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# Early Detection of Recurrence in Patients With Locally Advanced Non–Small-Cell Lung Cancer via Circulating Tumor Cell Analysis

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## Abstract

**We investigated the potential usefulness of sequential circulating tumor cell (CTC) analysis for patients treated for locally advanced non–small-cell lung cancer (LA-NSCLC). We found that a CTC level increase gave median and mean lead time notice of progression of disease of approximately 6 months ahead of radiographic evidence. This telomerase-based CTC assay might thus complement conventional imaging for post-treatment monitoring of patients with LA-NSCLC.**

**Background:** Assays to identify circulating tumor cells (CTCs) might allow for noninvasive and sequential monitoring of lung cancer. We investigated whether serial CTC analysis could complement conventional imaging for detecting recurrences after treatment in patients with locally advanced non–small-cell lung cancer (LA-NSCLC). **Patients and Methods:** Patients with LA-NSCLC (stage II-III) who definitively received concurrent chemoradiation were prospectively enrolled, with CTCs from peripheral blood samples identified using an adenoviral probe that detects elevated telomerase activity present in nearly all lung cancer cells. A "detectable" CTC level was defined as 1.3 green flourescent protein-positive cells per milliliter of collected blood. Samples were obtained before, during (at weeks 2, 4, and 6), and after treatment (post-radiation therapy [RT]; at months 1, 3, 6, 12, 18, and 24). **Results:** Forty-eight patients were enrolled. At a median follow-up of 10.9 months, 22 (46%) patients had disease recurrence at a median time of 7.6 months post-RT (range, 1.3-32.0 months). Of the 20 of 22 patients for whom post-RT samples were obtained, 15 (75%) had an increase in CTC counts post-RT. In 10 of these 15 patients, CTCs were undetectable on initial post-RT draw but were then detected again before radiographic detection of recurrence, with a median lead time of 6.2 months and mean lead time of 6.1 months (range, 0.1-12.0 months) between CTC count increase and radiographic evidence of recurrence. One patient with an early recurrence (4.7 months) had persistently elevated detectable CTC levels during and after treatment. **Conclusion:** These results indicate that longitudinal CTC monitoring in patients with LA-NSCLC treated with chemoradiation is feasible, and that detectable CTC levels in many patients meaningfully precede radiologic evidence of disease recurrence.

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## Introduction

Lung cancer remains the second most commonly diagnosed cancer in men and women, with an estimated 228,150 new cases of lung cancer expected in 2019, and accounts for approximately 13% of all cancer diagnoses. It is responsible for more deaths than any other type of cancer in men and women and is the leading cause of cancer death in men and women older than 40 years of age.[1] Non–small-cell lung cancer (NSCLC) accounts for >85% of these cases and the 5-year survival rate for locally advanced NSCLC (LA-NSCLC) range decreases precipitously from 53% for stage IIB disease, to 36% for stage IIIA disease, to 26% for stage IIIB disease.[2,3]

New developments in radiation therapy (RT), imaging techniques, chemotherapy, surgery, and biological agents offer renewed hope for prolonging survival and improving outcomes. Optimizing monitoring of disease status to guide treatment throughout each patient's clinical course and after treatment completion, including with novel biomarkers, could allow for improved efficacy of the combined modality therapies and improved outcomes for salvage therapies.[4-6]

Currently, the standard of care for patients having completed chemoradiation treatment for LA-NSCLC usually includes serial history and physical examinations, along with follow-up imaging, such as positron emission tomography (PET) or chest computed tomography (CT) scans. However, post-radiation changes and fibrosis often render detection of recurrent disease difficult to identify on imaging. In such situations when conventional imaging is unclear, circulating tumor cell (CTC) assays could be illuminating. There is a relative paucity of information regarding CTC trends in patients with localized, nonmetastatic lung cancer, including monitoring for treatment response or assessing for tumor recurrence after definitive chemoradiation. We therefore performed a prospective clinical trial with a primary end point to assess CTCs as a potential biomarker for patients with LA-NSCLC definitively treated with chemoradiation. A secondary goal was to assess whether CTC trends might precede conventional imaging in detecting recurrences.

## Patients and Methods

### *Patient Eligibility*

All consecutive patients with LA-NSCLC (group stage II-III, determined according to the seventh edition of the American Joint Committee on Cancer staging system) prospectively enrolled in this institutional review board-approved clinical trial were included in the analysis. Enrollment criteria included patients age 18 years or older who were planned to receive concurrent chemoradiation. Patients with a previous active malignancy in the past 5 years were ineligible. All patients involved were discussed in Thoracic Malignancy Multi-disciplinary Clinic and evaluated for surgery, but were deemed unresectable by consensus typically because of tumor location, extent, or excessive predicted perioperative risks, generally because of preexisting cardiopulmonary comorbidities and/or limited performance status.

### *Circulating Tumor Cell Assay*

The CTC assay used in this study has been previously described for patients with NSCLC, glioma, melanoma, bladder, and other types of solid tumors.[7-10] It uses a replication-competent adenovirus that detects elevated telomerase expression manifested by almost all tumor cells (but which is not elevated in most normal cells). The replication of the vector is regulated by the human telomerase reverse transcriptase promoter element, ultimately driving green flourescent protein (GFP) expression that can be imaged and quantified using fluorescence microscopy. The preclinical validation process included testing of the probe in peripheral blood samples from healthy volunteers with and without spiking them with NSCLC cells.[7] A logistic regression-based classifier was applied to these data to define the threshold for "CTC positivity" at 1.3 GFP-expressing cells per mL of collected blood. Key aspects of the validation process and assay involve depletion of erythrocytes, lymphocytes, granulocytes, and mononuclear cells via Oncoquick gradient centrifugation[8] gating via cell size to further exclude the smaller normal cells (such as monocytes),[9] and using a low titer of virus. These steps are essential to minimize false positive results and are far superior to simple red blood cell lysis (see Supplemental Figure 1 in the online version and data not shown).

### *Collection of Blood Samples From Patients*

Peripheral blood samples (1 tube, typically 7-10 mL) were obtained from patients to assess for CTCs using the previously mentioned assay. Patients were assayed before chemoradiation (pre-RT), at 3 time points during concurrent chemoradiation (weeks 2, 4, and 6), and after chemoradiation completion (post-RT; months 1, 3, 6, 12, 18, and 24). All care providers and laboratory personnel were blinded to quantitative and qualitative CTC analysis, and no treatment decisions were made on the basis of the results of these assays.

### *Treatment and Follow-Up*

Patients were treated to 6660 cGy in 37 fractions of external beam RT (180 cGy per fraction). In all cases, the RT was delivered to the primary tumor and to sites of nodal metastatic disease on the basis of size criteria, PET avidity, or pathologic confirmation of nodal metastasis. In all cases, patients received concurrent chemotherapy that was a platinum-based doublet, either cisplatin and etoposide or carboplatin and paclitaxel. Patients then underwent serial radiographic surveillance with either PET/CT or CT scans obtained just before each of their follow-up appointments (every 3 months in years 1 and 2, and every 4-6 months thereafter), or with additional imaging at the discretion of the patient's physicians, all of whom were blinded to the results of the CTC analyses. The time to local failure was defined as at least a 20% increase in the diameter of the treated lesion or histologic confirmation of disease and was marked from the date of histologic confirmation or empiric treatment of disease, whereas local control was defined as absence of local failure. Nodal and distant control were defined as absence of disease in the lymph nodes or absence of extrathoracic metastases, respectively. Date of death was as recorded on the death certificate or as noted in the electronic medical records of each patient.

### *Statistical Analysis*

Overall survival was defined as the duration from the start of radiation treatment to the date of death or last follow-up.

**Table 1** Characteristics of the 48 Patients Who Were Sequentially Enrolled

| Characteristic | Value |
|---|---|
| **Sex, n (%)** | |
| Male | 26 (54) |
| Female | 22 (46) |
| **Ethnicity, n (%)** | |
| White | 33 (69) |
| African American | 10 (21) |
| Other | 5 (10) |
| **Age, Years** | |
| Median | 66 |
| Mean | 66 |
| Range | 31-84 |
| **Smoking History, n (%)** | |
| Former | 37 (77) |
| Current | 10 (21) |
| Never | 1 (2) |
| **Tumor Size, cm** | |
| Median | 3.7 |
| Mean | 3.98 |
| Range | 1.5-10 |
| **Tumor $SUV_{max}$** | |
| Median | 13 |
| Mean | 13.9 |
| Range | 1.6-30 |
| **Histology, n (%)** | |
| Adenocarcinoma | 22 (46) |
| Squamous | 23 (48) |
| Other | 3 (6) |
| **Stage, n (%)** | |
| IIA | 1 (2) |
| IIB | 5 (11) |
| IIIA | 26 (54) |
| IIIB | 16 (33) |
| **T-Stage, n (%)** | |
| T1 | 13 (27) |
| T2 | 14 (29) |
| T3 | 14 (29) |
| T4 | 7 (15) |
| **N-Stage, n (%)** | |
| N0 | 6 (13) |
| N1 | 2 (4) |
| N2 | 29 (60) |
| N3 | 11 (23) |

Abbreviation: $SUV_{max}$ = maximum standardized uptake value.

Progression-free survival was measured from the start of radiation treatment to the date of any disease event or last follow-up. Survival curves were calculated via Kaplan–Meier methods. Statistical analysis was performed using SAS/STAT software, version 9.4 of the SAS System (SAS Institute Inc, Cary, NC).

## Results

### *Patient Characteristics*

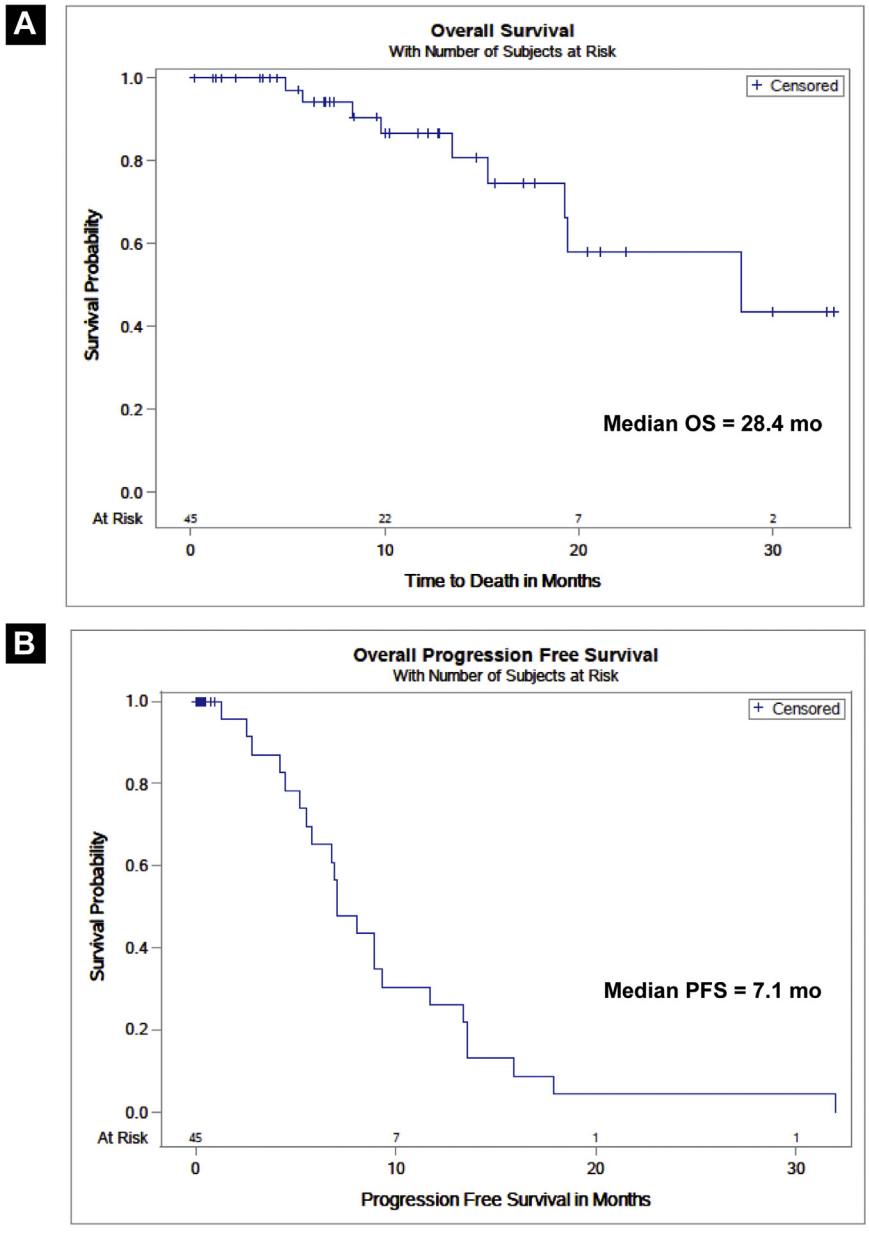
Forty-eight patients were sequentially enrolled. Patient composition included the following: male (54%), Caucasian (69%) or African American (21%), and former (77%) or current (21%) smokers. The median age of the patients was 66 years (range, 31-84 years). Adenocarcinoma (46%) or squamous cell carcinoma (48%) comprised most of the histologies of the tumors. The primary tumor median size was 3.7 cm (range, 1.5-10.0 cm). All patients were established to have histological confirmation of NSCLC and clinically have LA-NSCLC, defined as clinical group stage II or III. Patients predominantly had stage IIIA (54%) or IIIB (33%) disease, and they had cN2 (60%) or cN3 (23%) nodal disease (Table 1). The median overall survival for this patient population was 28.4 months (Figure 1A), a duration comparable with the best arm survival rate seen in the Radiation Therapy Oncology Group 0617 clinical trial.[11] Median progression-free survival was 7.1 months, within the range of 6 to 12 months often described for LA-NSCLC patients (Figure 1B).[11-14] The number of patients at each stage and the corresponding blood draws are shown in Supplemental Figure 2 in the online version.

### *Circulating Tumor Cell Analysis Provides Lead-Time Notice of Clinical Recurrence of Disease*

Thirty-five of the 48 patients (73%) had detectable levels of CTCs in their pretreatment samples, and all of these patients noted decreases in CTC counts after completion of treatment. At a median follow-up of 10.9 months, 22 of 48 patients (46%) had disease recurrence at a median time of 7.6 months post-RT (range, 1.3-32.0 months). There was a near even distribution between adenocarcinoma and squamous histology in these patients and no obvious difference in outcomes between the 2 histologies was observed. Post-RT blood samples for CTC analysis were obtained in 20 of 22 recurrent patients (2 patients were not able to return for CTC analysis). Of these 20 patients, 15 (75%) had a detectable increase in CTC counts post-RT. In 10 of these 15 patients (67%), CTC counts were undetectable on initial post-RT draw and increased to detectable levels before radiographic detection of recurrence, with a median lead- time of 6.2 months and mean lead time of 6.1 months (range, 0.1-12.0 months) between the CTC increase and radiographic evidence of recurrence. In these 10 patients, an average of 182 days (approximately 6 months) elapsed between when CTC increases were detected and when disease recurrence was clinically noted. Specific details about the 10 patients in whom CTC level increases preceded disease recurrence are included in Table 2.

Of the 5 patients in whom the number of CTCs increased into the detectable range after radiographic detection of recurrence, 1 patient with an early recurrence (4.7 months) had persistently detectable CTC levels during and after treatment. The CTC counts in the 4 remaining patients decreased to a nadir, but then had radiographic evidence of disease progression or recurrence before the increase in CTCs. It should be noted, however, that several of these patients missed clinic appointments or had follow-up care conducted at outside facilities and so, were unable to undergo blood draw and CTC analysis at protocol-specified intervals. Although

**Figure 1** Overall Survival (OS) and Progression-Free Survival (PFS). The Median OS for This Patient Population Was 28.4 Months (A), and the Median PFS Was 7.1 Months (B)




these missed serial CTC assays were not classified as protocol violations, it is plausible that at least some of these would have also resulted in lead time notice preceding radiographic evidence of disease progression or recurrence.

We also assessed whether CTC status before the start of treatment might have bearing on recurrence. As mentioned previously, 13 patients did not have detectable CTCs before treatment; of these 5 (or 38%) were found to have radiographic evidence of recurrence. In contrast, 17 of the 35 (or 49%) patients with CTCs detectable before treatment have had disease recurrence. The difference did not appear to be statistically significant. Furthermore, because CTCs tend to be rare, additional pretreatment blood draws would likely increase the probability of detection. Other potential reasons for the lack of CTC detectability are addressed in the Discussion.

### *Illustrative Clinical Vignette*

A 70-year-old man presented with stage IIIB squamous cell lung cancer (T3N2M0). On pretreatment PET/CT, the patient was noted to have a 1.5-cm mass with a standardized uptake value of 13.9 in the left lung, with fluorodeoxyglucose avidity in the prevascular and left hilar nodes, and detectable CTCs before treatment. At the end of chemoradiation, CTCs were no longer detectable, and

**Table 2** Details of 10 Patients in Whom CTC Increases Preceded Radiographic Evidence of Disease Recurrence

| Date RT End | Post-RT CTC Nadir (cells/mL) | Date CTC Increase Noted | CTC Count (cells/mL) at Time of Increase | Date of Recurrence | Days Elapsed: CTC Increase to Recurrence | Vital Status | Histology |
|---|---|---|---|---|---|---|---|
| June 17, 2013 | 0.3 | January 19, 2014 | 2.5 | January 19, 2014 | 3 | Deceased | Squamous |
| December 3, 2013 | 0 | January 13, 2015 | 1.5 | January 20, 2015 | 7 | Deceased | Squamous |
| March 30, 2015 | 0.7 | June 25, 2015 | 1.7 | November 1, 2015 | 129 | Alive | Adeno |
| September 16, 2015 | 0.1 | December 2, 2015 | 1.5 | May 18, 2016 | 168 | Alive | Adeno |
| August 27, 2015 | 0.1 | December 17, 2015 | 2 | June 6, 2016 | 172 | Alive | Adeno |
| June 22, 2015 | 0.7 | January 12, 2016 | 2.3 | August 1, 2016 | 202 | Alive | Adeno |
| December 26, 2013 | 0.4 | January 4, 2016 | 36.5 | August 24, 2016 | 233 | Alive | Squamous |
| December 29, 2014 | 0 | June 24, 2015 | 1.4 | February 15, 2016 | 236 | Deceased | Squamous |
| January 2, 2015 | 0 | June 24, 2015 | 5.9 | April 29, 2016 | 310 | Alive | Adeno |
| December 26, 2014 | 0 | June 25, 2015 | 1.7 | June 23, 2016 | 364 | Deceased | Squamous |

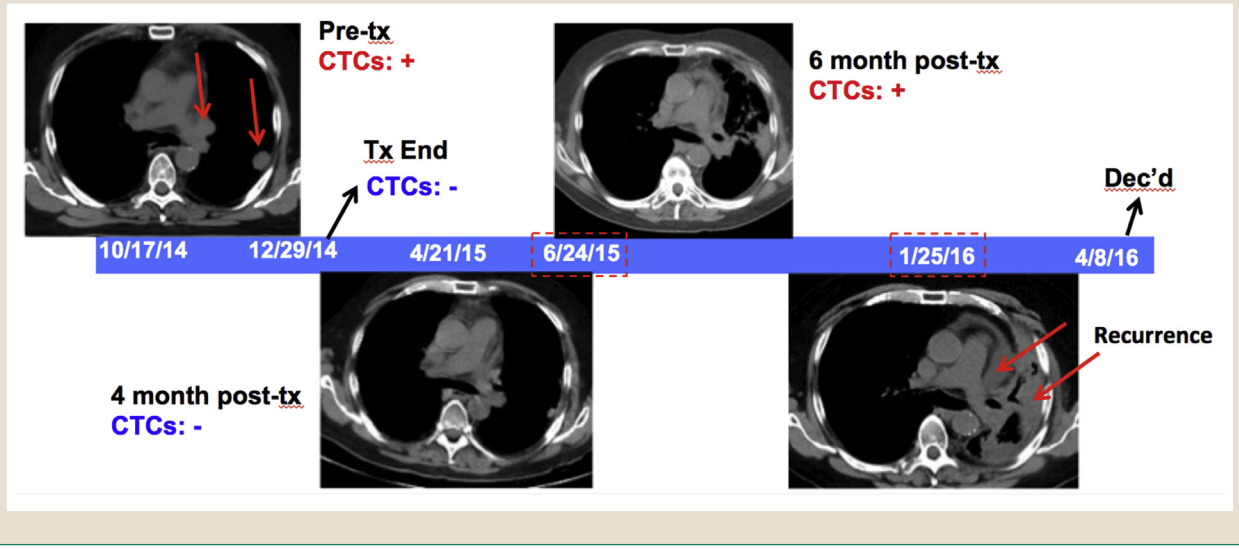
Abbreviations: Adeno = adenocarcinoma; CTC = circulating tumor cell; RT = radiation therapy.

they continued to be undetectable during the first 2 post-treatment CTC evaluations, with a post-treatment CTC nadir of 0. At the patient's 6-month post-treatment follow-up visit, however, CTCs were again detectable (1.4 CTCs per milliliter). However, the official interpretation of the CT scan obtained for this visit was: "Evolution of radiation changes and increase in prominence of mediastinal lymphoid tissue may be reactive." Seven months later, the patient was unambiguously deemed to have recurrent disease with CT scans now showing widespread progression in the left lung and lymphadenopathy. One month later, the patient developed brain metastases and passed away 2 months after that. In this patient's case, 236 days passed between the time when a positive CTC count was first noted and when the patient was clinically deemed on the basis of imaging to have disease recurrence. A graphical representation of this case, with imaging scans, is shown in Figure 2.

## Discussion

This pilot trial is, to our knowledge, the largest prospective study to assess CTCs in patients with LA-NSCLC undergoing chemoradiation and the first compelling report to indicate that CTC monitoring in such patients is feasible and clinically useful. CTC level elevations into the detectable range in many patients meaningfully precede radiologic evidence of disease recurrence. The 6-month lead-time notice of recurrence afforded by CTC analyses in this study (and in a population of patients with median progression-free survival of only approximately 7 months)

**Figure 2** Temporal Graphic Illustrating the Disease Course of a Representative Patient (Clinical Vignette), With Serial Circulating Tumor Cell (CTC) Assays and Computed Tomography Imaging Scans Showing the Status of the Tumor. Periods When the CTC Counts Were Elevated ("CTCs: +" in Red) or Not ("CTCs: −" in Blue) Are as Indicated, Along With When Disease Was Detected Radiographically (Red Arrows). CTCs Became Detectable Again on June 14, 2015, Preceding CT Scans Showing Disease Recurrence on January 25, 2016




Abbreviations: Dec'd = deceased; tx = treatment.

potentially might allow the initiation of re-treatment or early salvage strategies at a time when tumor burden remains lower.[15]

The earliest possible detection of recurrences aided by CTC assays would allow the greatest number of patients to be eligible for effective salvage treatment. For example, an increasing body of prospective data indicate that reirradiation for local and regional NSCLC recurrences allows for long-term survival, aided by recent advances in RT techniques and technology.[16] Such treatment is, however, contingent on smaller treatment volumes to minimize the risk of complications. Prompt institution of effective salvage therapy might, therefore, lead to better outcomes and better preservation of quality of life.[14] The development and introduction of immunotherapy and other novel effective treatments such as immune checkpoint inhibitors offer further opportunities for extending survival with early detection of recurrences. At the time of analysis, no subject in this trial had received immunotherapy. The demonstrated efficacy of durvalumab, a programmed death ligand 1 (PD-L1) inhibitor, in the recently reported A Global Study to Assess the Effects of MEDI4736 Following Concurrent Chemoradiation in Patients with Stage III Unresectable Non-Small Cell Lung Cancer (PACIFIC) trial suggests the kind of additional salvage treatment options that might be rapidly implemented with early detection of recurrence afforded by CTC analyses.[17]

A number of factors might have accounted for the lack of detectable CTCs before treatment in some of the patients. The concentration of CTCs is likely to be very low with estimates of 1 CTC per every 1 billion nucleated cells and thus a degree of sampling error might be possible, especially when the volume of blood collected is low.[18] In support of this possibility, wide variations in the rate of CTC release have been observed with real-time continuous bloodstream monitoring in preclinical studies.[19] Because of discontinuous shedding of CTCs from the primary tumor, it might be ideal in future studies to include additional pretreatment draws for each patient to achieve maximal sensitivity.[20] We speculate that there might also be alterations in physiology or biology (eg, poorly perfused tumors, such that tumor cells have difficulty migrating into the systemic circulation), and such factors might also account for why increases in CTC counts were not observed in a subset (5 of 20 [25%]) of patients with clear evidence of disease recurrence.

It should also be noted that there were often logistical limitations in the time points used to collect follow-up CTC samples. The protocol was designed for serial CTC assays during a year after completion of treatment (at 1, 3, 6, and 12 months). However, after completion of treatment at our quaternary care center, many patients elect to have most or all of the follow-up care performed closer to home because of expense, convenience, and other logistical factors. Consequently, for 3 of the 4 patients for whom an increase in CTC counts was detected after conventional imaging, CTC collection time points were missed because of logistical reasons such as follow-up visits or diagnostic imaging scheduled outside the home institution during which sample collection for CTC analysis was not possible. In future studies, fewer time points could be missed if CTC collection could be performed in community settings. This would require methods of shipping and handling that allow for the stable survival of cells, such as through temperature-controlled shipping containers (different versions of which are commercially available now). We are actively exploring such options.

Although these CTC assay results are promising, other circulating tumor materials are also being investigated for diagnostic or prognostic monitoring of cancer, such as cell-free DNA/circulating tumor DNA and exosomes.[21-24] Future studies might indicate strategies through which CTC assays might complement assays of these other circulating tumor material. Furthermore, the standard of care for these patients now would be to receive 1 year of consolidation therapy with the PD-L1 checkpoint inhibitor, durvalumab, which could affect the time to first increase in CTCs or the lead time in detecting recurrences, and further investigation in immunotherapy-treated patients is indicated. Ultimately, further analytical validation and testing are warranted for any of these assays to guide treatment and management decisions to reduce lung cancer-associated morbidity or mortality. On the basis of these compelling findings, an NRG Oncology National Cancer Institute-funded cooperative group study, "NRG-LU004: Phase I Trial of Accelerated or Conventionally Fractionated Radiotherapy Combined With MEDI4736 (durvalumab) in PD-L1 High Locally Advanced Non–small-cell Lung Cancer (NSCLC) (ARCHON-1)" has just been activated that will incorporate CTC analyses in all patients, and which aims to provide multicenter validation of our findings and test the assay in immunotherapy-treated patients.

## Conclusion

Serial circulating tumor cell analyses in patients with LA-NSCLC might provide early notice of tumor recurrence after chemoradiation therapy. An increase in CTC counts from a treatment-induced nadir provided on average approximately a 6-month lead time notice compared with conventional imaging. CTC assays might therefore complement conventional imaging in the follow-up of treated LA-NSCLC patients.

### *Clinical Practice Points*

- Analyses of circulating tumor material, including CTCs, have attracted much recent interest. These assays are on the basis of peripheral blood samples and are thus noninvasive and can be performed sequentially throughout a patient's treatment course. The clinical usefulness of CTC analysis for LA-NSCLC, however, is not yet defined.
- We investigated a CTC assay that detects the elevated telomerase activity in almost all cancer cells and which helps confer lack of senescence and immortality. In contrast, telomerase activity is not elevated in almost all normal cells so normal cells undergo senescence.
- We used CTC assay for consecutive patients treated with chemoradiation for stage II/III LA-NSCLC (standard fractionation and fields with concurrent platinum-based chemotherapy) before, during, and at months 1, 3, 6, 12, 18, and 24 after RT completion. Patients underwent serial radiographic imaging with either PET/CT or CT scans before treatment and at each of their follow-up appointments.
- Of 48 patients, 22 (or 46%) had disease recurrence at a median time of 7.6 months post-RT. Fifteen of 20 (75%) patients for whom post-RT samples were obtained had a increase in CTC counts. In 10 of these 15 patients, CTCs were undetectable on initial post-RT draw but were then detected again before

radiographic detection of recurrence, with a median lead time of 6.2 months and mean lead time of 6.1 months between CTC increase and radiographic evidence of disease progression.

- Use of the CTC assay might enable implementation of salvage treatment at the earliest time of tumor recurrence or progression.

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## Disclosure

The University of Pennsylvania has filed patent applications on the basis of associated research and is ultimately the license owner. Drs Hahn, Dorsey, and Kao are cofounders of Liquid Biotech USA through the University of Pennsylvania UPSTART Program. The remaining authors have stated that they have no conflicts of interest.

## Supplemental Data

Supplemental figures accompanying this article can be found in the online version at https://doi.org/10.1016/j.cllc.2019.04.011.

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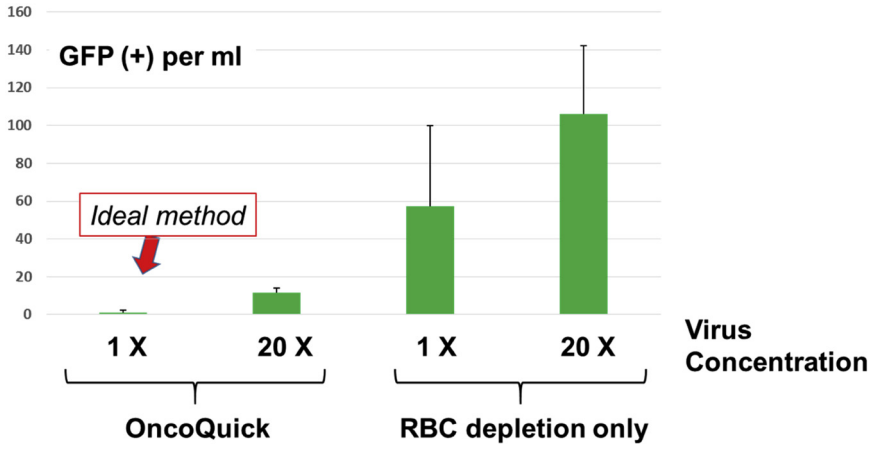
## Supplemental Data

**Supplemental Figure 1** Testing of TelomeScan Virus With Blood From Healthy Volunteers to Establish Ideal Assay Conditions Associated With Minimal "False Positive" Background

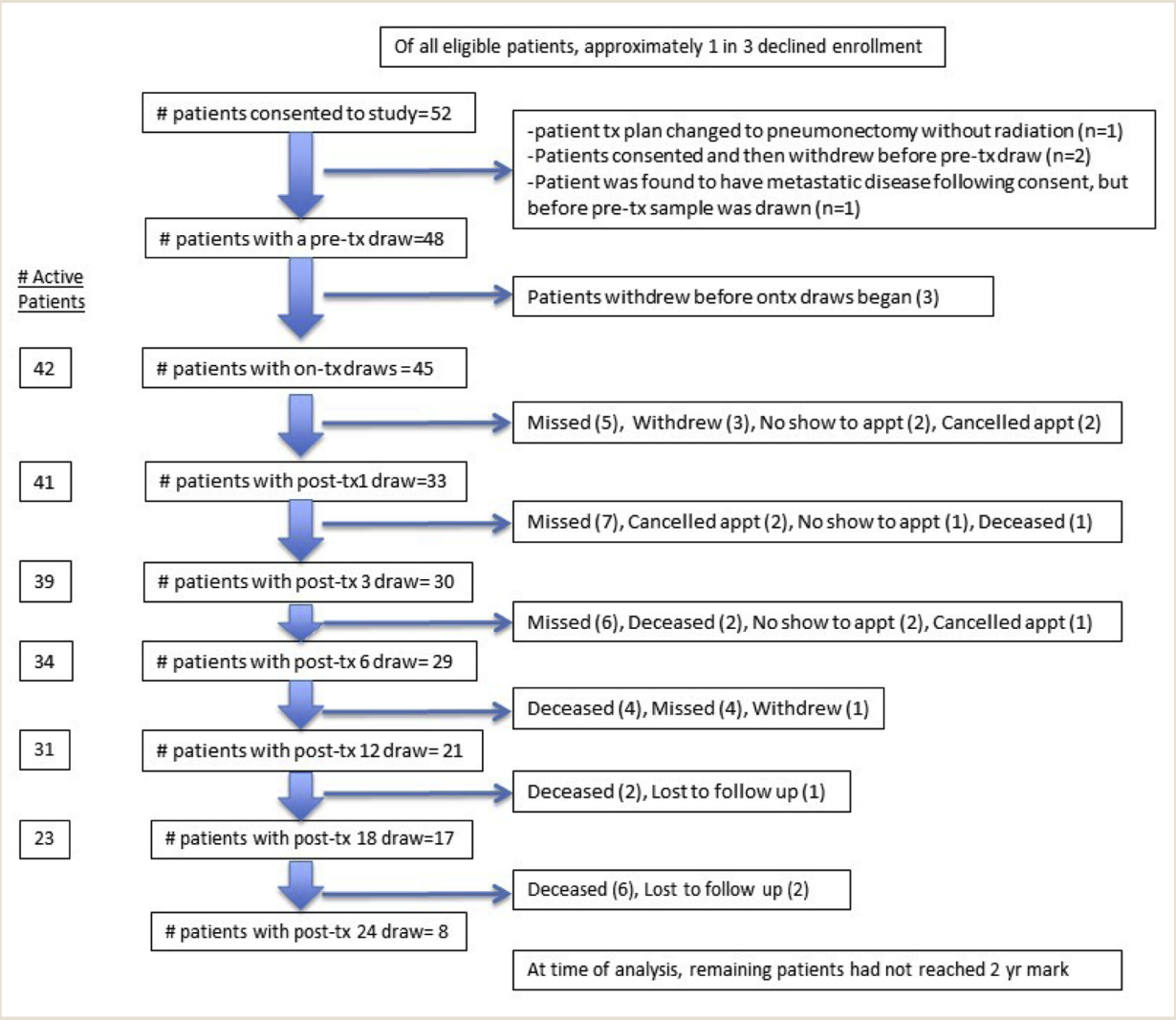



| Virus Conc | GFP (+) per ml | Method |
|---|---|---|
| 1 X | 1.0 | OncoQuick |
| 20 X | 11.6 | OncoQuick |
| 1 X | 57.3 | RBC removal |
| 20 X | 106.0 | RBC removal |

The use of OncoQuick tubes for processing and the appropriate concentration of virus (1X in the experiment) lead to an appropriately minimal GFP-positive cells below threshold.

Abbreviations: GFP = green fluorescent protein ; RBC = red blood cells.

**Supplemental Figure 2** Consort Diagram: Systematic Delineation of the Patients Sequentially Enrolled in the Study and the Number Active as the Study Progressed




Abbreviation: tx = treatment.