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

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8. PERFORMING ORGANIZATION REPORT NUMBER

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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)

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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?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

1.

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: 90% complete. Analysis of the data for validation in ongoing. Estimated completion by January 2021.

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: 40% Complete

E. To translate potential molecular targets into therapeutics using an in-vivo PDX model (Responsible PI, Harpole-Duke) – 67% complete
Expected completion: 24-36 months
Actual completion: 67% complete

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

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.

We have identified statistically robust candidate gene tests for clusters 1 and 4 to have validated with 192 EPP cohort and translated into FFPE with the 250 non-military cases. We have identified statistically robust candidate gene tests for clusters 1 and 4 to have validated with 192 EPP cohort and translated into FFPE with the 250 non-military cases. We have

also identified candidate gene tests for clusters 2 and 3; however, through this analysis and other work we have determined that clusters 2 & 3 represent threshold groups that exist on a continuum of EMT between the more robust clusters 1 and 4. For this reason, we are working on a framework to characterize cluster 2 and 3 samples in terms of the more robust clusters 1 and 4, which will be completed by October. Independently we found that the expression of the gene RERG is related to cluster membership and will use that in parallel to assess in the cohorts.

We have assembled 192 cases representing EPP patients. We've obtained gene expression data from 90 of them and prepared RNA from the remaining 102 which is being sent to a contract site for expression analysis to expedite the process and ensure uniformity. We expect the data to be analyzed for validation by December. We also have the matching FFPE blocks pulled and available for this cohort as we are actively working to transfer the winning signatures.

We've identified from our Mesothelioma cases 402 non-military and pulled 265 to confirm a final total of 250. RNA is already made from 102 and ready for analysis, and we are in the process of preparing RNA from the other 163.

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)

Gene expression analysis of 250 non-military and 250 military exposure mesothelioma cases is ongoing. Expression analysis for validation is expected to be completed and analyzed by January 2020. BAP1 IHC and NF2 FISH are completed and the database is currently being annotated. A DDX3 antibody has been identified which works well and IHC with this antibody has begun. We explored SETD2 expression with multiple available antibodies, but they gave poor results. Additional antibodies are currently being tested by our core. We have slides ready from all the specimens and once the best antibodies are chosen these will also be stained and annotated

D. Identification of Novel Therapies

In order to test the candidate drivers of mesothelioma and developing novel therapies, in this sub-aim, we are using mouse genetic approach. Table below shows number of mice obtained with single and multiple floxed genes (all homozygous) with Wt1-CreER promoter. We have obtained these mice after months of breeding. These mice have also been administered tamoxifen. No signs of distress have been observed even after 30 weeks of tamoxifen administration. We plan to harvest lungs at different time points. First, we will harvest lungs at 30 weeks from at least one mouse of each genotype. We will do the H&E staining and this

will help us in tracking the pathogenesis of the disease. Moreover, if no signs of distress are noted, we plan to wait a little longer and harvest the lungs at 35 and 40 weeks as well. 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 (8/24/2020)

1	Wt1-creER; NF2 fl/fl	14	1/28	7 months (30 weeks)
2	WT1-creER; NF2 fl/fl; CDKN2a fl/fl	6	1/28	7 months (30 weeks)
3	Wt-creER; NF2 fl/fl; P53 fl/fl	8	1/28	7 months (30 weeks)
4	WT1-creER; NF2 fl/fl; CDKN2a fl/fl; P53 fl/fl	12	1/28	7 months (30 weeks)
5	WT1-creER; NF2 fl/fl; CDKN2a fl/fl; P53 fl/fl	8	5/1	16 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.

Last, 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.

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

To date, BWH reports that Seventeen PDX models have completed p0 and 11 of these so far have passed QC. Twelve of the seventeen models have completed passage 1, and nine have been sent to Duke's animal facility for therapeutic agent testing, consisting of 6 biphasic, 2 epithelioid, and 1 sarcomatoid. Five additional PDX models are in progress in p1 passage. Two additional models that were in p1 failed QC and have been excluded from further analyses. The BWH PDX Core no longer passages tumors to p2 because they have determined that they can generate enough cryovials from p0 and p1. This saves the cost of mice. To date, Duke reports a sarcomatoid PDX line in progress in passage 3. Duke also has a predominantly epithelioid variant PDX line in progress in passage 1 and passage 2. Both PDX lines have developed tumors and are being monitored. Duke has implanted an additional 10 unique tumors.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased

knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to Report

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We have received a no cost extension to complete the following from the SOW.

Major Task 2: RNA-seq analyses on the prospectively-collected, fresh-frozen MPM tumors**.**

Major Task 3: We will continue working on a framework to characterize cluster 2 and 3 samples in terms of the more robust clusters 1 and 4.

Major Task 4: We plan to harvest lungs at different time points. First, we will harvest lungs at 30 weeks from at least one mouse of each genotype. We will do the H&E staining and this will help us in tracking the pathogenesis of the disease. Moreover, if no signs of distress are noted, we plan to wait a little longer and harvest the lungs at 35 and 40 weeks as well. We also plan to do the Kaplan Meier analysis (survival assays) for these various genotypes

Major Task 5: We will continue working on the following subtasks:

- Continue to divide tumors from passages 2 and 3 to be frozen/immortalized for future targeted drug evaluations and for formalin-fixation and embedding for histologic and molecular verification as identical as to the original patient specimen.
- Create 5 PDXs from 5 different patients from Cluster 4

- The two most promising agents identified from Aim 2 will be interrogated in the PDX models specific for Types 1 and 4 of MPM.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

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?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

6.

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. CHANGES/PROBLEMS:

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.

Due to COVID19 stay-at-home advisory and shutdown of laboratories we have been delayed with this work. To resolve this we requested and were granted a one-year no-cost extension, and now that restricted numbers of personnel are allowed to resume laboratory activities we will prioritize the DOD work over other projects.

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

No significant changes to the approach

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

Describe problems or delays encountered during the reporting period 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.

Due to COVID19 stay-at-home advisory and shutdown of laboratories we have experienced additional delays with this work. To resolve this we requested and were granted a one-year no-cost extension, and now that restricted numbers of personnel are allowed to resume laboratory activities we will prioritize the DOD work over other projects.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

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**

  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to Report

  8.

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to Report

  Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year*

(international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

Nothing to Report

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

  Nothing to Report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

  Nothing to Report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

  Nothing to report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*

- *clinical interventions;*
- *new business creation; and*
- *other.*

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: Mary N Dao (BWH)
Project Role: Technician
Nearest person month worked: 2
Contribution to Project: Performing technical effort in preparing samples for analysis
Funding Support: DoD W81XWH-17-1-0372

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*

- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

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

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

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Not Applicable

12.