Feasibility of Establishing Patient-Derived Organoids for Rectal Cancer

A Biospecimen Collection Protocol

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RESEARCH SUMMARY AND PROTOCOL

1. PURPOSE

To determine the feasibility of establishing patient-derived organoids from pre-treatment rectal adenocarcinoma biopsies.

Primary Objective(s):

1. To determine the feasibility of establishing patient-derived organoids from pre-treatment rectal adenocarcinoma biopsies. The primary endpoint is the percent of biopsies that form spherical organoids.

Exploratory Objectives:

1. To describe the radiation response of patient-derived organoids and compare it to the patient's clinical response to neoadjuvant radiation.
2. To describe the radiation response of orthotopic organoid transplants in mice and compare it to the patient's clinical response to neoadjuvant radiation.
3. To describe the histopathologic and molecular characteristics of patient-derived organoids.

Hypotheses

1. 70% of patient-derived organoid attempts will be successful. This protocol will be hypothesis-generating. Data obtained from this protocol will guide future studies on the utility of patient-derived organoids for assessing radiation response in rectal cancer.

2. BACKGROUND AND SIGNIFICANCE

The standard of care for stage II-III rectal cancer is neoadjuvant chemoradiation or short-course radiation followed by total mesorectal excision.[1] Response to neoadjuvant radiation varies across patients, with some having minimal response to 10-30% having a pathologic complete response. Pathologic complete response is associated with improved clinical outcomes including resectability, sphincter preservation, local control, and overall survival.[2-4] Predicting response to neoadjuvant radiation may inform prognosis and guide more individualized therapy. Pretreatment markers including gene expression profiles and radiomic features have been investigated but are not used clinically due to limitations such as cost and generalizability.[5-7] Preclinical models such as patient-derived tumor cell lines and xenografts have also been used to evaluate individual response to radiation.[8,9] Limitations of patient-derived cell lines include loss of genetic heterogeneity due to inefficient generation and selective pressure as well as loss of tumor morphology in 2D growth conditions. Patient-derived xenografts retain genetic and phenotypic features, but also have limitations including high resource consumption.

Tumor organoids have emerged as another relevant preclinical model.[10] Organoids grow in a 3D matrix, recapitulate tumor genetic and morphologic features, and have relatively efficient generation and expansion from patient tissue.[10] The Hubrecht Institute generated patient-derived organoids from untreated colorectal cancer in 20 patients with an 80-90% success rate and

demonstrated similar morphology and somatic mutations between primary tumor biopsies and tumor organoids by H&E staining and whole exome sequencing.[11]

More recently, several studies have explored the use of tumor organoids to predict response to chemotherapy and radiation, including three colorectal studies published in 2019. A study from the University of Wisconsin generated tumor organoids in multiple cancer types with a 76% success rate for colorectal cancer.[12] Seven primary tumors and corresponding organoids demonstrated similar morphology and mutations by next-generation sequencing, and colorectal cancer organoids had heterogeneous responses to 5-FU and radiation measured by organoid diameter and metabolic imaging. Additionally, as proof-of-principle, organoids from one patient with metastatic colorectal cancer were treated with 5-FU and oxaliplatin to predict whether the patient might respond to retreatment with FOLFOX.[12] Another study from Memorial Sloan Kettering generated rectal cancer organoids from 58 patients, both pre- and post-treatment, with a 77% success rate.[13] Similar histopathology was demonstrated between primary tumors and corresponding organoids. To demonstrate preserved genetic heterogeneity in the organoids, the mutational landscape across 31 tumor organoids by targeted next-generation sequencing was shown to be similar to that of an independent rectal cancer cohort. Radiation of 19 rectal cancer organoids resulted in varying viability and dose-response curves. In six patients, organoid radiation response measured by the area under the dose-response curve appeared to correlate with patient radiation response measured by pre- and post-radiation tumor size on endoscopy. Four patient organoids were also engrafted into the mouse rectum to create a parallel preclinical model, and 5-FU and FOLFOX response of these transplanted organoids appeared to mirror clinical patient responses.[13] Lastly, a study from Fudan University collected tumor samples from 112 patients with newly-diagnosed rectal cancer enrolled in a phase III trial (NCT02605265) evaluating the addition of irinotecan to neoadjuvant chemoradiation and generated organoids with a 86% success rate.[14] In line with prior studies, similar histopathology was observed in primary tumors and organoids, and whole exome sequencing in 18 primary tumors and organoids demonstrated similar genomic alterations. For 80 patient-derived organoids, response to 5-FU, irinotecan, and radiation was measured by cell viability and organoid diameter and compared to patient clinical responses. Organoid response to at least one of the treatments generally correlated with patient's AJCC tumor regression grade. However, in 19 patients who had a good response to chemoradiation, the corresponding organoids were actually resistant to radiation, and the authors suggested that in the future, such patients might receive neoadjuvant chemotherapy alone.[14]

These studies using patient-derived rectal cancer organoids to model individual radiation responses are promising and merit further investigation. Our institution and the investigators on this protocol have experience in generating organoids from human colorectal tumor biopsies, endoscopy-guided transplantation of organoids in mice, and pelvic radiation in mice.[15-18] Our institution also routinely evaluates and treats rectal cancer patients, from the initial work-up including proctoscopy to neoadjuvant radiation therapy for stage II-III patients to resection. Therefore, we believe that it would be feasible to generate patient-derived organoids from a rectal tumor biopsy obtained at proctoscopy. As proof-of-principle, we will also aim to measure the radiation response of these organoids *in vitro* and also of engrafted organoids in the mouse rectum *in vivo* and compare to patient clinical responses.

3. DESIGN AND PROCEDURE

For the primary objective, patient biopsies will be used to generate organoids as follows. One additional research biopsy will be obtained at the time of standard-of-care proctoscopy in clinic 3-2. The biopsy will be given a unique de-identified reference code. The de-identified biopsy will be placed in a sterile conical tube containing MACS Tissue Storage Solution (Miltenyi Biotec), then transported to Dr. Hsu's lab on wet ice by a research technician from Dr. Hsu's lab. Dr. Hsu will generate spherical organoids from the research biopsy as previously described.[19] Briefly, the biopsy will be manually minced with a razor blade or scalpel, transferred to a sterile conical tube containing enzymatic solution with Collagenase I (Roche), then spun in a MACSmix Tube Rotator (Miltenyi Biotec) in a 37°C incubator for 30-60 minutes. Then, the sample will be washed and centrifuged for 5 minutes, the supernatant will be discarded, and the tumor cell pellet will be resuspended in basal media and filtered through a 70 μm strainer. The cells will be counted, centrifuged again for 5 minutes, resuspended in a 1:1 Matrigel:PBS mixture (Corning), plated in a 24-well plate, and incubated at 37°C for 15-20 minutes, after which 1 mL media is added to each well. The cells will be assessed daily for organoid formation. Media will be changed every 3-4 days, and organoids will be passaged when the Matrigel reaches 80% confluency. Organoids will be passaged until the third passage, at which point the organoids will be transferred to Dr. Roper.

For the exploratory objectives, the de-identified and passaged organoids will be given to Dr. Roper to assess the radiation response of organoids and organoid transplants. For organoid radiation response, plated organoids will receive a single fraction of radiation ranging from 0 – 15 Gy, then cell viability will be assessed by Resazurin assay to generate a radiation dose-response curve, and the AUC will be calculated. Designated members of the study team with access to the master log will be able to compare this organoid radiation response with the patient's clinical response to neoadjuvant radiation from the surgical pathology report.

For organoid transplantation, organoids will be gently dissociated and resuspended in 3D matrix with minimal medium and injected into the lamina propria of the mouse rectum as previously described.[17] Transplanted tumors will be visible four to six weeks after injection. [20] Mice will then receive pelvic radiation of about 20-30 Gy in 4-5 fractions using a single arc, using previously described radiation methods.[18] To measure radiation response, mice will be followed with periodic endoscopy and the percent change in tumor size index after radiation will be measured as previously described.[20] The tumor size index is the tumor area divided by the colorectal lumen area. Designated members of the study team with access to the master log will be able to compare the radiation response of organoid transplants with the patient's clinical response to neoadjuvant radiation from the surgical pathology report.

The last exploratory objective will be to describe the histopathology and molecular characteristics of patient-derived organoids. Histopathology may include hematoxylin and eosin staining as well as immunohistochemistry of markers such as CK20, CDX2, and β-catenin. Recent studies have demonstrated a diversity of morphologies in patient-derived organoids that appear to retain characteristics of the primary tumor.[12-14] Molecular studies may include targeted next-generation sequencing and whole-exome sequencing. Recent studies have used high throughput sequencing to demonstrate that patient-derived organoids also preserve specific mutations compared to the primary tumor biopsy and also have similar total mutational loads and copy number variations.[12-14] Any findings from organoid histopathology or molecular studies

will not be used for clinical intervention. Only designated members of the study team with access to the master log will be able to make any comparisons between organoid histopathology or molecular studies and patient histopathology or molecular studies.

This protocol involves collection of biopsy samples and analyses of data extracted from the electronic medical record. The variables, endpoint measures, and other data elements that will be extracted include:

- Duke MRN
- Date of birth
- Sex
- Race
- Pathologic diagnosis date
- Clinical TNM stage
- Pre- and post-neoadjuvant therapy proctoscopy findings
- Neoadjuvant radiation therapy dose, fractionation, and dates
- Neoadjuvant chemotherapy type, dose, and dates
- Surgery date
- Biopsy and surgical pathology findings including histology, microsatellite stability, genomic profiling (e.g. FoundationOne)

Eligible subjects are identified as described below in "Subject Recruitment and Compensation" section, and are selected for inclusion in subsequent analyses based on the criteria described below in the "Selection of Subjects" section.

Timeframe to complete subject identification: 24 months
Timeframe to complete data extraction and entry: 6 months
Timeframe to complete quality assurance check on extracted data: 1 month
Timeframe to complete data analyses: 3 months
Timeframe to complete manuscript and submission to journal: 12 months
Timeframe to disseminate conclusions via conference/meeting, if applicable: 6 months
Timeframe to protocol termination: 36 months

4. SELECTION OF SUBJECTS

The study population is patients with newly diagnosed, stage II/III rectal adenocarcinoma who are undergoing standard-of-care proctoscopy and planned neoadjuvant therapy (including neoadjuvant radiation) and total mesorectal excision. Patients who meet the criteria below will be approached for enrollment, and patients who consent or e-consent to this study will undergo an additional tumor biopsy at time of standard of care proctoscopy. If a patient is scheduled for a standard of care tumor evaluation in the endoscopy suite or the operating room and meets all eligibility criteria, the additional tumor biopsy may be obtained during the standard of care tumor evaluation. The target enrollment is 20 patients with stage II or stage III rectal cancer. We are requesting permission to consent up to 25 subjects to obtain an evaluable sample size of 20.

Inclusion
1. Age ≥18 years old.
2. Able to provide informed consent; either paper or e-consent is permitted
3. Undergoing proctoscopy.

Exclusion
1. Patient not planned to receive neoadjuvant radiotherapy

5. SUBJECT RECRUITMENT AND COMPENSATION

Potential subjects with biopsy-proven stage II/III rectal adenocarcinoma (ICD-10 C20) or suspected stage II/III rectal adenocarcinoma with pending diagnostic biopsy be prospectively identified through MaestroCare.

Subjects will not be compensated for inclusion in this protocol.

6. CONSENT PROCESS

The informed consent form will be written in a manner that is understandable to the subject population. Prior to its use, the informed consent form and e-consent, if applicable will be approved by the IRB.

The Principal Investigator or authorized key personnel (other than Dr. Hsu) will discuss with the potential subject the purpose of the research, methods, potential risks and benefits, subject concerns, and other study-related matters. Initial introduction of and any further discussions regarding the study will take place in an exam room in the Duke Cancer Center (for paper consent) or by telephone (for e-consent).
Shiaowen (David) Hsu MD PhD founded a start-up company called Xilis. Duke holds the IP license for microfluidic technology owned by Xilis that may be applied in analyses of the research samples collected on study. This potential COI will be disclosed to all future subjects in the revised ICF submitted via amendment to the IRB on March 21 2022.
Signing of the paper consent will occur in a private room in the Duke Cancer Center. All meetings and discussions regarding study participation will take place in a private room or by telephone. Potential subjects will have the opportunity to contact the Principal investigator or authorized key personnel with questions and have all questions answered before signing the informed consent.

For e-consent, the Research Nurse Study Coordinator will discuss the study with the subject and review the study consent by telephone. Subjects will provide an email address which will be used to generate and deliver the REDCap study consent form. Subjects are given the nurse coordinator's phone number to call with any additional questions or concerns. If the patient elects to participate, he/she will complete the consent via a secure REDCap link and select "Yes, I have read the consent form and I wish to participate in the study." Subjects who do not want to participate will select "Yes, I have read the consent form and I DO NOT wish to participate in

the study." Patients may also review the e-consent and opt to sign a paper consent at their clinic visit.

Subjects will be given as much time as possible to make an informed decision about participation in the study. However, because consent will likely occur on the same day as the proctoscopy procedure, the amount of time available for consent review may be limited. Prospective subjects will be clearly informed of the voluntary nature of the study and freely allowed to decline participation.

The original informed consent form will be stored with the subject's study records, and a copy of the informed consent form will be provided to the subject. The Principal Investigator or authorized key personnel will be responsible for asking the subject whether the subject wishes to notify his/her primary care physician about participation in the study. If the subject agrees to such notification, the Principal Investigator will inform the subject's primary care physician about the subject's participation in the clinical study.

7. STUDY INTERVENTIONS

This study will involve collection of additional tumor biopsy at time of standard-of-care proctoscopy for generation of patient-derived organoids, which are described in Design and Procedure section. No other clinical intervention will be performed.

8. RISK/BENEFIT ASSESSMENT

There is a certain amount of risk to collection of an additional biopsy, which is generally about 3-5 mm in size, for research purposes. Biopsy risk will be minimized by collecting the research biopsy at the same time as the standard-of-care proctoscopy procedure. The research biopsy will be collected at the treating physician's discretion and only a single research biopsy sample will be obtained. There are also risks that privacy and confidentiality may be compromised. However, every reasonable effort will be made to limit breaches of privacy and confidentiality, as described in the "Privacy, data storage, and confidentiality" section below.

Subjects will not benefit directly from this protocol. However, data and conclusions derived from this protocol are expected to improve the current understanding of rectal adenocarcinoma and possibly influence future cancer patient care or cancer protocol designs.

9. COSTS TO SUBJECTS

This protocol does not involve any cost to subjects.

10. DATA ANALYSIS AND STATISTICAL CONSIDERATIONS

This is a specimen and clinical data collection protocol.

For the primary objective, all enrolled patients are evaluable, and we estimate the evaluable sample size will be 20 patient biopsies. For the exploratory objectives, evaluable patients are

those with successful patient-derived organoids, which we estimate will be about 14 of 20 initial biopsies.

Only descriptive statistical analyses will be performed. The primary objective will be reported as the percent of patient biopsies that form spherical organoids, and the 90% one-sided lower confidence limit will be reported. For instance, if we generate spherical organoids from 14 of 20 (70%) biopsies, the 90% one-sided lower confidence limit will be 56%. Laboratory procedures will follow group standard operating procedures (SOPs). Given the anticipated success rate of generating organoids of about 70%, if we are not successful at generating organoids from 3 of the first 5 patients, we will place the research protocol on hold, and all laboratory SOPs will be reviewed and modified.

The exploratory objectives will be described qualitatively given the anticipated small sample size.[13] For the organoid radiation dose-response experiments, we will compare the dose-response AUC in patients who did or did not achieve a pCR. For the organoid transplantation experiments, we will compare the percent change in tumor size index in patients who did or did not achieve a pCR.

11. DATA SAFETY AND MONITORING

This clinical research study will be monitored both internally by the PI, and institutionally by the Duke Cancer Institute (DCI). In terms of internal review the PI will continuously monitor and tabulate adverse events. Appropriate reporting to the Duke University Medical Center IRB will be made. If an unexpected frequency of Grade III or IV events occur, depending on their nature, action appropriate to the nature and frequency of these adverse events will be taken. This may require a protocol amendment, dose de-escalation, or potentially closure of the study. The PI of this study will also continuously monitor the conduct, data, and safety of this study to ensure that:

- Interim analyses occur as scheduled;
- Stopping rules for toxicity and/or response are met;
- Risk/benefit ratio is not altered to the detriment of the subjects;
- Appropriate internal monitoring of AEs and outcomes is done;
- Over-accrual does not occur;
- Under-accrual is addressed with appropriate amendments or actions;
- Data are being appropriately collected in a reasonably timely manner.

DCI Protocol Review and Monitoring systems (PRMS) review of this protocol begins with an initial review by the Cancer Protocol Committee (CPC). CPC new protocol review focuses on scientific relevance, study design, adequacy of biostatistical input, protocol prioritization, feasibility of completing the study within a reasonable time frame and risk assessment of the trial. The PI will abide by CPC assessment of the level of risk, which will determine the intensity of subsequent DCI monitoring. CPC also conducts annual scientific progress reviews on protocols that are open to enrollment and focus on protocol prioritization, accrual and scientific progress. These reviews are conducted at the time of IRB annual renewals and documentation of all CPC reviews will be maintained in eIRB/iRIS systems.

A determination for the degree of monitoring conducted by the DCI monitoring team is made at the time of initial CPC approval to commensurate with the type and level of intervention, phase, endpoints, degree of risk, size and complexity of the protocol. A formal, independent monitoring will be conducted by the DCI monitoring team according to the risk level and monitoring plan assigned by the CPC until the study is closed to enrollment or subjects are no longer receiving study drug or other interventions that are more than minimal risk. Additional monitoring may be prompted by findings from monitoring visits, unexpected frequency of serious and/or unexpected toxicities, or other concerns. Monitoring visits may also be initiated upon request by DUHS and DCI Leadership, CPC, SOC, a sponsor, an investigator, or the IRB.

The DCI monitoring team reviews the adequacy of informed consent, enrollment of eligible patients, implementation of protocol-specified procedures and treatment, adequacy of data collection, and appropriateness of adverse event monitoring and reporting. The DCI monitoring team presents final monitoring reports to the DCI Safety Oversight Committee (SOC) highlighting safety concerns and unresolved issues. The SOC, at a convened meeting, assigns an overall rating of satisfactory, marginal, or unsatisfactory to reflect the overall quality of data, regulatory, consent, eligibility, study conduct and AE reporting. Corrective action plans (CAPs) are developed, implemented, and evaluated as indicated. The SOC will notify the sponsor-investigator and DUHS IRB when significant safety concerns are identified.

The SOC in concert with DCI monitoring team conducts data and safety monitoring for DUHS sponsor-investigator phase I and II, therapeutic interventional oncology studies that do not have an independent DSMB. These reviews occur at a minimum every 6 months and more frequently for the high risk studies. The SOC safety reviews include review of safety data, enrollment status, stopping rules if applicable, accrual, toxicities, reference literature, and interim analyses as provided by the sponsor-investigator. The SOC, at a convened meeting, assigns a rating of satisfactory when adequate accrual with lack of excessive toxicity is present.

12. PRIVACY, DATA STORAGE, AND CONFIDENTIALITY

The Principal Investigator will ensure that subject privacy and confidentiality of the subject's data will be maintained. RDSPs will be approved by the appropriate institutional Clinical Research Unit.

To protect privacy, every reasonable effort will be made to prevent undue access to subjects during the course of the study. Prospective participants will be consented in a private room or by telephone, if using e-consent. All research related interactions with the participant will be conducted by qualified research staff who are directly involved in the conduct of the research study.

To protect confidentiality, subject files in paper format will be stored in secure cabinets under lock and key accessible only by the research staff. Subjects will be identified only by a unique study number and subject initials. Electronic records of subject data will be maintained using a dedicated REDCap database which is housed in an encrypted and password-protected Duke file server. Access to electronic databases will be limited to the PI and members of the study team. Subject data may be stored temporarily on encrypted and password-protected portable memory devices such as flash drives and external hard drives, but only when absolutely necessary. Data

stored on portable memory devices will be de-identified. Subject data will be deleted from the portable memory device at the earliest opportunity. The security and viability of the IT infrastructure will be managed by the DCI and/or Duke Medicine.

Any cell lines or organoids derived from the patient samples will be given a unique de-identified reference code and will not contain any subject PHI. A secure master log document linking the patient identification and specimen information will be generated during specimen collection. Only the PI and designated members of the study team will have access to the master log.

Upon completion of the study, research records will be archived and handled per DUHS HRPP policy.

Subject names or identifiers will not be used in reports, presentations at scientific meetings, or publications in scientific journals.

13. REFERENCES

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