Developing a Novel Technique for Culturing of Circulating Tumor Cells in Pancreatic Cancer patients for Realtime Assessment of Circulating Disease.

Ammar Asrar Javed, MD
Assistant Professor, Hepatobiliary and Pancreatic Surgery
Department of Surgery, NYU Langone Health
Ammar.Javed@nyulangone.org

Significance of research

Effective control of systemic disease remains the greatest challenge to improving survival in pancreatic cancer (PDAC). Despite improvements in systemic therapies their impact on survival has been modest at best. High rates of systemic recurrence have been reported in patients who undergo surgical resection coupled with systemic therapies. Even in patients achieving complete response to therapy in the primary tumor the rate of recurrence is >40%, suggesting inadequate control of circulating disease i.e. circulating tumor cells (CTCs). The current proposal is aimed at developing a novel technique for culturing of CTCs in PDAC. In the past this was limited by low yield of CTCs and inability to isolate them in a viable state. A new ISET device now allows for live cell isolation of CTCs and novel techniques have shown promise in culturing them in other cancer types. If we are able to do so in PDAC it would have a significant impact on the management and outcomes of these patients. We will be able to perform drug sensitivity analysis on circulating disease as opposed to the primary tumor and identify potential therapeutics that each patient's circulating disease is responsive to. Furthermore, expansion of CTC populations would allow for studies to identify aggressive clones and potentially develop novel therapies that specifically target these cells. We believe that if achieved we will develop a tool that will be immediately clinically applicable and allow for improved control of systemic disease resulting in a higher proportion of patients achieving long-term survival and cure.

Background information

PDAC is one of the leading causes of cancer related mortality worldwide and the only potentially curative therapy is local control through oncologic resection and systemic control through chemotherapy. However, even after resection a majority of patients experience treatment failure predominantly in the form of distant progression. **Effective control of systemic disease remains the greatest challenge to improving long-term survival.**

Circulating tumor cells (CTCs) have been identified as the seeds of metastasis and in the past our group has successfully been to isolate them from PDAC patients and demonstrated phenotypic heterogeneity and utility as biomarkers for non-invasive near "real-time" assessment of disease and predicting outcomes. Recent data suggest that primary tumor biology is distinct from that of circulating disease. While multiple studies have been performed on primary tumor specimens including sequencing, transcriptomic analyses, and organoid modelling for drug sensitivity analyses, limited work has been performed on circulating disease. In the past this was limited by low yield of isolated CTCs and isolation techniques being limited to fixation of cells which

did not allow for live cell capture. The latter is now possible with the new ISET device however the former still needs to be addressed. The ability to isolate and culture CTCs would allow us to perform drug sensitivity on circulating disease to identify the most effective chemotherapeutics to treat circulating disease in each patient in a personalized manner and provide sufficient volume of cells to perform sophisticated analyses to understand the biology of CTCs to identify potential novel therapies.

Preliminary observations

Preliminary data pertinent to this study are summarized as follows:

1. **Circulating tumor cell biology is distinct from the primary tumor.** Systemic dissemination of disease involves transformation of tumor cells through the well-established EMT process. These cells have unique properties allowing them to escape and survive in circulation, evade host immune system, and seed distant organs. Our lab and others have demonstrated phenotypic heterogeneity with CTCs differentially expressing epithelial, mesenchymal or stem cell-like markers. Mutationally, disconcordance in KRAS mutations variants has been reported between CTCs and the primary tumor.
2. **Circulating tumor cell characteristics are associated with treatment response and outcomes.** Our group has previously demonstrated correlation between CTC enumeration and phenotype with early disease recurrence, overall survival, and presence of occult metastases, proving that CTCs provide insight into tumor biology independent of primary tumor characteristics. We have also reported that the transitional subtype of CTCs is most strongly associated with more aggressive biology. Limited data on single cell RNA sequencing suggest that further subtypes of CTC may exist.
3. **CTC culturing and pharmacoptying has been described in other cancer types.** CTC culturing and pharmacotyping has been described for other cancer types (breast, lung, prostate, and colorectal) with success however these studies are limited by their isolation techniques which are either suboptimal or nonreproducible and need further investigation. Lastly, in case of successful cultures pharmacotyping of primary PDAC tumor derived organoids has been shown to be associated with patients' clinical response to therapy.

Methods & designs

We aim to develop and validate a novel approach to culture CTCs for characterization and drug sensitivity analysis.

Aim 1. Assessment of the most efficient technique to culture CTCs. Methods: In the initial phase we will spike normal blood samples (N=40) with PDAC cell lines. Tumor cells will be isolated using Isolation by Size of Tumor cells (ISET) device and cultured using three different techniques (Figure 1): (1) 3D Organoids: Tumor cells collected from the ISET will be resuspended in Matrigel and plated with Human Complete Feeding Medium (HCFM), (2) Serum enhanced 3D Organoids: A similar process to technique 1 will be employed, however, the matrigel and feeding media will be enhanced with serum from PDAC patients, and (3) Chicken Chorioallantonic Membrane (CAM) cultures (Figure 2): A small hole will be created in the shell of an egg at day-10 of the fertilization. Tumor cells isolated using ISET will be engraft on the CAM which supports embryo development. The embryo will be euthanized between day 16 and 19 and the tumor cell will be isolated for

further analysis. In the second phase, techniques showing most promise will be applied to 50 patients with PDAC to assess the feasibility of culturing CTCs.

Aim 2. Real-time drug sensitivity screening and definition of tumor heterogeneity. Drug sensitivity analysis will be performed on cultures obtained in the second phase of the study and compared to patients' clinical course to assess the accuracy of CTC pharmacotyping in predicting clinical response to therapy and outcomes.

Data analysis

The primary objective of the current study is to identify the most efficient technique to culture CTCs in PDAC. To assess this, a threshold of 50% success will be set for each technique. This is based on prior studies on CTCs culturing on other tumor types and primary tumor organoid culturing in PDAC. Techniques demonstrating ≥50% success will be applied to human samples. In these, success will be defined as >50% rate of establishing cultures with >500 cells that expand beyond three passage. This was determined based on consideration for the lowest cell count required for pharmacotyping and that this information would be clinically meaningful if obtained within four weeks of sampling. Clinicopathological factors associated with successful culturing will be identified. For the second aim pharmacotyping on developed CTC cultures will be performed. Established protocols will be used to assess sensitivity of CTCs to chemotherapeutic agents frequently used for PDAC. In these patients chemosensitivity will be defined as biochemical or imaging-based evidence of response to therapy or stable disease, while chemo-resistance will be defined as progressive disease. The results obtained on pharmacotyping of the CTC cultures will be compared to the clinical course of disease in these patients and the ability of pharmacotyping to predict clinical course will be assessed using ROC analysis with an acceptable AUC of >0.80 and compared to the performance of CA19-9 in predicting treatment response. Statistical significance will be set for a p-value at or below 0.05.

***PRELIMINARY PHASE*-40 NORMAL BLOOD SAMPLES SPIKED WITH PDAC CELL LINES**

CTCs isolation for enumeration, characterization

CTCs isolation for culturing

1 Organoids culture

2 SE-Organoids culture

3 CAM model

***CLINICAL PHASE*-50 METASTATIC and LA PDAC PATIENTS**

CTCs isolation for enumeration, characterization

CTCs isolation for culturing

Culture technique will be defined based on the preliminary phase results

Figure 1. Study design: In the preliminary phase three techniques for CTCs culturing will be assessed: (1) 3D Organoids, (2) Serum enhanced 3D Organoids, and (3) Chicken Chorioallantonic Membrane cultures. In the clinical phase the most feasible technique identified in aim 1 will be applied to CTCs culturing in human samples.

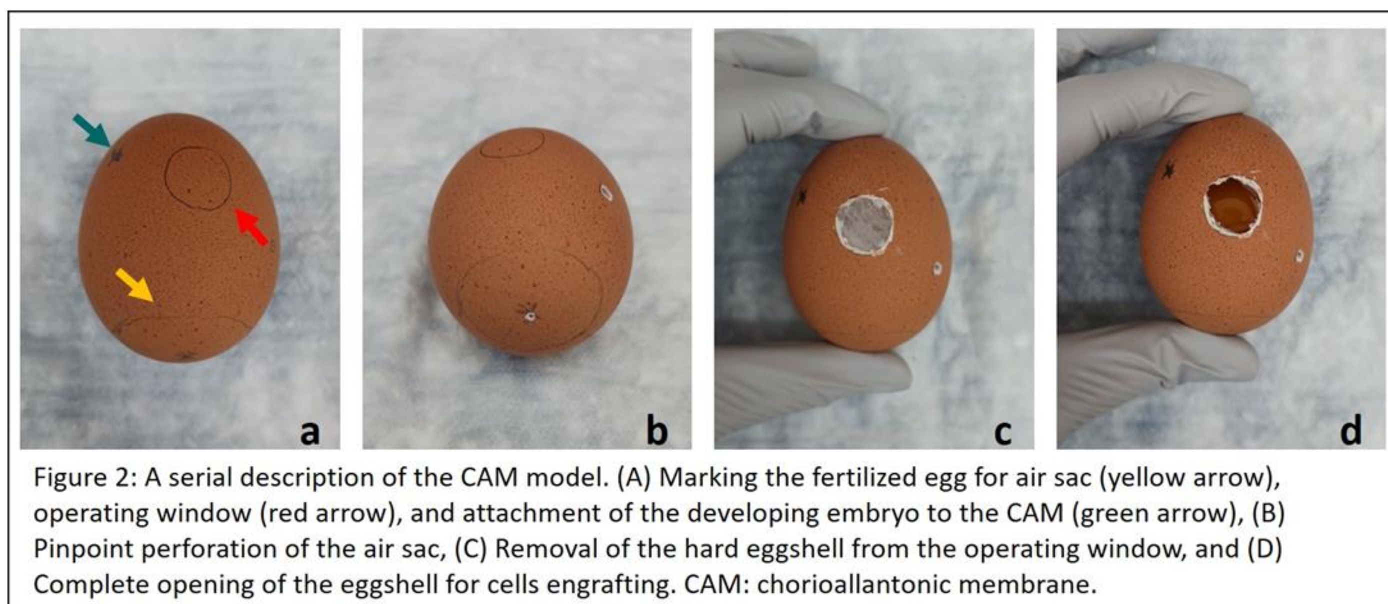

Figure 2: A serial description of the CAM model. (A) Marking the fertilized egg for air sac (yellow arrow), operating window (red arrow), and attachment of the developing embryo to the CAM (green arrow), (B) Pinpoint perforation of the air sac, (C) Removal of the hard eggshell from the operating window, and (D) Complete opening of the eggshell for cells engrafting. CAM: chorioallantonic membrane.