

Award Number: W81XWH-16-1-0568

TITLE: RUNX1T1 Amplification Induces "Small Cell" Cancer

PRINCIPAL INVESTIGATOR: Afshin Dowlati, M.D.

CONTRACTING ORGANIZATION:

Case Western Reserve University
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REPORT DATE: September 2017

TYPE OF REPORT: Annual

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

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REPORT DOCUMENTATION PAGE		<i>Form Approved</i> <i>OMB No. 0704-0188</i>	
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1. REPORT DATE: Sept 2017		2. REPORT TYPE: ANNUAL	
		3. DATES COVERED (From - To) 1 Sep 2016 - 31 Aug 2017	
4. TITLE AND SUBTITLE: RUNX1T1 Amplification Induces "Small Cell" Cancer		5a. CONTRACT NUMBER	
		5b. GRANT NUMBER W81XWH-16-1-0568	
		5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S): Afshin Dowlati, M.D. Afshin.Dowlati@case.edu		5d. PROJECT NUMBER	
		5e. TASK NUMBER	
		5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Case Western Reserve University 10900 Euclid Ave Cleveland, OH 44106		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 Small cell lung cancer (SCLC) is one of the deadliest cancers encountered by oncologists, with 5-year survival rates of less than 2% for patients with metastatic disease. Current thinking is that small cell lung cancer (SCLC) arises from a small population of specific neuroendocrine-like cells in the lung and is driven principally by concurrent mutation of two genes, TP53 and RB1. While this may be true for the majority of ‘every-day’ SCLC patients, there are two other clinically-important subgroups of cancer patients with ‘small cell’ disease; so-called combined small cell lung cancer and extra-pulmonary small cell cancer. In combined SCLC the tumors consist of both a SCLC component and a second subtype of lung cancer, such as adenocarcinoma, and it is believed that the second, more differentiated component has transformed into a small cell cancer. Similarly, extra-pulmonary small cell tumors have primary tumors that arise outside the lung, such as in the prostate or GI tract, and transform into a small cell cancer. So in reality the term ‘small cell’ simply describes a microscopic appearance, or phenotype. Clinically, however, this ‘small cell’ phenotype is of great importance because it is treated the same, regardless of whether it is pulmonary, combined or extra-pulmonary and predicts the same aggressive disease course with high mortality.

Here we seek to validate one potential pathway leading to the formation of a ‘small cell’ phenotype: through amplification of a gene called RUNX1T1, which we observed only in the ‘small cell’ component of two combined SCLC tumors. We will do this by investigating more combined SCLC tumors for RUNX1T1 amplification, as well as by over-expressing RUNX1T1 in various cancer cell lines to see if it transforms them into a ‘small cell’ phenotype. These studies may provide new ideas for treating SCLC itself, as well as other ‘small cell’ cancer subtypes outside the lung, such as some prostate cancers, which have broad military relevance beyond smoking-related diseases.

15. SUBJECT TERMS Small cell, lung cancer, RUNX1T1, non-small cell, amplification					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 14	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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1. Introduction:

The goal of this study was to explore a potential role for *RUNX1T1* amplification as a mechanism for transforming non-small cell lung cancer (NSCLC) into small cell lung cancer (SCLC). This idea was based on our finding of *RUNX1T1* amplification in the SCLC component of two ‘combined’ SCLC tumors that was not present in the matching NSCLC component. This was of interest because *RUNX1T1* plays an oncogenic role in some forms of leukemia as the C-terminal partner of the fusion protein ETO and because *RUNX1T1* demonstrates much higher mRNA expression levels in SCLC compared to NSCLC cells in the Cancer Cell Line Encyclopedia (CCLE) database (<https://portals.broadinstitute.org/ccle>). Therefore, the aims of this study were to: 1) determine the effect of *RUNX1T1* overexpression in NSCLC cell lines, and 2) investigate *RUNX1T1* amplification in a larger cohort of >22 archived ‘combined’ SCLC tumors by CNV analyses. This grant has received a no-cost extension for a year to allow us to continue pursuing these aims, thus this represents only an annual report. We describe below our progress for Year 1 as it relates to our SOW and some of the unexpected, yet interesting problems, we have encountered.

2. Keywords:

Small cell lung cancer, SCLC, combined small cell lung cancer, combined sclc, *RUNX1T1*, ETO, gene amplification, phenotype, non-small cell lung cancer, NSCLC, copy number variation, CNV, transformation

3. Accomplishments:

- **What were the major goals of the project? What was accomplished under these goals?** The goals are listed as the grant Specific Aims/tasks and are followed by relevant accomplishments.

Specific Aim 1: To overexpress *RUNX1T1* in NSCLC cells as a surrogate mechanism for gene amplification and look for phenotypic changes consistent with a SCLC phenotype:

*Major Task 1: To obtain and prepare *RUNX1T1* lentiviral constructs and make stable clones of six NSCLC cell lines:*

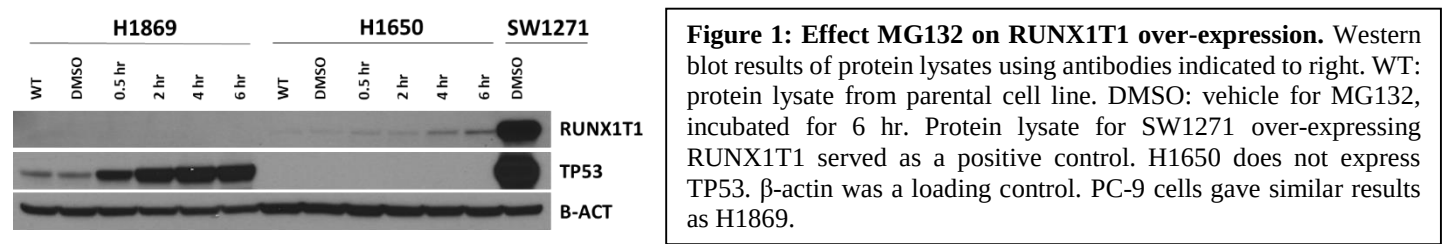
*Subtask 1: Obtain and prepare *RUNX1T1* lentivirus.* We purchased pre-made lentivirus particles from GeneCopoeia that carried a lentiviral vector encoding variant 3 of *RUNX1T1* with an N-terminal FLAG tag and the *neo* antibiotic selection marker. A complicating factor was that *RUNX1T1* has at least 15 mRNA transcripts encoding 6 different protein isoforms, all differing in the N-terminus. We chose to over-express variant 3 because it represented the shortest protein isoform while still retaining all of the annotated protein functional domains of *RUNX1T1*. There remain several more lentiviral vectors encoding longer *RUNX1T1* protein isoforms available for purchase if needed in future experiments.

*Subtask 2: Isolate stable clones expressing *RUNX1T1*.* Initially we infected three NSCLC cell lines; PC-9, H1650 and H1869. We isolated stable pools of cells by antibiotic selection. We did not observe any obvious change in cell phenotype in these pools of selected NSCLC cells. We then used qPCR to validate over-expression in these stable pools and found that while endogenous *RUNX1T1* mRNA levels were nearly undetectable in parental cells ($C_T \sim$ cycle 35-36), the pools of selected NSCLC cells demonstrated a robust ~256-fold over-expression of *RUNX1T1* mRNA relative to parental cells ($C_T \sim$ cycle 26-27). For comparison, the C_T for β -actin was about 25 for all NSCLC cells tested, both parental and stable pools.

*Major Task 2: To analyze NSCLC cells stably over-expressing *RUNX1T1*:*

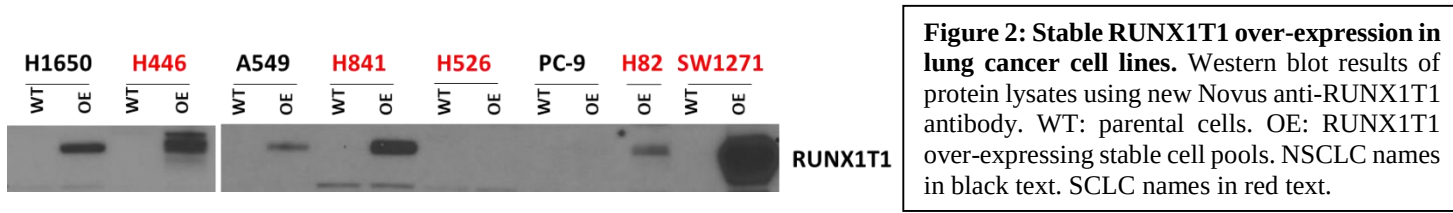
Subtask 1: Make protein lysates for western blot analyses. We examined whole cell protein lysates from parental and *RUNX1T1* over-expressing NSCLC cells and could not demonstrate any *RUNX1T1* expression in either the parental or *RUNX1T1* over-expressing NSCLC cell lines using either a Cell Signaling (CST) anti-

RUNX1T1 antibody or an anti-FLAG antibody. Subsequently, as a control, we infected HEK293T cells with *RUNX1T1* lentivirus and obtained cells with robust *RUNX1T1* protein expression, validating our protein detection methods (data not shown). As another control, we infected two SCLC cell lines, H446 and SW1271, with *RUNX1T1* lentivirus and after G418 selection obtained pools of cells with increased *RUNX1T1* mRNA ($C_T \sim$ cycle 22-24) and protein expression, particularly in SW1271 cells (see Figures 1, 2).



Taken together these results suggested that RUNX1T1 protein expression was under post-translational repression in NSCLC cells, but not in HEK293T, H446 or SW1271 cells. To explore this idea further, we incubated all three NSCLC cell lines over-expressing *RUNX1T1* mRNA with the proteasome inhibitor MG132 (10 μ M) for increasing amounts of time. We found that while RUNX1T1 expression was increased to detectable levels in H1650 cells, this was not true for H1869 or PC-9 cells (see Figure 1). The increase in RUNX1T1 protein expression in H1650 cells, however, was small compared to the baseline over-expression of RUNX1T1 in SW1271 cells. We used expression of TP53 as a control to show the efficacy of proteasome inhibition. We concluded that although RUNX1T1 protein may undergo proteasomal degradation, this is not the main reason for its lack of over-expression in NSCLC cells.

Based on these unexpected results we infected additional lung cancer cell lines with *RUNX1T1* lentivirus and made stable pools of both NSCLC (A549) and SCLC (H841, H82, H526) cells to determine if RUNX1T1 protein could only be over-expressed in SCLC, but not NSCLC cells. At the time of these experiments, we had also improved the sensitivity of our RUNX1T1 protein detection in western blots by switching to an anti-RUNX1T1 antibody from Novus. Once again we found that all new stable pools of cells, both NSCLC and SCLC, demonstrated robust increases in *RUNX1T1* mRNA compared to parental controls by qPCR. One major difference, however, was that SCLC cell lines demonstrated endogenous *RUNX1T1* mRNA expression ($C_T \sim$ cycle 26-27) whereas NSCLC cells did not ($C_T \sim$ cycle 35), validating the results in the CCLE database. Interestingly, RUNX1T1 protein was variably expressed among *all* cells (see Figure 2-endogenous expression of RUNX1T1 protein in SCLC cells could not be detected at this exposure). We concluded that RUNX1T1 protein is more likely to be over-expressed in SCLC cells than in NSCLC cells, although with great variability.



To date, we have not observed any obvious phenotypic changes in NSCLC or SCLC cells infected with *RUNX1T1* lentivirus. In NSCLC, this likely is due to little/no detectable exogenous RUNX1T1 protein expression. Never-the-less we did look by western blotting for an increase in expression of neuroendocrine genes typical of SCLC (INSM1, synaptophysin and NEUROD1) in H1650 cells, a NSCLC cell where RUNX1T1 protein over-expression is detectable (see Figure 2), but no expression was observed (data not shown). We could, however, detect a decrease in HES1 expression, indicating a potential decrease in NOTCH signaling, which we proposed as a possible mechanism behind a *RUNX1T1*-induced NSCLC to SCLC transformation. We are reluctant to follow up this interesting observation, however, because we only have one other NSCLC cell line (A549) with detectable

RUNX1T1 over-expression in which to confirm this result and would like several more to pursue this line of investigation further.

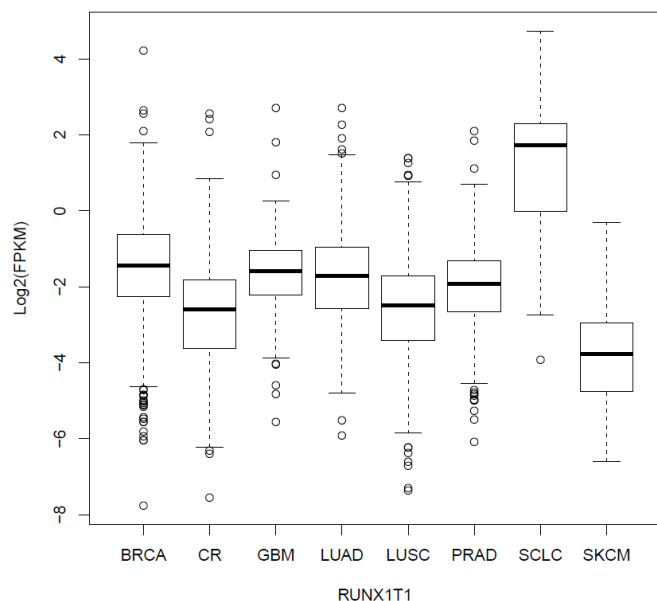
Subtask 2: Isolate RNA and perform gene expression profiling. Because of the difficulty in obtaining RUNX1T1 over-expression in NSCLC cells, we have not yet started these studies until we can obtain a few more NSCLC cells with over-expression similar to H1650 cells. Over-expression in four additional NSCLC cell lines is underway.

Subtask 3: Bioinformatic analysis of gene expression profiling. This will begin once our studies in Subtask 2 are completed.

Specific Aim 2: To interrogate our SCLC cohort of >22 combined SCLC samples for evidence of RUNX1T1 amplification at the DNA and protein levels:

Major Task 1: Obtain tissues blocks of tumor specimens and quality control using IHC markers. With the help of our collaborating thoracic pathologist, Dr. Michael Yang, we have begun searching for tumor specimens from patients annotated in our SCLC database as having ‘combined’ SCLC. Starting from a list of 14 initial patients, the records showed that 8 had possible archived tumor tissue available. When we looked further into these 8 samples it became clear that while archived tissue blocks were often available for the NSCLC component, the SCLC component was often only available as pre-cut slides, although one SCLC block was available. On a practical level this meant that it was unlikely that we could make tissue microarrays (TMAs) from the ‘combined’ tumor components, as we had originally proposed in the grant, and we may also have to develop methods to study *RUNX1T1* expression in individual slides of tissue rather than in ‘chunks’ of tissue. This means we may not be able to extract RNA/DNA for mRNA/CNV analysis, but rather develop in-situ hybridization (ISH) techniques for mRNA/CNV analyses. Never-the-less, more patients diagnosed with ‘combined’ SCLC remain in our database that will be looked up for tissue availability. This will continue into Year 2 of the project.

Major Task 2: Prepare TMAs of tumor specimens. See response to Major Task 1. However, we are currently constructing a new TMA consisting of only lung cancer cell lines (29 SCLC, 15 NSCLC, 6 mesothelioma, 1 normal), to help us trouble-shoot future IHC, RNAscope and ISH detection methods for *RUNX1T1* expression.



Major Task 3: Perform IHC and RNAscope analyses of tumor specimens. See response to Major Task 1. We initially attempted to determine RUNX1T1 protein expression using an IHC-only anti-RUNX1T1 antibody (LS BIO) using a lung cancer TMA we had previously constructed (22 SCLC, 12 NSCLC, 3 normal lung specimens, with 2-4 cores from each specimen). Unfortunately, Dr. Michael Yang did not find the staining

Figure 3: *RUNX1T1* mRNA expression in tumors. Determined from RNAseq data. BRCA: breast cancer, CR: colorectal cancer, GBM: glioblastoma, LUAD: lung adenoma NSCLC, LUSC: lung squamous NSCLC, PRAD: prostate adenoma cancer, SKCM: skin melanoma cancer.

to be specific for SCLC compared with NSCLC and it was also not nuclear, as we expected since RUNX1T1 is a transcriptional co-repressor. Thus, we sought to confirm the differential expression of *RUNX1T1* in SCLC compared to NSCLC tumors, as well as to other cancers. First, we used RNAseq data from the TCGA as well as a SCLC genomics study (Rudin et al, Nat Genetics 44:1111-

1116, 2012) and found that SCLC easily expressed the highest levels of *RUNX1T1* mRNA among all tumors examined (Figure 3).

We next sought to confirm the specific expression of endogenous *RUNX1T1* protein in SCLC vs NSCLC. We used western blotting with the sensitive Novus antibody on whole cell protein lysates, as well as nuclear fractions, prepared from 14 SCLC and 4 NSCLC cell lines. The results, some of which are shown in Figure 4, demonstrated that the majority of SCLC cell lines express endogenous *RUNX1T1* protein, although to variable levels, whereas no endogenous *RUNX1T1* protein was ever detected in any NSCLC cell line. The level of endogenous *RUNX1T1* protein in SCLC, however, was much less than that observed for over-expressed *RUNX1T1* in SW1271 cells. These results were consistent with the mRNA expression data in the CCLE and validated the use of our lung cancer cell line TMA under construction as a good tool to trouble-shoot future IHC, RNAscope and ISH detection methods for *RUNX1T1* expression.



Figure 4: Endogenous *RUNX1T1* expression in lung cancer cell lines. Western blot results of protein lysates using new Novus anti-*RUNX1T1* antibody. Wcl: whole cell lysate. Nuc: nuclear fraction. Space: empty lane. OE SW1271: stable pool of *RUNX1T1* over-expressing cells used as positive control. NSCLC names in black text. SCLC names in red text.

Major Task 4: Isolate DNA from FFPE sections. See responses to Major Tasks 1 and 5.

Major Task 5: Perform CNV analysis of DNA. See response to Major Task 1. We are now thinking this must be done by RNAscope (mRNA determination) and/or ISH (CNV determination) and we will use our lung cancer cell line TMA to validate our methods before attempting this in more precious tumor samples. We have already purchased a kit to perform RNAscope detection of *RUNX1T1* mRNA and *MYC* mRNA (to be used as a positive control since 30% SCLC cells show *MYC* amplification and expression). This work will continue in Year 2.

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

Although this project was not intended to provide training opportunities, a major portion of the research results have been supplied by Tian He, a graduate student in the Department of Biochemistry at Case Western Reserve University as part of her PhD thesis.

▪ **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?**

Specific Aim 1: Our main goal is to create more NSCLC cell lines with abundant, stable *RUNX1T1* over-expression. These cells will then be analyzed using gene expression microarrays to identify novel pathways activated by *RUNX1T1* in NSCLC cells that would not be expected to induce obvious changes in phenotype or neuroendocrine expression, which has been unsuccessful to date. These pathways may regulate colony formation, cell migration and invasion, metastasis or chemo-resistance.

Specific Aim 2: Our main goal is to finally obtain all the possible clinical tumor specimens of combined SCLC and look for *RUNX1T1* over-expression in the NSCLC component by RNAscope, ISH or IHC. This will require us to first perfect our techniques on the SCLC cell line TMA that is nearing completion.

4. Impact:

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

Taken together, we have confirmed the specific expression of *RUNX1T1* in SCLC, but not in NSCLC or many other cancers; however, it remains unclear what the function of *RUNX1T1* is in SCLC, let alone if it can drive a phenotypic transformation of NSCLC to SCLC in combined SCLC.

- **What was the impact on other disciplines?**

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 change.**

Because of the difficulty in obtaining *RUNX1T1* over-expression in NSCLC cells, we have begun exploring what this protein does in SCLC; that is, is it essential for maintaining the neuroendocrine phenotype? Does it play a role in cell proliferation, metastasis, survival, or chemo-sensitivity? This shift is driven largely by the fact that so

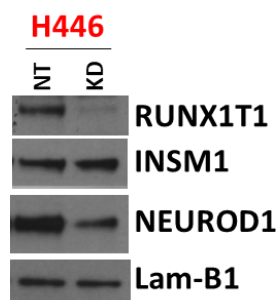


Figure 5: Effect RUNX1T1 knockdown on SCLC neuroendocrine expression. Western blot results of nuclear fractions using antibodies listed at right (new Novus anti-RUNX1T1 antibody). NT: non-targeting siRNA. KD: incubation with 1 μ M siRUNX1T1 for 3 days. Lamin B1 was used as a loading control.

little is known about the functional role of *RUNX1T1* as a *non-fusion* protein, that is, separate from ETO. We intend to explore these questions using both gene knock-down (KD) studies (siRNA/CRISPR) of endogenous *RUNX1T1* (see Figure 5) as well as over-expression studies, outlined above. This new strategy will give us many more cell lines to work with and thereby allow us to determine the generality of our findings. We can also expect to obtain opposite effects from these two lines of experimentation. To

date, we have found that KD of *RUNX1T1* decreases expression of endogenous *NEUROD1*, but not *INSM1*, in H446 cells (see Figure 5). We are following this up in other SCLC cell lines (H82, H2171) and looking at more neuroendocrine genes. Similar to our experiments with NSCLC cells over-expressing *RUNX1T1*, we may also use gene microarrays to explore the function of *RUNX1T1* in SCLC. These new proposed experiments have been added as two new Major Tasks 3 and 4 of a revised SOW added to the Appendix.

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

The only delay has been in our ability to obtain tumor specimens of combined SCLC from the Pathology archives. Although we may only be able to obtain <10 tumor specimens, this should be more than enough to draw conclusions about *RUNX1T1* expression in this subtype of SCLC.

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

Nothing to report.

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

Nothing to report.

6. Products:

Nothing to report.

7. Participation & other collaborating organizations:

- **What individuals have worked on the project?**

Name:	Afshin Dowlati, MD
Project role:	PI
Researcher identifier:	Orchid # 0000-0003-4535-6839
Nearest person months worked:	12
Contribution to project:	PI- oversight and direction.
Funding support:	This and grants listed below and University Hospitals of Cleveland research support.

Name:	Karen McColl, BS
Project role:	Lab manager
Researcher identifier:	N/A
Nearest person months worked:	12
Contribution to project:	Ms. McColl has worked on all aspects of this project.
Funding support:	This and University Hospitals of Cleveland research support.

Name:	Tian He, BS
Project role:	Graduate student.
Researcher identifier:	N/A
Nearest person months worked:	9
Contribution to project:	Ms. He has worked on all aspects of this project.
Funding support:	University Hospitals of Cleveland research support.

Name:	Gary Wildey, PhD
Project role:	Program manager
Researcher identifier:	Orchid # 0000-0001-7105-1313
Nearest person months worked:	12
Contribution to project:	Research advise and report writing.
Funding support:	University Hospitals of Cleveland research 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?**

Yes, two new grants were funded, one was discontinued:

New Active:

U24 (Rudin, PI) 02/20/17-01/31/22 0.6 calendar
NIH/NCI \$75,000 (sub only)

Small-cell Lung Cancer (SCLC) Consortium: Coordinating Center

Subproject: *Construction of SCLC tissue micro-arrays*

Here we propose to construct multiple tissue micro-arrays from de-identified small-cell lung cancer tumor specimens and provide matching genomic profiling data on tumor specimens with sufficient tissue. Additional data on patient and tumor features will be provided for each tumor specimen placed in the TMA.

Role: Leader, tissue micro-array subproject.

Overlap: None- no funds are requested to make the TMAs used in the current application, as stated in the Budget Justification.

Concept Award (Dowlati, PI) 09/01/17-08/31/18 1.2 calendar
DOD \$100,000

Targeting Chemo-resistance in Small Cell Lung Cancer

This proposal is focused on a gene, *HEPACAM2*, which appears to be uniquely and highly expressed only in SCLC. Because *HEPACAM2* likely is required for cell division and is poly(ADP-ribosylated) by the enzyme tankyrase, we propose that disruption of its function by tankyrase inhibitors may provide a unique strategy to selectively inhibit SCLC growth and proliferation.

Overlap: None.

Discontinued:

2P30 CA043703-24 (Gerson, PI) 08/01/97-03/31/18 0.84 calendar
NIH/NCI \$3,088,653

Case Comprehensive Cancer Center Support Grant

The objectives of the Center are: 1) to improve the prevention, diagnosis, and therapy of cancer through research; 2) to stimulate and support innovative, coordinated, interdisciplinary research on cancer diagnosis, treatment, and control; 3) to develop clinical applications of research discoveries and to make these applications available as quickly as possible; and 4) to develop cancer prevention and control activities to contribute to the reduction of cancer morbidity and mortality in Northeast Ohio and the surrounding region and nation.

Role: Co-Leader, Developmental Therapeutics Program

Overlap: None.

- **What other organizations were involved as partners?**

Nothing to report.

8. Special reporting requirements:

Nothing to report.

9. Appendix:

Revised SOW.

STATEMENT OF WORK – 02/12/2018
PROPOSED START DATE Sept 01, 2016

Site 1: Case Western Reserve University
 11100 Euclid Ave
 Cleveland, OH 44106
 PI: Afshin Dowlati

Specific Aim 1:		Timeline
To overexpress RUNX1T1 in NSCLC cells as a surrogate mechanism for gene amplification and look for phenotypic changes consistent with a SCLC phenotype:		(Months)
Major Task 1:		
To obtain and prepare RUNX1T1 lentiviral constructs and make stable clones of six NSCLC cell lines:		
Subtask 1: Obtain and prepare RUNX1T1 lentivirus.	1-2	Karen McColl
Subtask 2: Isolate stable clones expressing RUNX1T1.	3-18	Karen McColl
Major Task 2:		
To analyze NSCLC cells stably over-expressing RUNX1T1:		
Subtask 1: Make protein lysates for western blot analyses.	5-18	Karen McColl
Subtask 2: Isolate RNA and perform gene expression profiling.	18-20	Karen McColl
Subtask 3: Bioinformatic analysis of gene expression profiling.	20-24	Gene Expression core
Major Task 3:		
To analyze SCLC cells stably over-expressing RUNX1T1:		
Subtask 1: Make protein lysates for western blot analyses.	6-18	Tian He
Subtask 2: Isolate RNA and perform gene expression profiling.	18-20	Tian He
Subtask 3: Bioinformatic analysis of gene expression profiling.	20-24	Gene Expression core
Major Task 4:		
To analyze SCLC cells with stable knockdown of RUNX1T1:		

This generic Statement of Work document is intended to assist applicants with the format preferred by CDMRP. This particular SOW does not contain any specific scientific information and is intended to be easily modifiable for any project. Not all components will be applicable for every project; please consult your Program Announcement for specific award requirements.

Subtask 1: Make protein lysates for western blot analyses.	13-18	Tian He
Subtask 2: Isolate RNA and perform gene expression profiling.	18-20	Tian He
Subtask 3: Bioinformatic analysis of gene expression profiling.	20-24	Gene Expression core
Milestone(s) Achieved:		
Specific Aim 2: To interrogate our SCLC cohort of combined SCLC samples for evidence of RUNX1T1 amplification at the DNA and protein levels:		
Major Task 1: Obtain tissues blocks of tumor specimens and quality control using IHC markers.	1-20	Dr. Dowlati, Pathology core
Major Task 2: Prepare TMAs of SCLC and NSCLC cell lines for methods development.	12-20	Dr. Dowlati, Pathology core
Major Task 3: Perform IHC, RNAscope and ISH analyses of tumor specimens.	20-24	Dr. Dowlati, Pathology core
Milestone(s) Achieved:		
Milestone Achieved: HRPO/ACURO Approval:	05/05/16	