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The Detection of Circulating Tumor Cells (CTC) in Patients with Non-small cell lung cancer (NSCLC) Undergoing Definitive Radiotherapy or Chemoradiotherapy

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LIST OF ABBREVIATIONS

CACs	cytogenetically abnormal cells
CTC	circulating tumor cells
NSCLC	non-small cell lung cancer
PCR	polymerase chain reaction
WBC	white blood cells

1. ABSTRACT

Non-small cell lung cancer (NSCLC) is the most common cause of cancer mortality in the United States with approximately 180,000 patients diagnosed each year. Systemic disease and relapse after definitive therapy (surgery, chemotherapy and radiation) are common. There is a need to develop new approaches for treating patients with NSCLC. There is also a need to develop methods for predicting response to treatments, monitoring response to therapy, and early detection of relapse and/or systemic disease. There is little known about the numbers, time course, and effect of therapy on CTCs in NSCLC patients. The primary objective of this protocol is to describe the pattern of CTC detection in NSCLC patients undergoing definitive radiation therapy. Exploratory studies on the isolation of circulating tumor DNA (ctDNA) and its contributory value to the collection of circulating tumor cells will also be conducted. Patients with NSCLC who undergo radiation as part of a definitive course of treatment will be enrolled. Blood collections will be obtained before, during, and after the completion of radiotherapy. In patients who are also treated with the novel signal transduction inhibitor, nelfinavir, in addition to chemoradiotherapy, an additional blood sample will be obtained 4-6 days after starting nelfinavir and prior to starting chemoradiotherapy. We will collect demographic and treatment data and explore & describe the pattern of CTC detection in all patients.

2. BACKGROUND

NSCLC

Non-small cell lung cancer (NSCLC) is the most common cause of cancer mortality in the United States with approximately 180,000 patients diagnosed each year. Systemic disease and relapse after definitive therapy (surgery, chemotherapy and radiation) are common. There is a need to develop new approaches for treating patients with NSCLC. There is also a need to develop methods for predicting response to treatments, monitoring response to therapy, and early detection of relapse and/or systemic disease. There is little known about the numbers, time course, and effect of therapy on CTCs in NSCLC patients (Katz et al., 2010)

Current Methods of Circulating Tumor Cells Detection and Inherent Limitations

The absolute number of CTCs that have been reported from cancer patients has varied greatly from one study to another (Katz et al., 2010). Technical constraints may lead to an underestimation of CTCs in some studies. Tumor cells can adopt a wide range of shapes and sizes that may be indistinguishable from or overlap with normal cells, including white blood cells. CTC-detecting techniques based on morphology or size alone may therefore fail to isolate CTCs that mimic normal cells in size or shape. Such tumor cells however can be uncloaked by staining for tumor-specific antigens, including cell surface markers. Expression levels of such markers may, however, vary substantially between CTCs of different histologies or even possibly within the cells from a single patient. The specific CTC analysis technique employed, how the patient-derived materials are handled and stored, and the thresholds set for detection not to mention the patient's disease or treatment status may all potentially affect the sensitivity by which CTCs are detected.

Size exclusion filtration is a common CTC detection technique based upon the observation that tumor cells are in general larger than non-cancerous circulating cells. An advantage of this method

is that it may isolate CTCs of any histology, not just of epithelial origin, unlike other technologies currently being investigated and it uses polycarbonate membrane filters which are relatively inexpensive. However, a disadvantage is that the trach-etching process that is employed to create the filter pores can occasionally create two pores that overlap, resulting in larger cavities that allow all cells to pass through, including those that otherwise would not be able to pass through a single pore. This in turn leads to CTC loss and thus decreased sensitivity.

Density Gradient Separation Techniques attempt to separate CTCs from peripheral blood cells based on their density. The OncoQuick ® method (Greiner Bio-One, Frickenhausen, Germany) is one example of this technique. This method relies on the fact that erythrocytes and neutrophils have greater densities than mononuclear cells and CTCs. The method uses density gradient separation fluids such as Ficoll-Paque ® or Percoll ® through which erythrocytes and granulocytes will pellet while mononuclear cells and CTCs remain in the interphase layer above the fluid and below the plasma (Fizazi et al., 2007). It is unclear, however, if all CTCs have the appropriate buoyant density for capture via density gradient separation methods.

Other techniques have been described to distinguish CTCs from WBCs or to distinguish between subsets of CTCs expressing different biomarkers. For example, *laser scanning cytometry* is a technique in which pre-stained cells are subject to laser light illumination and then scanned under microscopy to detect and count cells that stain brightly. WBCs and CTCs are then differentially detected based on different degrees of light scatter, size, or other criteria (Pachmann et al., 2008). *Polymerase chain reaction* (PCR) is a sensitive method of detecting the expression of specific mRNA sequences, including those characteristic of CTCs. However, there is often uncertainty regarding whether CTCs or WBCs or other sources are the origin of the detected mRNA, which thus confounds the analysis. (Obermayr et al., 2010). Another method that relies on *antibody-based detection of epithelial cell antigens* expressed on the cell surface to identify CTCs is the EPISPOT (epithelial immunospot), in which live cells are incubated for 48-hours to ensure maximize cell surface protein expression, followed by the addition of primary anti-epithelial protein antibody and then a method to detect the binding of the primary antibody to identify CTCs (Alix-Panabières et al., 2007). The CellSearch System (by Veridex LLC, so far the only method currently approved by the FDA for clinical assays) relies on ferrofluid particles coated with anti-EpCAM (EpCAM = Epithelial cell adhesion molecule) antibodies to purify CTCs (Allard et al., 2004). The ferrofluid particles coated with anti-EpCAM antibodies bind to CTCs, and the entire complex is then removed via magnets. There may, however, be cellular components (debris) such as membranes retaining EpCAM that can be found freely circulating. Consequently, the magnetically captured material is further assessed using a panel of markers to confirm the presence of cells of tumor origin. These secondary markers include the DNA stain 4,6-diamino-2-phenylindole (DAPI), cytokeratins (CKs), and CD-45 (the latter two are detected via specific antibodies raised against the respective antigens). The CellSearch System is heavily reliant on EpCAM expression. Consequently there are many tumor types, especially those of non-epithelial origin, which would not be detectable via this system. Furthermore, it remains possible that loss of EpCAM, or lowered levels of expression such as that due to mutation or downregulation (epithelial-to-mesenchymal transition), would likely impede the ability to isolate even cancer cells of epithelial origin. The panel of secondary markers also requires regular validation and the

appropriate negative and positive controls. Commercial antibodies are often derived from animal sources, with attendant batch-to-batch variability in sensitivity and specificity.
Relatively few studies have been published describing the detection of CTCs in NSCLC. (Wu et al., 2009) described a technique using depletion of CD45 cells from blood with magnetic beads followed by CK18/19 staining of enriched cells. Relatively few CTCs were detected although the number of CTCs correlated with stage. Katz reported on the development of a quantitative antigen-independent fluorescent *in situ* hybridization-based (FISH) method for detecting cytogenetically abnormal cells (CACs) in the peripheral blood of NSCLC patients (Katz et al., 2010). This FISH-based assay detected losses and gains of selected chromosomal regions in the nuclei of cells regardless of their lineage. These investigators detected mean number of CACs ranging from 7.23 ± 1.32/μL for deletions of 10q22.3/CEP10 to 45.52 ± 7.49/μL for deletions of 3p22.1/CEP3.

Novel Methods of CTC Detection
There is evidence that alternate techniques of CTC detection, and in particular detection of viable tumor cells which have lost epithelial cell markers, are possible. Kojima and colleagues have described one such method, in which a green fluorescent protein (GFP)-expressing attenuated adenovirus (OBP-401) is used to selectively label cells with high activity of telomerase. (Kojima et al., 2009).This method is independent of cell-surface markers and therefore is independent of epithelial to mesenchymal transformation. The Kojima method labels only those cells susceptible to adenovirus transduction and with high levels of telomerase activity. In the peripheral blood there exists a paucity of cells, other than CTC, in which adenovirus transduction may occur. Additionally, while it is established that telomerase is activated in the majority of human cancer cells, other circulating cells, including hematopoietic cells, show low telomerase activity.(Kojima et al., 2009). This combination of factors makes this an ideal vector for the labeling and detection of CTCs.

To our knowledge a telomerase-based method of CTC detection has yet to be applied systematically to a NSCLC cancer population. Our group, however, has experience in measuring CTCs from lung tumors as part of the tissue collection/registry study in the Department of Radiation Oncology, thereby demonstrating the feasibility of this approach.

Among 23 patients with non-small cell lung cancer (NSCLC) undergoing definitive chemoradiation the range of CTCs detected per ml of blood ranged from 0.57 to 570.7 cells (mean 62.7, median 9.1, Standard Error 28.4). The rate of background, non-CTC cells, detected via the telomerase-based method in 4 normal, healthy controls ranged from 0.1 to 1.7 (mean 0.76, median 0.72).

Figure 2 shows the change in the number of CTCs detected among patients prior to and following definitive chemoradiation for NSCLC. Patient #1 decreased from 56.9 CTC/ml prior to treatment to 0.9 CTC/ml following treatment. Patient #2 decreased from 9.1 to 1.8 and patient #3 from 1.2 to 0.2. Percent decreases ([pre-post/pre]*100) for Patients 1-3 were: 98%, 80% and 83%.

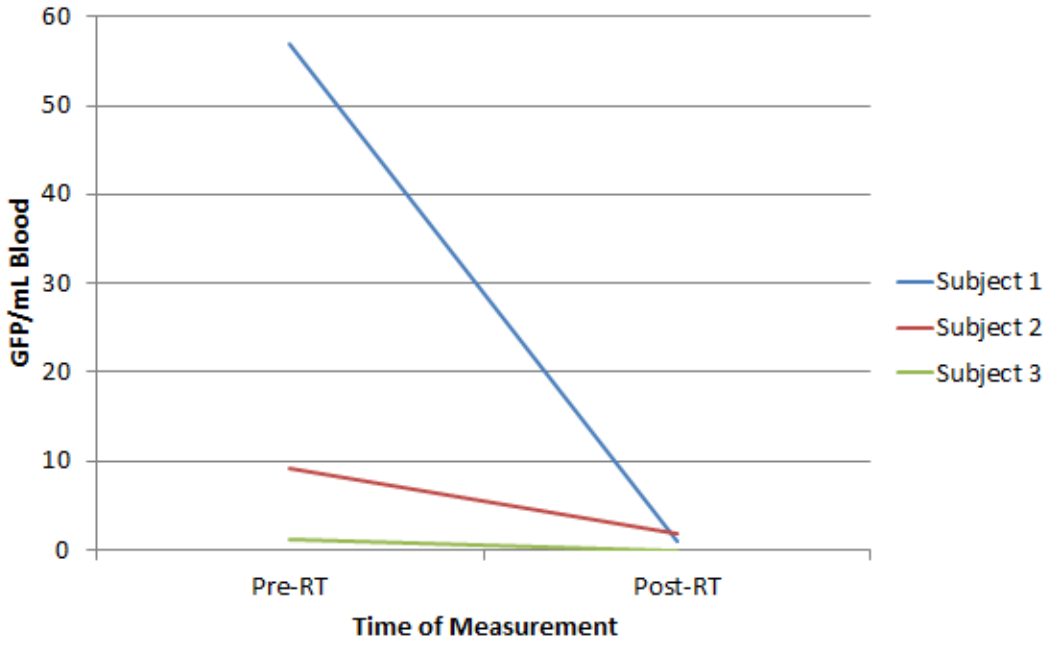

Figure 2: Number of green fluorescent protein labeled (GFP) cells detected per mL of blood in patients with confirmed non-small cell lung cancer (NSCLC) pre-radiation therapy (RT) and post-RT. All subjects were observed to have a decrease in the number of detected GFP cells following RT.

In summary, we have used novel methodology to detect CTCs in patients with NSCLC. In this protocol, using this novel CTC detection assay, we will investigate the pattern of CTC detection in patients with NSCLC undergoing definitive radiotherapy as a part of their treatment. We will systematically and prospectively collect demographic, treatment, and CTC data over a two-year period in each patient. These data will be exploratory and hypothesis-generating.

Isolation of Circulating Tumor DNA (ctDNA)

Circulating tumor DNA analysis may also be performed to provide additional correlated data. Following the isolation of the cells from the cell layer in the blood sample, the plasma from the rest of sample will be used to isolate circulating tumor DNA (ctDNA). This DNA will then be amplified by PCR and analyzed for specific mutations associated with lung cancer (such as K-Ras, ALK, EGFR, etc), based on published methodologies (CAPP-Seq, Newman et al., Nature Medicine 20, 548–554, 2014).

3. OBJECTIVE

3.1 Primary Objectives

1. To determine the intra- and inter-patient variability at baseline and test/re-test reliability of the method
2. To describe the pattern of CTCs before, during and after definitive radiotherapy for each treatment stratum

3.2 Secondary Objectives

1. To explore the relationship between baseline CTC and metabolic tumor response

2. To determine the change in CTC during treatment (i.e., slope from baseline to week 5 during radiotherapy) and metabolic tumor response
3. To explore the changes in CTCs during treatment and changes after radiotherapy
4. To determine the pattern of EGFR mutations in CTCs
5. To determine the pattern of signaling pathway expression of relevant biomarkers of CTCs before, during and after definitive radiotherapy in patients receiving Nelfinavir
6. To explore the contributory value of isolating circulating tumor DNA (ctDNA) in addition to the collection of CTCs.

4. SUBJECT POPULATION

We plan to enroll patients into seven distinct cohorts:

1. Patients with early stage NSCLC undergoing stereotactic radiotherapy
2. Patients with locally advanced NSCLC undergoing standard chemoradiotherapy
3. Patients with stage I-III NSCLC undergoing, fractionated radiotherapy alone
4. Patients with locally advanced NSCLC undergoing standard chemoradiotherapy with the novel signal transduction inhibitor, nelfianvir.
5. Patients with resectable stage IIIa NSCLC undergoing pre-operative chemoradiotherapy
6. Patients with suspected (no tissue diagnosis) early stage NSCLC undergoing stereotactic radiotherapy
7. Patients with locally advanced NSCLC undergoing stereotactic radiotherapy followed by concurrent mediastinal chemoradiotherapy.

Eligibility

1. Patients with biopsy-proven (except for Cohort 6) NSCLC who are undergoing definitive radiotherapy as a part of their treatment regimen.
2. Age 18 or older
3. Signed informed consent. The consent process can be completed in person or via Telemedicine per University of Pennsylvania Health Systems telemedicine policy and procedures.
4. Patients who are incapable of providing informed consent are excluded from participating in this study.

5. STUDY PROCEDURES

We will collect blood samples and determine CTC values in the seven cohorts of patients with NSCLC, as described below:

Cohort 1: Patients with early stage NSCLC undergoing stereotactic radiotherapy
Cohort 2: Patients with locally advanced NSCLC undergoing standard chemoradiotherapy
Cohort 3: Patients with stage I-III NSCLC undergoing, fractionated radiotherapy alone
Cohort 4: Patients with locally advanced NSCLC undergoing standard chemoradiotherapy with the signal transduction inhibitor, nelfinavir.
Cohort 5: Patients with resectable stage IIIa NSCLC undergoing pre-operative chemoradiotherapy*
Cohort 6: Patients with suspected (no tissue diagnosis) early stage NSCLC undergoing stereotactic radiotherapy

Cohort 7: Patients with locally advanced NSCLC undergoing stereotactic radiotherapy followed by concurrent mediastinal chemoradiotherapy.

*Often a patient cannot be fully evaluated as a surgical candidate until the completion of chemoradiotherapy. This means that the patients originally placed in cohort 2 can be moved to cohort 5 and vice versa upon the discretion of the PI.

Blood Collection Procedure

The procedure of blood collection for each patient will be as follows: up to 5 mL of blood will be drawn into a waste tube (to avoid collecting skin material and potential contaminating skin stem cells.) The waste tube will either be discarded or used to investigate other laboratory methods involving CTC virus transduction. This could lead to the use of a more effective virus for CTC analysis or the need for smaller subject blood samples in the future. Subsequently 10 mL of blood will be collected into heparinized tubes, and immediately placed on ice in a prepared plastic bag and transported to the laboratory for processing within approximately one hour. A 10 mL sample may also be collected (typically in an EDTA tube) for the isolation of ctDNA. These samples will be collected via venipuncture or by utilizing a patient's previously place central/peripheral line being used for other purposes (i.e. chemotherapy).

The total volume of blood obtained with each collection would therefore ideally be at least 25 cc. Blood collections will, be obtained up to 2 times before the start of radiation, during weeks 1-2, weeks 3-4, and weeks 5-6 of radiotherapy or as clinically indicated. Following the completion of radiotherapy blood draws will occur at approximately: 1 month, 3 months, 6 months, 12 months, 18 months and 24 months. In patients who are also treated with nelfinavir an additional blood sample will be obtained 4-6 days after starting nelfinavir and prior to starting chemoradiotherapy. Blood collections are performed exclusively for research purposes.

Ideal collection time points generally include:

- A maximum of two pre-radiotherapy samples (ideally within approximately 30 days of each other) prior to the first fraction of radiation.
- Approximately one to two weeks after the initiation of radiation therapy (for cohort 7 this will be obtained during the course of stereotactic radiotherapy)
- Approximately three to four weeks after the initiation of radiation therapy (this collection is omitted for cohort 1 and 6 because the treatment course of those cohorts is generally shorter than four weeks, for cohort 7 this will be obtained approximately three to four weeks after the initiation of concurrent mediastinal chemoradiotherapy)
- Approximately five to six weeks after the initiation of radiation therapy (this collection is omitted for cohort 1 and 6 because the treatment course of those cohorts is generally shorter than four weeks. This collection may also be omitted for other cohorts if the investigator decides to use a hypofractionated treatment plan, for cohort 7 this will be obtained approximately five to six weeks after the initiation of concurrent mediastinal chemoradiotherapy).
- Approximately one month after the completion of radiation

- Approximately three months after the completion of radiation
- Approximately six months after the completion of radiation
- Approximately twelve months after the completion of radiation
- Approximately eighteen months after the completion of radiation
- Approximately twenty-four months after the completion of radiation

Due to unanticipated side-effects of treatment, scheduling issues or logistical difficulties, the above schedule will be modified at the discretion of the PI. If a sample is unable to be obtained at a specified time point it will be collected at the next opportunity. Every attempt will be made to collect samples at normally scheduled standard of care appointments. In cases where this is not possible and the participant is willing, a sample may be obtained at a research visit only.

*cohort 5 patients who require follow up radiotherapy or chemoradiotherapy will have an additional CTC sample obtained prior to follow-up radiotherapy (approximately 7 days from the initiation of radiation) and then will follow the schedule of cohort 2 thereafter.

For patients on Nelfinavir, the two pre-radiotherapy collection samples will consist of:
(1) before start of Nelfinavir and before initiation of radiotherapy
(2) after start of Nelfinavir but before initiation of radiotherapy

An additional blood sample may be requested from the patient if he/she develops clinical or biopsy-proven recurrent disease or progression of disease at any time along with continued collection of samples at the same time points already determined per protocol.

***Adverse events will not be followed or reported for this protocol.**

Off Study Criteria

Patients will be taken off the study for 1) patient refusal; 2) extraordinary medical circumstances or 3) at the discretion of the PI.

Table 1. Outline of Study Blood Collection

	Pre-Treatment		On Treatment			Follow-up (by month)					
Cohort	#1	#2*	1/2 wks	3/4 wks	5/6wks	1	3	6	12	18	24
1	X	X	X			X	X	X	X	X	X
2	X	X	X	X	X	X	X	X	X	X	X
3	X	X	X	X	X	X	X	X	X	X	X
4	X	X	X	X	X	X	X	X	X	X	X
5	X	X	X	X	X	X	X	X	X	X	X
6	X	X	X			X	X	X	X	X	X
7	X	X	X**	X***	X***	X	X	X	X	X	X

*In cases where it is not possible to obtain two pre-treatment samples due to logistical considerations and planned treatment start date only one pre-treatment sample will be obtained.
**For cohort 7 this sample will be obtained during the course of stereotactic radiotherapy.

*** For cohort 7 these samples will be obtained approximately three to four weeks and five to six weeks after the initiation of concurrent mediastinal chemoradiotherapy.

6. STATISTICAL CONSIDERATIONS

6.1 Design:

This is a pilot study to establish that circulating tumor cells (CTC) can be detected in blood samples of patients with NSCLC undergoing definitive radiotherapy. CTCs will be detected by a method which uses a telomerase-specific replication-selective adenovirus expressing the molecular marker, GFP. Patients will be assigned to one of seven strata, as defined by their treatment. A total of 280 patients will be enrolled, 10 in cohort 4, 60 per stratum in cohorts 1,2, and 6 and 30 per stratum in cohorts 3, 5, 7.

Treatment Stratum
1: Early stage NSCLC undergoing stereotactic body radiotherapy
2. Locally advanced NSCLC undergoing standard chemoradiotherapy
3. Stage I to III NSCLC undergoing radiotherapy alone
4. Locally advanced NSCLC undergoing Nelfinavir + standard chemoradiotherapy
5. Stage IIIa NSCLC undergoing pre-operative chemoradiotherapy
6. Non-biopsy proven early stage NSCLC undergoing stereotactic body radiotherapy
7. Locally advanced NSCLC undergoing stereotactic radiotherapy followed by concurrent mediastinal chemoradiotherapy.

Up to two blood samples will be obtained at baseline (ideally within approximately 30 days of each other). Thereafter, samples will be obtained at approximately weeks 1/2, 3/4 and 5/6 during radiotherapy and at approximately 1, 3, 6, 12, 18 and 24 months after completion of radiotherapy. The sampling schedule varies slightly for patients who undergo SBRT or surgery or who receive Nelfinavir. EGFR mutations and signaling pathway expression of relevant biomarkers detected in CTCs will be measured at baseline, at week 5/6 during radiotherapy and at 3 and 6 months after completion of radiotherapy. Metabolic tumor response will be assessed by PET/CT when possible if performed as standard of care at 3 months after completion of radiotherapy and if clinically indicated.

6.2 Objectives:

6.2.1 The primary objectives are to determine:

1. intra- and inter-patient variability at baseline and test/re-test reliability of the method
2. the pattern of CTCs before, during and after definitive radiotherapy for each treatment stratum

6.2.2 Secondary objectives are to describe the relationship between:

3. baseline CTC and metabolic tumor response
4. changes in CTC counts during treatment (i.e., slope from baseline to week 5/6 during radiotherapy) and metabolic tumor response
5. changes in CTC counts during treatment and changes after radiotherapy
6. CTCs and the isolation of ctDNA

And the pattern:
7. of EGFR mutations in the detected CTCs
8. of genetic mutations and other biomarkers in the detected CTCs before, during and after definitive radiotherapy
9. in patients receiving Nelfinavir

6.3 Preliminary data:
CTCs were measured in 23 NSCLC patients undergoing definitive therapy. Substantial inter-patient variation was observed, with a coefficient of variation of 212%. Thus estimation of intra-patient variation and inter-patient variation at baseline in a prospectively identified patient cohort will be extremely important, in order to identify credible treatment-related changes in CTCs during and after radiotherapy.

Mean±SE	Median	Standard Deviation	Minimum	Maximum	Coefficient of variation
62.7±28.4	9.1	133.2	0.57	570.7	212%

6.4 Endpoints:
1. Circulating tumor cells will be detected by imaging for green fluorescent protein (GFP) which measures cells which high levels of telomerase activity after adenovirus transduction. CTCs are measured in units of number of GFP-positive cells /mL.
2. Metabolic tumor response will be determined by PET/CT (when performed as clinically indicated) at 3 months post-treatment and dichotomized as responder (CR/PR) or nonresponder (<PR).
3. Change in CTC will be measured by fold change (post/pre) and percent change from baseline (i.e., [pre-post)/pre]*100).
4. EGFR mutations will be detected by IF staining with EGFR mutation-specific antibodies.
5. The presence or expression of other biomarkers and pathways involved in tumor biology will be assessed via such methods as RT-PCR for genetic mutations.
6. In patients treated with Nelfinavir, the levels of and changes in signaling pathways and other relevant biomarkers will also be assessed.

6.5 Plans for Data Analysis:
Descriptive statistics will support hypothesis generation and the design of adequately powered future studies. Most analyses will be conducted separately for each treatment stratum.

Descriptive statistics/Graphs: Baseline CTC will be characterized by descriptive statistics (e.g., mean, median, SD, range, coefficient of variation) and reliability analysis (see below description). At each time point during and after radiotherapy, the CTC mean±SE and the fold change mean±SE (to account for each patient's baseline) will be calculated. Plots of mean and fold change over time will be generated. Test/re-test reliability analysis. Since repeated observations of CTC are obtained at baseline, a plot of Visit 1 and Visit 2 values will be constructed, Pearson correlation and linear regression (i.e., test of slope = 1) will be initial analyses to characterize overall test/re-test reliability for the study population, pooling over the 7 strata. Test-re/test reliability will be further evaluated by the within-patient coefficient of variation. Defining real change. To determine whether fold changes observed during and after treatment are beyond random variation, we will compute the ratio of Visit 1 to Visit 2 for each patient and then compute the standard deviation of

the distribution of the ratios. Values of fold change in CTC during and after treatment which fall outside the interval [1.0-2SD, 1.0+2SD] will be considered credible changes.

Repeated measures ANOVA and linear models: Because we hypothesize CTCs will decrease during radiotherapy, changes over time will be characterized by modeling slopes. Log transformation and piecewise linear models may be employed, as needed. Repeated measures ANOVA will be used to compare mean CTC values at each time point during and after radiotherapy to mean CTC at baseline, although these exploratory analyses may be underpowered. Group comparisons: At 3 months post-treatment, metabolic response will be determined. Descriptive statistics of baseline CTC will be generated for responders and nonresponders within each treatment stratum. Comparison of responders to nonresponders will be conducted by pooling over the 7 strata. The affect of Nelfinavir in combination with chemoradiotherapy will be evaluated by mixed effects models which test the fixed effect of stratum (#2 vs #4) while modeling the time course of CTC values. Similarly, the affect of standard radiotherapy versus stereotactic radiotherapy will be evaluated by mixed effects models which test the fixed effect of stratum (#1 vs Stage I patients in #3). Correlations: Relationships between CTC and biomarker expression or presence of genetic mutations will be evaluated at fixed time points, by scatter plots and correlations. Time courses of CTC, EGFR mutations and biomarkers will also be jointly plotted to investigate temporal relationships.

Sample Size: A total of 10 subjects will be enrolled in cohort 4, 30 subjects will be enrolled into cohorts 3, 5, & 7 and 60 subjects will be enrolled into cohorts 1, 2, & 6. Any subject who does not complete a full course of radiotherapy will be removed from the study and replaced.

Study Duration:
The study will enroll a total of 280 subjects. This study will likely complete enrollment in 48 months.

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