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TITLE: **Cellular Plasticity and Heterogeneity of EGFR Mutant Lung Cancer**

PRINCIPAL INVESTIGATOR: **Katerina Politi, PhD**

CONTRACTING ORGANIZATION: **Yale University**
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14. ABSTRACT

Phenotypic changes have been observed in EGFR mutant lung cancers that become resistant to targeted therapies. This grant aims to test the hypothesis that LUAD cells can transdifferentiate upon TKI treatment and to determine the cellular and molecular mechanisms that regulate this process. We proposed to trace the cellular origin of EGFR mutant SCLC in genetically engineered mouse models by using lineage tracing to test whether EGFR mutant LUAD cells can transdifferentiate along the neuroendocrine lineage and to establish the molecular mechanisms that cause TKI resistance in EGFR mutant SCLCs. To date, we have made progress towards generating mouse models for the proposed lineage tracing experiments and have begun genomic studies of pre- and post-treatment EGFR mutant tumors that transformed to SCLC following TKI treatment.

15. SUBJECT TERMS
lung cancer, EGFR mutations, drug resistance, targeted therapies, mouse models, phenotypic changes

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1. INTRODUCTION:

Lung cancer is the leading cause of cancer death worldwide. In recent years, significant progress has been made in understanding the genetic alterations present in lung cancer. Importantly, drugs that specifically target several of these alterations have been developed and benefit a subset of patients. However, these targeted therapies are almost always effective only for a limited amount of time. Drug resistance can emerge for a variety of reasons and effective strategies to counter this problem have yet to be developed. For example, a protein called the Epidermal Growth Factor Receptor (EGFR) is altered in 10-15% of lung adenocarcinomas, a subtype of lung cancer. Patients with tumors that have this alteration respond very well to therapies that prevent the EGFR from functioning in cancer cells. However, on average after a year of drug-treatment the tumors begin to grow again because they have acquired resistance to these therapies. One of the mechanisms of resistance involves a phenotypic change in the tumor cells from lung adenocarcinoma to small cell lung cancer. How this transition occurs is unknown. In this proposal, we are studying the molecular and cellular mechanisms that cause this transition to occur in preclinical models and patient specimens. Our goal is to discover new targets that can inform the design of clinical trials to counter these phenotypic conversions.

2. KEYWORDS:

- lung cancer
- EGFR mutations
- drug resistance
- phenotypic changes
- targeted therapies
- mouse models

3. ACCOMPLISHMENTS:

a. What were the major goals of the project?

The major goals of the project are to trace the cellular origin of EGFR mutant SCLC in genetically engineered mouse models and to establish the molecular mechanisms that cause TKI resistance in EGFR mutant SCLCs. Estimates of the % completion for each of the major tasks in the SOW for the grant are shown below.

Specific Aim 1 *Trace the cellular origin of EGFR mutant SCLC in genetically engineered mouse models*	**Timeline (months)**	**Percent accomplished**
Major Task 1 Generate experimental animals for experiments proposed in Aim 1 to determine whether transdifferentiation from lung adenocarcinoma to SCLC occurs.	4	75%
Major Task 2 Analyze the phenotype of *TetO-EGFR^{L858R}*; *Sftpc-CreER^{T}; CAG-LSL-rtTA-IRES-mKate*; *Ascl1-GFP* and *TetO-EGFR^{L858R}*; *Nkx2.1-cre; CAG-LSL-rtTA-IRES-mKate*; *Ascl1-GFP* mice.	4-12	25%

Specific Aim 2 *Establish the molecular mechanisms that cause TKI resistance in EGFR mutant SCLCs*		
Major Task 3 Perform RNA sequencing of *EGFR* mutant lung adenocarcinomas and SCLC from patient and mouse samples	0-12	75%
Major Task 4. Functional characterization of genes that contribute to the resistance and transdifferentiation of EGFR mutant lung cancers	4-12	25%

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

The objective of this grant was to test the hypothesis that lung adenocarcinoma cells can transdifferentiate to small cell lung cancer in the presence of tyrosine kinase inhibitors and to determine the cellular and molecular mechanisms that regulate this process. We developed two aims to achieve this objective.

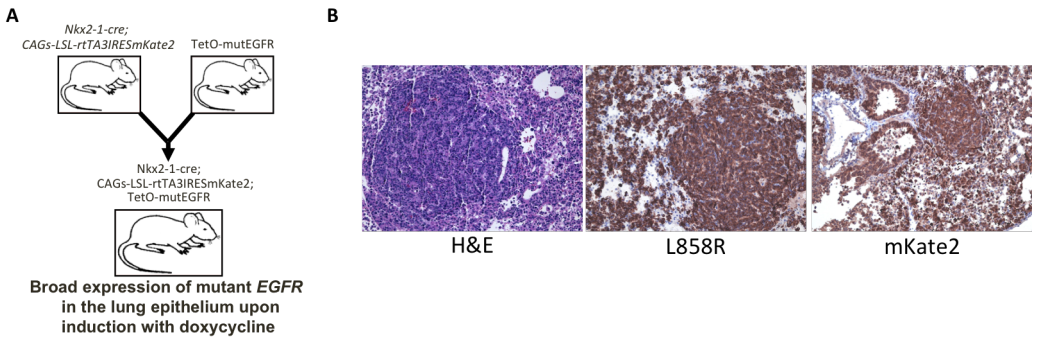

Figure 1. ***N***kx2-1-cre; ***C***AGs-LSL-rtTA3IRESmKate2; TetO-***E***GFR^{L858R} (NCE) mice develop lung adenocarcinomas upon induction with doxycycline. **A.** Schematic representation of the cross used to generate NCE mice. Upon Nkx2-1 directed cre-mediated recombination, rtTA is expressed and turns on mutant EGFR expression in Nkx2-1-expressing cells when doxycycline is present. **B.** Hematoxylin and eosin-stained section of a lung tumor in an NCE mouse (left). Immunohistochemical staining of a lung from an NCE mouse for EGFR^{L858R} and mKate2 (time on doxycycline = 6 weeks). Images taken at 10x magnification.

Specific Aim 1: Trace the cellular origin of EGFR mutant SCLC in genetically engineered mouse models. We proposed to set-up up crosses of appropriate mouse models that will allow us to perform lineage tracing and identify the cellular origin of EGFR mutant SCLC. We have made progress in generating the mice for these studies. First of all, we have established that *TetO-EGFR^{L858R}; CAG-LSL-rtTA-IRES-mKate2* mice in the presence of cre recombinase and doxycycline develop lung adenocarcinomas similar to the very well-studied tumors in *TetO-EGFR^{L858R}; CCSP-rtTA* mice (**Figure 1**). This important step was followed by the acquisition of *Sftpc-CreER^{T}* mice. We did encounter delays in the acquisition of *Ascl1-GFP* mice due to the presence of a viral infection in the colony of the donating lab. We were able to obtain these mice by purchasing and having them re-derived from Jackson labs and can now perform the proposed experiment.

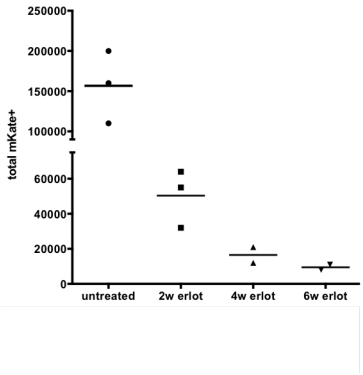

Figure 2. Successful sorting of mKate2 positive cells from the lungs of untreated and erlotinib-treated tumor-bearing mice.

We have also optimized our technique for fluorescence activated cell sorting of lung cells and have shown that we can efficiently detect mKate2 in the lungs of mice even after 6 weeks of treatment with erlotinib (**Figure 2**). This very important result highlights how we can retrieve rare cells after treatment from our mouse models for downstream analysis.

Specific Aim 2: Establish the molecular mechanisms that cause TKI resistance in EGFR mutant SCLCs. To achieve this goal, we have been working to characterize the genomic, transcriptomic and epigenomic landscape of EGFR mutant SCLCs and their corresponding pre-treatment LUADs. These are very rare specimens. Through our Yale rebiopsy program however, we have collected specimens from six patients that fall into this category. Whole exome sequencing has been performed on three of the cases and has revealed that all of the EGFR mutant SCLCs harbor TP53 mutations and 2/3 harbor RB1 mutations. Indeed, it is likely that RB loss is a critical event in the transformation of the LUADs to SCLC. In two pre- and post-treatment pairs we identified 10-30 acquired mutations present in the SCLC but not present in the pre-treatment NSCLC. Amongst the most promising candidates that we have identified and that could potentially-through mutation- contribute to the small cell transformation are: 1) proteins that control transcription including ALX4, EWSR1 and NELFA, 2) signaling proteins such as PPFIA, IBTK, PRKCI, PTPN13 and 3) chromatin modifiers like MLL3 and SETDB1. As next steps we plan to evaluate which of these mutated genes contribute functionally to the transformation of the lung adenocarcinomas to SCLC. In addition, we are also sequencing the additional cases. Importantly from the new cases, we are attempting to generate patient-derived xenografts that could be instrumental for us to gain an understanding of the molecular mechanisms that underlie the conversion of NSCLC to SCLC. In our original proposal, we had planned to perform RNA sequencing of the SCLC transformation cases. Of the 6 cases on hand, one has been RNA sequenced, 2 had insufficient material and the 3 newer cases are pending. Once we have data from these latter cases, we will analyze the data collectively and compare the expression profiles of non-transformed TKI resistant tumors with those of the transformed tumors. Moreover, in collaboration with Charlie Rudin at MSKCC, we are analyzing the epigenomic profiles of these specimens.

In summary, we have made substantial progress towards our goals and importantly this funding allowed us to set-up the models, systems and obtain samples to perform the very high-risk, high-reward experiments that we had proposed. We are now in the very strong position (with the preliminary data generated here) to apply for additional funding to complete this study.

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

Although this project was not formally intended to provide training and professional development opportunities, several of the personnel who have worked on this project have benefitted from it. Amlak Bantikassegn and Maserame Gaefele, for example, worked in the lab in between college and medical school. Through this opportunity they learned about oncology from both a clinical and science perspective and gained laboratory skills that position them well for med school. In addition, everyone working on the project, including myself, have many occasions for professional development by attending weekly lab meetings, a weekly translational lung cancer meeting at Yale, weekly cancer center and pathology grand rounds amongst others.

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?**

Not applicable. We plan to use the data and models obtained through these studies to apply for additional funding to complete the project.

4. IMPACT:

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

Nothing to report.

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.

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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 encountered three major delays. The first, was in obtaining the Ascl1-GFP mice important for our proposed crosses. These mice currently exist in a facility that has had a viral infection and therefore it would be problematic to import them. We identified another source of the mice and obtained them from there, where however they had to be re-derived from embryos which took additional time. We now have the mice in our colony. The second delay, had to do with the fact that RNA sequencing on FFPE specimens is notoriously problematic. However, we were able to optimize the process and obtain high quality data recently. Therefore, we will be performing RNA sequencing on samples of EGFR mutant SCLC in the next couple of months. Third, we did not identify additional cases of transformation to SCLC until recently when we found 3 additional cases, therefore we now have a larger set of samples on which to conduct our analyses.

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

Due to the delays in obtaining some of the mouse models and in optimization of the RNA sequencing pipeline, we did not expend all of the funds during the first year. Funds were present to cover a no-cost extension and we expended these funds during the current year.

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

Nothing to Report

e. **Significant changes in use or care of human subjects**

Nothing to Report

f. **Significant changes in use or care of vertebrate animals.**

Nothing to Report

g. **Significant changes in use of biohazards and/or select agents**

Nothing to Report

6. PRODUCTS:

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

i. **Journal publications.** Nothing to Report

ii. **Books or other non-periodical, one-time publications.** Nothing to Report
iii. **Other publications, conference papers, and presentations.** Nothing to Report

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**

Data generated from whole exome sequencing and RNA sequencing of tumors that have undergone phenotypic conversions at resistance and their pre-treatment specimens is being collected. Upon publication these data will be made available to the research community. Similarly, any cell lines generated in our studies will also be shared. The EGFR transgenic mouse models used in this study are already available through the NIH or directly from my laboratory.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name	*Katerina Politi*
Project Role	*Principal Investigator*
Researcher Identifier	
Nearest person month worked	*2*
Contribution to Project	*Dr. Politi proposed the work for this project and directs the laboratory in which this project is being conducted. She provides direct oversight to all personnel involved in this project's research efforts.*
Funding Support	*NIH/NCI* *Lung Cancer Research Foundation* *AstraZeneca* *Kolltan Pharmaceuticals* *Roche*

Name	*Amlak Bantikassegn*
Project Role	*Post-graduate Research Associate*
Researcher Identifier	

Nearest person month worked	*7*
Contribution to Project	*Mr. Bantikassegn performed the MR imaging of the animals and optimized the lung epithelial cell separation protocol during the first year of the award.*
Funding Support	*NIH/NCI* *AstraZeneca* *Kolltan Pharmaceuticals*

Name	*Mmaserame Gaefele*
Project Role	*Post-graduate Research Associate*
Researcher Identifier	
Nearest person month worked	*1*
Contribution to Project	*In Year 2 of the award, Ms. Gaefele managed the animal colony including the mice allocated to this research project.*
Funding Support	*NIH/NCI* *Kolltan Pharmaceuticals*

Name	*Mary Ann Melnick*
Project Role	*No Change*
Researcher Identifier	
Nearest person month worked	
Contribution to Project	
Funding Support	

Name	*Anna Wurtz*
Project Role	*No Change*
Researcher Identifier	
Nearest person month worked	
Contribution to Project	
Funding Support	

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

Listed below are updates to the active other support of the PI, Katerina Politi, since this award was activated on September 1, 2014. Table 1 provides a status update of the active and pending awards that were reported at the time of this award's activation. Table 2 provides information on awards that were activated during the course of this award, but had not been previously reported as "Pending Support" at the time of this award's activation.

Table 1: Updates to funding support status reported at the time of award activation

			Status at time	

Funding Agency	*Award Number*	*Project Title*	*of award activation*	*Current status*
NIH/NCI	*R01CA120247*	*Mutant EGF Receptor-dependent Lung Cancer in Human Cell Lines and Transgenic Mice*	*Active*	*Completed – May 31, 2015*
NIH/NCI	*R01CA121210*	*Overcoming Acquired Resistance to EGFR Inhibitors in Lung Cancer*	*Active*	*Active*
Labrecque Foundation	*Not Applicable*	*A Translational Pilot Study on Serum Biomarkers of Lung Cancer Using Transgenic Mouse Models of Lung Adenocarcinoma*	*Active*	*Completed – December 31, 2013*
Lung Cancer Research Foundation	*Not Applicable*	*The Influence of Tumor Cell-of-Origin and Heterogeneity on Acquired Resistance to Targeted Therapies in Cancer*	*Active*	*Completed- October 31, 2015*
NIH/NCI	*P50CA196530*	*Targeting the EGF Receptor Pathway in Lung Adenocarcinomas (Yale SPORE in Lung Cancer Project 3)*	*Pending*	*Active*

Table 2: Additional funding support activated during the course of the current award

Funding Agency	*Award Number*	*Project Title*	*Award Activation Date*	*Current Status*
AstraZeneca	*Not Applicable*	*Evaluation of EGFR TKIs Combined with Immunotherapy*	*September 14, 2014*	*Completed- September 18, 2016*
Kolltan Pharmaceuticals	*Not Applicable*	*Establish the Efficacy of KTN3379 in Preclinical Models of EGFR Mutant Lung Cancer*	*February 3, 2015*	*Active*
NIH/NCI	*UM1CA186689*	*Preclinical Studies to Support AZD9291 Project Team Efforts*	*March 1, 2015*	*Active*
NIH/NCI	*Not Applicable*	*A Randomized Phase II/III Trial of Afatinib Plus Cetuximab Versus Afatinib Alone in Treatment Naïve Patients with Advanced, EGFR Mutation Positive Non Small Cell Lung Cancer*	*April 1, 2015*	*Active*
NIH/NCI	*R01CA195720*	*Targeting the Immune System in Mouse Models of Lung Adenocarcinoma*	*April 16, 2015*	*Active*
Roche	*Not Applicable*	*Roche pRED Cancer Immunotherapy and Yale University Collaboration*	*May 1, 2016*	*Active*

What other organizations were involved as partners?

Organization Name:	*Duke University*
Location of Organization:	*353 Nanaline Duke Building* *Box 3709* *307 Research Drive* *Durham, NC 27710*
Partner's contribution to the project:	*In-kind support – provided two breeding pairs of the Sftpc-CreER^T mice*
Organization Name:	*Rudin Lab, MSKCC*
Location of Organization:	*1275 York Avenue* *New York, NY*
Partner's contribution to the project:	*Collaborating in research on the Epigenomic analysis of EGFR mutant SCLCs*

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:** Not applicable
- **QUAD CHARTS:** Not applicable

9. APPENDICES:

- Not applicable