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TITLE: HSV1 Oncolysis-Immune Checkpoint Inhibition Therapy for Breast Cancer Meningeal Metastases

PRINCIPAL INVESTIGATOR: Dr. Kumudu D. Kuruppu

CONTRACTING ORGANIZATION: Massachusetts General Hospital, Boston, MA

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Dr. Kumudu Darshini Kuruppu

E-Mail: dkuruppu@mgh.harvard.edu

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14. ABSTRACT

Meningeal metastasis is a terminal disease classified under stage IV breast cancer which affects 10-15% of patients. Currently there is no cure limiting survival to less than 4 months. The research conducted during this period explored a novel treatment approach which had hitherto not been investigated for breast cancer meningeal metastases – that of combination therapy with oncolytic HSV1 and immune checkpoint inhibition. Based on our initial investigation, the combination treatment approach was therapeutic for breast cancer meningeal metastases in our murine model. The tumor growth was inhibited following combination therapy survival was prolonged together with a remarkable recovery of neurological symptoms. Cancer-immune pathways regulated during combination therapy for this disease were identified for further investigation.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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1. INTRODUCTION (one para)

Meningeal metastasis is a terminal disease, categorized under stage IV breast cancer. It affects 10-15% of patients. Currently there is no cure limiting survival to less than 4 months. The research conducted during this period explored a novel treatment approach which had hitherto not been investigated for breast cancer meningeal metastases – that of combination therapy with oncolytic HSV1 and immune checkpoint inhibition. In this approach the meningeal metastases microenvironment was primed with oncolytic HSV1 in preparation for immune-checkpoint inhibition in an attempt to eradicate resilient tumor cells. The study was conducted in our syngeneic murine model of meningeal metastases. Based on our initial investigation, the combination treatment approach proved to be highly effective. Tumor growth was inhibited and the length of survival increased compared to the groups which received oncolytic HSV1 or anti-PD1 alone. The combination treatment group displayed a remarkable recovery of neurological symptoms. The cancer-immune pathways regulated during combination therapy were identified leading the investigation on a path to understand gene signatures that are regulated during treatment.

2. KEYWORDS (limit 20 words)

Breast cancer, meningeal metastases, Immune checkpoint inhibition, Anti-PD-1 therapy, oncolytic HSV1, combination therapy, Herpes Simplex Virus Type-1 (HSV1), Firefly Luciferase (Fluc), Immunohistochemistry (IHC), Magnetic Resonance Imaging (MRI), cancer-immune therapy, cancer immune-pathways.

3. ACCOMPLISHMENTS

What were the major goals of the project?

The major goals of the project as stated under the approved SOW are given below together with the expected milestones and target dates and their accomplishments to date.

<table>
<tr><td>Months</td><td>1</td><td>2</td><td>3</td><td>4</td><td>5</td><td>6</td><td>7</td><td>8</td><td>9</td><td>10</td><td>11</td><td>12</td></tr>
<tr><td colspan="13">Year 1</td></tr>
<tr><td>Task-1</td><td colspan="12">Investigate the therapeutic effect of combined oncolytic HSV1 and anti-PD-1 immune checkpoint inhibition</td></tr>
<tr><td>Sub-Task-1</td><td colspan="8">Study tumor growth response to combination therapy (with Gd-MRI)</td><td colspan="4"></td></tr>
<tr><td>Accomplishment</td><td colspan="6">Delayed due to Covid-19 lockdown – restricted facility access post-lockdown social distancing</td><td colspan="6">Commenced sub-task 1 (85% completed)</td></tr>
<tr><td>Sub-Task-2</td><td colspan="3"></td><td colspan="6">Define inflammatory gene signatures expressed during combination therapy (with Nanostring)</td><td colspan="3"></td></tr>
<tr><td>Accomplishment</td><td colspan="8">Delayed due to Covid-19 lockdown –limited access to imaging facilities</td><td colspan="4">Commenced sub-task2 (75% completed)</td></tr>
<tr><td>Sub-Task-3</td><td colspan="5"></td><td colspan="7">Correlate gene signatures with imaging scans and immune checkpoint and inflammatory markers (PD-L1, PD-1, CD8+, CD4, and IFNɤ) in ex vivo brains</td></tr>
<tr><td>Accomplishment</td><td colspan="7">Delayed as Sub-tasks 1 was delayed due to Covid-19 lockdown</td><td colspan="5">Sub-task 3 is yet to be completed - IHC staining of ex vivo brains remain to be done. (30% completed)</td></tr>
</table>

◦**What was accomplished under these goals? ▪For this reporting period describe:**

1) Major Activities;

(a). Animal Protocol Approval by ACURA. This was the first activity – imperative commence work on the proposed research. The animal experimental protocol which was approved by MGH-SRAC was submitted to ACURA. Following their review, the recommended changes were addressed, prior to approval. Commencement of laboratory work for this reporting period was delayed due to Covid-19 lockdown of the Institution research facilities and restricted facility access after reopening. The imaging facilities for animal experimentation reopened towards the year end. Work on the proposed project commenced as soon as these facilities reopened. During this period of lockdown the PI worked on obtaining ACURA approval, animal clearance, and PI registration at the MGH animal facility for imaging. The stereotaxic equipment a necessity for induction of meningeal metastases in the model for the study was purchased.

(b). Perform the first task in the proposed investigation. This involved waiting on a booking system following Covid-19 to use the animal facilities to induce tumors and for subsequent molecular imaging with MRI. The investigational activities were carried out: induce meningeal metastases in mice; image with Gd-MRI for baseline data; treatment administration; monitor tumor growth at subsequent time points after treatment with Gd-MRI alongside the non-treated tumor mice; study oncolytic virus replication with temporal bioluminescence imaging in the treatment groups that received virus (sub-task 1). The brains from the non-treated and treated groups were harvested for (i) RNA preparation for gene sequencing (sub-task 2) and (ii) prepared for ex vivo determination of relevant proteins expressed by IHC (sub-task 3).

2) Specific Objectives;

Specific objectives include:
(a). Investigate the outcome of a novel treatment concept – that of oncolytic HSV1 with immune checkpoint inhibition for breast cancer meningeal metastases
(b). Determine cancer-immune pathways regulated during this novel treatment option –combination therapy.
(c). Identify gene signatures and correlate the genes profiles with IHC staining of ex vivo brains.

3) Significant Results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or other achievements.

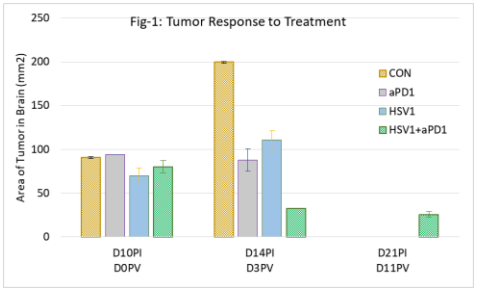
Our investigation on combined oncolytic HSV1 and immune checkpoint inhibition with anti-PD1 for breast cancer meningeal metastases generated the very first data for this novel therapy as described in this section, presented under the relevant sub-tasks. Tumor response to combination therapy, virus replication, physical and neurological response is detailed under sub-task 1 and inflammatory and cancer gene pathways under sub-task 2.

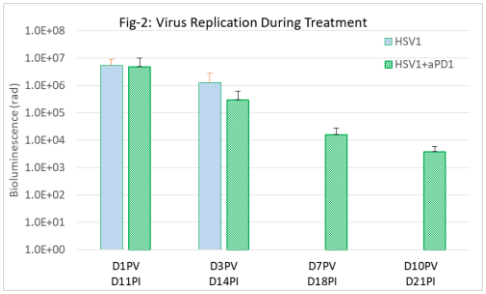
Sub-Task 1:
(1). Tumor response to combination therapy:
Meningeal metastases were induced in four groups of mice in preparation for the study. Ten days after induction, tumors were imaged with Gd-MRI to obtain baseline data before their respective treatment schedule. The groups include: (i) control (non-treated), (ii) anti-PD1, (iii) HSV1, (iv) HSV1+anti-PD1. Tumor response to treatment was determined by Gd-MR images obtained at selected time points after treatment. Tumors in the control (non-treated) group increased drastically by 2 weeks leading to severe neurological symptoms at which

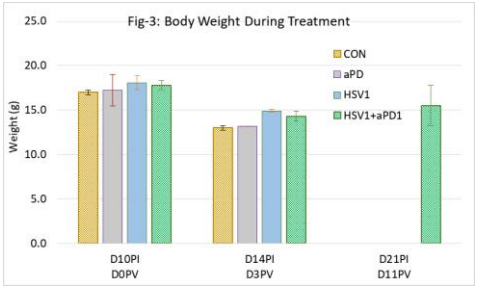
point the animals were sacrificed. Tumors in the combined treatment group decreased significantly by the 4th day after treatment and remained dormant over the course of one week. During this period the mice recovered from their symptoms of neurological impairment. The mice were sacrificed at this point – one week after the control group. The mice in the anti-PD1 and HSV1 treatment groups were sacrificed one day and three days after the control group. Tumor areas for selected time points for each treatment group are graphed in Fig-1.

(2) Virus replication during combination therapy:

Virus replication was monitored during treatment over selected time points. The oncolytic HSV1 which expresses the firefly luciferase (Fluc) was imaged with bioluminescence using luciferin as the substrate for Fluc. The high bioluminescence expressed a day after injection gradually declined over time, yet remaining active even ten days after treatment in the combination treatment group. Virus replication in the two treatment groups are graphed in Fig-2.







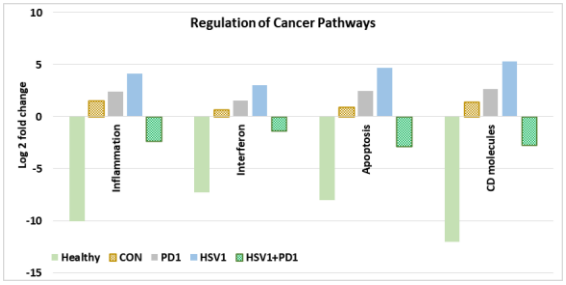
(3). Physical and neurological response to combination therapy:

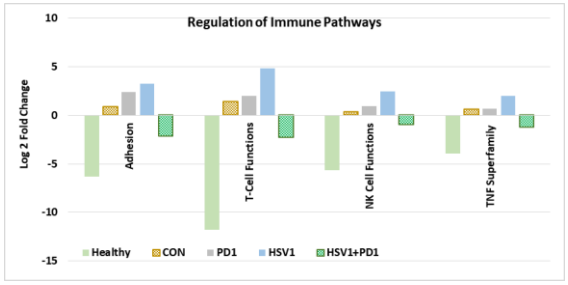
The physical and neurological features were observed during treatment in the respective groups. The body weights of each mouse were obtained over time. The control mice displayed symptoms of CNS involvement such as lethargy, loss of appetite, hind limb paralyses, inability to move, and lost nearly 18% of their weight at which time the animals were euthanized. These symptoms of CNS involvement were present in the anti-PD1 group and they were also euthanized early in the study. The group that received virus displayed symptoms of viremia but regained their normal physical activity within a few days. The combination treatment group regained their body weight to the healthy level by 3 weeks. Mice recovered displaying healthy habits such as responding to the external environment and exercising in their cage. The brains of these mice had very few to no tumors ex vivo. The body weights are graphed in Fig-3.

Sub-Task 2:

Inflammatory gene signatures expressed during combination therapy:

The brains of mice in the treated and control groups were excised for in vitro data analyses. RNA was prepared from these mice for probe hybridization and gene signature analyses, with Nanostring Cancer Immunology probes. Healthy mouse brain (that had not undergone any experimentation) was included in the analyses for a non-tumor (healthy) control.





The data obtained was phenomenal. Following a laborious task of studying data analyses software in consultation with Nanostring, we identified several cancer-immune pathways which are regulated during combination therapy. This data further compares pathway regulation during each of the treatments. We identified four cancer pathways and four Immune pathways which are regulated as presented in the two graphs above. In a normal healthy brain these pathways are dormant and are upregulated during cancer growth. While the single therapy potentiates these responses, interestingly, the combination treatment reverses regulation to restore the pathways to their healthy status observed in the healthy brains.

Include a discussion of stated goals not met:
The commencement of the investigation was delayed due to Covid-19 *lockdown* and subsequent restrictions once reopened. As a result we were able to accomplish the outlined sub-tasks at different levels of completion. For submit-tasks 1 and 2, we need to add numbers to the group (as stated in our protocol) for it to be fully completed. The tissues are prepared and sectioned to commence ex vivo immunohistochemical staining for the described proteins in question. This sub-task was delayed as it was dependent on obtaining tissue after the previous subtasks. We aim to complete these sub-tasks within the next few months.

◦What opportunities for training and professional development has the project provided?
Conference Attended:
(i). American Association for Cancer Research, April 10-15, 2021 (Virtual Meeting)
Abstract: Kuruppu D, Bhere D, Farrar C, Brownell A, Shah K, Mahmood U, Tanabe KK. “HSV1 oncolytic therapy for breast cancer meningeal metastases” Poster
Novel Drug Delivery Systems; Presentation No 1309.

(ii). Immuno-Oncology Virtual Summit, Oct 6-9. 2020.

▪ **Describe how the results were disseminated to communities of interest.**
The results we have obtained thus far are novel and would be considered “highly significant” in terms of understanding the biology and therapeutic response of meningeal metastases to combination therapy with oncolytic HSV1 and immune checkpoint inhibition. The gene sequencing study generated a complex set of data that remain to be further analyzed to understand the relevant genes involved in the pathways regulated and further investigated. Although there is significance even with small numbers, we would need to include the numbers initially considered in these experiments before we would disseminate these results to the communities via research manuscripts which will be published and presentation at conferences.

◦**What do you plan to do during the next reporting period to accomplish the goals?**
▪**Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.**
During the next reporting period our goal is to conduct IHC staining of ex vivo brains (sub-task 3); conduct a confirmation experiment on the combination treatment with HSV1 and anti-PD1 (sub-task1) to add more numbers to the treatment groups *(as explained in section 5)*. We will conduct Task 2: to Investigate the therapeutic effect of repetitive oncolytic HSV1 for the treatment of meningeal metastases.

4. **IMPACT**

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

This is the first ever investigation of the novel concept – that of sensitizing cancer cells with oncolytic virus and the use of immune checkpoint inhibition – in the search for a treatment for breast cancer meningeal metastases, a disease which has an extremely grim prognosis. Our research presents: (i) the overall therapeutic potential of combination therapy; (ii) the impact of oncolytic virus replication in tumors; and (iii) the combined influence in regulating immune and cancer pathways to inhibit tumor growth. First; the data has shown that combination therapy with oncolytic HSV1 and anti-PD1 therapy significantly decreased tumor volume, inhibited tumor growth and prolonged survival in our murine model. This suggests that combination therapy has the potential to harness tumor resilience enabled by their microenvironment. Second; the presence of replicating virus throughout the course of the study suggests that there exists a state of tumor sensitization by the virus for immune checkpoint inhibition (which needs to be confirmed by in vitro IHC), to offer a novel approach for meningeal metastases which are resilient to existing therapies. As such the data provides immense therapeutic potential for clinical translation for patients who are awaiting new treatment approaches. Third: The identification of immune and oncology pathways that are regulated during this novel therapy provides a large data set that remains to be dissected to understand the genes which regulate this treatment outcome. This will contribute to our understanding of treatment with combination therapy to device urgently needed treatment. This information is bound to lead to scientific advances in understanding cancer progression and treatment. The data generated in this study has a high potential to be translated to the clinic – with direct applicability. As such it will offer a new therapeutic approach, even hope for a cure, extend patient lifespan and greatly improve their quality of life.

◦**What was the impact on other disciplines?**

The results pave way for a potential therapeutic strategy for breast cancer meningeal metastases. However, the scope of applying these results to other disciplines is widely open. It could be a potential treatment for other cancers and their metastases which are resistant to existing forms of therapy. Besides, as gene sequences will be identified in our subsequent task this may give an insight to understand these treatments for other cancers.

◦**What was the impact on technology transfer?**

- "Nothing to Report."

◦**What was the impact on society beyond science and technology**?

The results generated from this year's investigation show that combination therapy with oncolytic HSV1 and immune checkpoint inhibition reduce tumor burden, extend survival and reverse debilitating symptoms in our animal model. As such, this preliminary data from this investigation is likely to have a phenomenal impact on the society at large –for patient with terminal stage IV breast cancer meningeal metastases. As systemic therapies help breast cancer patients overcome their primary cancer to survive longer, more patients are diagnosed with meningeal metastases. For these patients, the data from this novel research will provide a potential treatment option, hope for a cure, extend their lifespan and greatly improve the quality of life.

5. CHANGES/PROBLEMS:

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

Commencement of laboratory work for this reporting period was delayed due to Covid-19 lockdown of the Institution research facilities and restricted facility access after reopening. The imaging facilities for animal experimentation reopened towards the year end. Work on the proposed project commenced as soon as these facilities reopened. However, most of the tasks were accomplished and sub-task 3 is in place to be completed within the next few months.

◦**Changes that had a significant impact on expenditures**

Due to Covid-19 lockdown and restricted access, there was a temporary cease on hiring. As activities in our research facilities returned to normal we were able to conduct an interview for a potential hire to commence next month.

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

In the study conducted we encountered a challenge that needed to be addressed. After virus administration the mice develop viremia. Upon consultation with our vet, an action plan was put in place that describes viremia symptoms as a guide to monitor the mice very closely during the first few days –until they recover. The amendment has been approved by the MGH-IACUC on June 25th, 2021.

6. PRODUCTS

List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state
"Nothing to Report."

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

◦**What individuals have worked on the project?**

Name: Kumudu D. Kuruppu
Project Role: PI
Nearest person month worked: 10.8
Contribution to Project: Conduct animal surgeries and experiments, relevant imaging studies, ex vivo tissue processing and handling, in vitro cell culture, IHC, data analyses

Name: Christian T. Farrar
Project Role: Collaborator
Nearest person month worked: 0.9
Contribution to Project: Gd-MRI imaging and data analyses of acquired MRI scans.

Name: Howard Kaufman
Project Role: Collaborator
Nearest person month worked: 0.6
Contribution to Project: Gene analyses and Nanostring study

Name: Kenneth Tanabe
Project Role: Collaborator
Nearest person month worked: 0.6
Contribution to Project: Project discussion

Name: Lida Hariri
Project Role: Collaborator
Nearest person month worked: 0.1
Contribution to Project: Statistical data analyses

◦**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?**

▪No other organization was partnered; "Nothing to Report."

8. SPECIAL REPORTING REQUIREMENTS

Not Applicable

9. APPENDICES