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PRINCIPAL INVESTIGATOR: Venu Pillarisetty

CONTRACTING ORGANIZATION: University of Washington, Seattle, WA

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Venu Pillarisetty

E-Mail:vgp@uw.edu

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University of Washington
4333 Brooklyn Ave NE
Box 359472
Seattle, WA 98195-9472

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our proposal focuses on a unique form of liver cancer, fibrolamellar carcinoma (FLC). This debilitating disease affects young individuals between their teenage years and mid-30's. Importantly, this cancer afflicts young men, and women in the prime of their life with none of risk factors associated with chronic liver diseases.
Our multidisciplinary team of clinical and basic science investigators have put together a comprehensive experimental strategy to address 3 fundamental questions pertaining to FLC: 1) How does the DNJA-PKAc chimera initiate liver tumorigenesis? 2) What downstream signaling steps are necessary to promote FLC growth? and 3) How does FLC evade the host immune response? These questions have forged the inception of three related projects that will define new therapeutic targets at each stage of tumor development.

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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INTRODUCTION: Fibrolamellar Hepatocellular Carcinoma (FLC) is the most common form of liver cancer in adolescents and young adults between the ages of 15 and 35 in the absence of underlying liver disease. The diagnosis in these otherwise healthy individuals is often delayed until signs and symptoms occur, leading to an overall 5-year survival rate of ~30%. The disease is refractory to both conventional chemotherapy and current targeted therapies.

This proposal is devoted toward developing therapeutic solutions for FLC. Most, if not all, cases of FLC stem from the overexpression of a novel fusion protein, DNJA-PKAc in the liver, but little is known about the mechanisms by which DNAJ-PKAc initiates malignant transformation, steps necessary for tumor progression, and the host response to a tumor that expresses this neoantigen.

Our multidisciplinary team of clinical and basic science investigators have put together a comprehensive experimental strategy to address 3 fundamental questions pertaining to FLC: **1)** How does the DNJA-PKAc chimera initiate liver tumorigenesis? **2)** What downstream signaling steps are necessary to promote FLC growth? and **3)** How does FLC evade the host immune response?

KEYWORDS: liver, children, cancer, DNAJ, PKAC, Hsp70, inhibitors, kinase, organotypic, immunotherapy.

ACCOMPLISHMENTS:

Project 1: Role of Hsp70 in DNAJ-PKAc Function in FLC

The work in Project 1 was conducted at the University of Washington involving the partnering P.I. John D. Scott.

Transformative advances in our understanding of the molecular basis of Fibrolamellar carcinoma offer renewed hope to treat this disease. Hence project 1 focuses on the molecular mechanisms that underlie FLC. The molecular signature of most FLC tumors is expression of the DNAJ-PKAc fusion (see Figure below). While overwhelming evidence links this chimeric protein to FLC, the molecular mechanism(s) that underlie DNAJ-PKAc action are unknown. **We have begun to deploy innovative chemical-biology approaches to ascertain if kinase activity or the ability to recruit Hsp70 chaperonins is the oncogenic driver of DNAJ-PKAc action**.

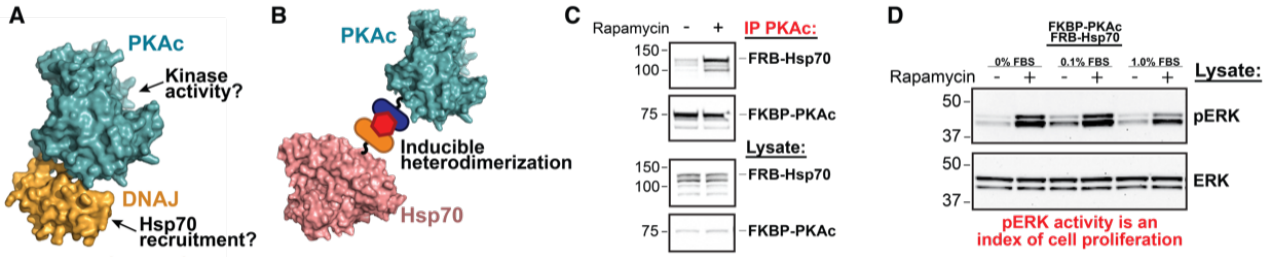

Figure. Preliminary data for aim 1. A) *Space filling model of DNAJ-PKAc. Kinase domain (teal) and chaperonin binding site (gold) are indicated.* ***B)*** *Drug-inducible heterodimerization creates stable PKAc/Hsp70 complexes.* ***C)*** *Biochemical proof of rapamycin responsive formation of PKAc/Hsp70 complexes and* ***D)*** *enhanced ERK activity in AML12 hepatocytes.*

Stabilization and refolding of protein kinases by chaperonins contributes to cancer progression. In FLC, the J domain of DNAJ-PKAc recruits the chaperonin Hsp70 in proximity to protein kinase A. Hence, we tested if bypassing the J domain by fusing Hsp70 directly to PKAc was sufficient to enhance hepatocyte proliferation (Fig 4B). Rapalog-inducible dimerization was initially used to form PKAc-Hsp70 chimeras in AML12 and HepG2 cells[14] (Fig 4B-D). Lentiviral vectors encoding FRB-Hsp70 and FKBP-PKAc were used to express these proteins (Fig. 4B & C, left). Treatment with 50 nM rapamycin (or the rapalog AP-21967) induced heterodimerization (Fig 4B) as assessed by immunoblot detection of FRB-Hsp70 in PKAc immune complexes (Fig 4C, right). Immunofluorescence imaging and proximity-ligation assays confirmed induction of Hsp70-PKAc adducts *in situ* (data not shown). Preliminary studies show that induced formation of Hsp70/PKAc clusters upregulate ERK signaling as compared to controls (Fig 4D). These molecular studies have provided a sound rationale to further explore if proximity of Hsp70 to PKAc underlies the aberrant cell signaling that is presumed to proceed through DNAJ-PKAc. **We anticipate that these studies will be completed in during the next funding cycle.**

Project 2: Systems-based Approaches to Identify Molecular Drivers of FLC

The work in Project 2 was conducted at the University of Washington and Fred Hutchinson Cancer Research Institute involving the P.I., Raymond Yeung, and co-PI, Taran Gujral.

Specific Aim 1: Validate kinases and their inhibitors predicted by KIR to be important for cell growth. ***This aim has been completed.***

Specific Aim 2: Validate KIs in human-derived FLC models

Major Task 1: Kinase inhibition in tumor slice culture (TSC)

Due to COVID, no human FLC specimen was collected over the past year. Hence, we were unable to perform experiments using human-derived tumor slice culture.

Major Task 2: **Novel patient-derived models of FLC**

The paucity of preclinical models such as immortalized primary human FLC cell lines has precluded many investigators. To address this significant gap, our team has established two new model systems: patient-derived cell lines bearing the FLC mutation, and patient-derived xenograft (PDX) mice. **Notably**, **we have established the first patient-derived FLC cell lines confirmed to harbor DNAJB1-PKAc fusion (Fig. 1).** These cells are cultured on extracellular matrix support such as collagen or synthetic matrigel to preserve FLC cell morphology and expression of FLC fusion protein. Thus, our proposal will utilize both an innovative systems-pharmacology-based approach and newly developed state-of-the-art preclinical FLC models to develop and test novel therapeutics for FLC to offer patients a chance for cure.

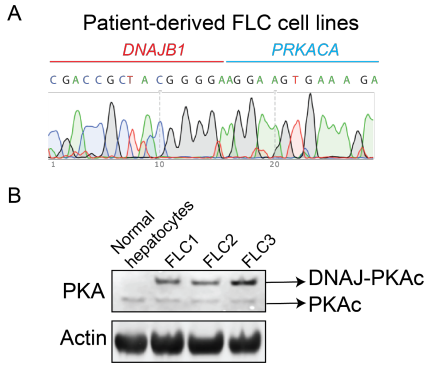

Figure. 1. Generation of patient-derived FLC cell lines. A. DNA sequence of the fusion protein DNAJ-PKAc from patient-derived FLC cell lines is shown red and blue. **B.** Immunoblot detection of wild-type PKAs and DNAJ-PKAc fusion in normal hepatocyte (AML12 cells), three FLC cell lines.

Kinase inhibition in PDX mice. In preliminary studies, we evaluated a single dose of PLK1 inhibitor, BI-2536 (50mg/Kg) in our FLC PDX model. Our data shows that PLK1 inhibition significantly decreased FLC tumor growth and tumor weight in a PDX model (**Fig. 2**). Further, we observed a significant decrease in Ki67 staining suggesting reduced cancer cell proliferation in BI-2536 treated tumors (**Fig. 2**). Motivated by this encouraging data, a detailed characterization and a more thorough investigation of PLK1 in FLC cells and PDX models will be carried out in the upcoming year.

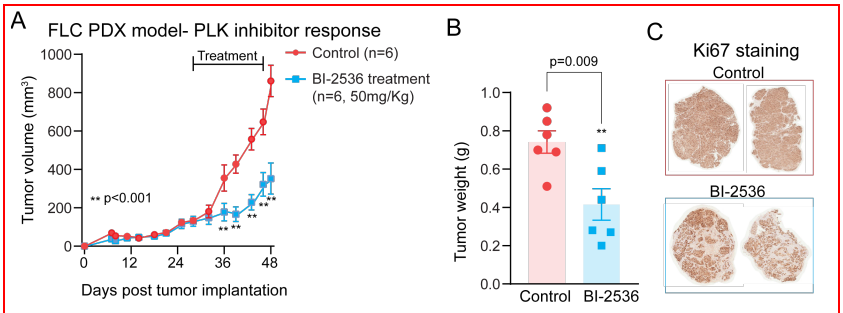

Figure 2. **PLK inhibitor treatment significantly decreases FLC growth and tumor weight (right) in PDX model**. **A.** A plot showing tumor growth of passage 2 FLC PDX model. Lines denote individual mouse. **B**. Representative images showing H&E, and IHC staining for indicated proteins. **C**. A plot showing expression of Prkaca, dnajb1, ulk1 and autophagy-related genes measured by RNA-seq in passage 2 FLC PDX model.

Project 3: Overcoming tumor immune evasion

The work in Project 3 was conducted at the University of Washington by the P.I., Venu Pillarisetty.

In Project 3, we have continued to define the immune response to fibrolamellar carcinoma (FLC) and test hypotheses on improving anti-tumor immune responses. The challenges posed by the COVID-19 pandemic substantially slowed our work over the past year, but we have been able to make progress on the project. Our manuscript entitled "*Reversing the immunosuppressive tumor microenvironment of fibrolamellar carcinoma*" is nearly ready for resubmission.

Specific Aim 1

Major Task 1: Define spatial relationship of tumor immune cells

We performed mIHC for CK18, CD8, CD4, FoxP3, CD68, HLA-DR, and DAPI on tumors from 6 patients. This demonstrated that T cells were more densely located within the stromal bands and tumor interface than in the main tumor compartment.

Major Task 2: Single-cell RNAseq (scRNAseq) analyses of FLC

We have collaborated with the University of California San Francisco in our current manuscript to include scRNAseq data from a single FLC tumor.

Specific Aim 2

Major Task 1: Combining CXCR4 and PD1 inhibition

We have performed in vitro treatment with combination CXCR4 and PD-1 inhibition, as well as IL-10 blockade, in slice cultures from three tumors. The data from these will be presented in our manuscript.

Major Task 2: Live imaging of immune-mediated killing
We performed live imaging of the slice culture experiments described above.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
Nothing to report

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

CHANGES/PROBLEMS

Changes in approach and reasons for change
Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them
Work has been significantly restricted over the past year due to COVID-19.

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
N/A

Significant changes in use of biohazards and/or select agents
N/A

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
Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on this project?
No change

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

***COLLABORATIVE AWARDS*:**
There are 3 projects that make up this Award, each with its own P.I., working closely together to maximize scientific engagement and productivity. The overall P.I., Raymond Yeung (University of Washington), is largely responsible for the work performed in Project 2 with the assistance of a Co-P.I., Taran Gujral. Project 1 is headed by John Scott at the University of Washington working closely with Shao-En Ong and Kimberly Riehle (University of Washington). Venu Pillarisetty (University of Washington) heads the third Project. Each of the P.I. and Co-P.I. crafted the progress reports for their respective Projects and are glued together as a single document.

APPENDICES

none