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14. ABSTRACT

During the second funding period we completed Major Tasks 1 and 2 and partially completed Major Task 3. The areas of interest were identified in 22 squamous cell lung cancer patients and isolated by LCM. Genomic DNA was isolated from these areas and whole exome sequencing was performed. The data has been analyzed for the progression-associated, premalignant- and malignant-specific mutations. The mutational data is being analyzed in the pathway context. Gene copy number alterations were identified by the analysis of the sequencing data. Multiplex immunofluorescence staining to determine the spectrum of infiltrating immune cells in tumor and premalignant microenvironment is ongoing.

15. SUBJECT TERMS
Lung cancer, premalignancy, immune microenvironment, progression, driver mutations, neoantigens, whole exome sequencing (WES)

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1. INTRODUCTION:

Lung cancer is the leading cause of cancer death among US Veterans as well as the world's leading cause of cancer death. Squamous cell carcinoma (SCC) accounts for 23% of non-small cell lung cancer cases in the US, however, to date no studies have focused on the mutational landscapes relevant to progression from premalignancy to invasive SCC. One of the major driving forces of carcinogenesis is somatic mutagenesis. Over 75% of lung cancers bear driver mutations that are causally implicated in cancer development, while the remainder of lung cancers does not bear mutations in known oncogenes or tumor suppressors. Bronchial epithelium of many SCC patients and subjects at risk for developing lung cancer often contain small proliferative premalignant lesions, such as squamous metaplasia (SM), dysplasia (SD), basal cell hyperplasia (BCH) and carcinoma *in situ* (CIS). Current studies suggest that SM and SD may be precursors of SCIS and, subsequently, to invasive pulmonary SCC. Factors that determine the fate of a premalignant lesion, i.e. whether it will progress to cancer or recede, remain enigmatic. Patients might be at increased risk for progression from these early premalignant lesions for years before developing clinically detectable lung cancer. Uncertainty about the clinical behavior of a premalignant lesion can lead to either inappropriate inaction or inappropriate aggressive treatment, either of which can result in harm to the patient. Unravelling the factors that determine whether premalignant lesions will progress to cancer or recede, including the modulation of cellular immunity, may change the way premalignant lesions are approached clinically. We anticipate that these studies could ultimately lead to the development of novel the approaches for lung cancer interception through immunoprevention and treatment of the very earliest phase of the disease.

2. KEYWORDS:

Lung cancer, premalignancy, immune microenvironment, progression, driver mutations, neoantigens, whole exome sequencing (WES).

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1(specified in proposal)	Timeline	Site 1	Site 2	Status after Year 2
Major Task 1	Months			
Subtask 1: Review the slides to identify the areas of interest for LCM and IHC	1-5	Drs. Wallace and Dubinett	Drs. Huang and Dubinett	100%completed
Subtask 2: To isolate areas of interest by LCM	2-6	Dr. Krysan		100% completed
Subtask 3: To isolate genomic DNA and perform quality control	7-8	Dr. Krysan		100% completed
Milestone(s) Achieved: Specific Aim 1				100% completed
Local IRB/IACUC Approval: Active, IRB#17-000726-CR-00001	1	Dr. Dubinett	Dr. Dubinett	Completed
Major Task 2				
Subtask 1: To construct sequencing libraries and perform exome enrichment	9	Dr. Krysan		100% completed
Subtask 2: To perform next generation sequencing	10-11	Sequencing Core facility		100% completed
Subtask 3: To perform data analysis and identify progression-associated mutations and copy number variations	11-14	Drs. Krysan and Tran		100% completed
Milestone(s) Achieved: Specific Aim 2				100% completed
Major Task 3				
Subtask 1: To perform multi-color IHC, slide	15-21	TPCL, Dr.		67% completed

scanning and image analysis		Wallace		
Subtask 2: To relate the expression of immune regulators to the mutational landscapes of the tissues	21-24	Drs. Tran and Krysan		60% completed
Milestone(s) Achieved:				Ongoing

What was accomplished under these goals?

During the second funding period we completed Major Tasks 1 and 2, and partially completed Major Task 3. Whole exome sequencing (WES) has been performed on a total of 104 samples from 20 SCC patients. The sequencing data from 9 cases repeatedly did not pass quality control due to poor quality of genomic DNA, and the data presented in this report is comprised of 11 cases (56 lesions) that had complete sets of normal, premalignant and malignant lesions sequenced with enough depth for downstream analysis (**Table 1**).

Case ID	Normal	SM	SD	SCIS	SCC
P36588	1	4	1	0	2
P09024	1	1	0	1	1
P22150	1	1	0	0	1
P17914	1	0	0	1	1
P22103	1	0	1	0	1
P00344	1	0	1	0	1
P07681	1	0	1	6	1
P23665	1	2	2	0	2
P18082	1	0	1	0	1
P36655	1	3	0	3	1
P16212	1	0	1	2	1

Table 1. Summary of regions sequenced for each case.

Mutational analyses as well as the analysis of the gene copy number variation have been performed. We identified the premalignant- and malignant-specific, as well as progression-associated non-synonymous somatic mutations, which were in accord with our recently published DOD CDMRP-funded study of lung adenocarcinoma (PMID: 31142513). Our analysis revealed that mutational burden is associated with disease progression in SQCC (**Figure 1**). This finding suggests that accumulation of somatic mutations could be a driving force for progression from squamous premalignancy to invasive cancer. We are in the process of evaluating the progression-associated mutations in the signaling pathway context and comparing them between the squamous and adenocarcinoma datasets.

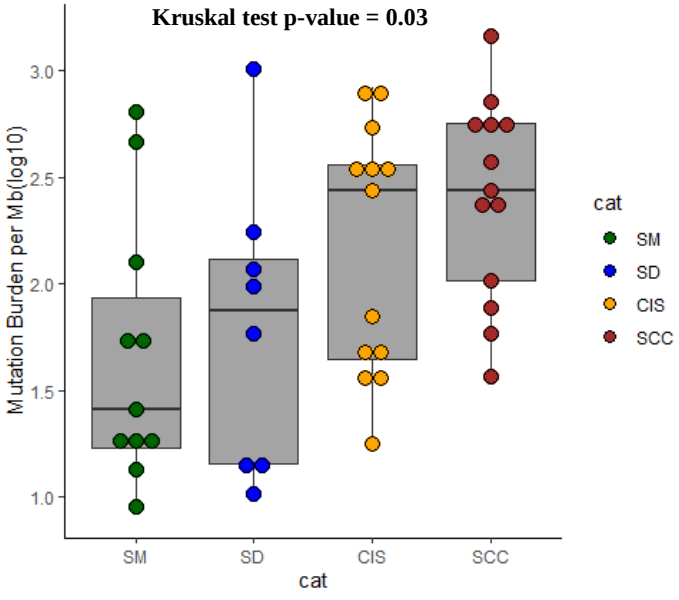

Figure 1. Total mutational burden per megabase DNA increases with squamous cell lung cancer progression.

To explore the relationship between sequenced lesions for each individual patient, phylogenetic trees were constructed (**Figure 2**, upper panel). In the majority of cases, the mutational profiles of SCC (brown

labels) were closely related to the profiles of SD (blue labels) and CIS (orange labels), but not SM (green labels), suggesting that SM is genetically more distant from the invasive cancer.

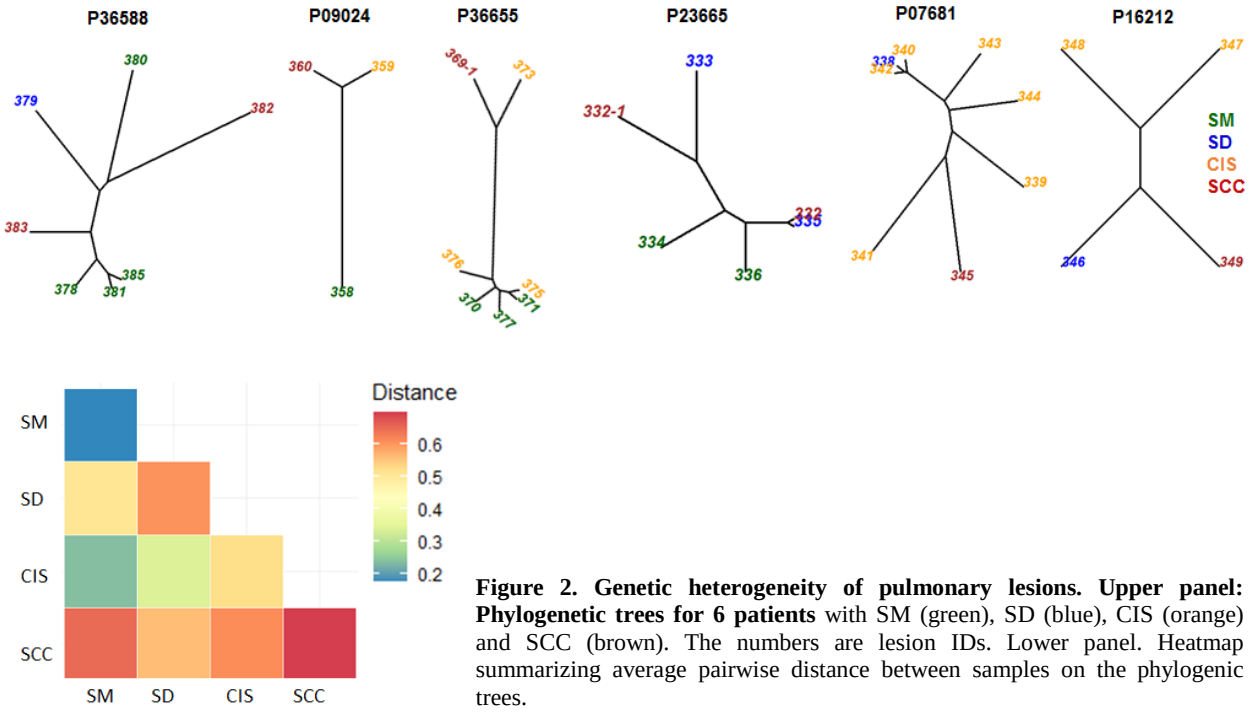

Figure 2. Genetic heterogeneity of pulmonary lesions. Upper panel: Phylogenetic trees for 6 patients with SM (green), SD (blue), CIS (orange) and SCC (brown). The numbers are lesion IDs. Lower panel. Heatmap summarizing average pairwise distance between samples on the phylogenic trees.

Then, we determined the pairwise distance between lesions from the same cases on the phylogenic trees (**Figure 2**, lower panel). We found that: SM lesions from the same cases were closely related (shortest distance) to each other. On the contrary, multiple SCC lesions from the same case (n=2 cases) were least related (longest distance) to each other. Premalignant SD and CIS lesions were more closely related to SM lesions than to others of the same stage, thus suggesting that these lesions evolved independently from each other.

Next, we evaluated the progression-associated somatic mutations (PAM). PAM were defined as the mutations found in both premalignant and malignant lesions from the same case. Consistent with our previous findings in lung adenocarcinoma, we found that PAM levels were similar among SD, CIS and SCC (**Figure 3**).

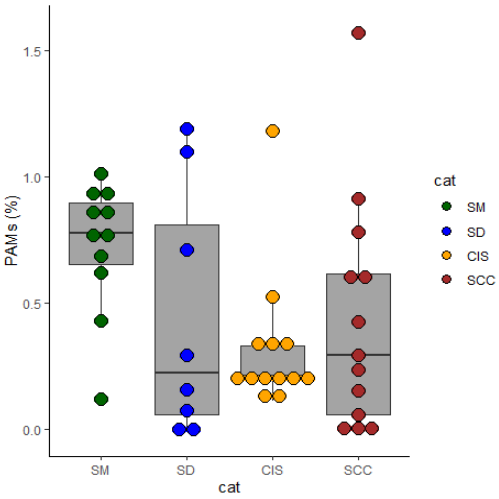

Figure 3. PAM levels are similar among SD, CIS and SCC.

We analyzed the frequency of mutations of the lung cancer driver genes from TCGA database in our cohort of patients and found that, in accord with lung adenocarcinoma, in squamous cell lung cancer most driver mutations occurred in CIS and SCC (**Figure 4**). Very few mutations (1/11) on the driver genes were PAM. These findings suggest that malignant

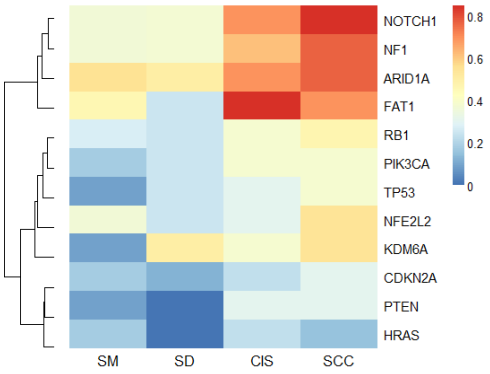

Figure 4. TCGA driver genes are frequently mutated in CIS and SCC.

progression was induced by the driver mutations occurring in some, but not all, premalignant lesions.

Next, we analyzed our sequencing data for copy number alterations (CNA). Recurrent CNAs were identified for 60 samples from 12 patients (i.e. an additional case was included in the analysis compared to the mutation analysis). We identified 164 recurrent regions with higher amplification frequency (>0.1) in either pre-malignant or malignant lesions compared to normal lung tissue (**Figure 5**). Thirty-two recurrent amplifications were found to be progression associated CNAs (PM-CNAs), which were amplified in premalignant lesions and cancer. The amplified genes included such drivers as EGFR, TP63 and CDK14. This suggests that CNAs but not mutations of the driver genes might be the driving force for the progression from premalignancy to invasive disease in squamous cell lung cancer.

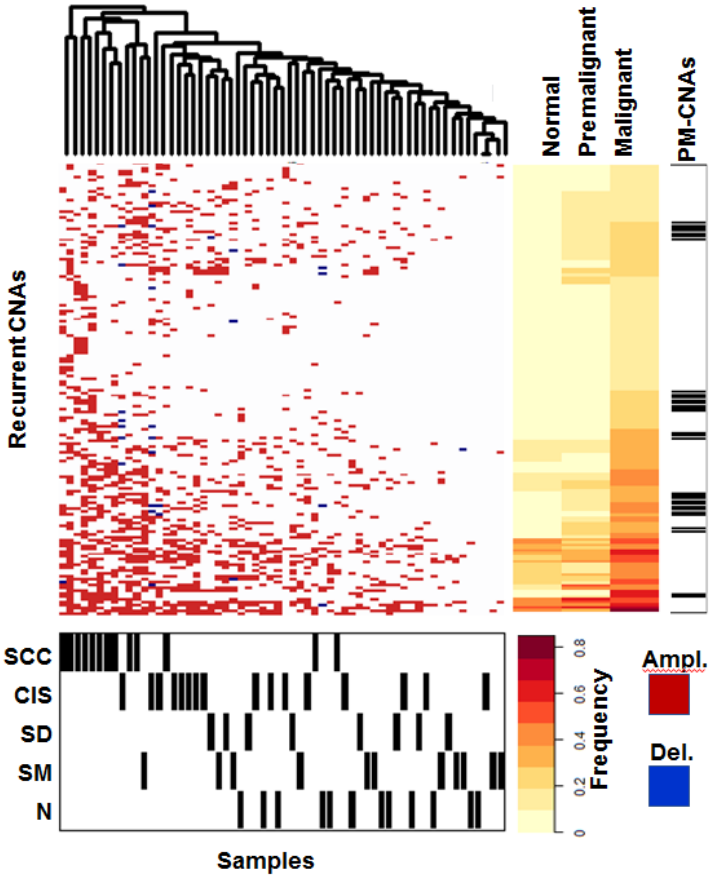

Figure 5. Copy number alterations are frequently found in CIS and SCC. Ampl. — amplification, Del. — deletion.

Toward the multiplex immunofluorescence (MIF) studies proposed in Specific Aim 2 (Major Task 3), we developed and optimized the staining protocols for three 6-marker panels that allow us to assess a wide range of immune cell types in the premalignant and

Panel		
1	**2**	**3**
CD3	CD4	CD3
CD8	CD8	CD11c
CD68	Granzyme B	HLA-DR
PD-1	FOXP3	LAG3
PD-L1	Ki67	TIM3
Pan-CK*	Pan-CK	Pan-CK
DAPI	DAPI	DAPI

Table 2. Multiplex immunofluorescence panels utilized to assess the immune microenvironment of squamous cell cancer continuum. *Pan-CK — pan-cytokeratin.

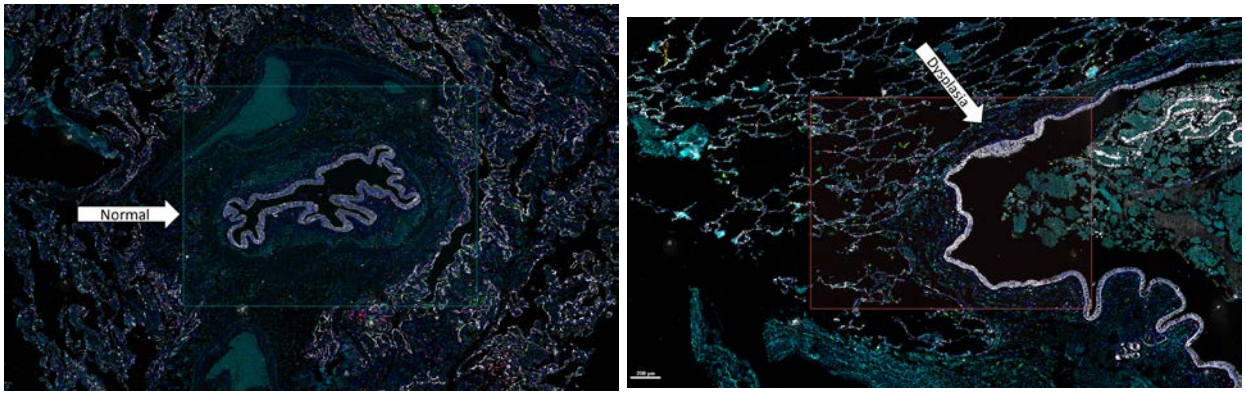

Figure 6. Six color MIF staining of normal airway epithelium (left panel) and squamous dysplasia (right panel). CD3 — cyan, PD-L1 — green, CD68 — yellow, PD-1 — orange, CD8 — red, PanCK — white, nuclei — blue (DAPI)

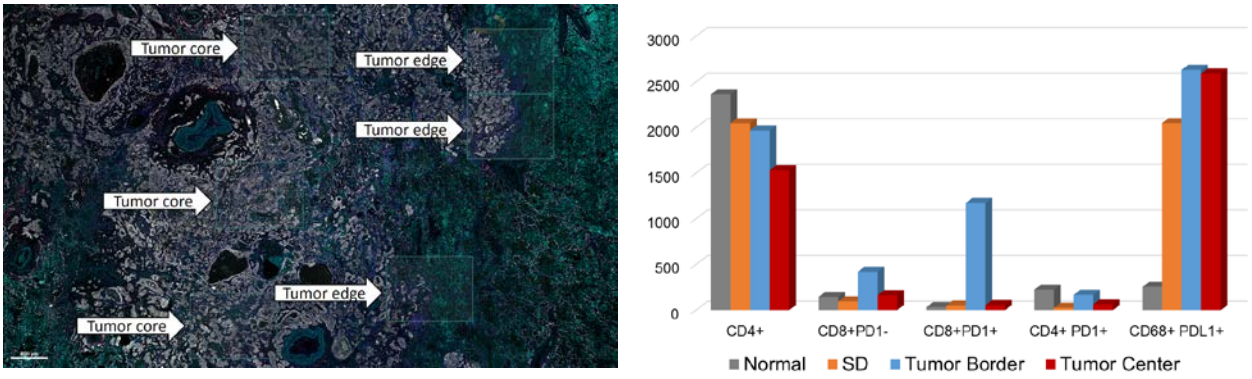

Figure 6, continued. Six color MIF staining of invasive SCC (left panel). Infiltrating immune call quantification in squamous dysplasia (SD), SCC and adjacent normal lung tissue (right panel).

malignant microenvironments (**Table 2**). The staining has been completed for Panels 1 and 2 for a subset of cases and is ongoing for Panel 3. A representative staining of an SCC case with the squamous dysplasia and invasive cancer regions for Panel 1 markers is shown in **Figure 6**. To analyze the microenvironment of large tumor sections we analyzed three areas of the tumor leading edge and three regions of the tumor core to improve the accuracy of the analysis. A comparison of infiltrating immune cell landscape in different lesions is shown in **Figure 6**. Our preliminary analyses show that progression is associated with an increase in immunosuppressive tumor-associated macrophages expressing PD-L1 ($CD68^+PD\text{-}L1^+$). Also, a spike in $CD8^+PD1^+$ T cells in the tumor border may suggest the initiation of T cell exhaustion. Further studies will determine if T cells in the tumor core are terminally exhausted. Overall, these data suggest an increased immunosuppressive microenvironment with squamous cell cancer progression and is in accord with our findings in lung adenocarcinoma. The analysis of the staining will be completed in 2020 utilizing funding from other sources.

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

Kostyantyn Krysan and Linh Tran participated in weekly conference calls related to pulmonary premalignancy. The teleconferences included investigators from other institutions involved in the MCL Pre-Cancer Atlas Program, as well as, the NCI Moonshot Pre-Cancer Atlas Program.

How were the results disseminated to communities of interest?

Our preliminary results have been discussed with members of the lung cancer community of researchers involved in the Lung SPORE, Stand Up 2 Cancer and HTAN programs.

4. IMPACT:

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

We anticipate that these results will add significant new insights into the pathogenesis of pulmonary squamous premalignancy. This turn will inform efforts related to the application of lung cancer interception.

What was the impact on other disciplines?

We anticipate that these studies, when completed, will have an impact in lung cancer screening, pulmonary medicine and thoracic oncology.

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:

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to report.

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.

Significant changes in use or care of human subjects
Nothing to report.

Significant changes in use or care of vertebrate animals
Nothing to report.

Significant changes in use of biohazards and/or select agents
Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**
  Nothing to report. Dr. Dubinett will be presenting results of these studies at the American Thoracic Society meeting in 2020.

  Journal publications.
  Nothing to report. Manuscripts in preparation.

  Books or other non-periodical, one-time publications.
  Nothing to report.

  Other 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 licenses**
  Nothing to report.

- **Other Products**
  Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Steven M. Dubinett - No change
Kostyantyn Krysan - No change
Linh M. Tran - No change
W. Dean Wallace - No change
Min Huang - No change

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.