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STUDY PROTOCOL

Application of circulating tumor DNA for dynamic monitoring of advanced non-small cell lung cancer treatment response: An open-label, multicenter, prospective, observational study protocol

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Abstract

Response Evaluation Criteria in Solid Tumors version 1.1 is currently considered the indicator to assess the curative effect of cancer. However, radiographic measurements often fail to detect tiny lesions or changes in the tumor burden, while tumor biomarkers possess low sensitivity and stability. Circulating tumor DNA (ctDNA) is a potential noninvasive approach that can be used to make an earlier diagnosis, monitor disease progress, and determine treatment efficacy. Previous studies of non-small cell lung cancer (NSCLC) have not clearly determined the optimum time to monitor ctDNA. Hence, the appropriate time to evaluate ctDNA to determine the curative effects of treatment in advanced NSCLC compared to conventional imaging and tumor markers should be explored in order to prevent unnecessary side effects and to avoid continuing ineffective therapies. This protocol outlines a prospective clinical trial in which advanced NSCLC patients will be recruited and longitudinal changes in ctDNA levels with changes in radiographic tumor size or tumor biomarkers will be assessed. ctDNA will be quantified by determining the allele fraction of cancer-associated somatic mutations in plasma using multigene next-generation sequencing assay. Conclusions will be drawn from data collection and analysis.

Introduction

Lung cancer is the leading cause of cancer-related death worldwide. Approximately 85% of lung cancers are non-small cell lung cancer (NSCLC), 60% of which are generally detected at advanced stages.[1]

At present, blood tumor markers (e.g. neuron specific enolase [NSE], Cyfra21-1, etc.) and Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1, which measure long diameters of solid tumors by radiological imaging, are considered the indicators for assessing the curative effect of cancer treatment.[2] However, radiographic measurements often fail to detect tiny lesions or changes in tumor burden, while biomarkers have low sensitivity and stability.[3,4] Misinterpretation of scans could lead to inappropriate discontinuation of a potentially effective therapy, or conversely, an ineffective treatment could be continued

hoping for a delayed response that never comes. Therefore, there is an urgent need to determine a complementary method to assess tumor burden that has both high sensitivity and specificity.

Circulating tumor DNA (ctDNA) exists in a number of body fluids, including blood and urine. It can be released into body fluids via tumor cell apoptosis and necrosis or spontaneous release by the whole tumor.[5] The amount of ctDNA in a patient's bloodstream depends on many factors, including clinical stage, tumor size, cancer type, and rate of cell turnover.[6] Furthermore, the ctDNA carries the same tumor specific sequence alterations as the solid tumor and can be used to detect clinically relevant gene mutations. Therefore, dynamic monitoring of the mutant allele fraction changes helps to evaluate tumor load, which can theoretically reflect the curative effect of antitumor therapy.

Numerous studies have shown that analysis of ctDNA is currently gaining momentum as an innovative methodology to characterize tumor genomes[7–10] and dynamically monitor the curative effect of treatments in NSCLC patients.[11–14] Swanton *et al.* analyzed 100 patients from the TRACERx cohort and conducted a phylogenetic approach to ctDNA profiling in early-stage NSCLC, showing that ctDNA was detected in 92.9% of relapsed cases 70 days prior to clinical computed tomography (CT) scan confirmation.[8] A recent study reported that the median time to assess immunotherapy efficacy among NSCLC patients is 24.5 days by ctDNA versus 72.5 days by imaging.[15] However, these previous studies did not detect plasma ctDNA at fixed time points. In other words, the appropriate time to detect ctDNA after antitumor therapy to determine the treatment effect is still unclear. In addition, the precise lead time required for ctDNA compared to RECIST 1.1 to evaluate the curative effect still needs to be verified. Thus, clinical trials are urgently required to determine the appropriate therapeutic effect time and the precise lead time of ctDNA assessment compared to biomarker and imaging after chemotherapy, radiotherapy, or targeted therapy. Assessment of ctDNA should be performed as early as possible to improve prognosis.

Study rationale

Current research shows that ctDNA could derive from either the apoptosis and/or necrosis of circulating cancer cells or micrometastases shed by the tumor. In addition, because ctDNA is a by-product of dying cancer cells, it is rapidly cleared from the circulatory system, possessing a half-life of two hours; as such, its levels provide a real-time snapshot of active tumor cell death rather than simply a measure of tumor burden.[6,16] Example cases from prior studies have shown that post-treatment (from chemotherapy, radiotherapy, targeting, etc.) ctDNA levels can spike as tumor cells are killed and thereafter rapidly decline after the initial wave of cell death has subsided.[17,18] Furthermore, the overall tumor burden is reduced. Therefore, in order to determine the effect earlier, it is necessary to identify the most appropriate time point to monitor ctDNA to determine when this test is most sensitive.

In addition, imaging cannot evaluate efficacy in all cancer patients. For example, in extensive pleural metastasis of stage IV lung cancer, it is not possible to visualize evaluable lesions on imaging, and lesion changes are difficult to evaluate. Therefore, another method is needed to evaluate the efficacy of treatment and supplement RECIST 1.1. A large number of clinical trials have proven that ctDNA screening offers a noninvasive clinical tool for real-time monitoring of primary tumor and metastatic disease burden, as well as therapeutic response.

Theoretically, ctDNA could be of great clinical value to evaluate the curative effect earlier, and can be used to supplement RECIST 1.1.

Hypotheses

1 ctDNA can be used to assess the efficacy of chemotherapy, radiotherapy, and targeted therapy after one to two weeks through a > 50% decrease in the mutant allele fraction from baseline.
2 When imaging evaluation is difficult, treatment response can be assessed by ctDNA with a > 50% decrease in the mutant allele fraction from baseline.

Previous studies

We performed a series of studies on ctDNA in lung cancer to confirm whether ctDNA detection in early stage NSCLC patients is feasible and may potentially be applied for clinical management. Recently, we performed a prospective observational study to determine dynamic changes in ctDNA after surgery.[19] Consecutive patients with suspected lung cancer who underwent curative-intent lung resection were enrolled. Blood samples were collected (10 mL) by intravenous puncture. Plasma samples were obtained before surgery (time A) and at a series of scheduled time points following tumor resection. DNA was isolated from 4 mL of purified plasma. A multiplex assay based on circulating single-molecule amplification and resequencing technology (cSMART) was used to simultaneously detect and quantify hot spot *EGFR*, *KRAS*, *BRAF*, *ERBB2*, *PIK3CA*, *TP53*, *ALK*, *RET*, and *MET* plasma DNA variants. Positive plasma mutations were validated in tumor tissue and normal lung tissue by targeted sequencing. The results showed that in most cases, plasma ctDNA concentration exhibited a rapid decrease from time A to time F. The median half-life of ctDNA was 35 minutes. Most patients who were positive at time F (3 days post-surgery)

experienced recurrence, while those who were negative at time F remained disease free. At time E (1 day post-surgery), all seven patients with positive ctDNA whose plasma ctDNA concentration dropped to 0% at time F exhibited a good prognosis at follow-up, suggesting that minimal residual disease detection one day after surgery may be affected by incompletely degraded ctDNA. Thus, three days after surgery was used as a base value for postoperative surveillance. This previous study revealed the elimination rate of ctDNA in radical resected lung cancer patients. In our forthcoming study, we will focus on non-resected lung cancer patients to investigate dynamic changes in ctDNA in response to systematic or local treatment.

Primary aims

1 To explore the appropriate time to measure chemo/radio/target effective time (CET/RET/TET) by monitoring ctDNA after anticancer therapies are applied in advanced NSCLC.
2 To determine the precise lead time (the interval between ctDNA detection and treatment response confirmed on clinically indicated imaging) for ctDNA compared to conventional biomarker or image evaluation to detect disease recurrence after radiotherapy, chemotherapy, or targeted therapy.

Secondary aims

1 To assess the accuracy of ctDNA when undetectable on imaging or when tumors are difficult to assess.
2 To determine the consistency of ctDNA for predicting the treatment response of measurable lesions after radiotherapy, chemotherapy, or targeted therapy compared to conventional image evaluation.
3 To explore the consistency of ctDNA for determining the efficacy of treatment in advanced NSCLC compared to tumor markers after radiotherapy, chemotherapy, or targeted therapy.

Study population

The study population will be drawn from the Oncology Departments of multiple centers, including the First Hospital of Shijiazhuang, Peking University People's Hospital, Hebei Medical University Fourth Hospital, Xingtai People's Hospital, and Han Dan Downtown Hospital. The eligibility criteria are: (i) patients with stage III–IV NSCLC according to the 8th edition of the International Association for the Study of Lung Cancer (IASLC) Tumor Node Metastasis (TNM) Classification; (ii) aged > 18 years; (iii) tissue specimens (fresh or wax blocks) were collected before this treatment; (iv) positive ctDNA detection at baseline stage; and (v) both treatment naïve patients and treated patients can be enrolled. The exclusion criteria are as follows: (i) multiple primary lung cancer; (ii) presence of any unstable systemic disease; (iii) histology that is not NSCLC; (iv) unqualified blood samples; and (v) patients lacking detection points.

Study design

This protocol outlines a prospective clinical trial in which advanced NSCLC patients will be recruited (Registration: NCT 03664843). Ethical approval was obtained from The First Hospital of Shijiazhuang Medical Ethics Committee (2018FH/HB-16), and all participants will provide written informed consent. The results will be disseminated through presentations at scientific meetings and publications in international peer-reviewed journals.

RECIST 1.1 will be used to assess imaging, while ctDNA level and biomarkers will be detected in patient plasma following a specific schedule. In particular, the study consists of two phases. The first phase, as an exploratory stage, will determine the most appropriate time to monitor ctDNA after chemotherapy, radiotherapy or targeted therapy by evaluating imaging data and changes in tumor markers. The second phase will determine the precise lead time of ctDNA relative to tumor biomarkers or image evaluation to determine the efficacy of advanced NSCLC cancer therapy.

The study will be observational and both stages will include three subgroups: chemotherapy, radiotherapy, and targeted therapy groups. The chemotherapy group will be treated with pemetrexed/gemcitabine/docetaxel/vinorelbine/etoposide with or without cisplatin/carboplatin following the lung cancer treatment guidelines. The radiotherapy group will be treated with three-dimensional conformal radiation therapy of pulmonary lesions. In cases with mutations, the targeted therapy group will be treated with corresponding targeted drugs, such as EGFR-tyrosine kinase inhibitors (TKIs).

During the first stage, histological specimens (fresh or paraffin blocks) of all enrolled patients will be obtained prior to treatment (Fig 1). In the chemotherapy group, blood samples for ctDNA and biomarker analysis will be collected before chemotherapy (time A1) and at a series of scheduled time points after chemotherapy (B1: 7 days; C1: 14 days; D1: 21 days; E1: 28 days; F1: 35 days; G1: 42 days), with serial blood samples collected every three months from a subset of patients. Meanwhile, imaging technology, such as CT scan detection, will be performed according to RECIST 1.1 at certain time points (D1: 21 days; G1: 42 days). If the treatment is judged as a complete response/partial response/progressive disease (CR/PR/PD) rather than stable disease (SD), we would gain

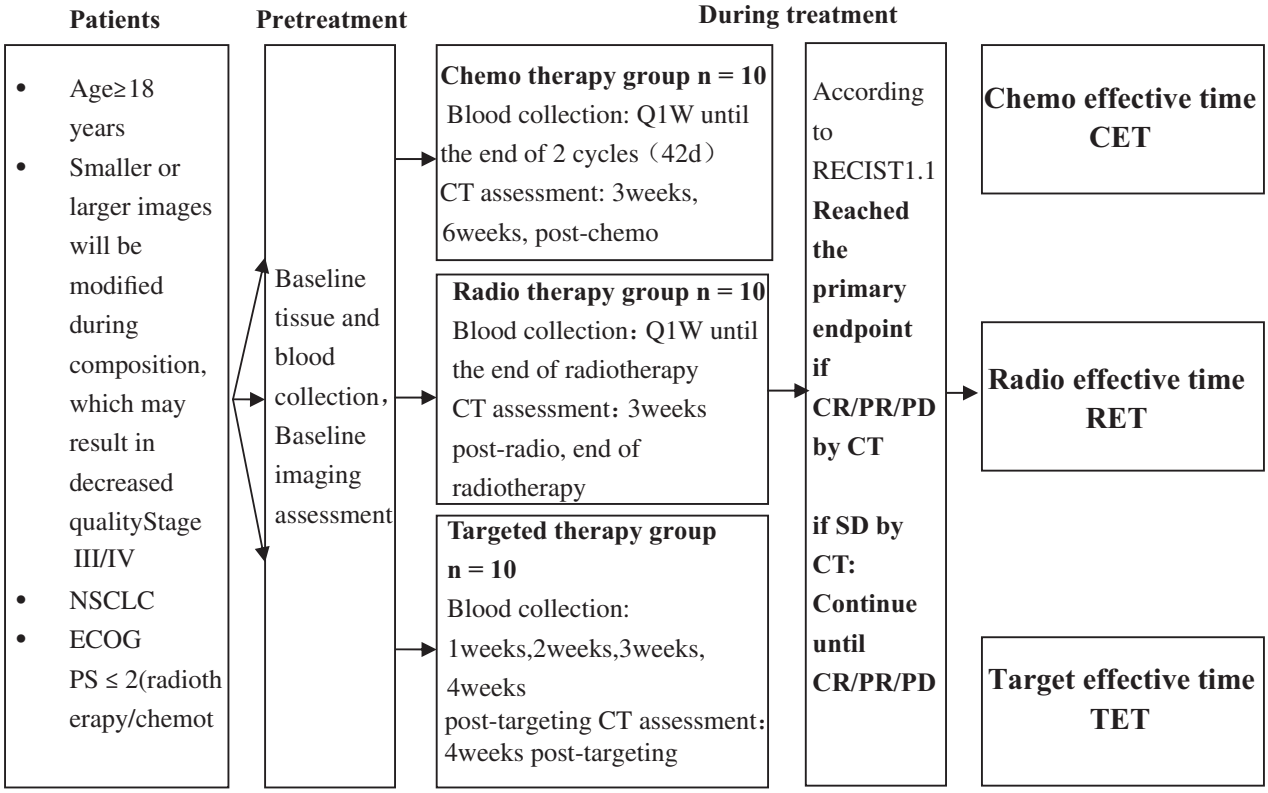

Figure 1 The first phase. CT, computed tomography; CR, complete response; ECOG PS, Eastern Cooperative Oncology Group performance status; NSCLC, non-small cell lung cancer; PD, progressive disease; PR, partial response; RECIST, Response Evaluation Criteria in Solid Tumors; SD, stable disease.

a lead time compared to imaging, and thus the patients will have reached the primary endpoint and further blood samples will not be required. Because of different treatment methods, the time points will be recorded discriminately.

The second phase, considered a continuation and amplification of the first, entails collection of the histological and blood samples obtained before treatment (Fig 2). CtDNA will be determined at the appropriate CET/RET/TET and the precise lead time will be explored. The consistency of ctDNA compared to traditional efficacy assessment methods will also be evaluated. Similarly, if treatment is judged as CR/PR/PD according to RECIST 1.1, patients will have reached the primary endpoint of the study.

Tissue and blood sample collection

Fresh tumor tissue collected prior to treatment will be directly placed into tissue preservation solution in a sample box. Paraffin samples will be cut into 6–10 micron slices and placed into slide boxes. The samples must be pathologically confirmed to contain no less than 20% tumor cells.

Ten milliliters of blood will be collected in ethylenediamine-tetraacetic acid

(EDTA)-K2 tubes (Vacuette; Greiner, Pleidelsheim, Germany) and centrifuged at 1800 g for 10 minutes, followed by immediate freezing at −80°C until needed.

Blood samples will be used to monitor tumor biomarkers, such as CEA, SCC antigen, CA125, CYFRA21-1, and NSE by electrochemiluminescence immunoassay.

DNA extraction, library preparation, and target enrichment

Genomic DNA will be extracted from tumor tissues and blood cells using the QIAamp FFPE Tissue Kit (Qiagen, Hilden, Germany), as instructed by the manufacturer. Peripheral blood samples will be collected using a cell-free DNA BCT tube (Streck, La Vista, NE, USA) and processed within 24 hours of sample collection. Plasma will be separated by centrifugation at 820 g for 10 minutes. Circulating cell-free DNA will be isolated from 1 mL plasma using the QIAamp Circulating Nucleic Acid kit (Qiagen) according to the manufacturer's instructions. DNA concentration will be measured using a Qubit fluorometer (Life Technologies,

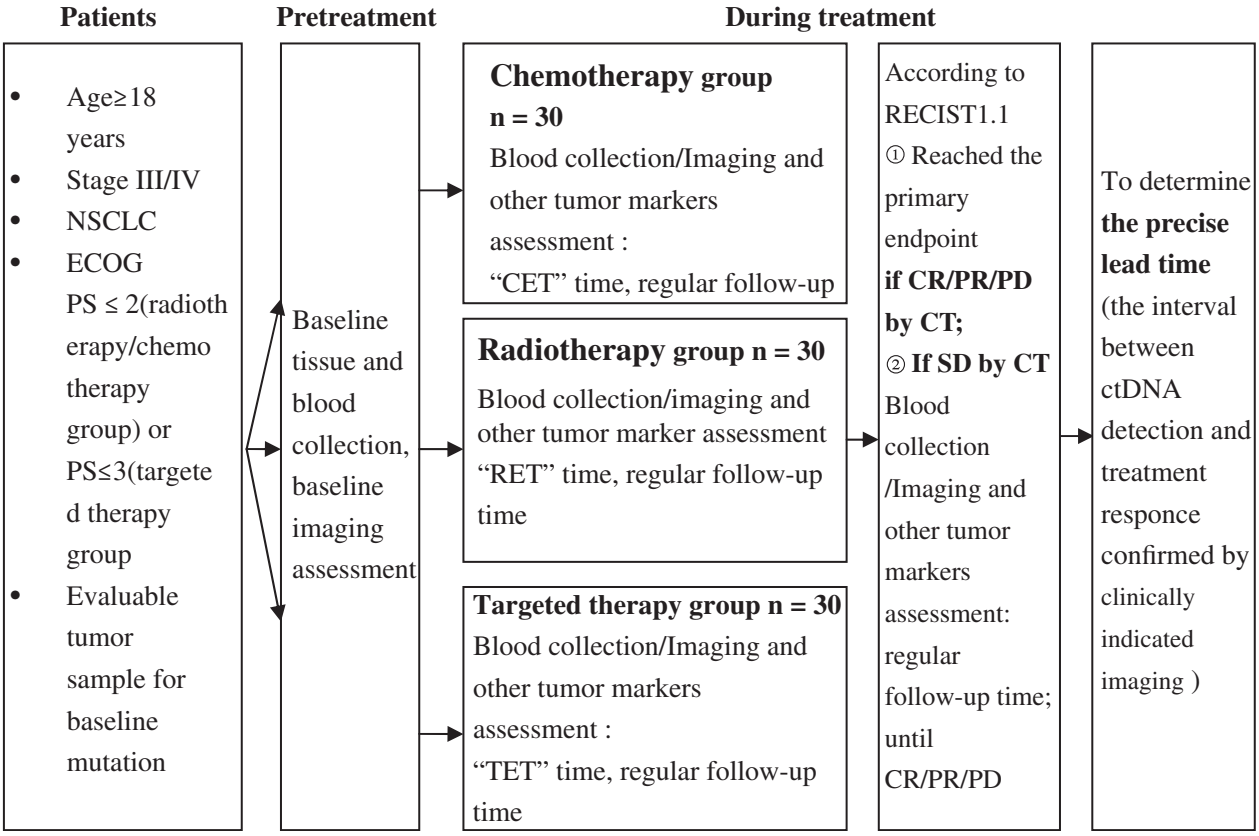

Figure 2 The second phase. CT, computed tomography; ctDNA, circulating tumor DNA; CR, complete response; ECOG PS, Eastern Cooperative Oncology Group performance status; NSCLC, non-small cell lung cancer; PD, progressive disease; PR, partial response; RECIST, Response Evaluation Criteria in Solid Tumors; SD, stable disease.

Gaithersburg, MD, USA), and 100 ng DNA will be sonicated for 30 seconds three times to obtain small DNA fragments 180–220 bp in size, using a Diagenode TM Bioruptor TM Pico Ultra sonicator (Diagenode, Liege, Belgium). DNA will be stored at −20°C. The DNA Library will be constructed according to the manufacturer's instructions. Genomic DNA (100 ng) will be amplified using a commercially available kit according to the manufacturer's recommendations (Kapa Biosystems, Wilmington, MA, USA). Each sample library will be ligated with a specific barcode index according to the manufacturer's protocol (Kapa) and the DNA libraries will then be pooled and captured using DNA capture probes (target 422 cancer-related genes, Geneseeq). Samples will be purified by AMPure XP beads, quantified by quantitative PCR (Kapa) and sized on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA).

High-throughput sequencing

Libraries will be normalized to 2.5 nM and pooled. Deep sequencing of approximately 416 cancer-related genes will be performed on an Illumina HiSeq 4000 using a PE150 V1 Kit (Illumina Inc., Madison, WI, USA). Cluster generation and sequencing will be performed according to the manufacturer's protocol. A previous study reported that tumor-specific mutations were detected in 84% of ctDNA samples of NSCLC patients, with a customized gene panel of 382 cancer-relevant genes.[4] Furthermore, previous studies have reported that a 416 cancer-related gene panel was broad enough to present lung cancer related genes for ctDNA assessment. The average sampling depth of tissue samples will not be less than 600X, while the average sequencing depth of ctDNA samples will not be less than 3000X. The mutant allele frequency from the baseline will be used to certify the ctDNA response.

Radiographic data and clinical outcomes

Patients will undergo CT scans before their first treatment and then typically at intervals of three to six weeks (chemotherapy and radiotherapy groups) or four weeks (targeted therapy group) thereafter. Scans will be evaluated according to RECIST version 1.1 by a radiologist blinded to ctDNA data. The radiographic tumor burden will be

quantified as the sum of the longest unidimensional diameters of target tumor lesions. Radiographic responses will be recorded as PR if the tumor burden decreased by at least 30%, PD if the tumor burden increased by at least 20% or if new lesions appear, or as SD if neither criterion is met. The treating oncologist will determine treatment duration.

Statistical analysis

A detailed statistical analysis plan will be completed prior to the beginning of the study. The proportion of ctDNA in circulating free DNA will be measured as ctDNA%. Quantitative data will be analyzed using the Student's *t*-test. Agreement between radiographic/biomarker and ctDNA responses will be assessed with Cohen's kappa coefficient. A McNemar test will be used to evaluate the sensitivity and specificity between the ctDNA and radiographic/biomarker responses. Tests will be two-sided, and $P < 0.05$ will be considered statistically significant. All analyses will be conducted using SPSS version 19.0.

Study management

The study will be managed by the Thoracic Surgery Department at The First Hospital of Shijiazhuang, Hebei Province, China.

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Disclosure

No authors report any conflict of interest.

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