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13. SUPPLEMENTARY NOTES

14. ABSTRACT
During the second funding period we partially fulfilled Major Task 3. The degree of lymphocyte infiltration was assessed in premalignant (n = 328) and malignant lesions (n = 15 AIS and 50 ADC), along with adjacent histologically normal areas (n = 50) in lobectomy specimens from 41 patients who had undergone surgery for early stage ADC. Lymphocyte infiltration was graded 0-to-3 based on H&E staining and was significantly increased in AAH vs. normal areas and became highest in AIS and ADC. Expression of regulators of cell-mediated immunity, including CD4, CD8, FOXP3, PD-1 and PD-L1 was quantified in AAH and ADC in a subset of 9 cases by immunohistochemistry. Quantitative multiplex immunofluorescent staining for 8 immune markers in the entire study cohort (41 cases) is ongoing and will be completed during the No Cost Time Extension.

15. SUBJECT TERMS
Lung cancer, premalignancy, progression, whole exome sequencing, driver mutations, neoantigens.

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1. Introduction

Lung cancer is the leading cause of cancer death in the U.S. and throughout the world with adenocarcinoma being the leading subtype in the US, Japan and other countries. 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. Distal airways of many lung cancer patients and subjects at risk for developing lung cancer often contain small focal proliferative lesions designated atypical adenomatous hyperplasia (AAH). Current studies suggest that AAH may be a precursor of adenocarcinoma *in situ* (AIS) and, subsequently, to invasive pulmonary adenocarcinoma (ADC). Factors that determine the fate of a premalignant lesion, i.e. whether it will progress to cancer or recede, remain enigmatic. Early attempts to evaluate somatic mutations in premalignant pulmonary lesions revealed mutations in known driver genes, such as *KRAS*, *EGFR* and *TP53*. Furthermore, clonal analysis demonstrated identical monoclonal patterns in AIS and AAH adjacent to it, strengthening the notion that AAH is a preneoplastic lesion rather than reactive hyperplasia. A recent study utilizing targeted sequencing of AAH lesions and related tumors identified mutations in other cancer-related genes as well as clonality between premalignant lesions and cancer. This study also highlighted the importance of the mutational landscape variations in progression from premalignancy to cancer, however, the genomic and microenvironmental determinants of progression have not yet been elucidated.

2. Keywords

Lung cancer, premalignancy, 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	Status after Year 1
Major Task 1	Months		
Subtask 1: Review the slides to identify the areas of interest for LCM and IHC	1-3	Dr. Wallace	Completed
Subtask 2: To isolate areas of interest by LCM	2-4	Dr. Krysan	Completed
Subtask 3: To isolate genomic DNA and perform quality control	5	Dr. Krysan	Completed
Milestone(s) Achieved			Completed
Local IRB/IACUC Approval: Active, IRB#10-001096-CR-00005		Dr. Krysan	Completed
Milestone Achieved: HRPO/ACURO Approval			Completed
Major Task 2			
Subtask 1: To construct sequencing libraries and perform exome enrichment (50 cases)	6-8	Dr. Krysan	Completed
Subtask 2: To perform next generation sequencing	9-11	Sequencing Core facility	Completed
Subtask 3: To perform data analysis and identify progression-associated mutations	12-14	Drs. Krysan and Tran	Completed
Milestone(s) Achieved:			All
Specific Aim 2			
Major Task 3			
Subtask 1: To perform multi-color IHC, slide scanning and image analysis	15-20	TPCL, Dr. Wallace	Ongoing
Subtask 2: To relate the expression of immune regulators to the mutational landscapes of the tissues	21-24	Drs. Tran and Krysan	Ongoing
Milestone(s) Achieved:			Ongoing

- **What was accomplished under these goals?**

During the second funding period we partially fulfilled Major Task 3. To evaluate the presence of the early adaptive immune response against pulmonary premalignancy, we first assessed the degree of lymphocyte infiltration in premalignant (n = 328) and malignant lesions (n = 15 AIS and 50 ADC), along with adjacent histologically normal areas (n = 50) in lobectomy specimens from 41 patients who had undergone surgery for early stage ADC described in the 1st year progress report. The median number of lesions evaluated per patient was six for AAH, and two for malignant lesions. Lymphocyte infiltration was graded 0-to-3 based on H&E staining and was significantly increased in AAH vs. normal areas (χ^2 test p<10-16), and became highest in AIS and ADC vs. AAH (χ^2 test p<10-14) (**Figure 1A**). We then assessed the expression of regulators of cell-mediated immunity, including CD4, CD8, FOXP3, PD-1 and PD-L1 in AAH and ADC in a subset of 9 cases by immunohistochemistry (**Figure 1B**). We found both infiltration of T effector and cytotoxic cells and expression of PD-L1 checkpoint in premalignancy, suggesting that cell-mediated immunity and possible recognition of neoepitopes occur in pulmonary premalignancy. Encouraged by the promising IHC staining data, we are currently performing the quantitative multiplex immunofluorescence staining of the entire cohort of samples for the following immune markers: CD4, CD8, CD11c, CD68, CD163, FOXP3, PD-1, PD-L1 and granzyme B. The staining and the data analysis will be completed during the No Cost Time Extension.

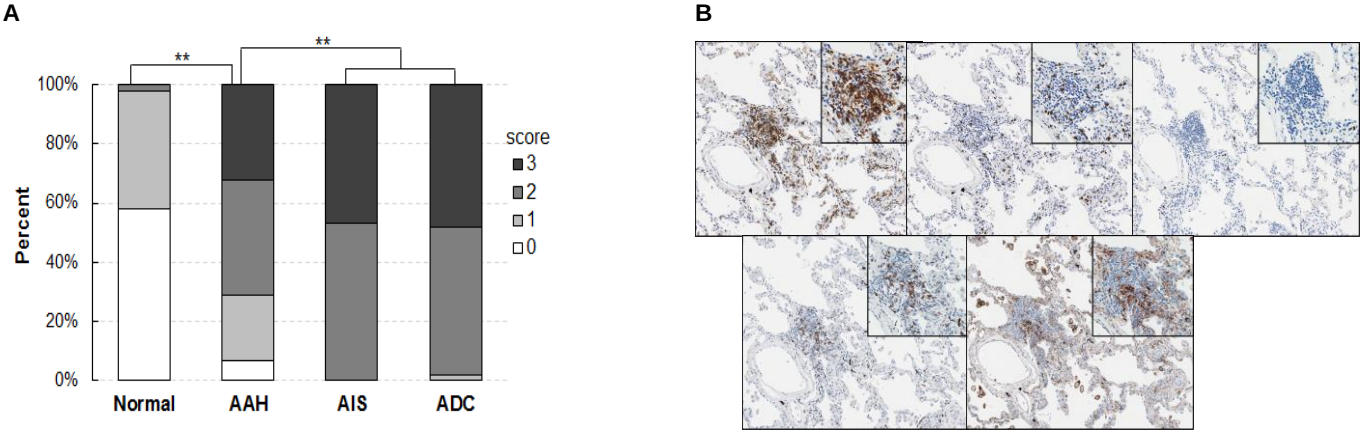

Figure 1. Immune cell infiltration in adenomatous premalignancy.
A) Local lymphocyte infiltration index (0 — lowest, 3 — highest) in adjacent normal tissue, AAH, AIS and ADC (**χ^2 test $p < 10^{-10}$).
B) A representative IHC staining of lymphocytic markers in an AAH lesion with local lymphocyte infiltration score = 2.

Next, we evaluated the association of neoantigen load (reported in the 1st year progress report) and the number and phenotypes of infiltrating immune cells by lesion- and patient-wise comparisons. The lesion-wise comparison evaluated neoantigens and infiltrating immune cell characteristics from the individual AAH lesions, while in the patient-wise analysis these endpoints were aggregated for the corresponding patient. At the lesion level, we found that the percentage of $CD8^+$ T cells infiltrating AAH correlated strongly with the percentage of PANs in the respective lesions (Kendall's τ = 0.56, p = 0.0003) (**Figure 2A**). Furthermore, AAH lesions with greater neoantigen loads had significantly more infiltrating $CD4^+$ T cells (Kendall's τ = 0.32, p = 0.05) (**Figure 2B**) and PD-L1 expressing cells (Kendall's τ = 0.44, p = 0.01) (**Figure 2C**).At the patient level, the percentage of progression-associated neoantigens (PANs) (i.e., neoantigens present in the entire spectrum of the disease from premalignancy to invasive cancer) significantly correlated with the average percentage of $CD8^+$ T cells infiltrating AAH lesions (Kendall's τ = 0.61, p = 0.02, **Figure 2D** top panel) but not to those infiltrating AIS/ADC (Kendall's τ = 0.14, p = 0.7, **Figure 2D** bottom panel). These results indicate that the high percentage of PANs promotes $CD8^+$ T cell infiltration in AAH lesions, whereas the overall neoantigen load in AAH is associated with $CD4^+$ T cell infiltration and PD-L1 expression.

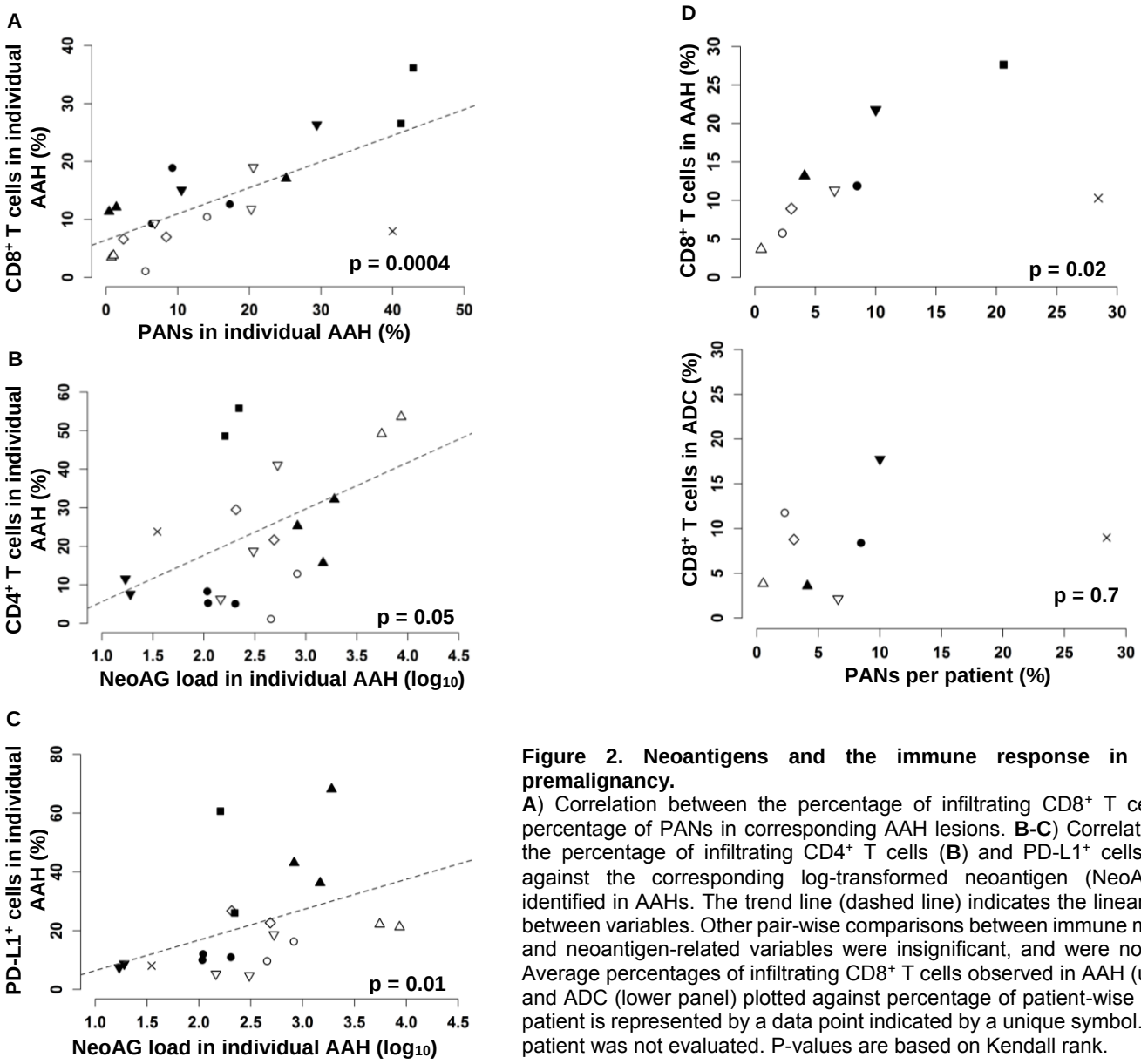

Figure 2. Neoantigens and the immune response in pulmonary premalignancy.
A) Correlation between the percentage of infiltrating $CD8^+$ T cells and the percentage of PANs in corresponding AAH lesions. **B-C**) Correlation between the percentage of infiltrating $CD4^+$ T cells (**B**) and PD-$L1^+$ cells (**C**) plotted against the corresponding log-transformed neoantigen (NeoAG) number identified in AAHs. The trend line (dashed line) indicates the linear association between variables. Other pair-wise comparisons between immune marker levels and neoantigen-related variables were insignificant, and were not shown. **D**) Average percentages of infiltrating $CD8^+$ T cells observed in AAH (upper panel) and ADC (lower panel) plotted against percentage of patient-wise PANs. Each patient is represented by a data point indicated by a unique symbol. ADC in one patient was not evaluated. P-values are based on Kendall rank.

The evidence of the apparent immune responses in lung cancer premalignancy and the notion that the somatic mutations can contribute to modulation of the pathways regulating the tumor immunity, prompted us to determine if the activity of such pathways was associated with outcomes in early stage ADC. The expression of genes involved in 16 pathways from the Molecular Signature Database was analyzed in the TCGA LUAD (lung adenocarcinoma) cohort (444 tumors and 58 normal samples). Gene Set Variation Analysis was utilized to estimate the activities of immune-modulating pathways in individual patients, and these were then subjected to unsupervised hierarchical cluster analysis to stratify samples. Based on the pathway activity, we identified three major groups (**Figure 3A**). Among them, group 0 (Gr0, annotated by black) had the highest levels of immune-related gene expression and included 51 tumors and the majority of normal samples (n = 52), whereas the other two groups included the remainder of the tumor samples (χ^2 test $p < 10\text{-}16$): Gr1 (n = 198, blue) with intermediate and Gr2 (n = 201, red) with lowest expression of immune-related genes. These groups were not significantly associated with tumour stage (χ^2 test p = 0.14 for stage I vs. stage II and higher), however, the overall survival was marginally higher in Gr1 compared to Gr2 (log-rank test (LRT) p = 0.063). Remarkably, the difference in survival between Gr1 and Gr2 was most prominent for stage I patients (LRT p=0.05, **Figure 3B**), but not for stage II and higher patients (LRT p = 0.44, **Figure 3C**). Together, these results suggest that modulation of the immune-related pathways, especially at the earliest stages of lung ADC, may critically affect the outcomes of lung cancer patients.

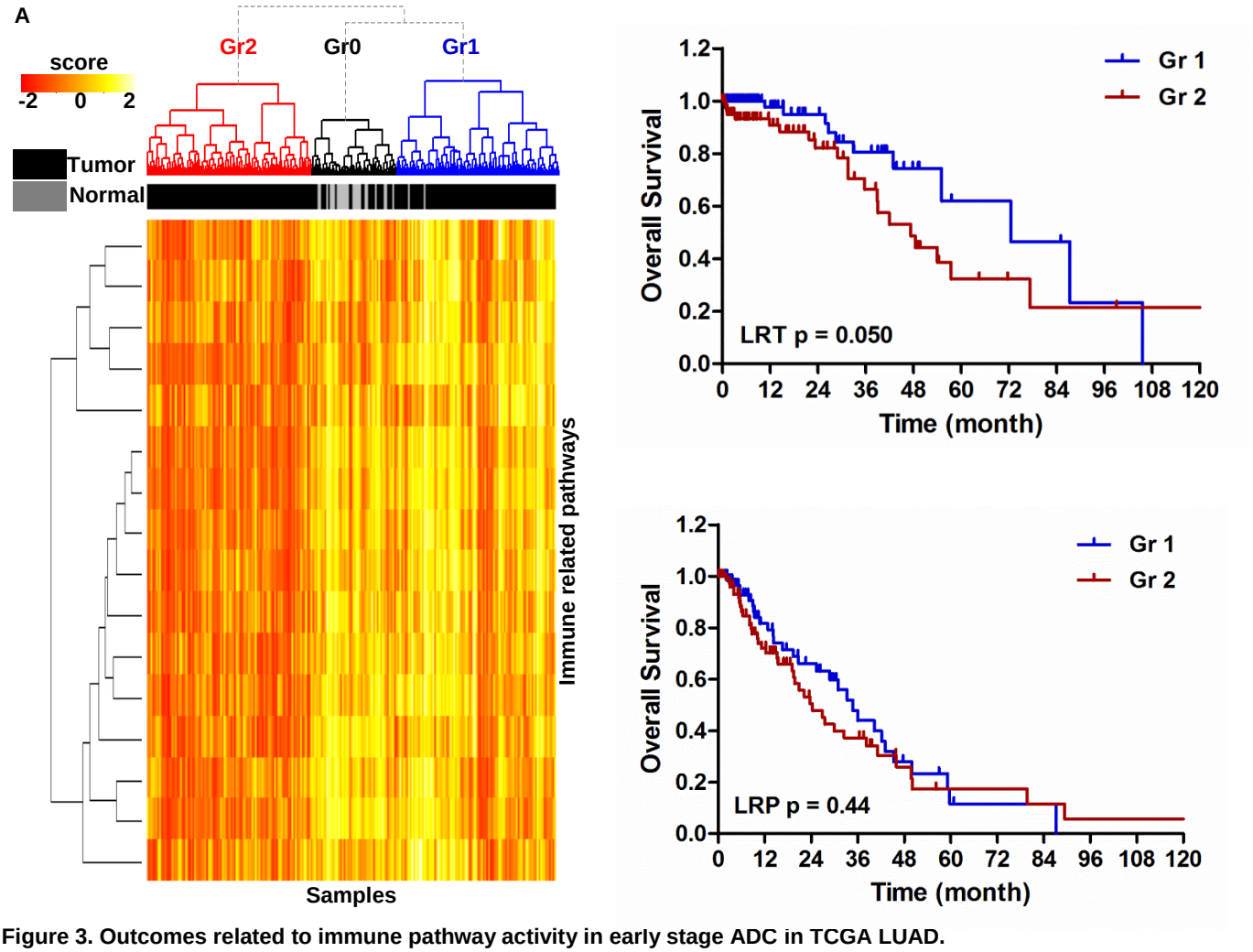

Figure 3. Outcomes related to immune pathway activity in early stage ADC in TCGA LUAD.
A) Heatmap of gene expression scores of 16 immune-related pathways in TCGA LUAD and normal lung samples. Serial sections stained for the indicated markers shown at 10x and 20x magnification. **B**) Kaplan-Meier survival curves of stage I, and **C**) stage II and higher patients from the groups identified in **Figure 3A**.

In accord with the cancer immunosurveillance theory, our findings support the concept that the immune system is capable of recognizing cancer precursors. Because evasion of immune surveillance has been implicated as an emerging hallmark of cancer development, future investigations will focus on stimulating specific immune responses. Thus, unleashing the immune response against pulmonary premalignancy may facilitate a blockade of the progression of premalignancy to invasive cancer at the earliest stages of disease. Our studies in the framework of the DOD Idea grant pave the way for developing novel therapeutic strategies, such as vaccines, targeting clinically actionable neoepitopes across the spectrum of premalignancy to invasive disease before the development of invasive cancer.

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

These preliminary results were presented as the oral presentation at the American Association for Cancer Research 2017 annual meeting in Washington, DC.

- **How were the results disseminated to communities of interest?**

As these results are preliminary, they have not yet been disseminated. After the completion of the project, the WES data will be deposited to the publicly available data repository.

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

During the No Cost Time Extension, we will complete the Major Task 3, including the multiplex immunofluorescence staining, slide scanning, image analysis and linking the expression of immune regulators to the mutational and neoantigen landscapes of the tissues

4. Impact.

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

In our preliminary studies we first demonstrated that heterogeneity between different AAH lesions from the same patient is significantly lower than between lesions from different patients. This leads to the occurrence of different PANs, which in turn will cause varied treatment responses and outcomes. On the other hand, low genomic complexity of pulmonary premalignancies raises hope that unleashing the immune response against them (as opposed to targeting the established tumors) may be a successful strategy for cancer prevention. These findings a) clearly demonstrate that cancer interception and prevention strategies will need to be tailored to individual patients, and b) will facilitate development of novel immunoprevention approaches for lung cancer treatment.

- **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**

No changes were made to the original research plan.

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

No problems have been encountered.

- **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**
  - **Journal publications.**

Manuscript in preparation, will be submitted by the end of August 2018.

  - **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?**

Kostyantyn Krysan.
No change.
Linh M. Tran.
No change.
William D. Wallace.
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

None.