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14. ABSTRACT

Enclosed is the second annual report
This proposal seeks to address the PRCRP topic area of colorectal cancer, and addresses the gap that exists in therapeutic options and genetic testing available for service members and their families at risk for early-onset colorectal cancer. Large population genetic studies have demonstrated an excess of rare, pathogenic mutations in the germlines of individuals. These germline mutations are routinely discarded in most cancer genomics workflows, and can often have driver effects in tumorigenesis even when not Mendelian in nature. Our preliminary data suggests that certain pathways in DNA damage repair are particularly enriched in individuals with early-onset colorectal cancer, and while potentially cancer-predisposing, may make subsequent tumors exquisitely sensitive to select precision chemotherapeutics, a relationship called synthetic lethality. Our overall hypothesis is that in addition to Mendelian cancer syndromes such as Lynch Syndrome, routine exploitation of synthetic lethal relationships derived from driver germline mutations in non-Mendelian cases of early-onset colorectal cancer will improve the efficacy and utilization of precision therapeutics. These data may provide proof-of-principle for routine precision cancer therapeutics based on patients' inherited genetic profile in addition to the molecular features of their tumor. If successful, this approach would represent a paradigm shift in genetic testing and treatment for early-onset colorectal cancer.

15. SUBJECT TERMS

PRCRP, early-onset colon cancer, precision medicine

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TABLE OF CONTENTS

Introduction.

Genome-wide characterization of somatic alterations in human cancers has led to hopes of "precision medicine" approaches to identify optimal molecularly-targeted treatments, particularly through exploitation of synthetic lethal (SL) interactions. Several computational strategies exist to identify SL interactions from genomic alterations found in tumors by subtracting the individual's genome from the tumor. As such, these approaches ignore the contribution of germline variation to disease pathogenesis. However, population-wide, exome sequencing projects have demonstrated that each person harbors several, rare loss-of-function (LoF) mutations in her germline. These germline alterations may have potentially profound therapeutic consequences as shown by the recent approval of PARP inhibitors for those with ovarian cancer and germline defects in homologous recombination (HR) DNA repair genes, such as *BRCA1/2* mutations. Thus, our overall hypothesis is that in addition to Mendelian cancer syndromes, routine exploitation of synthetic lethal relationships derived from driver germline variants in DNA-repair genes will improve the efficacy and utilization of precision therapeutics particularly for those individuals with early-onset colorectal cancer, whose incidence rates have been sharply increasing over the past decade. The work encompasses ex-vivo testing of human/normal early-onset colorectal cancers, and modeling of cancers generated from human tissue by genomic engineering techniques.

Keywords.

Early-onset colorectal cancer; organoid; exome-sequencing; DNA-repair pathways; homologous recombination

Accomplishments.

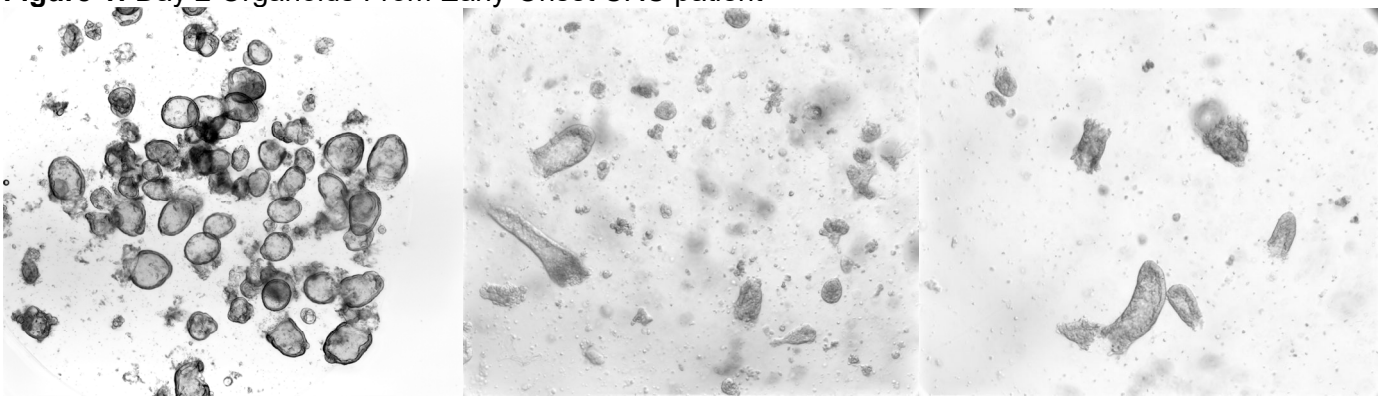
Major Goals for the Project (Aim #1):

Major Task 1: Host-genome based Organoid Drug Studies	Month	
Subtask 1: Local IRB modifications to accommodate DoD Regulations	1	Complete
Subtask 2: Regulatory Review and approval by the USAMRMC Human Research Protection Office (HRPO)	1-3	Complete
Milestone 1: HRPO Approval Received	3	Complete
Subtask 3: Organoid creation of tumor and normal tissue from resected colorectal cancers 50 patients will be enrolled, and 50 organoid pairs (human normal and cancer cell lines) will be generated. Human Anatomical Substances Used: Endoscopic and surgical resected tissue of normal colon and colon cancer will be harvested, isolated, and patient-matched organoids will be generated from each study participant. Organoids will be frozen after expansion and passage.	3-18	48/50 Patients recruited
Milestone 2: 50 patient-matched tumor/normal organoid pairs will be generated	18	48/50 complete
Subtask 4: Whole-Exome sequencing (WES) of 50 tumor and normal organoids. WES will be performed at 50x for germline DNA (derived from either normal colon organoids or blood/saliva specimen). Pathogenic germline mutations in DNA-repair genes will be identified. Human Anatomical Substances Used: Extracted DNA from organoids for tumor DNA and saliva collection from study participants (for germline DNA)	3-18	48 patients under analysis
Subtask 5: Tumor/Normal Toxicity Curves Organoid pairs with druggable, germline mutations in DNA-repair genes will be tested to see if targeted therapies lead to differential killing in tumor organoids versus patient-matched normal organoids (IC50). Student's t-test will be used to determine if differential killing is	6-22	Underway

statistically significant. Human Anatomical Substances Used: Organoids (normal and tumor) generated from enrolled participants		
Subtask 6: Tumor vs. Tumor Toxicity Curves Tumor organoids with germline targets will be matched with tumor organoids without germline mutations. Ordinal ranks will be calculated in a permutation test to assess if germline-based testing results in significant enhancement of killing. Human Anatomical Substances Used: Organoids (tumor) generated from enrolled participants	12-23	Underway
Milestone 3: Completion of *ex-vivo* testing complete	23	

What was accomplished under these goals (Aim #1): Despite delays instituted by the COVID19 lockdown and second surge in Boston, we were able to successfully construct patient-matched, paired tumor/normal organoid lines from 48 study participants (Figure 1). Our success rate has been ~90% in generation of tumors, while 100% of normal mucosa. Exome Sequencing of more germlines has revealed that approximately 10% of cases ae indeed Lynch Syndrome, and that ~10% of participants are Fanconi Anemia (FA) carriers.

Figure 1: Day 2 Organoids From Early-Onset CRC patient



(Left panel) High power magnification of Day 2 Organoids from Tumor. (Middle panel) Low power view of tumor organoids (Right panel) Lower power view of normal organoids.

While in our first year, we have developed a robust pipeline of germline exome sequencing, we have also developed robust pipelines to examine somatic tumor mutations, copy number variation, and calculate somatic signatures as well. While 10% of cases seem to be FA carriers, no LOH has been seen on tumors. Moreover, we have not seen any molecular features associated with homologous recombination deficiency (HRD) in such tumors (**Figure 2**). Single base substation 3 has been associated with HRD, and is absent in such tumors

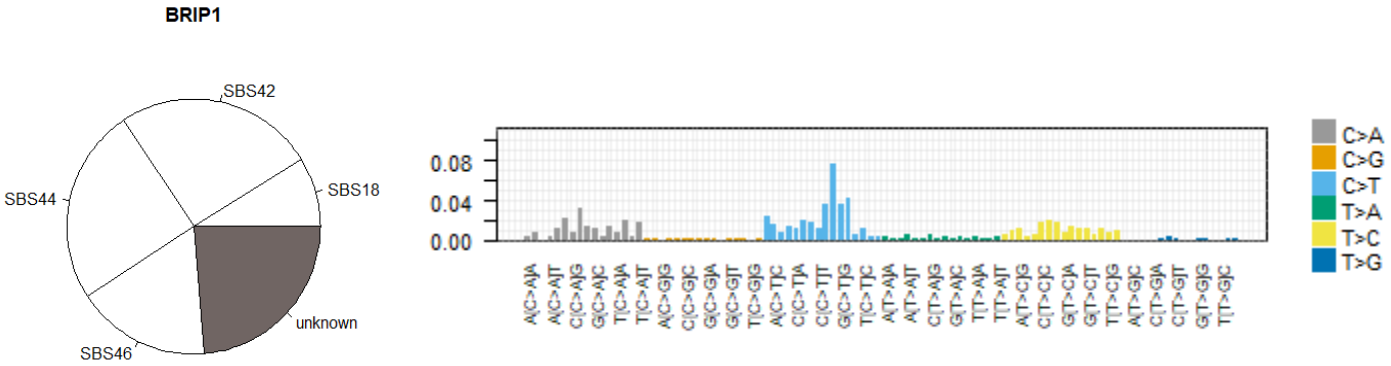
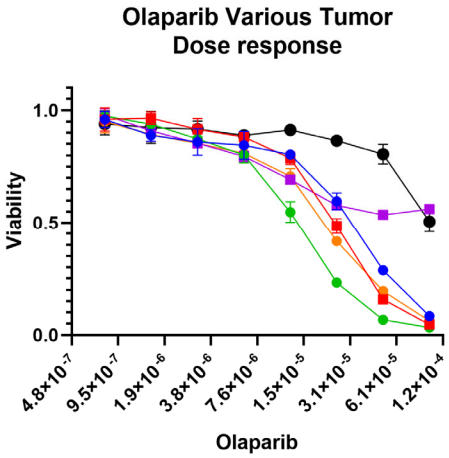


Figure 2: Single base Substition features of EOCRC with Germline *BRIP1*.

Underway, we have also began testing drug sensitivities for PARP inhibition, and these FA tumors do not appear to be PARP sensitive.

Figure 3: Panel of Tumors Responses to Olaparib.



Major Goals for the Project Aim #2:

Specific Aim 2: To identify CRC-relevant somatic signatures associated with germline mutations in homologous recombination (HR) and classical non-homologous end joining (cNHEJ) through CRISPR-Cas genome-edited of human colon organoids that recapitulate key germline and somatic driver events.		
Major Task 1: Engineering of Canonical DNA-Repair Pathway Deficient Organoids *Ex Vivo*	Month	
Subtask 1: Acquisition of 10 normal colon organoids from individuals without any family history of colorectal cancer. These samples have been pre-banked. Medical records of these individuals will be searched to ensure little possibility of germline mutations in canonical DNA-repair pathways (e.g. no evidence of personal or family history of breast/ovarian cancers). Human Anatomical Substances Used: Normal colon organoids from 10 study participants (already pre-existing)	3-4	Complete
Subtask 2: Ex-vivo CRISPR-Cas Genome Engineering to mimic germline mutations and common somatic mutations found in the adenoma to carcinoma sequence in the colorectal cancer. Viral vectors and or electroporation will be used. Resulting clones will be sequentially validated by targeted next-generation sequencing. 6 strains of modified tumor organoids are expected for each patient-derived, normal organoid for a total of 60 tumoroids. Human Anatomical Substances Used: Normal colon organoids from 10 study participants (already pre-existing)	4-12	75% Complete, difficulty with inducing TP53 knockout reliably. Some subclones are not knockout.
Milestone 4: Creation of 60 organoid strains mimicking germline mutations in HR and NHEJ and canonical somatic mutations in colorectal cancer (*APC, TP53)*	12	Delayed
Major Task 2: Accelerating Tumor Evolution *In Vivo*	Month	
Subtask 1: Review and approval by the USAMRMC Animal Care and Use Review Office (ACURO)	9-12	Delayed
Subtask 2: Endoscopic implantation of 60 engineered tumor organoids into the colon of mice. Each organoid strain is injected into one mouse and will allow to incubate for 2-3 months undergoing selection pressures seen *in vivo*. Human Anatomical Substances Used: 60 engineered organoids derived from 10 study participants Animals Used: Anticipate use of 75 female B6 NOD-SCID mice (NOD.CB17-Prkdc^{scid}/J) to achieve 60 implanted.	12-15	To be performed

Subtask 3: Harvesting of implanted engineered organoids and DNA extraction. Mice will be sacrificed and tumors will be isolated. DNA will subsequently be extracted from these tumors, in addition to histological assessments with H&E slides to assess if tumors appear histologically similar to human samples. Animals Used: Anticipate sacrifice of 60 female B6 NOD-SCID mice (NOD.CB17-Prkdc^{scid}/J)	15-18	To be performed
Milestone 5: Creation of 60 tumors directed by selection pressures *in vivo*.		

What was accomplished under these goals (Aim #2): We were able to begin genetic engineering of 10 human organoid lines. We elected to use a system of a doxycycline inducible Cas9 construct (integrated) through lentiviral transfection (Takara biosciences) (Figure 4):

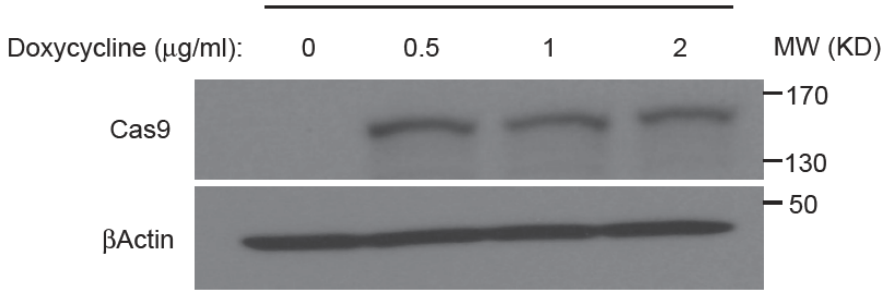


Figure 4: Inducible Cas9 in Organoids

We initially have transfected guide RNAs for APC and TP53. APC was easy to achieve with Wnt withdrawal. TP53 was thought initially successful with Nutlin selection, however, western blot demonstrated some residual expression, suggesting that these organoids developed resistance to this mechanism.
IACUC Approval has been granted and ACURO Approval is pending. IACUC was largely delayed due to the COVID-19 pandemic and policy regarding human specimens, which has been resolved.

Opportunities for training and professional development has the project provided: Nothing to Report

Results disseminated to communities of interest: Nothing to Report.

Plan to do during the next reporting period to accomplish the goals: For Aim 1, we will continue our ongoing activities as the cohort expands to 50 tumor/normal pairs. We will finish our drug sensitivities assays

For Aim #2, we will finish knockout of TP53, followed by shRNA of the target genes. Next, we will implant them into mouse colons and sequence the remaining clones.

Major Task 3: Derivation of Associated Somatic Signatures from Engineered Organoids	Months	
Subtask 1: Exome Sequencing of 60 engineered organoids and original 10 starting organoids. Extracted DNA from engineered tumor organoids will be sequenced using WES at a mean depth of 150X and normal organoids will be sequenced at a depth of 50X. The Genome Analysis Toolkit and Broad Institute Cancer Suite will be used to call somatic mutations. Human Substances Used: 60 engineered organoid lines and the 10 original ones used to derive them.	16	
Subtask 2: Calculation of somatic signatures Mutations calls from each of the 60 sequenced tumors will be analyzed using the R package Somatic Signatures to	18	MGH

derive signatures associated with each germline mutation. This signature will be compared to the pre-existing COSMIC signatures using the cosine similarity tests		
Subtask 3: Identify relevancy of derived signatures with human data. We will search deposited data in government and non-governmental repositories (TCGA and ICGC) for genomic data of colorectal tumors with germline mutations in *BRCA1/2, PRKDC, XRCC6* and compare somatic signatures with these tumors versus the engineered tumors we created. A cosine similarity test can be used to assess and quantify accuracy.	20	We have already

Impact.

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

While enriched in EoCRC, FA mutations do not confer PARP sensitivity and do not undergo LOH. Thus present commercial algorithms (e.g. Foundation One) that suggest Olaparib for such tumors are unlikely to be impactful. The lack of LOH in such tumors demonstrates that haploinsufficiency may be sufficient to increase risk.

We also have enrolled and tested additional Lynch Syndrome candidates, suggesting that there is a vulnerability to cytarabine in the redox environment of the surrounding conditions are favorable.

What was the impact on other disciplines?

The findings we have for MSI-H/ Lynch Syndome colorectal cancers may be possibly applicable to other tumor types. Also, our results may demonstrate that organoids are a viable testing platform prior to initiation of chemotherapeutics.

What was the impact on technology transfer?: Nothing to Report

What was the impact on society beyond science and technology?: Nothing to Report

Changes/Problems.

Changes in approach and reasons for change: Only one minor change in approach will be pursued in Aim #2, to generate organoids based on DNA-repair genes, we will use shRNA constructs in the last step. We have pursued this change due to the fact that *BRCA1/2, PRKDC, and XRCC6* do not have any selectable means pharmacologically. Moreover, single clone selection will be laborious. Using validated shRNAs in cancer cell lines, we can rapidly induce loss of protein and see the evolutionary aspects of tumor development in the context of these changes. It is possible that loss of these genes may result in slower growth, which would result in adverse selection by Crispr techniques.

Actual or anticipated problems or delays and actions or plans to resolve them: The COVID19 pandemic greatly affected our productivity during time, slightly delaying patient enrollment and delaying our ability to submit a protocol to our insitution's IACUC. We recently obtained approval and submitted to ACURO. Another issue has been obtaining proper TP53 knockout in organoids. We have used Nutlin as a selection agent for knockout, but found accommodation by these cells. We will need to re-introduce guides and apply more stringent selection.

Changes that had a significant impact on expenditures: The COVID19 pandemic resulted in experimental delays but challenges in still funding the relevant staff. We were able to work with limits on hours during the shutdown given the irreplaceable resource of the organoid creation.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select Agents: We report no significant changes other than outlined above.

Products.

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: Through this award, we have created a biobank of early-onset colon cancer organoids.

Participants and Other Collaborating Organizations.

Name: Manish Gala, MD
Project Role: PI
Researcher Identifier: 0000-0002-3126-0783
Nearest Person Month worked: 3 (over 1 year)
Contribution to Project: PI, Arranged for IRB amendments, and supervision

Name: Minyi Lee, ND
Project Role: Research Coordinator
Nearest Person Month worked: 3 (1 year)
Contribution to Project: PI, Edited IRB and achieved approvals, has built system to rapidly enroll patients once HRPO approval given.
Funding: Departmental Funds, K23 Award by Manish Gala, MD

Name: Rachid Zagani, PhD
Project Role: Senior research scientist
Nearest person month worked: 8 (over 1 year)
Contribution to Project: CRISPR- design and optimization; assembling reagents for organoids

Name: George Eng, MD, PhD
Project Role: Postdoctoral Fellow
Nearest person month worked: 1
Contribution to Project: Organoid work
Funding: NIH T32 held by MGH Pathology

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? Nothing to report.

What other organizations were involved as partners? Nothing to report.

Special Reporting Requirements: N/A

Appendences: N/A