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

PRINCIPAL INVESTIGATOR: **Dr. Raphael Bueno**

CONTRACTING ORGANIZATION: **Brigham and Women's Hospital, Inc., Boston, MA**

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Fort Detrick, Maryland 21702-5012

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6. AUTHOR(S)

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5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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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. 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.

15. SUBJECT TERMS
Malignant pleural mesothelioma (MPM)

16. SECURITY CLASSIFICATION OF: Unclassified			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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TABLE OF CONTENTS

1. Accomplishments:

What were the major goals of the project?

Major Goals Established in approved SOW	Projected Timeline	% Completion
1. Establish Consortium Collaborative Infrastructure	Months	100%
a. Finalize material transfer agreements and data use agreements between participating consortia members	1-3	100%
b. Develop and implement project data dictionary and case report form (CRF) for clinical data entry	1-3	100%
c. Develop and implement central RedCAP project database	1-3	100%
d. Implement standard operating procedures across the consortium sample submission for pathologic assessment of tissue samples	1-3	100%
e. Finalize HRPO proposals	1-3	100%
f. Finalize Animal use protocols	1-3	100%
2. Perform RNA-seq analyses on the prospectively-collected, fresh-frozen MPM tumors	*3-9*	*95%*
a. Unsupervised analysis to identify potential novel, distinct MPM subgroups that could be added to our 4 consensus clusters	*3-9*	100%
b. Complete validation analysis of our MPM consensus clusters in an independently-collected cohort of MPM tumors (n=192; BWH)	*3-9*	*90%*
3. 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)	6-18	60%
a. Validation analysis of the consensus clusters by rt-PCR	6-18	80%
b. Genotype specimens from military and civilian cohorts from the five most frequently mutated genes in MPM with the FFPE specimens	6-18	100%*
c. Assess the role of candidate genes in causing pleural malignancy by altering the GEMMS with the addition/deletion of specific genes to interrogate their role in tumorigenesis/invasion	6-12	20%
d. Assess pleural surfaces of mice 40 weeks after birth. The lungs and pleura will be dissociated into suspensions of single cells using mechanical and enzymatic protocols, and flow cytometry for GFP will be carried out.	6-12	10%
4. Identification of Novel Therapies	12-24	45%
a. Identify genetic drivers that result in neoplasia from genes identified in consensus clusters 1 and 4 by	12-24	45%

isolating wild-type and oncogenic cells that result in neoplasia.		
b. Grow colonies of wild-type and tumor-suppressor knockout cells.	12-24	100%
c. Transformed pleural cells that form tumors will be isolated and grown in matrigel plates for testing of small molecule inhibitors or known FDA-approved drugs (approximately 30 will be tested) and analyzed in an unbiased way using ImageJ software.	12-24	25%
d. The positive drugs will be interrogated to select the best two candidate targeted agents to be carried forward in Aim 3	12-24	0%
5. To translate potential molecular targets into therapeutics using an in-vivo PDX model.	24-36	70%
a. Utilizing tumor samples collected from patients verify the diagnosis of consensus clusters 1 and 4 tumors from patients from Duke University and BWH	24-36	100%
b. Obtain tumor cells from pleural effusion/biopsy from Duke University and BWH for the creation of PDXs in the Duke Animal Facility.	24-36	100%
c. Tumors from passages 2 and 3 will be divided into 2 sections. One section will be frozen/immortalized for future targeted drug evaluations. One section will be formalin-fixed and embedded for histologic and molecular verification as identical as to the original patient specimen.	24-36	75%
d. Create 10 PDXs from 10 different patients from Cluster 1	24-36	100%
e. Create 5 PDXs from 5 different patients from Cluster 4.	24-36	40%
f. The two most promising agents identified from Aim 2 will be interrogated in the PDX models specific for Types 1 and 4 of MPM. This will be determined by assessing the tumor doubling time (Td) (calculated by the exponential fit of the whole curve and treatment efficacy (T/C%; 100xT(tm)/C(tm), where T(t) and C(t) are the mean of the tumor volumes in the treated and control groups, respectively at any time t and tm was the observation time given the minimum T(t)/C(t) value), and absolute growth delay (ADG) and log cell kill (LCK), drug tolerability (based on animal weight loss, clinical observation and any mortality).	24-36	0%

** A pilot test performed indicated that the FFPE samples are not suitable for validation. Therefore, this metric is considered complete because it cannot be completed as written in the SOW. We are performing RNAseq in frozen samples, exome sequencing and immunohistochemistry as a replacement for this metric.*

What was accomplished under these goals?

Major Goals Established in approved SOW

1. 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.

2. 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

3. 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 will be completed during the current NCE. 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.

4. Identification of Novel Therapies

   In order to test the candidate drivers of mesothelioma and developing novel therapies, in this sub-aim, we are using a mouse genetic approach. We continue to try to generate conditional mouse lines with NF2, CDKN2a and p53 deletion in mesothelial cells (WT1-CreER). We have successfully obtained these single and multiple knockout mice. 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; P53 fl/fl	8	1/28	(54 weeks)
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)

In addition to this, we have procured sgRNA lentiviral particles against Bap1, SETD2 and PTEN. These lentiviral sgRNAs will be injected into the pleural space of WT1-creER; lsl Cas9 after tamoxifen administration, which will lead to inactivation of target gene in recombined cell. If this method works, we plan to test the effect of knockdown of other candidate genes as well.

Lastly, we have been optimizing the conditions to grow pleural cells in culture. Once established, we will grow these pleural cells from both wild type and mutant mice and use libraries of small molecule inhibitors or known DFA-approved drugs to perform high throughput drug-screening.

5. 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 in passage 5, implanted on May 3, 2021. There were no issues with the surgeries or recovery of the mice and all surgical sites are healed and healthy. Duke also has a predominantly epithelioid variant PDX line in progress in passage 3, implanted on April 13, 2021 Both established PDX lines have been developing/ growing at a faster pace than previous passages and are approaching its endpoint quicker due to size and tumor burden. Both lines are continually monitored. In addition to the established PDX, we have 3 unique lines of tumors ready for passage. Due to inventory issues with the vendor, our mice order has been delayed. We plan to implant these three within the next month. 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 is 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.

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. 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,

Describe the Regulatory Protocol and Activity Status (if applicable).
Describe the Protocol and Activity Status for sections a-c, as applicable, using the format described for each section. If there is nothing significant to report during this reporting period, state "Nothing to Report."

(a) ***Human Use Regulatory Protocols***

TOTAL PROTOCOLS: *State the total number of human use protocols required to complete this project (e.g., 5 human subject research protocols will be required to complete the Statement of Work."). If not applicable, write "No human subject research will be performed to complete the Statement of Work."*

1 protocol is required to complete this project.

PROTOCOL(S): *List the identifier and title for all human use protocols needed to complete the project. Include information about the approved target number for clinical significance, type of submission, type of approval with associated dates, and performance status.*

The following format shall be used:

Protocol (1 of 1 total):

Protocol A-20258

Title: Malignant Pleural Mesothelioma (MPM): An Innovative Translational Approach to Inform Novel Molecular-Targeted Treatment Development

Target required for clinical significance: 250 (Approved to review 300 records to obtain 250)

Target approved for clinical significance: 250 (Approved to review 300 records to obtain 250)

Submitted to and Approved by:

Provide bullet point list of protocol development, submission, amendments, and approvals (include IRB in addition to HRPO).

Duke

- *Duke IRB Submitted for Departmental Regulatory approval 6/27/2017*
- *Departmental Regulatory review completed 7/7/2017*
- *Departmental Regulatory approval received 7/27/2017*
- *Submitted to the Departmental IRB Chair for approval 7/27/2017*
- *Departmental IRB Chair approval received 9/6/2017*
- *Duke IRB Submitted for Cancer Protocol Center approval 9/8/2017*
- *Cancer Protocol Center approval received 9/21/2017*
- *Duke IRB recommended protocol for expedited review 9/22/2017*
- *Received Duke IRB approval 10/11/2017*
- *Approval of amendment to add Jason Lones as RedCAP database manager, 10/11/2017*
- *Duke HRPO Documents submitted11/15/2017*
- *Follow-up Duke HRPO information submitted 1/30/2018*

- *Duke HRPO approval – 2/28/1018*
- *DUHS IRB notification of continuing review approval – 9/27/2018*
- *Duke IRB amendment – Amendment allows for the use of mesothelioma samples collected under an additional collection protocol at Duke – Approved 11/10/2018*
- *DUHS IRB notification of continuing review approval through 9/13/2020*
- *DUHS IRB notification of continuing review approval through 9/16/2021*
- *DUHS IRB notification of continuing review approval through 10/22/2021*

BWH

- *BWH A-20259 HRPO Approval Memorandum (IRB Study Number 2017P001115/PHS, Proposal Log Number CA160891P1, Award Number W81XWH-17-1-0373; Protocol 18-006 Approval through 3/24/2024*
- *approved by the Partners Healthcare System Institutional Review Board (IRB) on 22 June 2017. The U.S. Army Medical Research and Material Command (USAMRMC), Office of Research Protections (ORP), Human Research Protection Office (HRPO) reviewed the protocol and found that it complies with applicable DOD, U.S. Army, and USAMRMC human subjects protection requirements 11/3/2017*
- *BWH IRB notification of continuing review approval through 9/24/2020*
- *BWH IRB notification of continuing review approval through 8/30/2021*
- *BWH IRB notification of continuing review approval through 8/30/2022*

Status:

Report (i) progress on subject recruitment, screening, enrollment, completion, and numbers of each compared to original planned target(s), e.g., number of subjects enrolled versus total number proposed; (ii) amendments submitted to the IRB and USAMRMC HRPO for review; and (iii) any adverse event/unanticipated problems involving risks to subjects or others and actions or plans for mitigation.

- *Duke IRB approval 10/11/2017*
- *Duke HRPO approval – 2/28/1018*
- *Duke IRB amendment* – Amendment allows for the use of mesothelioma samples collected under an additional collection protocol at Duke – Approved 11/07/2018HRPO approval for this protocol was received 2/28/2018. The study team will assess the actual number of samples available for analysis.

TOTAL PROTOCOLS: 1

(b) Use of Human Cadavers for Research Development Test & Evaluation (RDT&E), Education or Training

Not applicable to this study

(c) Animal Use Regulatory Protocols
TOTAL PROTOCOL(S): 3

PROTOCOL(S):

The Duke, BWH and UCSD all have approved local IACUC protocols and have all received ACURO approval.

What do you plan to do during the next reporting period to accomplish the goals and objectives?

We plan to perform clustering analysis on the data generated from whole exome sequencing for these samples, as well as a the “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. In addition, we plan to complete secondary validation of 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

Major Goal 4. We will continue to cross breed the correct genetically modified mice to obtain the mesothelioma preparation and begin gene interrogation.

Major Goal 5. Maintain current PDX models by continuting to passage and freeze the models currently active.

2. Products:

Nothing to report from this reporting period

3. 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:	Karla Ballman, PhD (Cornell) – 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

4. Changes/Problems:

a. Actual Problems or delays and actions to resolve them/

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.

b. Anticipated Problems/Issues

None at this time

5. Special Reporting Requirements:

Quad Charts: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.