HHS Public Access

Author manuscript

Int J Radiat Oncol Biol Phys. Author manuscript; available in PMC 2019 November 01.

Published in final edited form as:

Int J Radiat Oncol Biol Phys. 2018 November 01; 102(3): 536–542. doi:10.1016/j.ijrobp.2018.06.041.

Circulating Tumor Cell Assessment in Presumed Early Stage Non-small Cell Lung Cancer Patients Treated with Stereotactic Body Radiation Therapy: A Prospective Pilot Study

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Conflict of interest: University of Pennsylvania has submitted a patent application based on a component of the technology presented in this article. SMH, GDK, and JFD are co-founders of Liquid Biotech, USA.

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Abstract

PURPOSE: In patients treated with SBRT for presumed early stage non-small cell lung cancer (NSCLC), detection and monitoring of circulating tumor cells (CTCs) may be useful for assessing treatment response safely and non-invasively. No published reports of CTC trends in this patient population exist to date.

METHODS AND MATERIALS: Patients with clinically diagnosed stage I NSCLC treated with SBRT were eligible for this IRB-approved, prospective clinical trial. Peripheral blood samples were assayed for CTCs via a green fluorescent protein (GFP)-expressing adenoviral probe. “CTC positivity” was defined as 1.3 GFP-positive cells/mL of collected blood. Samples were obtained before (pre-radiation therapy [RT]), during, and after SBRT (post-RT; months 1, 3, 6, 12, 18, and 24). SBRT was delivered in <5 fractions (median 50/12.5 Gy) to a BED of >100 Gy in all cases.

RESULTS: 48 consecutive patients (T1a (73%), T1b (21%), and T2a (6%)) were enrolled; median follow-up was 14.2 months. Twenty (42%) patients had a positive CTC level pre-RT, with a median CTC count of 4.2 CTCs per ml (IQR, 2.2–18.7). Of these, 17 patients had evaluable post-RT CTC evaluations showing reduced CTC counts at 1 month (median 0.2, IQR 0.1–0.8) and 3 months (median 0.6, IQR 0–1.1). Three of these 17 patients experienced disease progression at a median of 19.9 months; all three experienced >1 positive post-RT CTC tests predating clinical progression by a median of 16 months (range, 2–17 months). In contrast, of patients presenting with CTC-detectable disease and for whom all post-RT CTC tests were negative, none experienced recurrence or progression.

CONCLUSIONS: CTC monitoring following SBRT for presumed early stage NSCLC may give lead-time notice of disease recurrence or progression. Conversely, negative CTC counts following treatment may provide reassurance of disease control. CTC analyses is thus potentially useful in enhancing clinical diagnosis and follow-up in this population.

Introduction

Each year, approximately 234,000 people in the United States will be diagnosed with non-small cell lung cancer (NSCLC), approximately one-sixth (or nearly 40,000) of whom present with localized disease.[1] This number is expected to rise as the increasing implementation of low-dose CT screening captures a greater proportion of early-stage tumors that previously would have gone undetected or presented with advanced disease.[2] Many early stage lesions can only have a presumed diagnosis of malignancy due to small tumor size, inaccessibility by transthoracic needle aspiration or bronchoscopy, non-diagnostic sampling, or competing co-morbidities (such as advanced emphysema), therein rendering the risk of tumor biopsy to be unfavorable.[3] Despite early detection, a substantial proportion of these patients will still recur or progress despite treatment, as five-year survival rates for clinical stage IA and IB NSCLC are 82% and 66%, respectively.[4]

Early stage NSCLC patients are increasingly being treated with stereotactic body radiation therapy (SBRT), also termed stereotactic ablative radiotherapy (SABR), due to the convenience, safety, and favorable local control efficacy that this modality offers.[5] However,

post-radiation effects and fibrosis can appear similar to recurrent tumor on imaging studies, and imaging findings often present more challenges than clarity in directing clinical decisions.[6–12] A reliable biomarker of disease status may be useful in patients with early stage NSCLC for complementing the presumed diagnosis of cancer, in addition to non-invasive monitoring of treatment response or disease recurrence.

CTCs are rare tumor cells in the peripheral blood stream and have garnered interest as a biologically relevant biomarker in NSCLC.[13–18] Methods of CTC detection have relied on surface markers, cell morphology analyses, or reverse transcriptase polymerase chain reaction (RT-PCR) detection of putative tumor expression patterns.[17–19] More recently, an assay that relies on the detection of high telomerase activity in tumor cells has been shown to be effective for detecting live CTCs derived from NSCLC.[13]

We herein report the application of this method for sequential CTC monitoring in patients with clinical stage I NSCLC (presumed to have cancer due to the inability to obtain tissue confirmation) treated with SBRT.

Methods

Circulating Tumor Cell Assay

The CTC assay has been previously described for patients with breast cancer, NSCLC, glioma, melanoma, and bladder cancer.[20–25] In brief, the *ex vivo* assay makes use of a replication-competent adenoviral vector whose replication is regulated by the human telomerase reverse transcriptase (hTERT) promoter element and contains a downstream CMV promoter that drives GFP expression. Cells with increased telomerase activity will rapidly replicate the vector and drive amplified GFP expression detected by fluorescence microscopy. This assay leverages the fact that telomerase is up-regulated in almost all tumor cells, but not in the majority of normal cells.[26] A pre-clinical validation process defined the threshold for CTC “positivity” at 1.3 GFP-expressing cells per mL of collected blood.[13]

Analysis of Patient Samples

Patients with clinically-diagnosed Stage I (T1a-2aN0M0)[27] NSCLC undergoing SBRT were considered for enrollment on this trial. The clinical diagnosis of NSCLC was determined in all cases via multidisciplinary consensus based on interval lesion growth over time, lesion size, lesion avidity, lesion radiographic appearance, and patient specific factors such as smoking status. Patients with a pathologically-confirmed diagnosis of NSCLC were excluded, although those who underwent biopsy with indeterminate pathology remained eligible. Patients with a prior active malignancy (except for non-melanoma skin cancer) in the last five years were ineligible. If patients had a previous diagnosis of NSCLC, their case was reviewed by the principal investigator to ensure only patients thought to be free of their initial disease and presenting with a new primary NSCLC were included. After identification, patients were prospectively approached to participate in an Institutional Review Board-approved biomarker trial (Clinicaltrials.gov number, NCT02135679) and provided written informed consent.

Pre-treatment specimens (pre-RT) were obtained at initial consult and/or simulation for radiation treatment planning. If a patient had two pre-RT samples, the greater of the two values was used in pre-RT analysis. One on-treatment (on-RT) sample was obtained following the delivery of at least one fraction but prior to completion of the full SBRT treatment course. Post-treatment (post-RT) samples were drawn at 1-, 3-, 6-, 12-, 18-, and 24-months follow-up appointments. All care providers and laboratory personnel were blinded to CTC quantitative and qualitative analyses.

Treatment and Follow-up

SBRT was delivered in 5 or fewer fractions (median course: 50 Gy delivered in four fractions of 12.5 Gy) to a BED of >100 Gy in all cases. To assess for disease control, patients underwent radiographic surveillance with either CT or PET/CT every 3–6 months. Local failure was defined as growth following initial tumor shrinkage or progression on 2 consecutive scans or histologic confirmation and was dated as the earliest scan to show progression. All such cases were confirmed histologically when possible. The date of death was determined either by death certificate or medical records from inpatient or outpatient encounters.

Statistical Analysis

Descriptive statistics were used to describe patient demographics, disease characteristics, and CTC trends. Associations between clinicopathologic characteristics and pre-RT CTC positivity (treated as a categorical variable) were explored using a t-test or Fisher's exact test, as appropriate.

Recurrence-free survival (RFS) was measured from the start of radiation therapy to the date of last follow-up, death, or disease recurrence (local, nodal, or distant). Progression-free survival (PFS) was measured from the start of radiation therapy to the date of last follow-up, death, or any disease event, including metachronous primary NSCLC. Overall survival was defined from the start of radiation treatment to the date of last follow-up or death. Survival curves were estimated by the Kaplan-Meier methods. Statistical analysis was performed using STATA 14 (StataCorp, College Station, TX). Statistical significance was defined as $p<0.05$, and all tests were two-sided.

Results

Patient Demographics and Disease Characteristics

A total of 48 consecutive patients with early stage NSCLC were enrolled onto the study from August 2013 to January 2017 and provided a pre-treatment CTC sample. Demographic and disease characteristics are detailed in Table 1.

CTC Detection Prior to Radiation Therapy (RT)

Of the total population, 20 of 48 patients (42%) had a positive pre-RT CTC test (defined as GFP cells >1.3 threshold of CTCs/mL), herein referred to as "CTC-detectable disease." There were no identifiable factors that significantly correlated with detectable CTCs prior to RT, including age, race, BMI, smoking status, tumor size, tumor SUV, and T stage except for

prior history of NSCLC (p=0.01) (Table 1). Notably, 40% (8 of 20) of CTC-detectable nodules were categorized as FDG-indeterminate (SUV max = 2.5 or less) prior to SBRT.

Correlations of CTC Positivity with Disease Status Following RT

Median follow-up for the cohort was 14.2 months. At last follow-up, 3 of 20 (15%) in the CTC-detectable group and 1 of 27 (4%) in the CTC-undetectable group had developed recurrence. Follow-up data were not available for the remaining CTC-undetectable patient due to the patient's withdrawal from the protocol prior to treatment. The median time to recurrence for the CTC-detectable cohort was 20.0 months (range, 17.4–28.3 months) and the single recurrence in the CTC-undetectable group occurred at 20.8 months (log-rank test, p=0.61). Taking into account both distant and local recurrences, 6 of 20 (30%) in the CTC-detectable group and 3 of 27 (11%) in the CTC-undetectable group had progression of disease. The median times to progression were 22.3 months (range, 14.4–30.9 months) and 9.7 months (range 9.2–20.8 months), respectively (log-rank test, p=0.68). In regards to overall survival, 19 of 20 (95%) in the CTC-detectable group and 25 of 27 (93%) in the CTC-undetectable group were alive at the time of analysis. The median overall survival was 24.2 months (range, 0.1–38.6 months) and 13.1 months (range, 1.7–33.3 months), respectively (log-rank test, p=0.29).

CTC Trends over Time

Sequential (both pre- and post-RT) CTC counts were available for 17 CTC-detectable patients. The median pre-RT CTC count (n=20) was 4.2 CTCs per mL. The median on-RT CTC count decreased to 0.6 CTCs per mL (n=17) and remained low with median post-RT counts at 1-month (n=11) and 3-months (n=15) follow-ups of 0.2 and 0.6 CTCs per mL, respectively. Figure 1 illustrates CTC trends in this population.

Of the patients in whom CTCs were detectable, 82% (9 of 11) and 80% (12 of 15) experienced a conversion to negative CTC levels at 1- and 3-months following SBRT, respectively. Notably, of the two patients who did not convert to negative CTC levels, one patient experienced oligometastatic disease to the kidney at 17 months and local progression at 30 months (Case 2, more details below); the second patient transferred care to an outside facility at 3 months and no follow-up was available until notification was received of that patient being diagnosed with stage IB melanoma at 21 months after completion of SBRT for the initial lung lesion.

Post-RT CTC Patterns in Relation to Treatment Failure

We assessed post-RT CTC trends relating to clinical outcomes. At a median follow-up of 25 months, three patients with CTC-detectable NSCLC experienced disease recurrence, all three having had at least one positive post-RT CTC test. These three patients included one that developed an isolated nodal failure, one that experienced concomitant locoregional and distant failure, and one that experienced distant metastasis to the kidney before later recurring locoregionally. In all cases, a positive CTC test in follow-up proceeded radiographic or pathologic detection of recurrence. Median lead-time notice of CTC elevation prior to recurrence on conventional diagnostic imaging was 16 months (range, 2–

17 months). Conversely, of those patients presenting with CTC-detectable disease and for whom all post-RT CTC tests were negative, none had experienced recurrence or progression.

For the CTC-undetectable cohort, one patient relapsed at both local and intrathoracic sites with CTCs remaining undetectable in follow-up.

Clinical Vignettes

Three patient vignettes are described below to illustrate the clinical application of sequential CTC analyses. (Figure 2A and B)

Case 1 is a 62-year-old with clinical stage IA (T1aN0M0) NSCLC and history of sarcoidosis. On pre-RT scan, the patient had a 1.4 cm FDG-avid (SUV max 4.3) primary nodule and had a pre-RT CTC count of 4.0 CTCs/mL. After SBRT, the PET/CT scan demonstrated interval decrease in size and metabolic activity of the nodule consistent with treatment response. A CT performed 13 months following SBRT re-demonstrated treatment response and radiation fibrosis. Post-RT CTC counts were negative at 3, 6, 12, and 15 months, although they increased to 5.7 at 26-months. A CT performed around that time demonstrated interval growth of the lung mass, with additional new pulmonary nodules concerning for intrathoracic metastases. The patient underwent a lung biopsy at 28 months post-RT that revealed recurrence of poorly differentiated carcinoma. Most recently, the patient has progressed on third line therapy and is alive at 37 months follow-up.

Case 2 is a 74 year old with clinical stage IB (T2aN0M0) NSCLC. On a pre-RT PET/CT scan, the patient was found to have a 3.0 cm FDG-avid primary nodule (SUV max 6.6) with two pre-RT CTC counts of 22.9 and 3.0 CTC/m. A CTC count at one month post-RT was positive at 1.5 CTC/mL, and the patient also had positive CTC counts at 6, 10, and 24 months following SBRT. A PET/CT scan at 3 months post-RT demonstrated an interval decrease in size and metabolic activity of the lung mass. The patient was found to have oligometastatic disease to the kidney at 17 months post-RT. He underwent SBRT to this lesion and started treatment with pembrolizomab. A PET/CT scan at 30 months demonstrated progression of tumor at the primary site with extension into the chest wall, as well as a nodal recurrence and progression of the kidney lesion. Palliative radiation was delivered for symptomatic chest wall invasion. The patient is currently alive with disease at 37 months follow-up.

Case 3 is an 89 year old with clinical stage IA (T1bN0M0) NSCLC. On pre-RT PET/CT scan, the patient was found to have a 2.2 cm FDG-avid (SUV max 2.6) primary lung nodule with an accompanying CTC count of 1.5 CTCs/mL. A PET/CT scan performed 3 months following SBRT re-demonstrated a 2.2 cm FDG-avid (SUV max 3.0) nodule with evolving radiation changes. Post-RT CTC counts decreased to 0.1 CTC/mL at 1 month following SBRT and remained negative at 3, 7, and 13 months; subsequent CTC samples were not obtained. The patient's CT scan at 15-months post-RT demonstrated continued treatment response and radiation fibrosis. The patient is alive at 36 months follow-up with no evidence of disease.

Discussion

To our knowledge, this is the first report of sequential CTC analysis in clinical stage I NSCLC patients treated with SBRT. Relying on a telomerase-based live CTC assay that is unaffected by changes in surface antigen expression, we found in this prospective trial that CTC counts converted to negative levels in the early months following SBRT in a majority of patients with CTC-detectable disease.

Following SBRT, monitoring for recurrent disease is often challenging due to imaging-related uncertainties. Distinguishing local recurrences from radiation-induced fibrosis by imaging studies is often difficult despite the identification and definition of high-risk CT-based features.[8–10,12] Clinical uncertainty may lead to excessive imaging and invasive testing and which incurs economic costs, inconvenience, and other risks to patients. Our data suggest that persistently negative CTC tests in follow-up can provide reassurance and confirmation of continued treatment response following SBRT. Consequently, sequential CTC monitoring as a biomarker for treatment failure may thus complement conventional imaging studies before treatment and in follow-up, guiding more frequent surveillance studies if and when a patient presents with a positive CTC test in follow-up.

CTC assays may also usefully complement PET scanning in this patient population. Lung injury following the ablative radiation doses delivered in SBRT is commonly associated with a metabolically active FDG-avid lesion, which may even exacerbate transiently in the immediate months following treatment.[6,7,11,28] The natural history of these imaging findings makes it difficult to apply standardized metrics, such as the PET Response Evaluation Criteria in Solid Tumors (PERCIST 1.0) criteria, to gauge treatment response in early follow-up.[29] Furthermore, some patients with early stage NSCLC may not have FDG-avid lesions prior to treatment where nodule growth was the main criteria driving the decision towards treatment. This was the case in our cohort, as 40% (8 of 20) of CTC-detectable nodules were categorized as FDG-indeterminate (SUV max = 2.5 or less) prior to SBRT.

We are enthusiastic regarding the ultimate value of CTC analysis for NSCLC patients. However, we note a number of limitations in this study. First, the follow-up is relatively short. Long-term results from RTOG 0236 suggest that patients continue to experience local-regional failure up to five or more years following SBRT.[30] Additionally, while we have demonstrated in both preclinical and pilot testing that this assay has excellent internal and intra-patient validity[13]- particularly in “positive” versus “negative” tests - we did observe a degree of variability in absolute CTC values, We believe some of this variability is related to time of day (i.e. a trend to lower values in the morning) and site (i.e. blood draws through a catheter versus directly from peripheral extremity) of blood draws, and we are planning to test these hypotheses in future trials.

Conclusion

This is the first report that sequential CTC monitoring is feasible in patients without pathologically- confirmed stage I NSCLC treated with SBRT. Following radiotherapy, negative CTC tests may provide reassurance and confirmation of continued treatment

response, thus complementing conventional imaging in a patient population in whom differentiating tumor recurrence from radiation fibrosis is often challenging. Based on these promising results, a larger prospective trial of CTC analysis in this population has been initiated.

Acknowledgements:

We acknowledge the Department of Radiation Oncology's Clinical Research Division and Clinical Research Coordinators for excellent assistance with the clinical trial described. This work was supported by the following grants from National Institutes of Health: grant R01 CA201071 from the National Cancer Institute and grant K08 NS076548–01 from the National Institute of Neurological Disorders and Stroke. J.F.D. was also supported by a Burroughs Welcome Career Award for Medical Scientists (1006792).

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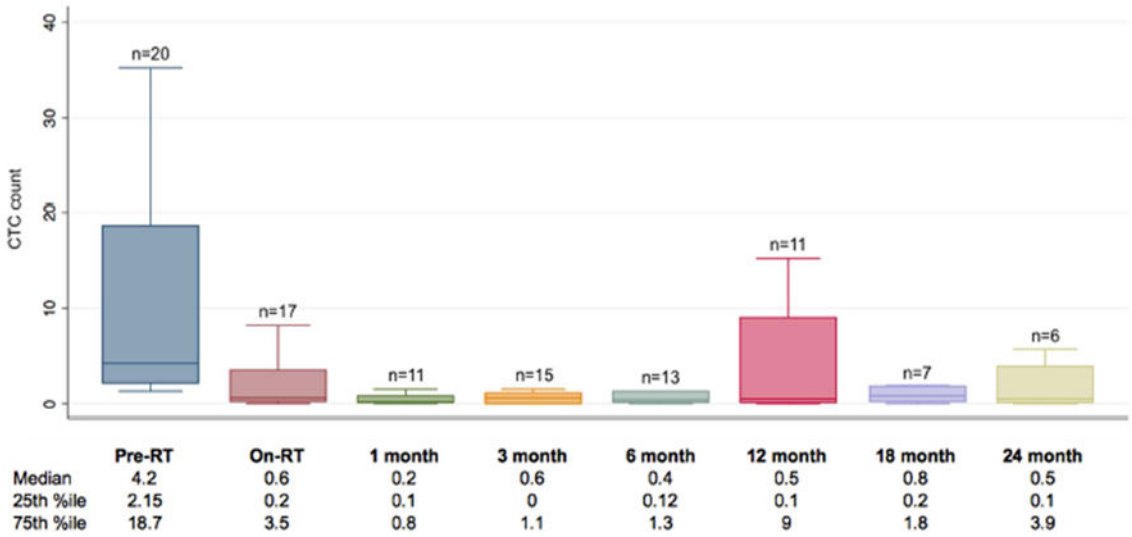
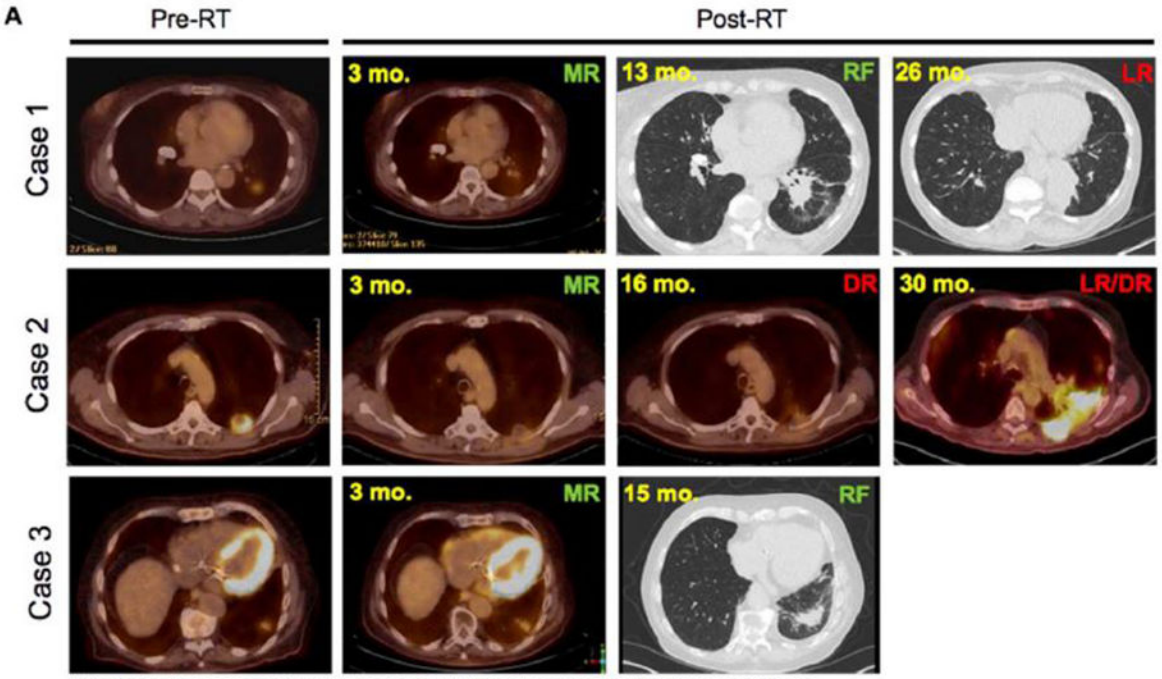


Figure 1.

Longitudinal CTC trends in presumed stage I NSCLC patients with CTC-detectable disease, outlying values outside of the 25–75th percentile were not included.



B

		Pre-RT	On-RT	Post-RT (follow-up, in months)				
				#1	#2	#3	#4	#5
Case	1	+	+		- (3)	- (6)	- (12)	+ (26)
	2	+	+	+ (1)	- (3)	+ (6)	+ (10)	+ (24)
	3	+	-	- (1)	- (3)	- (7)	- (13)	-

Figure 2.

Clinical vignettes illustrating the potential useful for CTC assays: A) Serial conventional imaging studies (MR: metabolic response (on PET imaging); RF: recurrence-free (on CT imaging); LR: local recurrence and DR: distant recurrence (either PET or CT imaging)) and B) CTC assay status in relationship to time periods during CTC tests.

Table 1

	Total	CTC-detectable	CTC-undetectable	*p*-value
	n=48	n=20	n=28	
Age at enrollment (median, range)	69.5 years (55–89 years)	70.5 years (59–89 years)	67.5 years (55–84 years)	0.63
Sex (female)	26 (54%)	14 (70%)	12 (43%)	0.08
Race				0.86
Caucasian	37 (77%)	16 (80%)	21 (75%)	
African American	9 (19%)	3 (15%)	6 (21%)	
Other	2 (4%)	1 (5%)	1 (4%)	
Smoking status				0.49
Current	12 (25%)	4 (20%)	8 (29%)	
Former	34 (71%)	16 (80%)	18 (64%)	
Never	2 (4%)	0 (0%)	2 (7%)	
Pack-years (median, range)	40 (3.5–165)	37.5 pack-years (4.5–75 pack-years)	45 pack-years (3.5–165 pack-years)	
Tumor size (median, range)	1.4 cm (0.5–3.8)	1.2 cm (0.5–3.2 cm)	1.75 cm (0.9–3.8 cm)	0.08
Tumor SUV (median, range)	3 (0.8–19.9)	2.6 (0.8–12.7)	3.1 (0.8–19.9)	0.40
T stage				0.31
1a	35 (73%)	17 (85%)	18(64%)	
1b	10 (21%)	2 (10%)	8 (29%)	
2a	3 (6%)	1 (5%)	2 (7%)	
Prior history NSCLC				0.01
Yes	10 (21%)	8 (40%)	2 (7%)	
No	38 (79%)	12 (60%)	26 (93%)	

Detection and monitoring of CTCs may be useful in patients with early stage NSCLC when definitive tissue diagnosis cannot be confirmed by biopsy. We present a pilot prospective assessment of 48 clinical stage I NSCLC patients treated with SBRT undergoing serial CTC monitoring with a novel, telomerase-based assay. Our results suggest that CTC monitoring in this patient population is feasible and potentially useful in enhancing clinical diagnosis and detection of recurrent or progressive disease.