AWARD NUMBER: W81XWH-17-1-0182

TITLE: Adhesion-Dependent Regulation of Mutant K-Ras Protein Levels in Lung Cancer

PRINCIPAL INVESTIGATOR: Anette Claudia Schafer

CONTRACTING ORGANIZATION: Massachusetts Institute of Technology

Cambridge, MA 02139

REPORT DATE: October 2018

TYPE OF REPORT: Final Report

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
October 2018	Final Report	1 Jul 2017 - 30 Jun 2018

4. TITLE AND SUBTITLE

Adhesion-Dependent Regulation of Mutant K-Ras Protein Levels in Lung Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-17-1-0182

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Anette Claudia Schafer
email:
cschafer@mit.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Massachusetts Institute of Technology

Cambridge, MA 02139

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

In many cancers, gain-of-function mutations in K-Ras cause enhanced proliferation and survival leading to tumorigenicity and increased risk of metastasis. Mutant K-Ras is a major driver of metastasis in lung cancer and is highly correlated with poor prognosis. Mutations that give rise to constitutively active K-Ras proteins elevate downstream pathways independent of growth factor stimulation. In clinical and translational cancer research little is known regarding whether the expression of the mutant K-Ras is heterogenous in the primary tumor and, if so, whether this affects metastatic progression. This study was designed to investigate differential protein expression levels of K-Ras in lung cancer and how this can contribute to malignant lung cancer progression. Even though the set-up of some in vitro experiments turned out to be very challenging, we are happy to report that the funds provided by this award have supported the generation of data that have set the stage for further in vivo investigation into the mechanisms that coordinate K-Ras protein levels and with K-Ras mRNA levels.

15. SUBJECT TERMS

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		19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Final Report - LC160614 - "Adhesion-Dependent Regulation of Mutant K-Ras Protein Levels in Lung Cancer":

1) Introduction: In many cancers, gain-of-function mutations in K-Ras cause enhanced proliferation and survival leading to tumorigenicity and increased risk of metastasis. Mutant K-Ras is a major driver of metastasis in lung cancer and is highly correlated with poor prognosis. Mutations that give rise to constitutively active K-Ras proteins elevate downstream pathways independent of growth factor stimulation. In clinical and translational cancer research little is known regarding whether the expression of the mutant K-Ras is heterogenous in the primary tumor and, if so, whether this affects metastatic progression. This study was designed to investigate differential protein expression levels of K-Ras in lung cancer and how this can contribute to malignant lung cancer progression. Even though the set-up of some in vitro experiments turned out to be very challenging, we are happy to report that the funds provided by this award have supported the generation of data that have set the stage for further in vivo investigation into the mechanisms that coordinate K-Ras protein levels and with K-Ras mRNA levels.

2) Keywords: K-Ras, lung cancer, KP mouse model, protein level, ubiquitination

3) Accomplishments:

What were the major goals of the project?:

The overall main goals of the project were to analyze levels of mutant K-Ras protein and mRNA in primary lung tumors and metastases from the KP mouse model relative to hypoxic areas and ECM properties and further to study the dynamic regulation of mutant K-Ras protein levels *in vitro* and in xenografts. Furthermore, we planned to identify adhesion-dependent ubiquitination events within the K-Ras protein in murine and human lung cancer cell lines and to validate that adhesion-dependent ubiquitination regulates mutant K-Ras protein levels and, consequently, cell motility and dissemination.

What was accomplished?: To investigate K-Ras protein heterogeneity, we reported originally differential expression levels in cancer cell lines obtained from the KP (K-Ras$^{LSL-G12D/+}$;p53$^{flox/flox}$) mouse model *in vitro* which was analyzed by by clonal separation and western blot analysis. To further evaluate heterogeneous *in vivo* K-Ras protein levels in tumor sections from the KP mouse model, tumors were localized by H&E as well as dtTomato staining (Fig. 1A). K-Ras protein levels were found to be very heterogeneous with the tumor. Such areas were also found to be positive for the epithelial tumor markers E-Cadherin and Cytokeratin 8 (Fig. 1B). To further evaluate the role of K-Ras mRNA and to visualize the spatial distribution throughout tumor, we used FISH staining to stain for mRNA. With this we were successful to image the mRNA levels in KP tumor. As shown in figure 1C, the K-Ras mRNA levels are localized to

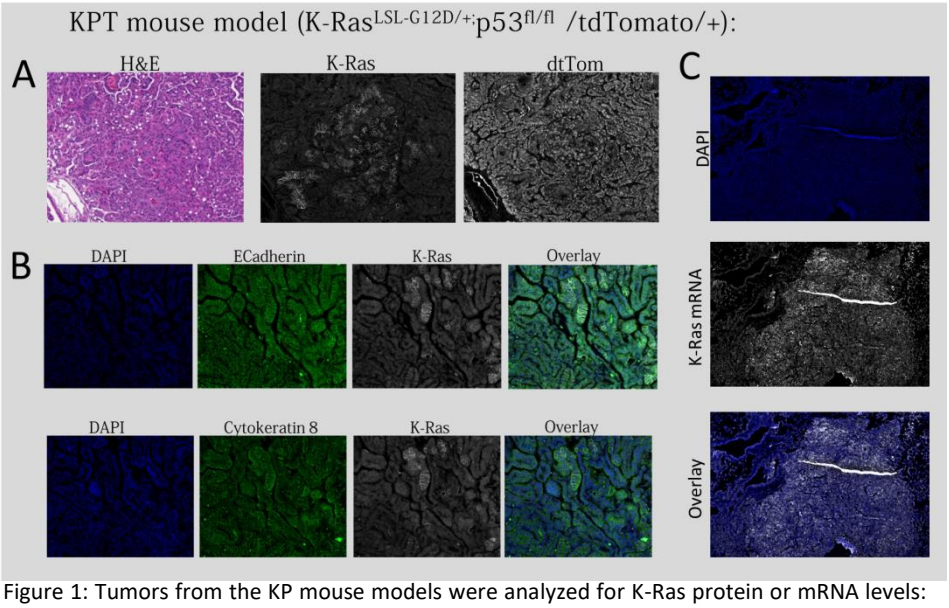
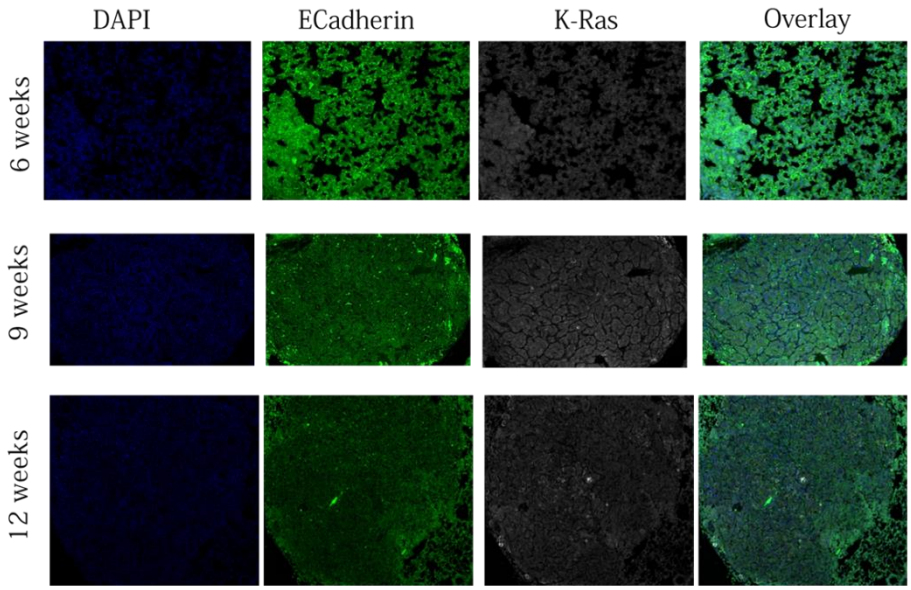


Figure 1: Tumors from the KP mouse models were analyzed for K-Ras protein or mRNA levels: A) KP tumors were stained for K-Ras to visualize heterogeneous protein levels throughout the tumor. To localize the tumor H&E as well as dtTomato staining was performed. B) Sequential tumor sections were stained for K-Ras in combination with E-Cadherin or Cytokeratin 8. Areas showing high K-Ras levels are also positive for epithelial tumor markers. C) FISH staining was performed to visualize K-Ras mRNA distribution in KP tumors.

the tumor areas with low staining in the healthy lung tissue. The signal appears to be not evenly distributed throughout the tumor but overall does not show similar heterogeneous distribution compared to the protein levels. Protein levels seems to display a higher degree of variation (compare Fig. 1A) suggesting at least an additional regulation mechanism controlling K-Ras protein levels.



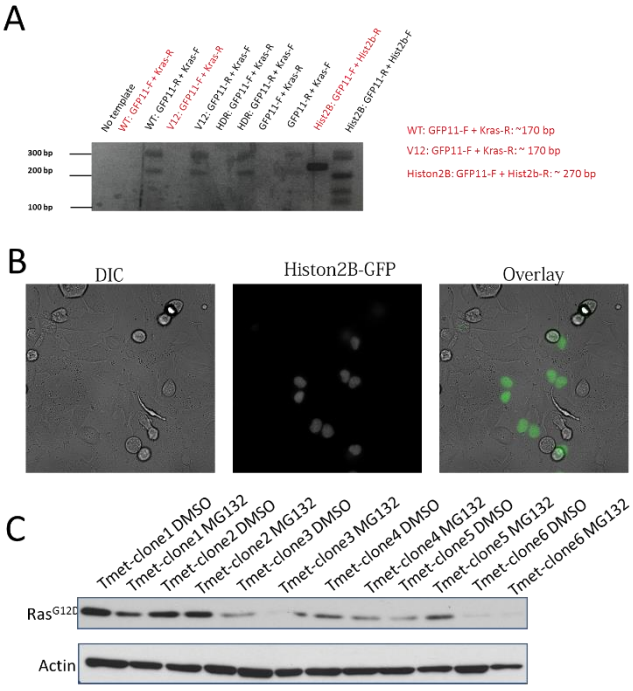
To further evaluate changes in K-Ras protein levels and distribution throughout KP tumors, K-Ras was stained in tumors 6, 9 and 12 weeks after initiation (Fig. 2). Furthermore, to mark tumor areas, E-Cadherin was co-stained. As shown in figure 2, no K-Ras staining is observed in early lesion (6 weeks) but intensities get increased after 9 weeks, as well as heterogeneous K-Ras distribution becomes apparent in progressed tumor after 12 weeks. This indicates that differential K-Ras protein levels correlate with late tumors in the KP model.

Figure 2: K-Ras protein levels change during disease progression in the KP mouse model: KP tumors were stained with K-Ras antibody and protein expression as well distribution changed in tumors from 6, 9 and 12 weeks after tumor initiations.

What opportunities for the training and professional development has the project provided?: *Nothing to report*
How were the results disseminated to communities of interest?: *Nothing to report*
What do you plan to do during the next reporting period to accomplish the goals? *Nothing to report*

4) Impact:

What was the impact on the development of the principal discipline(s) of the project?: *Nothing to report*
What was the impact on other discipline(s)?: *Nothing to report*
What was the impact on technology transfer?: *Nothing to report*



What was the impact on society beyond science and technology?: *Nothing to report*

5) Changes /Problems:

Changes in approach and reasons for changes: Nothing to report
Actual or anticipated problems or delays and actions or plans to resolve them: In order to visualize K-Ras protein on the endogenous level, we used the Crisp/Cas9 approach. Guide RNAs were designed for WT and the mutant G12V form of K-Ras. As a positive control Histon 2b was also tagged with GFP using a validated guide. Unfortunately, we were not able to

Figure 3: A) shows the result of the Crispr/CAS9 approach to tagged K-Ras WT and G12V mutant form. As positive control Histon2b was used. The gel shows that no product was successfully inserted into the locus for WT and mutant K-Ras, but Histon2b was tagged successfully, also shown in fluorescence images in B). C) Tmet clones show heterogeneous, non-conclusive response in their K-Ras protein levels upon incubation of proteasome inhibitor MG132.

tagged K-Ras WT or mutant fusion under the control of the endogenous promoters as shown by the missing product in figure 3A. Positive control showed a clear band with the right size and correct insertion of the GFP was additionally validated by fluorescence imaging (Fig. 3B). A positive GFP signal was recorded within the nucleus of the cells. Since this was a critical requirement for the further proposed experiments, we could not proceed with these.
Additional, we proposed an ubiquitination-dependent mechanism to upregulate K-Ras protein levels based on our previous preliminary data. To evaluate the existence of such a mechanism in the Tmet cells, various clones with differential K-Ras protein levels were treated with the proteasome inhibitor MG132, but heterogeneous responses were recorded (Fig. 3C).
We tried to change the treatment conditions but the response was never consistent and therefore the results were non-conclusive which suggest that the mechanism in these cells might not only depend on ubiquitination but also on other factors which were unknown and therefore we were unable to control for these.

Changes that had a significant impact on expenditures:
PI Anette Claudia Schafer took another position and grant was suspended early.
Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents: Nothing to report

6) Products:
Publications, conference papers, and presentations: Nothing to report
Website(s) or other Internet site(s): Nothing to report
Technologies or techniques: Nothing to report
Inventions, patent applications, and/or license: Nothing to report
Other products: Nothing to report

7) Participants & other collaborating organizations:
What individuals have worked on the project?: No changes
Has there been a change in the active other support of the PD/PI(s) or senior key personnel since the last reporting period?: Nothing to report
What other organizations were involved as partners: Nothing to report

8) Special reporting requirements: Nothing to report

9) Appendices: Nothing to report