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TITLE: Military Exposure-Related Pleural Mesothelioma: An Innovative Translational Approach to Inform Novel Molecular-Targeted Treatment Development

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Not applicable

14. ABSTRACT

Malignant pleural mesothelioma (MPM) is a highly aggressive form of cancer that develops within the pleural lining of the lungs. Asbestos-related malignancies dropped precipitously in military/veteran populations upon the removal of asbestos from naval ship construction. However, older naval vessels and military facilities still containing asbestos were still in use decades later, resulting in thousands of veterans suffering asbestos exposure. Indeed, it is estimated that military veterans account for one third of all MPM patients. Despite this estimation, there is little data on the phenotype of military exposure and MPM pathogenesis. During this funding cycle, we demonstrated at the molecular level, Clusters 2 & 3 represented a continuum, or "histo-molecular gradient", predominantly biphasic and sarcomatoid tumors, respectively. Correlated with the epithelial to mesenchymal transformation (EMT) process, the two more extreme clusters 1 and 4 were enriched for epithelioid and sarcomatoid tumors, respectively. We identified an association of single-pattern cytoplasmic staining with markers of EMT, suggesting a complex role for BAP1 in MPM. In fact, it appears that military exposed MPM patients have a unique phenotype compared to matched civilian cohort (unpublished data undergoing secondary validation). We successfully generated conditional mouse lines with NF2, CDKN2a and p53 deletions in mesothelial cells (WT1-CreER driver) as single and multiple knockout mice. The mice were sacrificed 1 year after administration of tamoxifen with successful pleural MPM tumor generation in all genotypes. The final aim of this funding cycle was accomplished with establishment of patient-derived xenografts (PDX) from 14 patients (including 2 rare sarcomatoid tumors and several biphasic tumors) utilizing modifications of existing techniques such that it should be possible to created immortalized tumors from most patients with triplicate implantation.

15. SUBJECT TERMS
Malignant pleural mesothelioma (MPM)

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1. INTRODUCTION:

Malignant pleural mesothelioma (MPM) is a highly aggressive form of cancer that develops within the pleural lining of the lungs. Asbestos-related malignancies dropped precipitously in military/veteran populations upon the removal of asbestos from naval ship construction. However, older naval vessels and military facilities still containing asbestos were still in use decades later, resulting in thousands of veterans suffering asbestos exposure. Indeed, it is estimated that military veterans account for one third of all MPM patients. Despite this estimation, there is little data on the phenotype of military exposure and MPM pathogenesis. We recently defined the mutational landscape of MPM and have identified the most commonly mutated genes as BAP1, NF2, TP53, and SETD2, as well as other frequent mutations. We have also classified MPM into 4 distinct molecular clusters that provide new opportunities to identify MPM patients with better prognosis as well as to rationally divide tumors based on distinct molecular/biochemical driving mechanisms. The objective of the study is to refine the classification of MPM into biologically and prognostically distinct sub-groups, relate these sub-groups to the military-exposed veterans and rationally design potential biomarker-selected targeted therapies for the military/veteran population for future human trials. This study aims to define and compare MPM tumors from military versus non-military cases for diagnosis and prognosis, using the type of mutations and cluster membership by RNA expression. This study also intends to identify potential novel therapies utilizing genetically-engineered mouse models (GEMMs) to interrogate MPM-specific tumorigenesis, invasion, and metastasis. Finally, this study plans to translate potential molecular targets into therapeutics using an *in-vivo* PDXs model. MPM tumors from civilian and military/veteran patients will be genotyped for the five most frequently mutated genes in MPM and will then be used to establish the distribution of mutations of all types in the 4 molecular cluster groups that have been classified. Frequently observed mutations or other genomic aberrations will be further interrogated using GEMMs to more completely understand MPM carcinogenesis and progression, as well as to identify potential targets for therapy. PDXs models will then be developed *in vivo* from the diagnostic/prognostic biomarkers that are identified in the civilian and military populations to focus pre-clinical therapeutics on the two extreme sub-types of MPM: 1 and 4.

2. KEYWORDS:

Malignant pleural mesothelioma (MPM)

genetically-engineered mouse models (GEMMs)

patient-derived xenograft (PDX)

3. ACCOMPLISHMENTS:

What were the major goals of the project?

A. Establish Consortium Collaborative Infrastructure (Responsible PI, Harpole-Duke)

Expected:1-3 months
Actual: 100% complete

B. Perform RNA-seq analyses on the prospectively-collected, fresh-frozen MPM tumors (Responsible PI, Bueno-BWH)

Expected: 3-9 months
Actual: 95% complete.

C. Investigate whether there are any genomic / genetic differences between civilian and veteran MPM tumors based on the consensus cluster expression and mutational genotyping (N=250 FFPE) (Responsible PI, Bueno-BWH)
Expected: 6-18 months
Actual: 60% complete.

D. Identification of Novel Therapies (Responsible PI, Harpole-Duke)
Expected: 12-24 months
Actual: 45% Complete

E. To translate potential molecular targets into therapeutics using an in-vivo PDX model (Responsible PI, Harpole-Duke) – 70% complete
Expected completion: 24-36 months
Actual completion: 70% complete

What was accomplished under these goals?

A. Establish Consortium Collaborative Infrastructure

This major goal was previously completed and reported in prior reports. There was nothing further to complete regarding this major goal. However, we are pleased to inform the DOD that we have successfully assembled the most comprehensive and largest collection of Mesothelioma cases and associated fresh Frozen and FFPE specimens with linked military service and clinical data in North America. This number is slightly above the one proposed in the grant, to allow for redundancy in case some of the specimens are not adequate.

B. Perform RNA-seq analyses on the prospectively collected, fresh-frozen MPM tumors.

Unsupervised analysis to identify potential novel, distinct molecular MPM subgroups (n=192; BWH). We validated at the molecular level, Clusters 2 & 3 represented a continuum, or "histomolecular gradient", consisting mainly of biphasic and sarcomatoid tumors, respectively. Correlated with the EMT process, the two more extreme clusters 1 and 4 were enriched for epithelioid and sarcomatoid tumors, respectively. The following tests were developed for Cluster diagnosis:

A. *Cluster 1 test (CHP1/ENAH): AUC 0.95, at 9.877 threshold: sensitivity 0.92 / specificity 0.89*
B. *Cluster 2 test (ANKRD50/FXYD6): AUC 0.90, at 0.989 threshold: sensitivity 0.80 / specificity 0.83*
C. *Cluster 3 test (KRT17/PLB1): AUC 0.79, at 0.2 threshold: sensitivity 0.95 / specificity 0.53*
D. *Cluster 4 test (DYSF/MISP): AUC 0.9, at 1.678 threshold: sensitivity 0.76 / specificity 0.95*

C. Investigate whether there are any genomic / genetic differences between civilian and veteran MPM tumors based on the consensus cluster expression and mutational genotyping (N=250 FFPE)

We analyzed both the military and the matching non-military cohort by RNA sequencing using frozen tissue to have more reliable data after pilot data indicated that the FFPE samples might not reproducible for validation due to distortion caused by some genes. We have used whole exome sequencing for these samples, as well as a "deeper dive" into the sarcomatoid tumors (n=72) with RNA sequencing, whole exome sequencing and the Saphyr (Bionano Genomics, San Diego, CA) DNA deletion/insertion panel. Clustering analyses from these data in progress. Our targeted exome sequencing panel (mutations and variants in BAP1, NF2, SETD2, SETBP1, TP53, CDKN2A) has been collected from all military-exposed veterans and MPM controls in our cohort. Initial analysis of 677 cases, 243 military and 392 non-military revealed not unexpectedly that the military population is almost entirely composed of men, whereas the non-military population includes 25% women. Also noted is the observation that in the military cohort, the non-epithelial histology is significantly higher 43% (104/243; 43% vs. 245/392; 36%, $p<0.05$). Our results suggests that the military patient population is different from the civilian one and we are currently completing final genomic analysis to understand why.

In addition, BAP1 IHC on a preliminary cohort has been performed using other funding sources, and NF2 FISH are completed. The BAP1 preliminary data were published (De Rienzo et al. *Journal of Pathology* 2021). The data from NF2 FISH analysis is currently being analyzed by a biostatistician. Oncopanel data, which detects mutations and variants in BAP1, NF2, SETD2, SETBP1, TP53, CDKN2A, has been collected from all patients consented to an IRB-approved protocol. Genomic mutation results are available for 240 patients.

c. Identification of Novel Therapies

In order to test the candidate drivers of mesothelioma and developing novel therapies, in this sub-aim, we used a mouse genetic approach. We continued to try to generate conditional mouse lines with NF2, CDKN2a and p53 deletion in mesothelial cells (WT1-CreER). Over this grant cycle, we have generated several novel mouse lines in which oncogenic stimuli are inducible expressed in mesothelial cells of the mouse pleura. Although breeding multiple floxed alleles to homozygosity is very complex and time-consuming, and the addition of the COVID pandemic that presented a unique challenge, we successfully generated the cell lines outlined in the table below. Table below shows number of mice obtained with single and multiple floxed genes (all homozygous) with Wt1-CreER promoter. These mice have also been administered tamoxifen. In order to determine the individual and combined effect of NF2, CDKN2a and P53 deletion on disease progression or tumor formation, we sacrificed one mouse from each genotype (Wt1-creER; NF2 fl/fl, WT1-creER; NF2 fl/fl; CDKN2a fl/fl and WT1-creER; NF2 fl/fl; CDKN2a fl/fl; P53 fl/fl) 35 weeks post tamoxifen injections. The H&E analysis showed that there was no apparent difference between the control and knockdown transgenic mice. So, we decided to wait little longer and we plan to harvest lung sections after 60 weeks post tamoxifen administration. We also plan to do the Kaplan Meier analysis (survival assays) for these various genotypes.

S.No.	Genotype	No of mice	Date Tamoxifen injection	Time since injection (Feb 13, 2021)
1	Wt1-creER; NF2 fl/fl	14	1/28	(54 weeks)
2	WT1-creER; NF2 fl/fl; CDKN2a fl/fl	6	1/28	(54 weeks)
3	Wt-creER; NF2 fl/fl;	8	1/28	(54 weeks)

	P53 fl/fl			
4	WT1-creER; NF2 fl/fl; CDKN2a fl/fl; P53 fl/fl	12	1/28	(54 weeks)
5	WT1-creER; NF2 fl/fl; CDKN2a fl/fl; P53 fl/fl	8	5/1	(41 Weeks)

All the above lines with a lox-stop-lox Cas9 allele that will allow Crispr knockout of additional genes after lentiviral delivery of sgRNAs to the pleural space. Characteristic images of the tumors demonstrating invasive mesothelioma growth in the mouse pleura after 12 months.

d. To translate potential molecular targets into therapeutics using an in-vivo PDX model.

BWH reports that eleven models have completed passage 1, of which one is Sarcomatoid and ten are Epithelioid or Biphasic. Nine have been sent to Duke's animal facility for therapeutic agent testing, consisting of 6 biphasic, 2 epithelioid, and 1 sarcomatoid. The BWH PDX Core no longer passages tumors to p2 because they have determined that they can generate enough cryovials from p0 and p1.

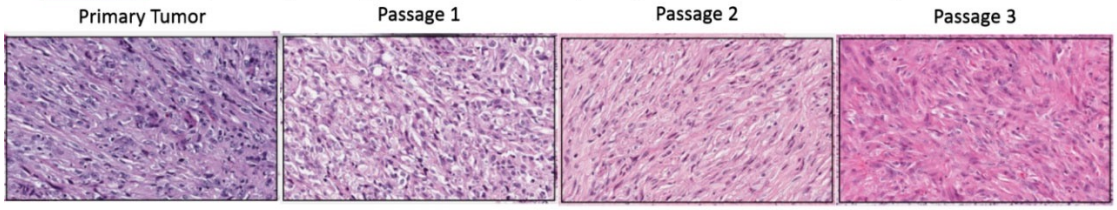
To date, Duke reports a sarcomatoid PDX line and a predominantly epithelioid variant PDX line. Both established PDX lines have been developed/ grew at a faster pace than previous passages have reached their endpoint quicker due to size and tumor burden. In addition to the established PDX, we established 3 additional, unique lines of tumors. However, due to inventory issues with the vendor, mouse orders were delayed and delayed the implantation and passage of these new lines.

To verify that the PDX immortalized tumor accurately represents the patient's tumor and environment, fresh tumor from two different primary tumors implanted in mice and a specimen from each subsequent passage were analyzed and compared to the original primary for concordance. Concordance was evaluated by: histopathologic (H/E, IHC) assessment, miRNA array assessment using FFPE-based qNPA Whole Transcriptome Assays (HTG, Inc) using other funding sources. We set a threshold of similar histology and >90% concordance for an acceptable representation of the primary MPM.

Fresh tumor from resection or biopsy is implanted in a mouse (passage 1), taking 6-9 months to grow to a size that requires harvesting and re-implantation to the next mouse (passage 2). This continues if the tumor remains intact. At each passage, part of the tumor is analyzed and compared to the original primary for concordance. To the best of our knowledge, this quality control process has never been utilized and includes histopathologic (H/E, IHC) assessment, miRNA array and mRNA array full genomic assessment using FFPE-based qNPA Whole Transcriptome Assays (HTG, Inc). We set a threshold of similar histology and >90% concordance for an acceptable representation of the primary MPM. Below are two examples: a rare sarcomatoid and a biphasic mostly epithelial tumor we successfully generated PDX models. Note in Figure 6 that the histology is similar up to the third passage collected more than 2 years after resection, with miRNA array (Figure 7) data demonstrating 0.92 correlation at passage 3 of the primary for the sarcomatoid tumor and 0.96 at passage 2 for the biphasic tumor we are focusing on in veterans. (mRNA arrays were delayed by Covid-19 laboratory shutdowns)

These data demonstrate the reproducibility and fidelity of our PDX models, allowing us the ability to continue with this platform in our proof of principle prospective trial.

6A. Sarcomatoid MPM (Cluster 4): Histologic Correlation between primary MPM and PDX tumors Passage 1 to 3



6B. Biphasic MPM (Cluster 2): Histologic between primary MPM and PDX tumors Passage 1 to 2

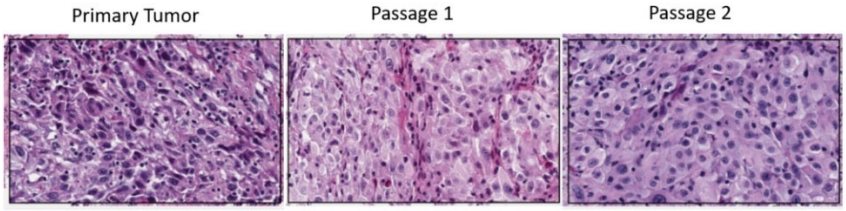


Figure 7a. Sarcomatoid MPM (Cluster 4): Correlation of miRNA Expression between primary MPM and PDX tumors Passage 1 to 3

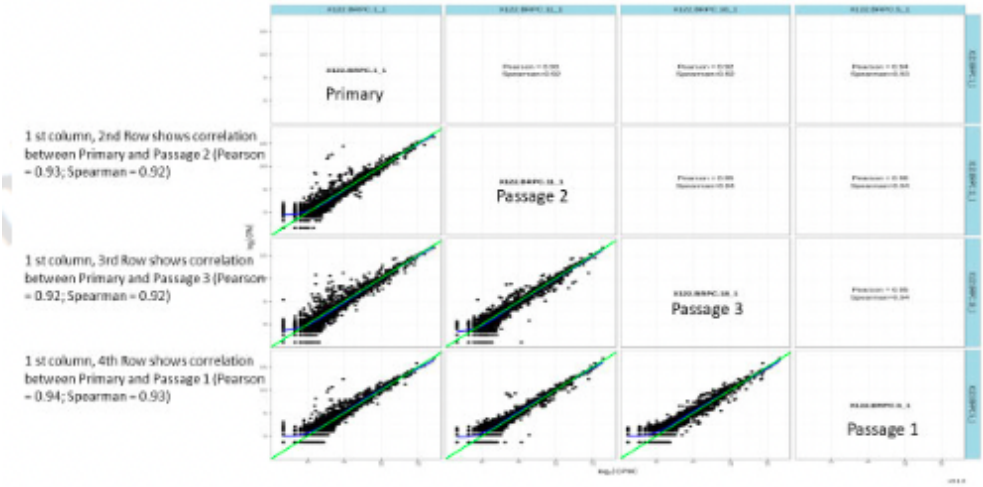
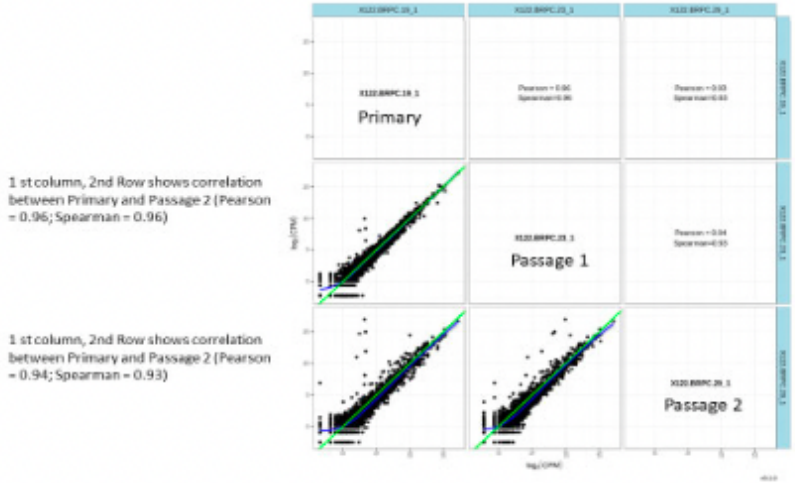


Figure 7b. Biphasic MPM (Cluster 2): Correlation of miRNA Expression between primary MPM and PDX tumors Passage 1 to 2



The histology is similar up to the third passage and was collected more than 2 years after the initial resection, with miRNA array data demonstrating 0.92 correlation at passage 3 of the primary for the sarcomatoid tumor and 0.96 at passage 2 for the biphasic tumor we are focusing on in veterans. These data demonstrate the reproducibility and fidelity of our PDX models.

Our Duke IACUC protocol#A043-18-02 was reviewed and approved for renewal on February 02, 2021. The new IACUC protocol number is A020-21-01 and was approved through January 31, 2024. This protocol was submitted to ACURO and gained approval on April 14, 2021. The BWH IACUC protocol (CA160891P1.e001) 3-year rewrite for the previously approved protocol 18-006 was approved on 3/24/2021 through 3/24/2024.

What opportunities for 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?

This is the final report for this grant. Therefore, there is nothing to report.

4. IMPACT:

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

We have assembled the most comprehensive and largest collection of Mesothelioma cases and associated fresh Frozen and FFPE specimens with linked military service and clinical data in North America, available for current and future analyses. This comprehensive proposal for molecular

characterization of mesothelioma whose goal is identification of novel targeted therapies specifically matched with genetically identified subsets of tumors study, seeks to identify genetic markers specific to military-related MPM. Thus, these findings will be relevant to thousands of military veterans who were exposed to asbestos. The identification of these markers could lead to earlier/enhanced diagnosis and treatment strategies for veterans afflicted with this deadly disease and improve patient survival.

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:

Cluster-4 Sarcomatoid Mesothelioma tumors are very rare, therefore it has become evident that we will not reach the stated goal of five Cluster-4 PDX models within this grant period.

Changes in approach and reasons for change

A pilot test that was performed indicated that the FFPE samples are not suitable for the validation planned under Major goal 3. Therefore, we are performing RNAseq in frozen samples, exome sequencing and immunohistochemistry as a replacement for this metric.

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

We anticipate that creating 5 PDX models from Cluster 4 may be challenging for the following reasons;

> Based upon the fact that BWH's PDX success rate has now been determined to be 3-6 tumors required for each one successful PDX model. Therefore, to get 5 Cluster 4 PDX models needed for this grant we will need to have approximately 15-30 Cluster 4 histology tumors available in the next one to two years. However, Cluster 4 tumors are rare - we might expect up to approximately 6 per year between Duke and BWH. To mitigate this, a high priority will be placed on any Cluster 4 tumors that become available for PDX models.

Cluster-4 Mesothelioma tumors are very rare, therefore we may not reach the stated goal of five Cluster-4 PDX models within this three year grant period.

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.

De Rienzo et al. *Journal of Pathology* 2021

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**

A. Collaborative mesothelioma clinical RedCAP database

B. Collaborative Biospecimen collection of fresh-frozen and FFPE mesothelioma tumor samples – samples are continuing to be added as the project progresses

C. Collaborative data repository of RNA-seq analyses on mesothelioma tumor samples – data is continuing to be added as analyses are completed

D. - Database of clinical data, outcomes, and military exposure on 500 patients.

E. - Physical collection of 250 fresh frozen non-military and matching FFPE samples, plus 250 Military FFPE samples.

F. - >10 PDX Mesothelioma mouse models.

G. Molecular signatures of potential clinical value

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: David Harpole, MD (Duke) – no change

Name: Mary-Beth Joshi, MPH (Duke) – no change

Name: Tam How (Duke) – no change

Name: Mark Onaitis, MD (UCSD) – no change

Name: Priyanka Chaudhary, PhD (UCSD) – no change

Name: Guangfang Wang (UCSD) – no change

Name: Raphael Bueno (BWH) – no change

Name: Corinne Gustafson (BWH) – no change

Name: David Severson, Ph.D. (BWH)
Project Role: Computational Biologist
Nearest person month worked: 1
Contribution to Project: analysis of RNAseq data, clustering

Name: Assunta De Rienzo, Ph.D. (BWH)
Project Role: Lab Director
Nearest person month worked: 6
Contribution to Project: Sample prep, data analysis

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

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Independent reports will be submitted by BOTH the Initiating PI and the Collaborating/Partnering PI.

QUAD CHARTS:

Not applicable

9. APPENDICES:

Not applicable