AWARD NUMBER: W81XWH-15-1-0243

TITLE: Development of a Prognostic Marker for Lung Cancer Using Analysis of Tumor Evolution

PRINCIPAL INVESTIGATOR: Edward F. Patz, Jr., M.D.

CONTRACTING ORGANIZATION: Duke University
Durham, NC

REPORT DATE: August 2016

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2016	Annual	1 Aug 2015 - 31 Jul 2016

4. TITLE AND SUBTITLE

Development of a Prognostic Marker for Lung Cancer Using Analysis of Tumor Evolution

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0243

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Edward F. Patz, Jr., M.D.

E-Mail: patz0002@mc.duke.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Duke University
Durham, NC 27705

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The goal of this project is to sequence the exomes of single tumor cells from tumors in order to construct evolutionary trees, the characteristics of which will be used to predict whether a tumor will metastasize or not. We established a procedure for isolating tumor cells from lung tumor tissue and observed high variability in yield. We also encountered loss of tumor cell viability after freezing. Despite these challenges, we will be able to obtain sufficient numbers of cells to proceed. The next step is whole genome amplification of the DNA from single cells as a prerequisite to sequencing. We encountered a low success rate of whole genome amplification from single sorted cells and have spent a great deal of time troubleshooting this process. We anticipate that exome sequencing of single tumor cells should begin shortly in order to reconstruct tumor cell evolution in tumors.

15. SUBJECT TERMS

NSCLC; tumor evolution; whole exome sequencing

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	9	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

1. Introduction

There is currently no consistent way to determine how aggressive or indolent a lung tumor will be even among patients with the same radiographic findings, histology, stage, or molecular markers. The purpose of this grant is to apply evolutionary analytical methods developed to study expansion and migration of populations to tumor biology in order to produce a prognostic marker in cancer. As with the Darwinian evolution of populations, the evolution of tumor cells within a tumor can be diagrammed on a phylogenetic tree. The more diverse a tumor's phylogenetic tree, the more likely it is that there are cells within it that have acquired the genetic alterations that allow them to proliferate at an increased rate, migrate, and metastasize. We will develop and validate a novel, objective, and measurable "prognostic score" based on the probability that some tumors will be *aggressive* and metastasize, and other tumors will be *indolent* and not metastasize. We first will perform whole exome sequencing of individual tumor cells from the tumors of a training set of patients (half early stage, half late stage). We will reconstruct each tumor's phylogenetic tree (a map of the clonal evolution reflecting divergence and heterogeneity), and compare the tree patterns from early stage NSCLC (indolent tumors without metastasis) to those from late stage disease (tumors with metastasis). We will use a combination of *tree features* (including branch length and tree shape) to generate a prognostic score (a continuous variable and a measure of tumor heterogeneity) that separates tumors with very different phenotypes (indolent vs. aggressive). We will derive the prognostic score by determining the probability of each individual tumor's outcome in the pilot training study, and then validate this strategy in an independent set of patients. An accurate prognostic score could significantly change clinical management and improve outcomes.

2. Keywords

NSCLC; tumor evolution; whole exome sequencing

3. Accomplishments

Specific Aim I: Isolate individual tumor cells from 10 patients with stage I non-recurrent NSCLC and 10 patients with advanced stage NSCLC.

Our initial studies have focused on optimizing the technical aspects of tumor dissociation, tumor cell isolation, sorting, and whole genome amplification (WGA). These studies are summarized below, with specific challenges and solutions noted.

Establishment of the initial procedure: Fresh lung tumor tissue was obtained from surgical pathology at Duke Hospital under our IRB protocol. All patients signed an informed consent prior to surgery. The tissue was weighed, transferred into 5 ml human tumor dissociation medium (Miltenyi, San Diego, CA), and processed on a Miltenyi gentleMACS™ Dissociator. The resulting cell/tissue suspension was then filtered through a 70 µm nylon screen and non-tumor cells removed using a tumor cell isolation kit (Miltenyi). The recovered tumor cells were counted using a Scepter cell counter (Millipore, Billerica, MA) and either stained for fluorescence activated cell sorting (FACS) or frozen in Bambanker cell freezing medium (Lymphotec Inc., Tokyo, Japan).

Prior to FACS, isolated tumor cells were stained with mouse anti-human CD45RA PE/Cy7 (clone L48, BD Biosciences, San Jose, CA) and Aqua Vital Dye (Invitrogen, Carlsbad, CA). CD45 is a blood cell marker used to separate blood cells from tumor cells. Cells gated as live/CD45RA^{neg} were sorted into the wells of a 96-well PCR plate, which was covered with plate sealing film and frozen at -80°C. Initial studies were carried out with 1, 2, 5, or 10 cells per well. Sorts were conducted with a BD FACSDiva with 70 psi sort pressure and a 70 µm nozzle.

Encountering inconsistency in tumor cell isolation: The processing of tumor specimens using Miltenyi's tumor dissociation kit and their GentleMACS Dissociator is fairly robust and required minimal optimization. The tumor dissociation kit includes various enzyme mixes in lyophilized form with instructions for reconstitution and storage of the enzyme mixes at -20°C for up to 6 months. The dissociation of the tumor specimens on the GentleMACS is largely automated with minimal hands-on time for incubation at 37°C at specified intervals. Following the dissociation procedure, the resultant cell/debris suspension is filtered, and the

cells are counted, incubated with the antibody mixes supplied with the tumor cell isolation kit, and applied to a magnetic bead column designed to deplete the suspension of non-tumor cells such as blood cells and fibroblasts. The recovered cells are counted again and either stained for sorting or frozen in freezing medium.

Counting the cells with the Scepter counter allows one to monitor the size range of the cells being counted. Ideally, the cell suspension obtained from the tumor dissociation contains a mix of blood cells of 3 to 8 µm diameter and other cells of around 10 to 20 µm diameter. After the magnetic bead column, the suspension consists of far fewer blood cells and proportionally more cells with a larger average diameter. In practice, however, some tumors yield cell suspensions that consist almost entirely of blood cells with very few or undetectable amounts of larger, and presumably, tumor cells. Since the yield of tumor cells from any given tumor cannot be predicted from the physical appearance of the tumor piece, unproductive tumor dissociations and cell isolations are frequent occurrences.

As can be seen in **Table 1**, tumor dissociation and tumor cell isolation demonstrate considerable variability from tumor specimen to tumor specimen. As shown in the table, twelve tumor specimens were obtained from the Duke Surgical Pathology Department and processed. Of these, three tumors yielded no tumor cells following magnetic bead depletion. Although specimens 16-009-T and 16-019-T were relatively small in size, which could help explain the absence of tumor cells recovered, 16-012-T was over 400 mg and should have resulted in successful tumor cell isolation. However, this tumor was highly vascularized and bloody. Hence, the great majority of the cells isolated were red blood cells and lymphocytes that could not be removed sufficiently to allow isolation of tumor cells.

We found that in general, the less vascularized a tumor is, the better the tumor cell isolation. However, this is not invariably true and thus cannot be used as a guideline to triage tumors for processing.

Table 1. Summary of tumors processed and cells sorted

Tissue ID	Processing Date	Total tumor cells isolated	mg tumor	cells/mg	Sorted
16-002-T	1/25/16	100,875	225	448	1/27/16
16-003-T	1/29/16	951,000	268	3549	
16-004-T	2/4/16	113,700	323	352	3/18/16
16-009-T	3/25/16	0	116	0	
16-011-T	4/7/16	17,000	380	45	
16-012-T	4/12/16	0	426	0	
16-014-T	4/28/16	6930	440	16	
16-017-T	5/3/16	2.80E+07	910	30,769	5/5/16
16-019-T	5/10/16	0	75	0	
16-020-T	5/12/16	1.77E+07	610	29,016	
16-022-T	6/13/16	4.00E+05	216	1,852	6/14/16
16-023-T	6/27/16	4.15E+04	46	911	

Encountering loss of tumor cell viability after freezing: Although the tumor cell isolation kit is designed to rid cell suspensions of non-tumor cells, the process has limitations. The result is that the final suspension of cells obtained still has appreciable numbers of non-tumor cells. In order to carry out the intended aims of this project (*i.e.*, to use next generation sequencing of individual tumor cells to generate phylogenetic trees for metastasis prediction), the isolation of bona fide tumor cells is crucial. Hence, we employ FACS as a further means of capturing tumor cells from the mixed populations of cells that are obtained from tumor dissociation and tumor cell isolation. FACS is also the most convenient way we are aware of to sort the single cells, which are key to the success of this project, into individual wells for analysis.

Sorting of cells individually into 96-well plates is done routinely at the Duke Human Vaccine Institute (DHVI), which is the facility that carries out cell sorting for this project. However, the DHVI facility has the most experience with sorting lymphocytes rather than tumor cells. Hence, some optimization is required to ensure that we have the best chance of capturing tumor cells with as little damage as possible.

It is not always possible to sort tumor cells into 96-well plates immediately after tumor dissociation due to the difficulty in obtaining FACS time on short notice. Hence, dissociated tumor cells that have been depleted of most blood cells with the Miltenyi tumor cell isolation kit must be frozen at -80°C until the FACS can take place. We use Bambanker cell freezing medium to freeze the tumor cells since we have years of experience with this medium and good viability can be obtained without liquid nitrogen. In spite of this, we still see appreciable cell death upon thawing the tumor cells. This is evident in **Figure 1**.

Cells gated as live CD45RA negative are contained within the pink box in each plot. Both unfrozen and frozen sorts contained about one million cells each, counted before freezing. The cells that were sorted before freezing

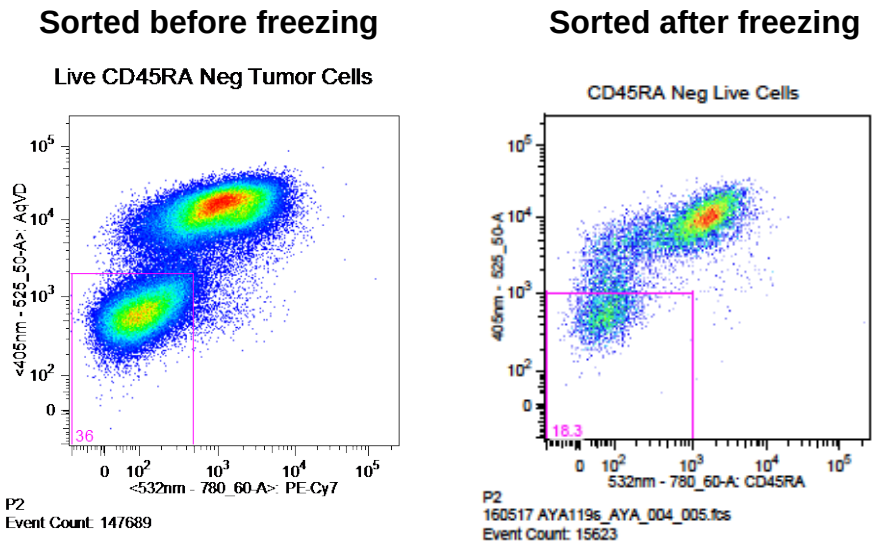


Figure 1. Effect of freeze-thaw on tumor cell viability.

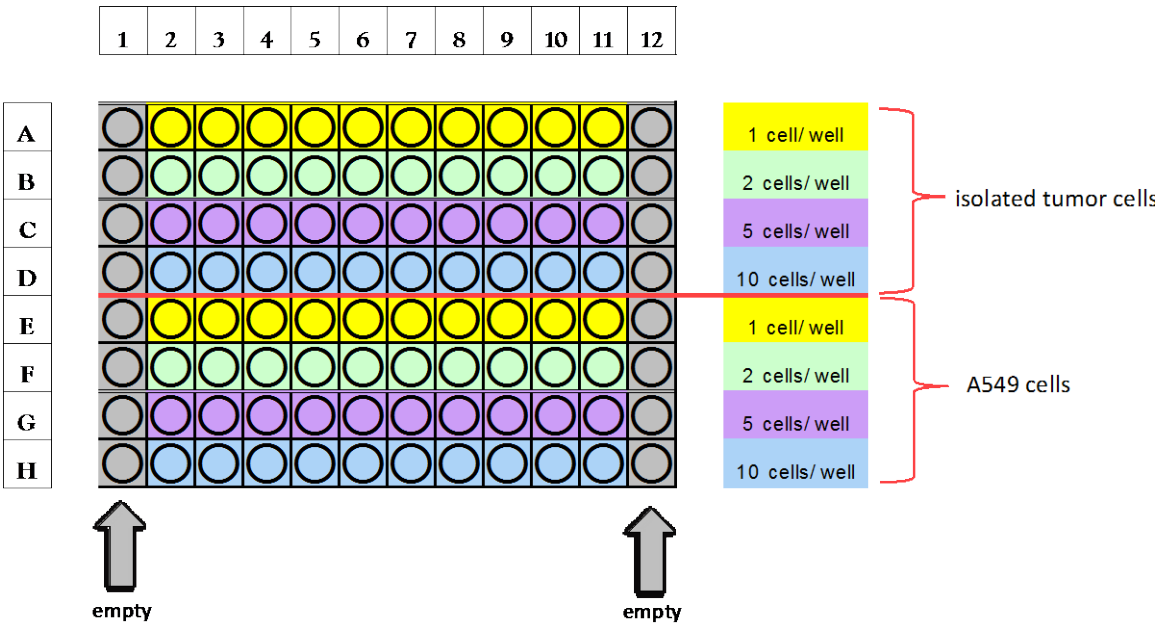
(left plot) are more abundant overall and have a greater proportion of live cells compared to those that were frozen and thawed prior to sorting. Although freezing does cause a marked decrease in the number of viable cells, the number of remaining cells should be sufficient to obtain the 40 cells per patient that are required for the project. In addition, our WGA data indicates that freezing does not result in problematic decreases in WGA success rate (see below). The upshot is that we will sort the cells prior to freezing when possible, and freeze them when we have to for sorting later.

Optimization of cell sorting medium and volume (with A549 cells): Once tumor cells are sorted into 96-well plates, the next step is single cell WGA followed by sequencing. WGA is carried out using a REPLI-g single cell kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. WGA is verified by PCR amplification of a region of the kinesin light chain 2 gene (KCL2) followed by agarose gel electrophoresis. Since we were unsure of the success rate of single tumor cell WGA, we wanted to first establish the WGA success rate with cultured lung tumor cells, and to determine the optimal volume and composition of the liquid into which the cells would be sorted. For this experiment, we used the lung adenocarcinoma cell line A549, which we sorted individually into 2 plates, each containing 80 cells in 80 wells. Prior to sorting, the wells of the plates were evenly divided to contain 10 or 20 µl of water or 0.1X TE. While this exercise showed 10 or 20 µl water to be approximately equivalent in their ability to support WGA (50% vs. 47.5% success, respectively), 10 or 20 µl 0.1X TE was found to be somewhat inferior (47.5% vs. 32.5% success, respectively). We chose to perform all future sorts into 20 µl water since the surface area of a 20 µl drop of water is larger than that of a 10 µl drop and would give each individually sorted cell a better chance of actually hitting the water.

Troubleshooting the low WGA success rate for individual tumor cells:

The dependence of WGA on cell number. Our initial trial of WGA of individually sorted tumor cells was disappointing in that only 4 wells out of 80 successfully amplified (5% success rate). Unsuccessful WGA could be due to (a) an inability of the REPLI-g single cell WGA kit to utilize low concentrations of DNA, (b) cell damage from freezing prior to sorting leading to a problem in the process, (c) the possibility that not all wells are receiving the intended numbers of cells during the sorting, or (d) a combination of these.

We did not know if the very small amount of DNA present in a single mammalian cell (~ 6 pg) could be a factor in the low WGA success rate. Therefore, we used FACS to place 1, 2, 5, or 10 cells in each well, and used A549 cells in addition to isolated tumor cells as the DNA source. If DNA quantity limits success of WGA, inclusion of greater cell numbers in the WGA reaction should increase the WGA success rate. Following WGA, PCR for KCL2 was performed as before. The plate map of the sort and the results of this experiment are shown



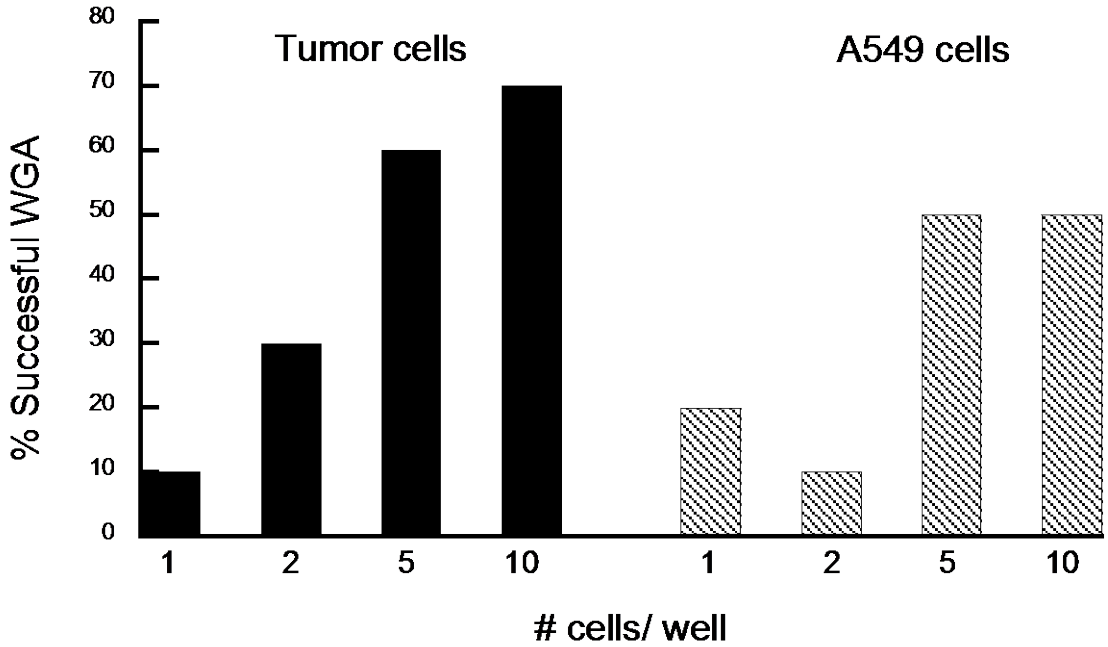


Figure 2. The dependence of WGA on cell number.

in **Figure 2**. The results indicate a clear trend toward a higher WGA success rate with increasing numbers of cells in each well.

The effect of tumor cell freezing on WGA. In order to address the possibility of issues with freezing, we isolated tumor cells from a lung tumor, froze half the cells prior to sorting, and sorted the other half fresh. WGA was then performed. The results are shown in **Figure 3**.

These data suggest that although freezing the cells prior to sorting may have some negative effect on the outcome of WGA, the effect is variable and relatively minor. Hence, if freezing must be done, the overall results should not have too detrimental an effect on WGA. However, these results also suggest that something other than freezing may be responsible for the low WGA success rate for single cells.

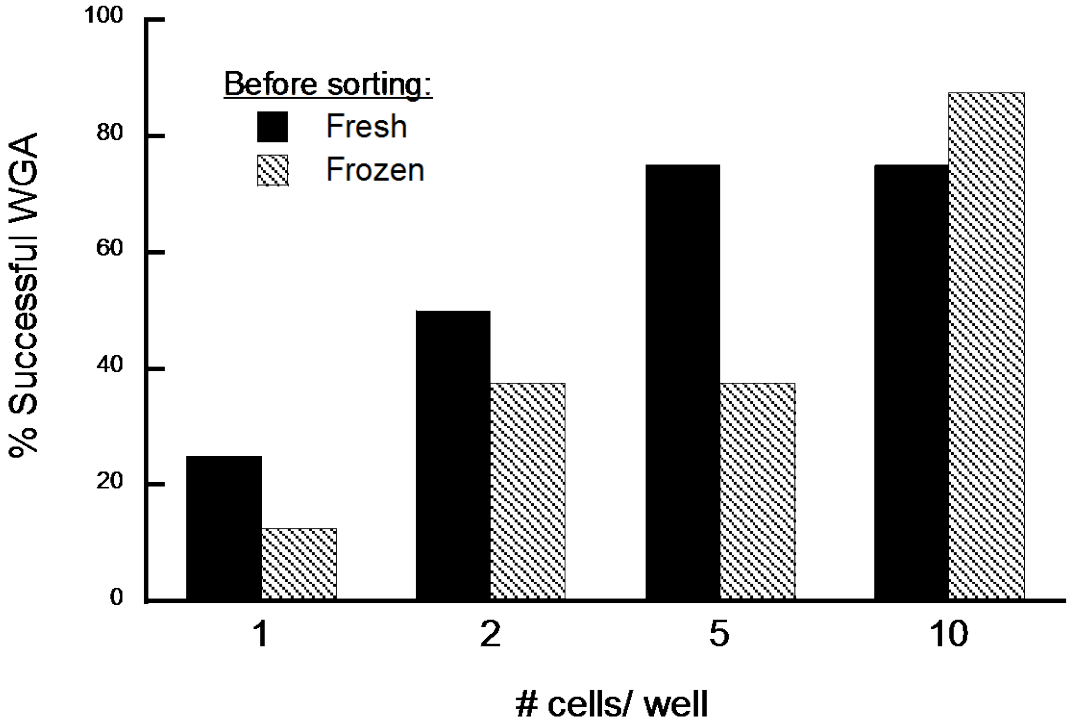


Figure 3. The effect of tumor cell freezing on subsequent WGA.

Maximizing cell placement in wells. We have recently begun altering the sorting parameters (sort pressure and nozzle diameter) with the aim of maximizing the placement of single cells in wells. (See plans for next reporting period.)

Specific Aim II. Perform single cell whole exome sequencing on 40 individual cells isolated from each tumor.
Nothing to Report.

Specific Aim III. Using the whole exome sequence data, analyze phylogenetic relationships of tumor cells and develop a prognostic classifier.
Nothing to Report.

Specific Aim IV. Validate the prognostic classifier developed in Specific Aim III in an independent blinded study.
Nothing to Report.

Opportunities for training and professional development
Nothing to Report.

How results were disseminated to communities of interest

Nothing to Report.

Plans for next reporting period
Prior to the initiation of whole exome sequencing on 40 individual cells from each tumor in Specific Aim II, we will optimize the processes related to single tumor cell capture and WGA. We have recently completed a sort of A549 (lung adenocarcinoma) and H460 (large cell lung cancer) cells using a 100 µm nozzle, 20 psi, and 3 different drop phases. This sort aimed for 1 cell/well, which we will confirm by RT-PCR for GAPDH. Not only does this circumvent the expense of WGA, but it permits us to determine the percentage of wells that actually receive a cell. If this shows that at least 50% of the wells contain a cell, we will then carry out a sort of isolated and CD45RA-stained tumor cells at 1 cell/well, using the same sort parameters that resulted in the greatest number of wells receiving a cell. The sort hit rate will be determined once again by GAPDH RT-PCR. A hit rate of at least 50% will allow us to proceed with tumor cell isolation, sorting, and WGA without GAPDH RT-PCR confirmation. We anticipate completing these experiments in the next several weeks, which will enable us to initiate the collection of whole exome sequence data on individual tumor cells for Specific Aim II.

4. Impact

Impact of the development of the principal discipline of the project
Nothing to Report

Impact on other disciplines
Nothing to Report

Impact on technology transfer
Nothing to Report

Impact on society beyond science and technology
Nothing to Report

5. Changes/Problems
The main challenge we have encountered relate to optimizing single cell capture and WGA of isolated tumor cells. Obtaining fresh tissue has not been an issue. We anticipate completing the technical issues of single cell capture and WGA within a month, at which time we will begin Specific Aim II. Given that the technical issues have taken longer than expected to optimize, it is possible that we may not be able to complete Specific Aim IV, the validation of the prognostic classifier, within the original time frame. We will, of course, carry out the validation but this may require an extension.

6. Products
None to date

7. Participants & Other Collaborating Organizations
E. B. Gottlin, investigator, performed tumor cell isolation, 2.4 cal. months
S.G. Gregory, investigator, supervised WGA, 0.48 cal. months
E.F. Patz, Jr., PI, 1.356 cal. months
EA Burns, Lab Assistant, 2.4 cal. months

No other collaborating organizations; no change in active support of the PI.

8. Special Reporting Requirements
None

9. Appendices
None