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TITLE: Evaluation of Biomarkers Predictive of Benefit from the PD-1 Inhibitor MK-3475 in Patients with Non-Small Cell Lung Cancer and Brain Metastases

PRINCIPAL INVESTIGATOR: Sarah B. Goldberg, MD

CONTRACTING ORGANIZATION: Yale University

New Haven, CT 06510

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PREPARED FOR: U.S. Army Medical Research and Materiel Command

Fort Detrick, Maryland 21702-5012

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Sarah B. Goldberg, MD

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

Yale University
333 Cedar Street
New Haven, CT 06520

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**14. ABSTRACT**

Immunotherapies inhibiting the Programmed Death-1 (PD-1) axis can result in dramatic responses and durable benefit in patients with non-small cell lung cancer (NSCLC). However, the overall response rate is only 20-30% and there is no clearly-defined biomarker that predicts which patients are most likely to benefit. Moreover, patients with NSCLC and brain metastases represent a population for which there are limited treatment options, and these patients are typically excluded from immunotherapy clinical trials or require local therapy prior to study enrollment. Therefore we conducted a trial of the PD-1 inhibitor pembrolizumab (MK-3475) in patients with NSCLC and untreated brain metastases. The objective of this proposal was to study the immunophenotypic characteristics of primary lung tumors, brain metastases and extra-cerebral metastases with the goal of determining the variability across sites, and to study tumor- and blood-based biomarkers to establish predictors of immunotherapy benefit. We hypothesized that identifying biomarkers predictive of benefit to immunotherapy in patients with NSCLC and brain metastases would result in improved patient outcomes. Over the first two years of the grant, we optimized the assays to be used to study, compiled the cohort of paired tumor samples, constructed the tissue microarray, accrued patients with NSCLC and untreated brain metastases to the clinical trial with pembrolizumab, obtained both blood and tumor tissue samples from these patients, and began to analyze these samples. Additionally, the PI had the opportunity to learn the laboratory skills necessary to complete this project. Over the past year, we have completed accrual to the clinical trial and collected the final tissue and blood samples. We tested the tissue microarray and trial patient samples for various immune biomarkers and analyzed them to determine whether they can predict for benefit from immunotherapy. We have also optimized additional assays to be used for further testing on this cohort of patients. Our work on the clinical trial and comparing tumor infiltrating lymphocytes and biomarker expression has been presented at national meetings, and several publications are preparation.

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1. **INTRODUCTION:**

Lung cancer is the leading cause of cancer death in the United States, resulting in more than 160,000 deaths each year. The majority of patients with lung cancer have non-small cell lung cancer (NSCLC) and present with disease at an advanced stage when cure is not possible. Approximately 30% of these patients develop brain metastases at some point during their clinical course. Typically these patients have more limited survival than patients without brain metastases, and many undergo surgery or radiation therapy that can have lasting neurologic toxicity. In recent years we have seen dramatic responses to a new class of therapeutics that target the immune system, specifically with agents targeting the PD-1 axis. Among these agents, the PD-1 inhibitor pembrolizumab has been found to be a safe and effective treatment for a subset of patients with NSCLC. Although the overall response rate is 20-30% with the PD-1 agents, it is unknown whether these agents benefit patients with brain metastases and there is no clearly defined predictive biomarker that determines which patients are most likely to benefit from treatment. We have conducted an investigator-initiated trial at our institution of the PD-1 inhibitor pembrolizumab (MK-3475) in patients with untreated brain metastases from NSCLC (NCT 02085070). The tumor biopsy specimens and blood samples from patients on the trial form the basis for this proposal with the goal of identifying predictive biomarkers for response to PD-1 inhibitors in patients with NSCLC and untreated brain metastases. Additionally, among the putative predictive biomarkers, it is unknown whether expression is consistent at various sites of disease, including in the CNS, where the tumor microenvironment may alter marker expression. Understanding biomarker variability is critical as we explore which patients derive benefit from treatment.

2. **KEYWORDS:**

- NSCLC
- Immunotherapy
- PD-1
- PD-L1
- Brain metastases
- Biomarker

3. **ACCOMPLISHMENTS:**

a. **What were the major goals of the project?**

The major goals of the project are to identify biomarkers that are predictive of response to PD-1 inhibitors in patients with NSCLC and untreated brain metastases, as well as to determine whether biomarker expression is consistent at various sites of disease.

Completion dates and estimates of the percentage of completion for each of the major tasks in the Statement of Work are as follows:

| | **Timeline** (months) | Percent accomplished |
|---|---|---|
| **Major Task 1:** Obtain HRPO approval | 1-6 | Completed |
| **Specific Aim 1: To examine the immunophenotype variability in lung cancer** | | |
| **Major Task 2:** Analyze the immunophenotypic pattern in CNS metastases compared to other distant sites of disease and primary versus metastatic disease sites. | 1-28 | 100% |
| **Specific Aim 2: To determine tissue- and blood-based biomarkers predictive of response to immunotherapy** | | |
| **Major Task 3:** Tissue-based predictive biomarker evaluation | 12-32 | 100% |
| **Major Task 4:** Blood-based predictive biomarker evaluation | 12-35 | 100% |

b. **What was accomplished under these goals?**

The objective of this grant is to identify biomarkers predictive of benefit to immunotherapy in patients with NSCLC and brain metastases and delineate immunphenotypic patterns at various sites of disease. We proposed two aims to achieve these objectives:

**<u>Specific Aim 1</u>: To examine the immunophenotype of NSCLC and the variation at different sites of disease.** To achieve this goal we planned to study expression of checkpoint stimulators and inhibitors in both tumor cells and tumor infiltrating lymphocytes (TILs) at various sites of disease to determine the variability across tumor sites.

During the first year of this grant, I learned the laboratory techniques necessary to carry out the tasks required for completion of the proposed studies, optimized the assays to use immunofluorescence to study immune markers, and started the process of building a tissue microarray (TMA) of tumor samples. We then reviewed potential tissue samples and confirmed the cohort of paired samples to be included in the tissue microarray. The

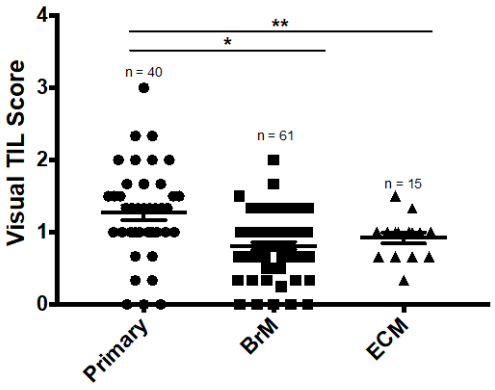
TMA was constructed and includes both paired and unpaired tissue samples. We reviewed patient demographic and treatment outcome information to correlate with the tissue analysis.

Over the course this grant, we utilized quantitative immunofluorescence to analyze this TMA. We had sufficient data from 94 patients with tissue samples including 40 primary lung cancers, 63 brain metastases, and 15 extracranial metastases. Paired samples included primary-brain metastases from 11 patients and brain-extracranial metastases from 12 patients. TIL density was determined by a semi-quantitative pathologist-based, scoring system using H&E preparations. Multiplexed quantitative immunofluorescence was used to evaluate PD-L1, CD4 for helper T-cells, CD8 for cytotoxic cells, and CD20 for B-lymphocytes. Signal for each marker was measured in marker-selected tissue compartments using the Automated Quantitative Analysis (AQUA) platform. We studied the association between markers and major clinicopathologic variables, including overall survival. This allowed for spatially resolved, multiplexed analysis to compare PD-L1 and major TIL subsets in primary lung cancers, brain metastases, and extracranial metastases.

We determined that of the 94 patients, histology included adenocarcinoma in 62.5%, squamous cell carcinoma in 11.5%, small cell in 9.4%, and other NSCLC in 16.7%. We found that TIL density by pathologist read was significantly lower in brain metastases compared with primary lung tumors ($p<0.0001$, Figure 1). Brain metastases had significantly lower levels of CD4+ T-cells ($p=0.0416$), CD8+ T-cells ($p=0.0003$), and CD20+ B-lymphocytes ($p=0.0058$) than primary lesions (Figure 2). Levels of tumor PD-L1 were comparable between brain metastases and primary lung tumors or extracranial ($p>0.05$, Figure 3). However, PD-L1:CD8 ratios were significantly higher in brain metastases compared with primary tumors ($p=0.0024$) or extracranial metastases ($p=0.0322$) without differences in PD-L1:CD4 ratios ($p>0.05$). We also analyzed that patients who had paired sample available and noted demonstrated similar trends, though statistical significance was not achieved given the relatively small sample size. Additionally, we assessed patient outcomes, and found that there was no association

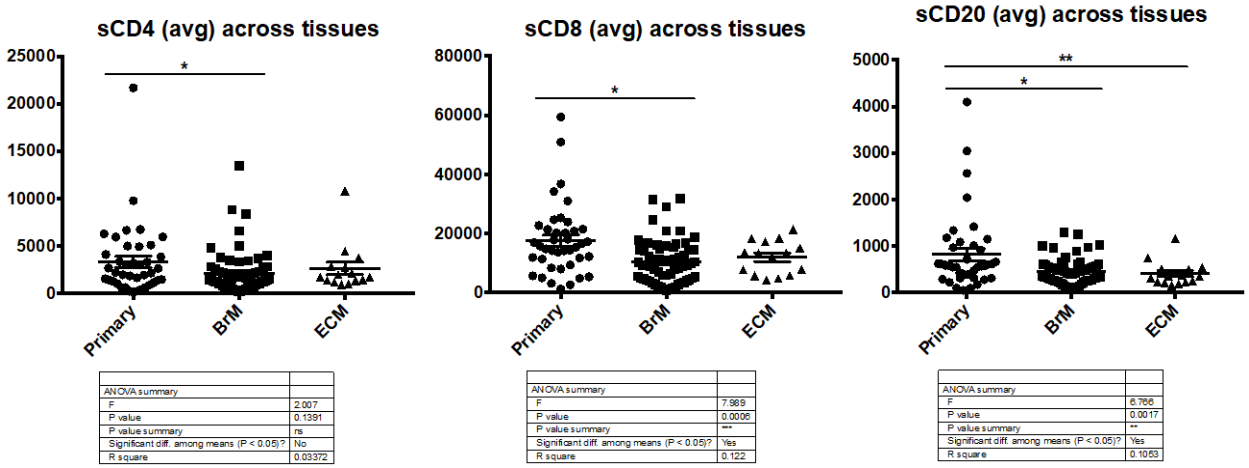
observed between overall survival and TIL density, levels of TIL subsets, or PD-L1 expression (Figure 4).

Figure 1. Tumor infiltrating lymphocyte (TIL) density by pathologist read




| ANOVA summary | |
|---|---|
| F | 10.53 |
| P value | <0.0001 |
| P value summary | **** |
| Significant diff. among means (P < 0.05)? | Yes |
| R square | 0.1571 |

Figure 2. Tumor infiltrating lymphocyte (TIL) subsets in the stroma including CD4, CD8 and CD20 by quantitative immunofluorescence (QIF)

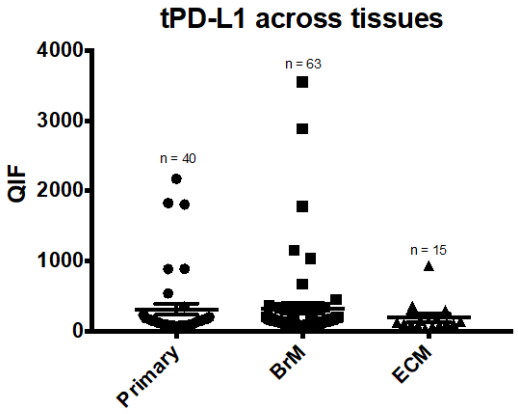



| ANOVA summary | |
|---|---|
| F | 2.007 |
| P value | 0.1391 |
| P value summary | ns |
| Significant diff. among means (P < 0.05)? | No |
| R square | 0.03372 |

| ANOVA summary | |
|---|---|
| F | 7.989 |
| P value | 0.0006 |
| P value summary | *** |
| Significant diff. among means (P < 0.05)? | Yes |
| R square | 0.122 |

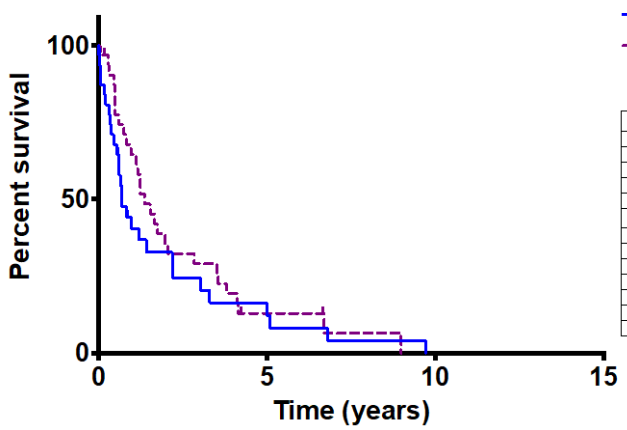
| ANOVA summary | |
|---|---|
| F | 6.766 |
| P value | 0.0017 |
| P value summary | ** |
| Significant diff. among means (P < 0.05)? | Yes |
| R square | 0.1053 |

Figure 3. PD-L1 expression in the tumor (tPD-L1) by quantitative immunofluorescence (QIF) in different tumor specimens including the primary lung mass (Primary), brain metastasis (BrM), and extracranial metastasis (ECM).




| ANOVA summary | |
|---|---|
| F | 0.3831 |
| P value | 0.6826 |
| P value summary | ns |
| Significant diff. among means (P < 0.05)? | No |
| R square | 0.006618 |

Figure 4. Overall survival of patients by tumor PD-L1 expression (tPD-L1) in the brain

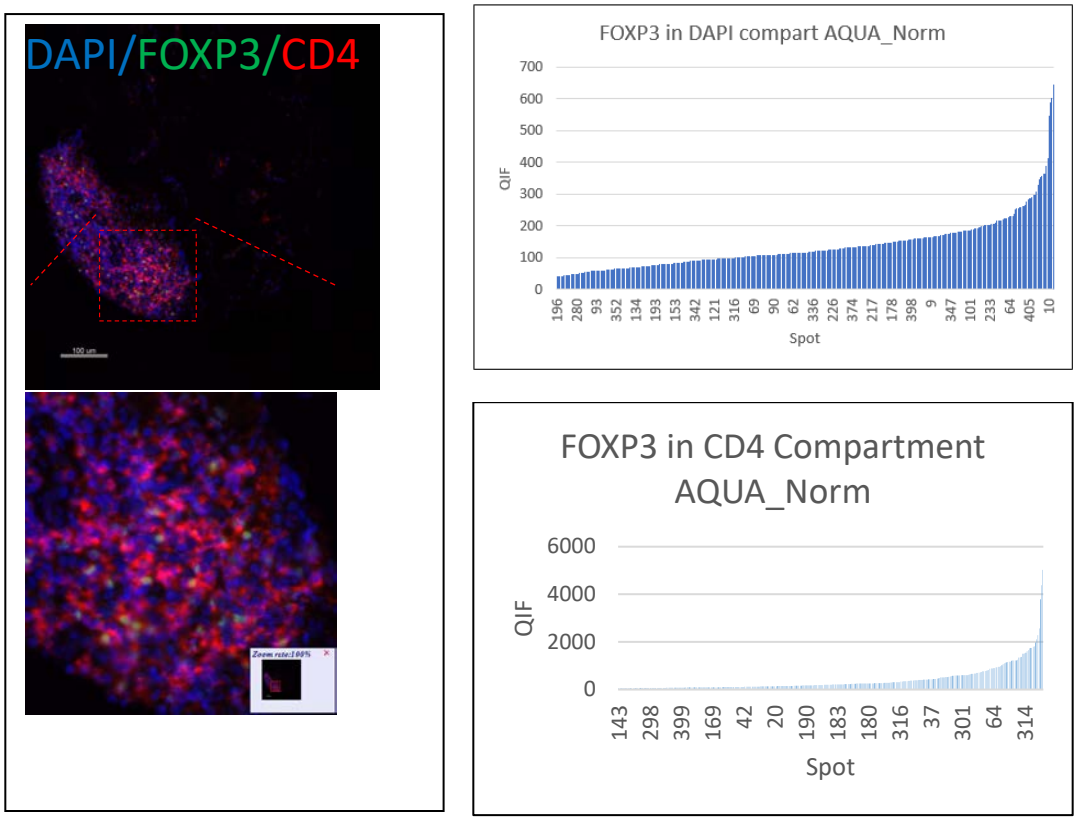



| | |
|---|---|
| Log-rank (Mantel-Cox) test | |
| Chi square | 0.8122 |
| df | 1 |
| P value | 0.3675 |
| P value summary | ns |
| Are the survival curves sig different? | No |
| | |
| Gehan-Breslow-Wilcoxon test | |
| Chi square | 2.647 |
| df | 1 |
| P value | 0.1037 |
| P value summary | ns |
| Are the survival curves sig different? | No |

From this data we have determined that despite having lower levels of major TIL subsets, lung cancer brain metastases displayed similar PD-L1 expression compared with lung primary cancers and extracranial metastases. The latter indicates differences in the adaptive immune modulation of PD-L1 in brain metastases compared with extracranial tumors, suggesting alternative TIL-independent mechanisms sustaining PD-L1 expression in brain metastases. We have presented this work at the ASCO annual meeting in 2018, and will also be presented an updated analysis at the SITC annual meeting later this year. We are preparing a manuscript for publication.

In addition to the data we have generated above, we have continued to validate additional targets that we think will be important to examine in the brain microenvironment. Specifically, we have successfully validated FOXP3, a marker of regulatory T cells. The assay has been challenging to validate and took many months. Fortunately, we were recently able to complete the validation and the antibody was used to stain the brain metastasis TMA (Figure 5). We will continue work on this project by analyzing this data to determine whether FOXP3 expression differs between various sites of disease.

Figure 5. Quantitative immunofluorescence (QIF) of FOXP3. Left panels demonstrating staining of DAPI in blue, FOXP3 in green, and CD4 in red. Right panels show the variable expression of FOXP3 in various tumor samples in the DAPI (top) and CD4 (bottom) compartments.

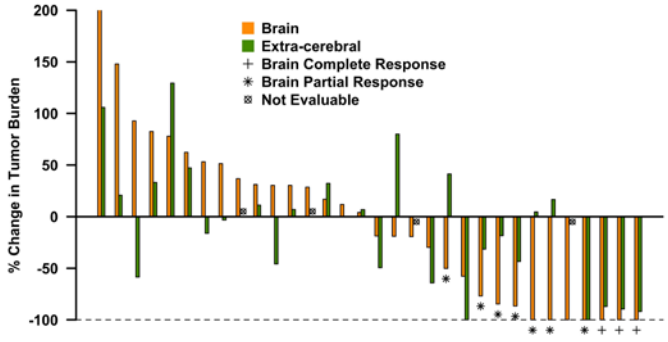


**<u>Specific Aim 2</u>: To determine tissue- and blood-based biomarkers predictive of response or resistance to the PD-1 inhibitor pembrolizumab in patients with NSCLC and untreated brain metastases treated on a prospective Phase II clinical trial.** We proposed to study the immunophenotypic pattern of NSCLC tumor samples including T-cell infiltration and immune marker expression as well as blood-based biomarkers.

Tissue and blood samples for these studies were obtained from patients on the clinical trial at Yale "A phase 2 study of MK-3475 in patients with metastatic melanoma and non-small cell lung cancer with untreated brain metastases." Since the trial opened in March 2014, we have screened 71 patients and enrolled and treated 41 lung cancer patients on the trial. To our knowledge, this is the largest cohort of patients treated on a prospective trial of immunotherapy for patients with lung cancer and untreated brain metastases.

We have found that pembrolizumab can have activity in the CNS (Figure 6) and may be a safe and active treatment option for patients with small, asymptomatic brain metastases. We observed durable benefit and long-term survival in patients with untreated brain metastases from NSCLC, with 31% of patients living more than 2 years after initiation of therapy, which is comparable survival to patients on other trials with pembrolizumab that required pre-treatment of brain metastases.

Figure 6. Brain metastasis and extracranial responses from patients with non-small cell lung cancer treated on the clinical trial with pembrolizumab for untreated brain metastases



We have analyzed pre-treatment tumor tissue from patients on this trial. We have reviewed the tissue samples to determine the quality of the tumor tissue and found that most cases had sufficient tissue for correlative studies. Using quantitative immunofluorescence (QIF), we assessed tumor infiltrating lymphocyte subsets and PD-L1 expression in tumor tissue from trial patients (Figure 7). We broadened our methods to included not just QIF, but also gene expression profiling using the NanoString nCounter Gene Expression Assay. This was chosen based on the small amount of genetic material needed and the potential for detecting expression of a large number of genes (~800). See Figures 8 and 9 for details.

Figure 7. Survival of patients on the clinical trial of pembrolizumab based on expression of tumor infiltrating lymphocytes (CD4, CD8 and CD20).

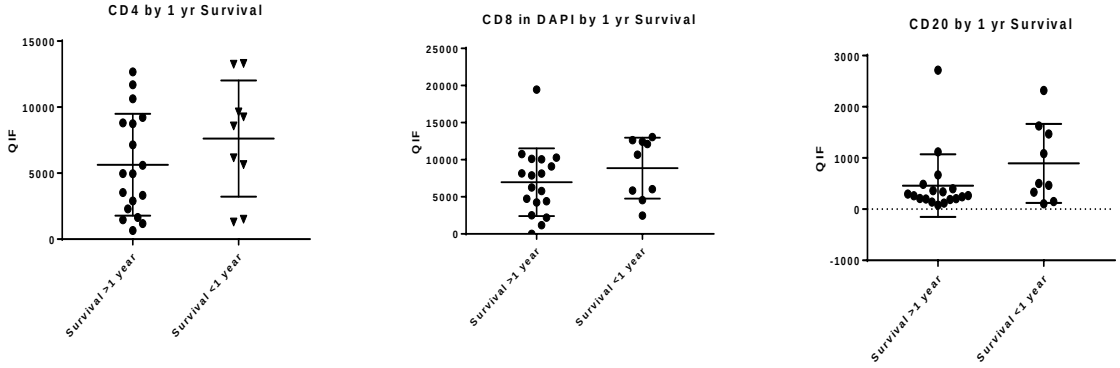


Figure 8. Transcriptome analysis of immune pathway genes in patients with brain metastases treated on the trial of pembrolizumab. Patients are divided by response in the brain, body and overall.

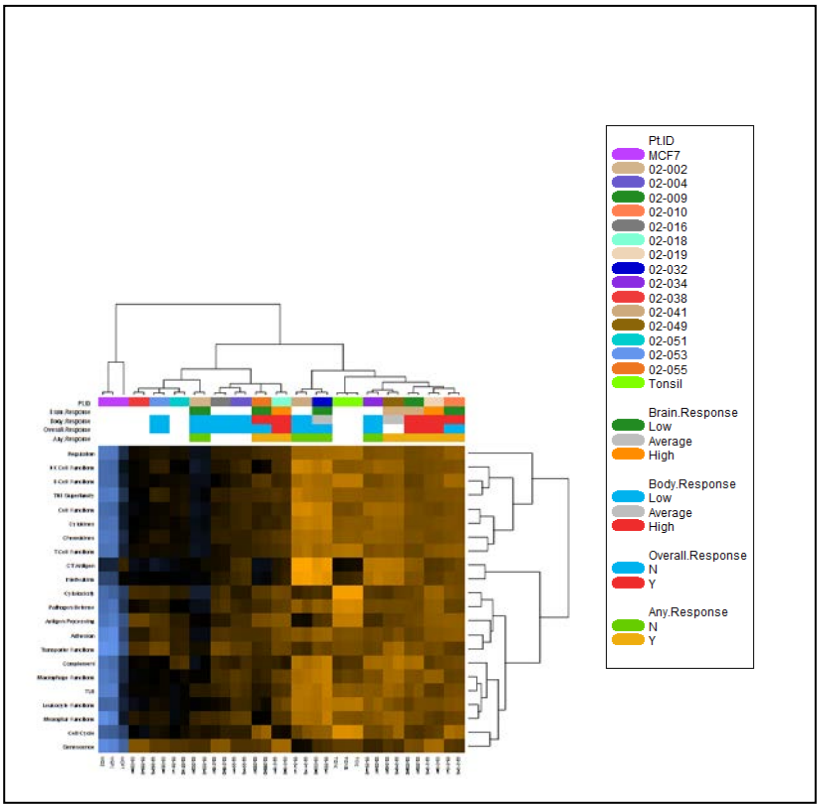
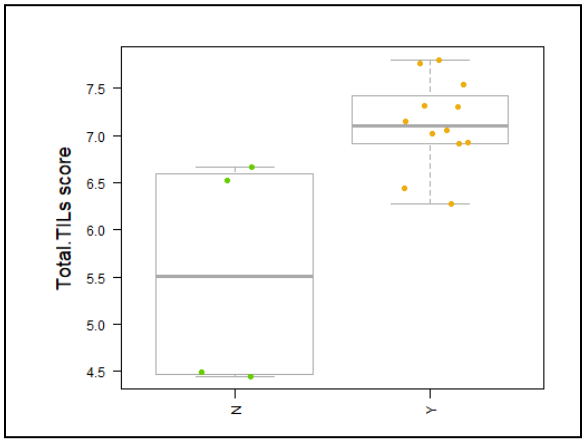
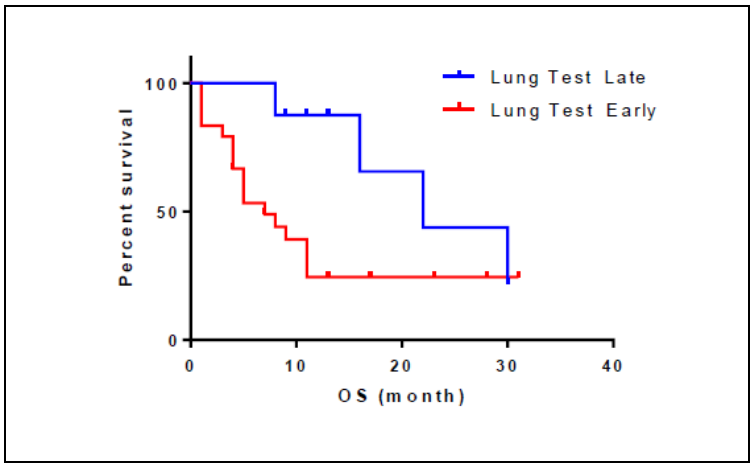



Figure 9. Transcriptome analysis of Tumor Infiltrating Lymphocytes (TILs) in patients with brain metastases treated on trial with pembrolizumab, divided by whether they achieved a response to therapy.



We have also examined blood-based predictive biomarkers in patients on the clinical trial of pembrolizumab. Blood was collected at multiple timepoints, including prior to the start of study treatment and throughout the course of treatment on trial. We analyzed pre-treatment samples from 32 patients to a mass-spectrometry-based proteomic test to determine whether we could predict clinical outcomes based on the results. The test divides patients into groups that potentially could predict for better or worse outcomes. We found that there was a trend towards predicting overall survival (see Figure 10 below) but it was not statistically significant, HR 0.357 (95% CI 0.118-1.081, p=0.0624), possibly because of the small sample size. We presented this data at the Society for Immunotherapy of Cancer Conference.

Figure 10. Overall survival in patients with brain metastases treated with pembrolizumab, divided into predictive groups based on a mass-spectrometry-based proteomic test.



*c.* **What opportunities for training and professional development has the project provided?**

I have had ample opportunity for training and professional development during the first two years of this project. I have dedicated 40% of my effort to this project as well as additional time to other research projects. Dedicated time at this early stage of my career is invaluable and has allowed me the opportunity to work towards my research goals in a mentored setting.

I have worked closely with my mentor Dr. Roy Herbst and have learned a great deal from his guidance. We continue to meet weekly during a one-on-one session to discuss my research progress and goals. Throughout this grant period he has guided me in my translational and clinical projects and has taught me a great deal about lung cancer research. Additionally, I have attended weekly meetings with the thoracic research team which includes participation by basic scientists, clinical researchers, and research staff.

I have also continued to work closely with my collaborators Drs. Harriet Kluger and Lucia Jilaveanu on the basic science aspects of this project. I meet frequently with Drs. Kluger and Jilaveanu to carry out the tasks for this grant and to learn the skills required.

I have had many opportunities for professional development during this grant period. I have participated in weekly translational lung cancer meetings at Yale and weekly Cancer Center Grand Rounds. I have attended the ASCO Annual Meeting, the IASLC World Conference on Lung Cancer, the IASLC Targeted Therapies in Lung Cancer Meeting, and the Society for Immunotherapy of Cancer Conference.

**d. How were the results disseminated to communities of interest?**

Nothing to report.

**e. What do you plan to do during the next reporting period to accomplish the goals?**

N/A, this is the final report

4. **IMPACT:**

   a. **What was the impact on the development of the principal discipline(s) of the project?**

The results from the clinical trial of patients with brain metastases from lung cancer or melanoma treated with pembrolizumab was published during the first year of this grant. This trial was the first of its kind to demonstrate that immunotherapy can be effective in the brain. Prior to this study, patients with untreated brain metastases were typically excluded from clinical trials with immunotherapy agents. The knowledge gained from our study is likely to make an impact on the field of oncology as we now know that patients with brain metastases can benefit from immunotherapy.

Since then we have presented additional work from this project at national meetings. We reported updated results from the clinical trial at the ASCO Annual Meeting 2018. We have also been exploring biomarkers from tumor tissue and blood that may be predictive of which patients are most likely to benefit from such treatment, and have presenting this work at SITC 2017 and ASCO 2018. We have an upcoming presenting at SITC 2018 and have several manuscripts in preparation to disseminate the exciting results that we have obtained.

b. **What was the impact on other disciplines?**

Nothing to report

c. **What was the impact on technology transfer?**

Nothing to report

d. **What was the impact on society beyond science and technology?**

Nothing to report

5. **CHANGES/PROBLEMS:**

a. **Changes in approach and reasons for change**

Nothing to report.

b. **Actual or anticipated problems or delays and actions or plans to resolve them**

We have been delayed in finalizing manuscripts for publication. The work has been prepared in abstract form, however we are still preparing the manuscripts. We expect to have 2-3 manuscripts completed and ready for submission in the next 3 months.

**Changes that had a significant impact on expenditures**

Nothing to report

c. **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to report.

6. **PRODUCTS:**

a. **Publications, conference papers, and presentations**

- **Journal publications.**

  Goldberg SB, Gettinger SN, Mahajan A, et al. Pembrolizumab for patients with melanoma or non-small-cell lung cancer and untreated brain metastases: early analysis of a non-randomised, open-label, phase 2 trial. *Lancet Oncol* 2016; 17(7):976-83.

- **Books or other non-periodical, one-time publications.**

  Nothing to report.

- **Other publications, conference papers, and presentations.**

  Yale Cancer Center Grand Rounds, March 2017. "Systemic therapy for brain metastases in non-small cell lung cancer."

  Society for Immunotherapy of Cancer Annual Meeting, November 2017, National Harbor, MD. S.B. Goldberg, L. Jilaveanu, H.M. Kluger, V. Chiang, A. Mahajan, B. Xia, M. Ribeiro, H. Roder, J. Roder, C. Oliveira, J. Grigorieva, M. Muller, A. Niemeijer, A. de Langen, R. Schouten, E. Smit. Poster Presentation: "Mass spectrometry-based test predicts outcome on anti-PD-1 therapy for patients with advanced non-small cell lung cancer, including those with brain metastases."

  American Society of Clinical Oncology (ASCO), Annual Meeting, June 2018, Chicago, IL B.Y. Lu, R. Gupta, M. Ribeiro, T. Stewart, V. Chiang, J.N. Contessa, A. Adeniran, H.M. Kluger, L. Jilaveanu, K.A. Schalper, S.B. Goldberg: Online Presentation: "PD-L1 expression and tumor-infiltrating lymphocytes in lung cancer brain metastases."

  American Society of Clinical Oncology (ASCO), Annual Meeting, June 2018, Chicago, IL S.B. Goldberg, S. Gettinger, A. Mahajan, R. Herbst, A. Chiang, R. Lilenbaum, L. Jilaveanu, E. Rowen, H. Gerrish, A. Komlo, W. Wei, V. Chiang, H.M. Kluger. Poster Discussion: "Durability of Brain Metastasis Response and Overall Survival in Patients with Non-Small Cell Lung Cancer (NSCLC) Treated with Pembrolizumab."

  Society for Immunotherapy of Cancer Annual Meeting, November 2018, National Harbor, MD. Accepted for Poster Presentation: B.Y. Lu, R. Gupta, H. Wyatt, M. Ribeiro, T. Stewart, V.L.S. Chiang, J.N. Contessa, A.J. Adeniran, H.M. Kluger, L.B. Jilaveanu, K.A. Schalper, S.B. Goldberg. "Quantitative evaluation of tumor-infiltrating lymphocyte subsets and PD-L1 expression in lung cancer brain metastases."

b. **Website(s) or other Internet site(s)**

Nothing to report.

c. **Technologies or techniques**

Nothing to report.

d. **Inventions, patent applications, and/or licenses**

Nothing to report.

*e.* **Other Products**

Nothing to report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

a. **What individuals have worked on the project?**

| | |
|---|---|
| Name: | *Sarah Goldberg* |
| Project Role: | *Principal Investigator* |
| Researcher Identifier (e.g. ORCID ID): | |
| Nearest person month worked: | *5* |
| Contribution to Project: | *Dr. Goldberg proposed the work for this project and is responsible for overseeing the studies performed. She has accrued patients to the clinical trial, identified cases to include in the cohort, and has gathered blood and tumor tissue that will be analyzed.* |
| Funding Support: | *NIH/NCI*<br>*Boehringer Ingelheim* |

b. **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Additional funding support activated since the previous report:

| Funding Agency | Award Number | Project Title | Award Activation Date |
|---|---|---|---|
| **NIH/NCI** | 1R01EB025468-01A1 | Quantitative Low-dose PET Imaging | 07/24/18 |
| **NIH/NCI** | 1R01CA224140-01A1 | Personalized Task-based respiratory motion correction for low-dose PET/CT | 07/02/18 |
| **NIH/NCI** | 5R01CA197486-03 | Circulating DNA as a Marker of Treatment Efficacy and Failure in Lung Cancer | 04/01/16 |

c. **What other organizations were involved as partners?**

Nothing to report

8. **SPECIAL REPORTING REQUIREMENTS**
   a. **COLLABORATIVE AWARDS:** Not applicable
   b. **QUAD CHARTS:** Not applicable
9. **APPENDICES:** Not applicable