular profiles is shown in Figure 1A. Additional patient characteristics are highlighted in Table 1.

To ascertain the clinical significance of cell-free DNA in cancer patients, we quantified the purified DNA extracted from patients and healthy volunteers. Figure 1B shows the comparison of results for patients at baseline. Within the different patient groups, there were insignificant differences in the quantity of cell-free DNA. The mean amount of DNA extracted from NSCLC patients was 8.2 ng (95% CI 7.7 ng to 8.8 ng). For healthy volunteers, we observed a significantly lower quantity of cell-free DNA as compared to cancer patients, using a *t* test (*p* value <0.001). Healthy volunteers registered a mean purified DNA of 4.4 ng (95% CI 3.2 ng to 5.5 ng). The difference in total cell-free DNA between healthy and diseased individuals could indirectly suggest that this is disease-related.

#### Concordance at baseline showed good clinical correlation

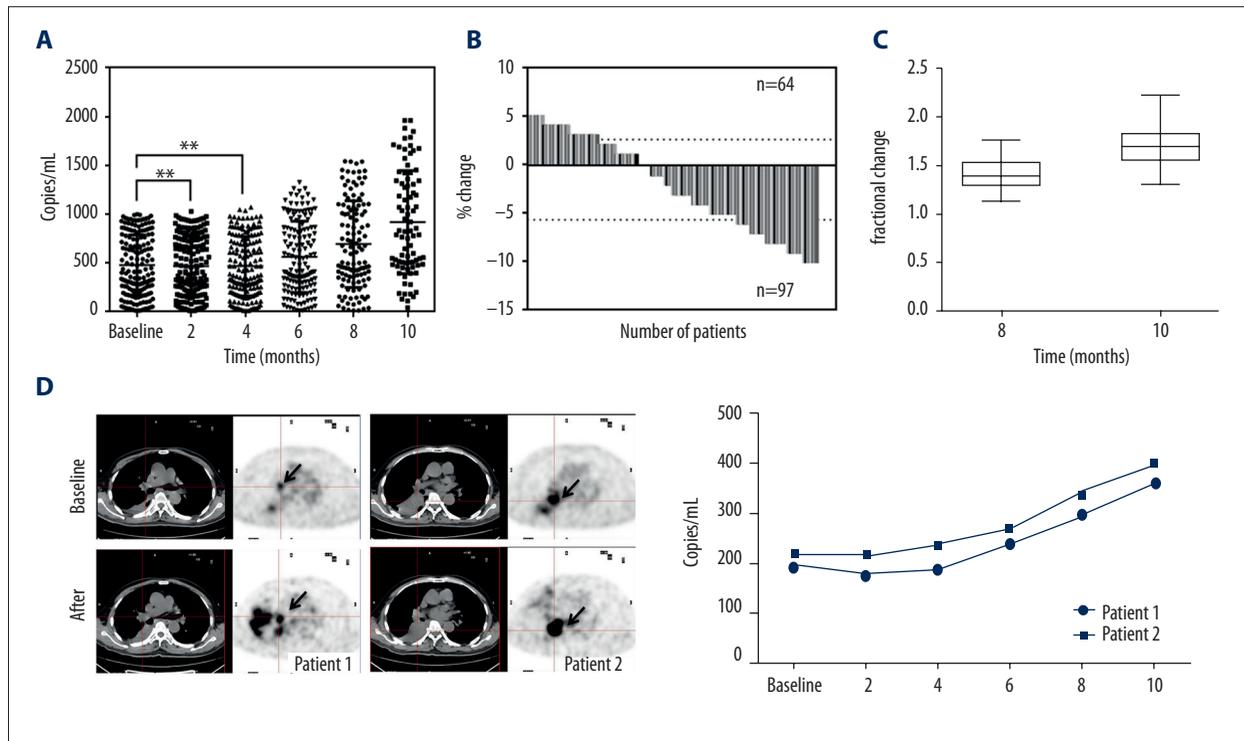
We analyzed the concordance of the EGFR profiles between mutant DNA in circulation and primary tissue biopsy to ascertain the clinical value of ctDNA. This also established the sensitivity for detecting various EGFR mutations using circulating DNA. Figure 2A summarizes the results. Of the tissue and blood plasma samples, the overall concordance was 84%. Healthy controls yielded entirely wildtype EGFR profiles. The breakdowns for subgroups of patients with different EGFR mutations were as follows: the L858R-positive patient cohort had 86% concordance rate with matched tissue samples, while exon 19 deletions-positive patients were 81% in accord to tissue biopsies. For the 3 cases of T790M-positive patients, the mutation was positively identified in all of them. We performed ROC analyses, as shown in Figure 2B, to determine the suitability of using ctDNA as a detection assay. The area under the curve (AUC) was 0.77 (95% CI 0.68 to 0.86), in comparing healthy volunteers to NSCLC L858R-positive patients. For comparison with

exon 19 deletions-positive patients, the AUC was 0.78 (95% CI 0.6884 to 0.8755).

#### EGFR tracking of NSCLC patients showed disease progression

To show the usefulness of ctDNA in disease monitoring and to gauge treatment response, we obtained serial measurement of ctDNA quantities from the entire concordant patient cohort. Baseline blood plasma analysis was performed prior to the initiation of TKIs and provided the reference point for each patient. Serial blood extraction was conducted at regular intervals of 2 months during patient follow-up visits. Figure 3A shows the trending at different measurement time points for all patients. At the onset of treatment, we observed ctDNA quantities fell, and the absolute decrease varies across different patients, as shown in Figure 3B. Using a paired *t* test, the decrease in ctDNA quantity was statistically significant at the first (*p* value <0.0001) and second (*p* value=0.0007) measurement time point (patients who died during this period were excluded from the analysis). The maximum registered drop in ctDNA quantities was 18%. An exception to the group was the 3 patients with *de novo* T790M mutations, where we observed an increase in ctDNA quantity.

From the fourth time point onwards, we registered an upward trend (Figure 3A) for the quantity of ctDNA. Figure 3C summarizes the variations in measured quantity among the entire patient cohort. The mean increase in ctDNA was 71%, with a maximum registered value of 123%. With consent from 2 patients, we performed further radiographic tests after the final measurement time point. These patients had maximum ctDNA quantity increase of 87% and 81% and were observed to have consistently increasing amounts of ctDNA in peripheral blood. Figure 3D compares the outcome prior to treatment and after the monitoring period. In both cases, the tumors registered a significant increase of size.



**Figure 3.** Serial monitoring of ctDNA variations during TKI treatment. **(A)** Summary of ctDNA concentrations for the entire study cohort. **(B)** Variation in ctDNA at the first measurement time points, where 97 patients experienced a drop and 64 patients had a rise. Average percentage change for different groups are reflected by the dotted lines. The rest experienced relatively little change in ctDNA quantity. **(C)** Fractional change in ctDNA concentration at later measurement time points. Deceased patients are excluded. **(D)** Radiographic comparison at baseline and after 10 months of ctDNA monitoring for 2 different NSCLC patients.

### Survival analysis showed ctDNA could be a useful prognostic marker for NSCLC EGFR-positive patients

We observed a number of emerging trends from monitoring each patient sample. Besides the variations in measured ctDNA quantities, we also observed the positive identification of T790M among more patients. We hypothesize that this will have impact on survival since EGFR T790M confers TKI resistance. The cumulative distributions of T790M mutation incidence rates among these patients are tabulated in Figure 4A. We observed that most secondary mutations occurred in the later part of our monitoring intervals. The median period was 6 months for the T790M-positive patient group.

We performed a number of subgroup analyses using overall survival (OS) as the endpoint measure. Firstly, we assessed whether higher ctDNA quantity has an effect on OS (Figure 4B). Taking a median split of the patient cohort based on the maximum detected quantity of ctDNA of each patient, we divided the cohort into 2 groups of equal sizes. Median OS for the subgroup with higher ctDNA quantity was 13 months compared with 14.5 months for the lower ctDNA quantity group. The hazard ratio was 0.84 (95% CI 0.56 to 1.19) and, using a log-rank test, there was

no significant difference between the 2 patient groups ( $p$  value=0.3396). We next analyzed patients with the largest change in ctDNA quantity with respect to the baseline measurements (Figure 4C). Similarly, a median split of the patients was performed after ranking their quantity change. Median OS for the subgroup with larger variations was 14 months compared with 16 months for patients who experienced lower variations. The hazard ratio was 1.483 (95% CI 1.14 to 2.17) and log-rank test showed a statistically significant difference between the 2 patient groups ( $p$  value <0.01). The final analysis was performed on patients based on T790M status (Figure 4D). Patients with T790M has a median OS of 2 months lower than in the wild-type group. The hazard ratio was 1.5 (95% CI 1.18 to 2.23) and using a log-rank test, this was significant difference between the 2 patient groups ( $p$  value <0.01). The results clearly demonstrate the usefulness of serial monitoring of patients on TKIs and its associated link to prognosis.

### Discussion

NSCLC patients who have activating EGFR mutations have benefitted greatly from the first-line treatment of TKIs [19].

However, the development of drug resistance limits usefulness of the therapy [20]. It is thus important that the molecular changes associated with therapy resistance are captured early [2]. Current disease management routines are insufficient to actively monitor such changes, and our study shows the feasibility of using an alternative source other than primary tumor biopsies. Potentially, this has clinical value to complement current cancer management regimens. CtDNA obtained less invasively presents an opportune use for real-time treatment monitoring.

The use of circulating DNA for clinical applications has been well investigated in recent years [21]. Used in non-invasive prenatal testing (NIPT), this provides screening of an unborn baby for any genetic abnormality [22]. In oncology applications, it is shown to be present in numerous cancers, including NSCLC [23–25], breast cancer [26–28], and prostate cancer [29,30]. For instance, in NSCLC, Newman et al. observed that levels of ctDNA were distinguishable between residual disease and treatment-related changes [23]. Higgins et al. reported that the loss or gain of *PIK3CA* mutations can occur in breast cancer patients with metastatic disease [26]. In prostate cancer, the plasma DNA levels were observed to be correlated with subgroups of localized and metastasized prostate cancer [29]. The approach is favored for its relatively less invasive means for sample extraction and the propensity for serial sampling [15]. Our work clearly demonstrates its effectiveness in monitoring of EGFR-positive NSCLC patients on first-generation TKIs. We investigated patients with sensitizing EGFR mutations L858R and exon 19 deletions; together, these mutations make up 85–90% of all EGFR mutations in the general population [31].

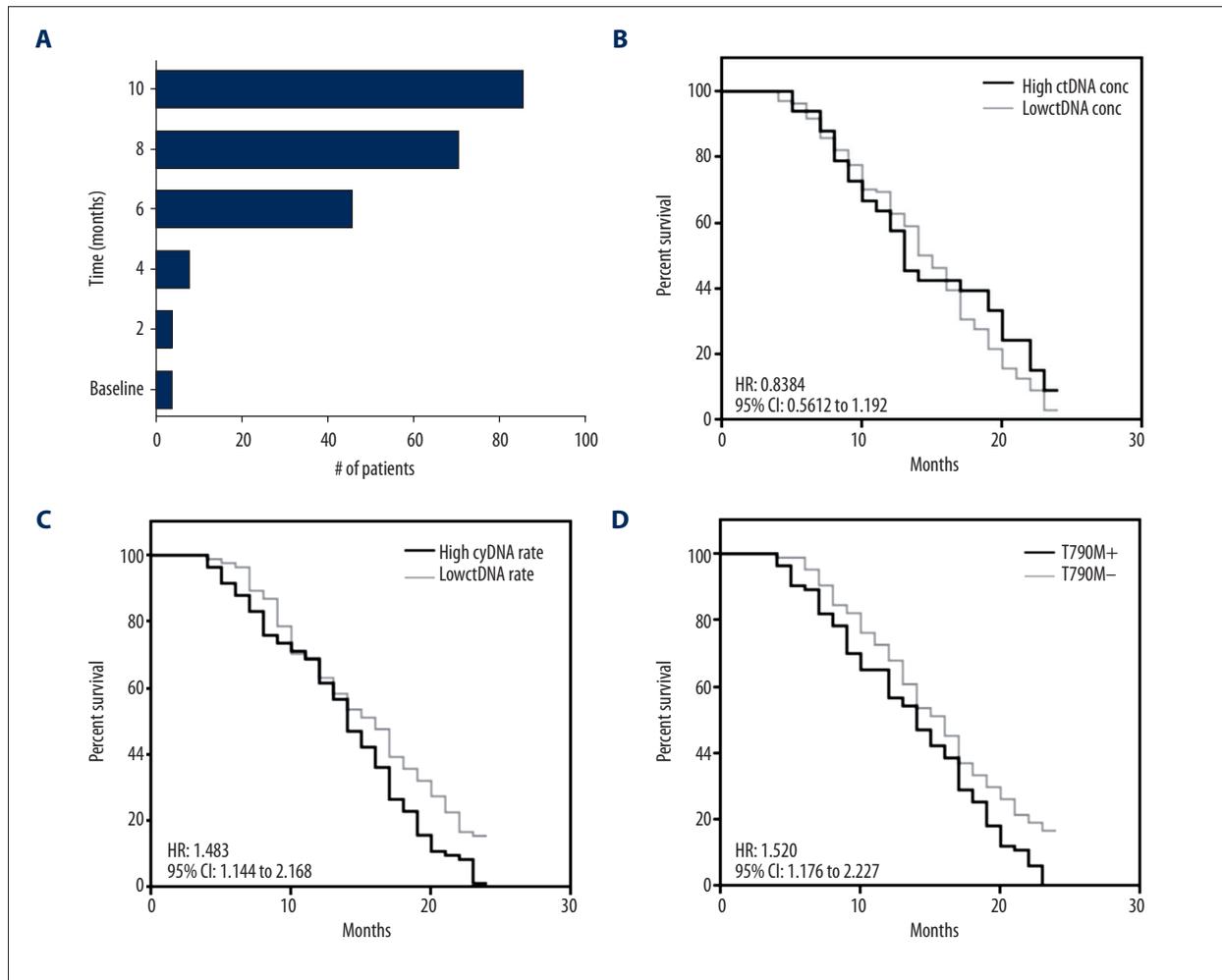
At baseline, we noted diseased patients tend to have higher quantity of cell-free DNA, and this is likely to be contributed to by tumor masses located close to the vasculature [32]. This contributes to the positive ctDNA signatures and the high concordance rate with the primary tumor at baseline. These results agree with other studies that similarly demonstrated the usefulness of cell-free DNA as a measure for diseased patients [13,29]. This establishes the clinical relevance of plasma cell-free DNA in NSCLC. Furthermore, in our ROC analysis, we determined a fairly high AUC, which potentially indicates good accuracy for using such test assays.

Our work extends further to address clinical monitoring of NSCLC first-line treatment for EGFR-positive patients. The dynamic responses of the disease complicate the treatment process, and ctDNA monitoring is shown to be useful for gauging treatment effects. In our analysis, we observed clear indications of ctDNA initially decreasing with the initiation of treatment. This is indicative of treatment efficacy at the initial phase, as patients with activating EGFR mutations tend to respond well

to TKIs [4,31]; in contrast, the 3 patients with *de novo* T790M mutations did not. T790M mutation has been linked to abolishing the effects of TKIs, and the upward trend for ctDNA could show higher tumor burden. We observed similar trends in subsequent monitoring of patient profiles corroborated by radiographic analysis. The mean increase in ctDNA was approximately 70% as compared to baseline for the entire cohort. In this period, we observed clear indications of molecular changes within the EGFR status.

Specifically, the acquired T790M mutations for a significant number of patients warrant greater attention. It is well established that a greater number of NSCLC patients on EGFR TKIs developed this mutations during treatment [2] and our results concur with this findings. Moreover, through our monitoring regimen, we could chart out exactly when the mutations appear and this could have led to more rapid response to alternative treatment. Unlike conventional disease management, the use of ctDNA allows faster clinical predictions of treatment resistance. Our study also showed how the resistance mechanism developed differently in the entire cohort (Figure 4A). Overall, 47% of the entire patient group had the mutation at the end of the monitoring process. This is consistent with observed trends in late-stage NSCLC patients [33]. To combat the emergence of this mutation, third-generation TKIs that involve entirely new classes of EGFR-irreversible inhibitors are recommended and have shown promising results [3]. It is thus important to be able to uncover the resistance mutation promptly with active genetic profiling.

To determine if the detection of ctDNA aids in disease prognosis, we performed a series of survival analyses. As larger amounts of ctDNA could be indicative of higher overall tumor burden in patients, we divided the entire patient cohort equally into 2 groups by ranking the absolute quantity of mutant DNA. We found that the result was not significantly different between the 2 patient groups. We postulate that the aggressiveness of the disease will play a bigger role in overall survival estimation instead of just relying on overall tumor burden. To confirm our hypothesis, we stratified patients based on the rate of increase of ctDNA during the monitoring period and performed a similar median split of the group. We found that the patient group with higher rate of increase had worse outcome. Our study is the first to associate such clinical parameters to NSCLC treatment monitoring, and may help to better identify patients at risk. Median survival for the subgroup with worse outcome was 2 months. As earlier discussed, the occurrence of T790M mutation emerging in TKI-treated NSCLC patients is a critical hallmark of TKI resistance [2]. Our study aimed to determine if the presence of the resistance mutation contributes to significant disease progression and affects survival. We grouped all patients during the monitoring period with *de novo* or acquired T790M mutation together, and



**Figure 4.** Real-time monitoring of the EGFR mutational profile and survival analysis of different patient groups. **(A)** Distribution of patients who acquired the secondary T790M mutation during the monitoring period. **(B)** Overall survival analysis comparing patient groups with high or low detected concentrations. **(C)** Overall survival analysis comparing patient groups with different rates of change in ctDNA concentrations. **(D)** Overall survival analysis comparing patients with T790M status.

performed Kaplan-Meier analysis. The results showed that the T790M-positive group had worse outcome, with median survival 2 months lower than the other group. This result is consistent with other studies that investigated the prognostic value of T790M detection [10]. The presence of T790M in treatment-naïve patients in one study showed adverse effects on progression-free survival [34].

Our study systematically analyzed the different aspects of using cell-free DNA in NSCLC. It clearly demonstrated the accuracy to detect and identify patients with disease progression and subsequently worse survival outcome. This evidently shows that ctDNA is useful and can potentially complement current NSCLC disease management for treatment monitoring. The easy sample extraction makes this an ideal body fluid for serial sampling and will also aid in cases where tissue biopsies fails. Another interesting insight was the association

of ctDNA to tumor burden. Our study presented early evidence through radiographic analysis, establishing the groundwork for future work to correlate tumor burden with ctDNA using serial radiographic scans. The main limitation of the current study is the low purity of ctDNA in the samples. Several patients at baseline who had discordant results between tissue biopsies and plasma DNA could likely be attributed to mutant DNA being masked in abundant wildtype cell-free DNA. The low purity became a limiting factor even for ddPCR to detect. This was revealed in our discordant patient group during randomly sampling. Many of these samples turned positive at later time points but were detected at very low concentrations (data not shown). This can potentially be resolved with more sensitive detection assays.

## Conclusions

The tracking of NSCLC patients on TKIs is important, and our study established a robust link for ctDNA to complement current disease management. Our analysis showed that it is highly correlated to NSCLC and can be utilized for treatment response and active genetic profiling. This helps to identify high-risk patients for more timely alternative therapy. Survival analysis highlighted a critical need for disease monitoring to detect key mutations, which severely affect survival outcomes. We also

showed that NSCLC patients with higher ctDNA increase during monitoring had significantly worse survival outcome. This work lays the foundation for future clinical investigations addressing the critical need for timely interventions in patients with resistance mutations.

## Conflict of interest

All authors declare no conflict of interest.

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