A novel and highly efficient biomimetic capture approach for the isolation and characterization of circulating metastatic cancer cell clusters from lung cancer patients

Kourosh Kouhmareh [1], Erika Martin [1], Darren Finlay [2], Anukriti Bhadada [3], Hector Hernandez-Vargas [4], Francisco Downey [3], Jeffrey K. Allen [3] and Peter Teriete [5,$]

1) PhenoVista Biosciences, 6195 Cornerstone Ct E STE 114, San Diego, CA 92121; 2) Sanford Burnham Prebys Medical Discovery Institute, 10901 N. Torrey Pines Rd., La Jolla, CA 92037; 3) TumorGen Inc., 6185 Cornerstone Ct E STE #101, San Diego, CA 92121; 4) Centre Léon Bérard, 28 Prom. Léa et Napoléon Bullukian, 69008 Lyon, France; 5) now IDEAYA Biosciences, 7000 Shoreline Ct STE #350, South San Francisco, CA 94080; $) corresponding author

Abstract

Capture and isolation of circulating metastatic cancer cell clusters (MCCC) is the essential first step to identify novel therapeutic targets focused on this important route of metastasis. We developed a unique biomimetic capture approach that allows selective isolation of these deadly clusters from the whole blood of lung cancer patients. Utilizing a microfluidic platform we developed a versatile smart-coating that combines biomimicry and immuno-capture for a highly efficient and selective isolation of viable MCCCs. Here we show the capture and characterization of multiple MCCCs from non-small cell lung cancer (NSCLC) patients. Table 1 shows the count of clusters detected in NSCLC patients based on a stringent criterion confirming their identification. The MCCCs were detected in every patient tested but none were detected in normal blood controls from donors of similar age. Isolated MCCC where characterized by immunofluorescence and submitted for genomic analyses.

Metastasis remains one of the leading causes of cancer related deaths worldwide. Insights into how the tumor propagates to pre-metastatic sites is necessary to develop focused therapeutic approaches. Circulating metastatic cancer cell clusters (MCCCs) have been established as a primary entity causing distal metastases. Significant advances have been made linking cluster integrity and viability to increased metastatic potential highlighting the unmet need for cluster focused therapies.

Our novel approach allows the rapid and easy capture of MCCCs paving the way for the discovery of novel or unique targets that eventually will bring new anti-metastatic therapies to the clinic.

What are MCCCs?

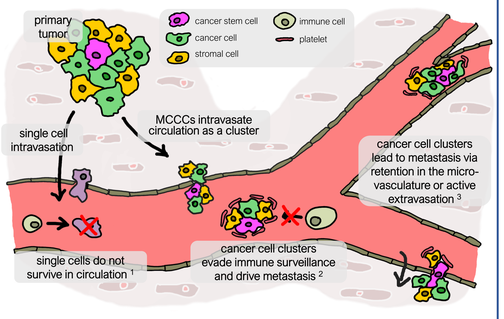

Figure 1: Basic concept of MCCC-driven metastasis. The primary tumor releases MCCCs and single circulating tumor cells, which travel through the bloodstream and localize elsewhere in the body forming potential metastatic seeds.

- MCCCs are also referred to as circulating tumor micro-emboli, CTC clusters, and collective cell migration.
- MCCCs migrate from the primary tumor as homotypic or heterotypic groups of cells intravasating into circulation.
- Contrasting to single cells and homotypic MCCCs, heterotypic MCCCs have been observed to preferentially escape immune-surveillance and survive in circulation, demonstrating a greater metastatic potential.
- MCCCs can get 'stuck' in micro-vasculature or actively extravasate into tissue forming pre-metastatic niches that may evolve into metastatic lesions.

Clinical significance of MCCCs

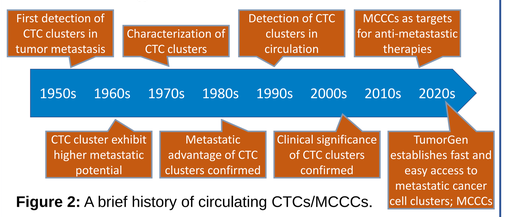

Figure 2: A brief history of circulating CTCs/MCCCs.

A snapshot of published clinical data correlating MCCCs with OS and PFS

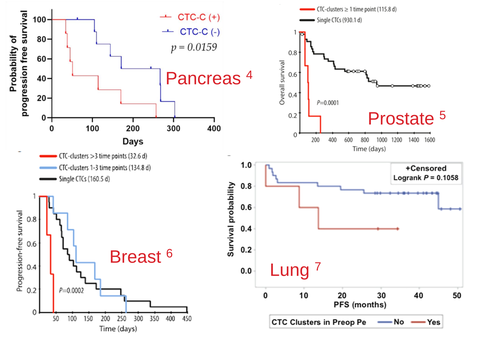

Figure 3: Selected OS/PFS graphs from recent key publications on CTC-clusters/MCCCs detected in clinical patient studies.

The presence of MCCCs has a dramatic impact on the Overall Survival (OS) or Progression Free Survival (PFS) for cancer patients across most solid tumor types.

Microfluidic Capture of MCCCs

- Current technologies require extensive 'pre-processing' of whole blood samples leading to significant loss of the rare MCCCs.
- Size based technologies run risk of sample loss due to varying sizes of MCCCs and the effect of the shear forces on cluster integrity.
- The CD44 surface antigen is an abundant marker of MCCCs and associated cell types.
- CD44 upregulation is associated with the formation and an increased metastatic potential of MCCCs.[8]
- During leukocyte extravasation the binding of CD44-presenting cells to endothelial hyaluronic acid (HA) is the first and essential step initiating margination, rolling, and extravasation.

Taking advantage of this existing biological principle, we have designed and manufactured a microfluidic chip with a SMART-Coating™ technology that combines a tunable biomimetic and immuno-based dual capture approach.[9]

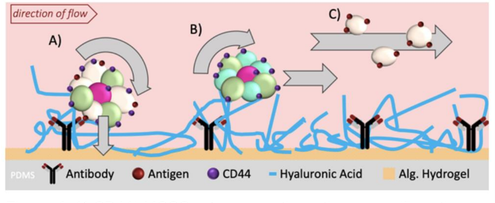

Figure 4: A) CD44⁺-MCCCs also presenting a tissue specific antigen of interest are captured by avidity-driven interaction with HA, marginate to the walls, and are immobilized by an antibody. B) CD44⁻-Clusters presenting the antigen and C) CD44⁺ hematopoietic cells lacking the tissue specific antigen are not captured.

Sample processing

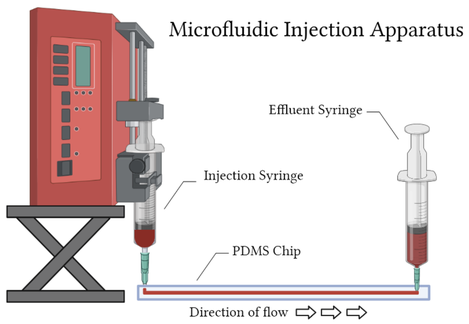

Figure 5: Schematic of sample processing. Syringe containing unprocessed patient blood sample is injected into MCCC capture chip at a flow rate of 100µL/minute. Effluent can be collected and stored for subsequent multiplexed analyses (ctDNA, exosome, *etc.*).

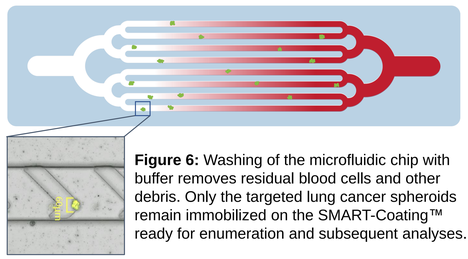

Figure 6: Washing of the microfluidic chip with buffer removes residual blood cells and other debris. Only the targeted lung cancer spheroids remain immobilized on the SMART-Coating™ ready for enumeration and subsequent analyses.

Capture optimization

- Co-cultured spheroids were generated by seeding A549 and HCC827 cells respectively, in combination with HFF-1 fibroblasts into a 384 well U-bottom plate.
- Spheroids were fluorescently stained and spiked into an 4mL freshly collected normal donor blood sample.
- The spiked blood sample was processed on a SMART-coated™ capture chip and washed with buffer.
- An accurate spiked spheroid count was taken and compared to the spheroids captured on the chip in order to calculate a capture efficiency percentage.
- Multiple normal blood donor samples without spiked clusters were processed and no clusters were observed.

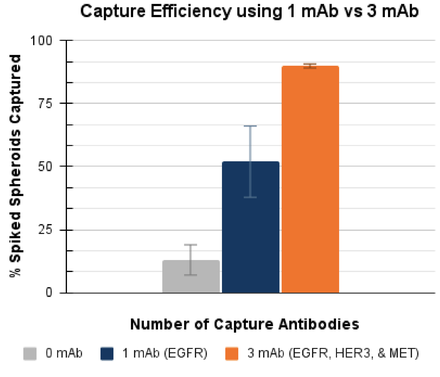

Figure 7: Capture efficiency comparison using varying numbers of tissue specific capture antibodies. The unique properties of the dual-capture approach allows targeting of multiple antigens concurrently.

Capturing MCCCs from patients.

Whole blood samples of patients[10] with confirmed lung cancers were collected and processed on a SMART-coated™ MCCC capture chip. Fast processing times keep MCCCs viable and naïve. Captured MCCCs were detected using automated bright field microscopy and either stained with immunofluorescence marker confirming MCCC-status or processed for genomic sequencing analysis.

Patient	Age/ Gender	Diagnosis	Stage	# clusters detected	Sample use
INMC-044.1	82 / M	lung adenocarcinoma	IVb	2	MCCC detection by bright field microscopy and confirmation by immunofluorescence staining for CD44, EGFR, Hoechst
INMC-044.2	53 / F	squamous cell lung carcinoma	IV	1	
INMC-044.3	59 / F	metastatic lung adenocarcinoma	IV	2	
INMC-044.4	65 / M	squamous cell lung carcinoma	IV	1	
INMC-044.5	67 / M	non-small cell lung carcinoma	IIIa	3	
INMC-044.6	64 / F	lung adenocarcinoma	IV	MCCC were not IF stained to preserve RNA quality	MCCC detection by bright field microscopy, samples processed for genomic analysis by RNA and DNA seq.
INMC-044.7	46 / F	non-small cell lung cancer	IV		
INMC-044.8	66 / F	non-small cell lung cancer	IV		

Table 1: Details of patient samples processed.

Immunofluorescence staining was performed *in situ* on the capture chip. This minimizes the sample handling and accelerates analysis. Imaging was performed on an automated Yokogawa CQ1high-content analysis system.
Detected cell clusters were classified into three groups, [Hoechst positive, EGFR positive, and CD44 negative], [Hoechst positive, CD44 positive, and EGFR negative], and [Hoechst positive, CD44 positive, and EGFR positive]. Cells positive for only Hoechst and CD44 were presumed to be of hematopoietic origin and disregarded. CD44/EGFR staining without positive nuclear Hoechst stain were disregarded. Only clusters positive for all three stains were considered potential MCCC's.

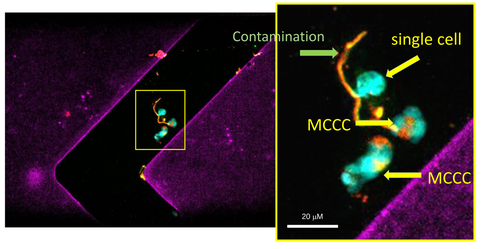

Figure 8: 40X of a microfluidic channel showing captured NSCLC MCCCs from a Stage IV NSCLC patient. Anti-EGFR-AF594 (red), anti-CD44-FITC (green), Hoechst 33342 stained nuclei (blue), SMART-Coating™ (purple).

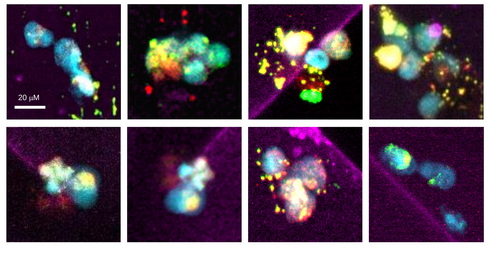

Figure 9: Gallery of MCCCs captured from patient whole blood samples. Heterogeneous shapes, sizes, and cell composition of the clusters are observed. The design of the chip and properties of the SMART-Coating™ allows culturing of viable MCCCs. Furthermore, we demonstrate efficient immunostaining procedures that enable advanced confocal imaging for detailed phenotypic characterization, and *ex vivo* drug testing.

RNA Seq on patient-captured MCCCs

RNA was extracted from sample INMC-44.6 (*i.e.* sample LC6). This sample corresponded to a 64-years old female patient, diagnosed by transthoracic biopsy with a TTF-1 positive stage IV lung adenocarcinoma that had metastasized to the brain. Three RNA aliquots of the LC6 sample were processed for library preparation using SMART-Seq® HT Kit (Takara) and Illumina Nextera reagents for tagmentation. RNA sequencing was performed at The Scripps Research Institute NGS core facility. Resulting FASTQ files were downloaded and imported into R. All subsequent analyses were performed using R/Bioconductor packages. Other datasets used in addition were: GSE51827: a study comparing gene expression profiles of clusters (CCLs) and single cells (SCs) in breast cancer.[5,11] GSE74639: RNA-Seq of lung tumor circulating single cells (SCs) and primary lung tumor cells (PTs) from an orthotopic lung xenograft model.[12]

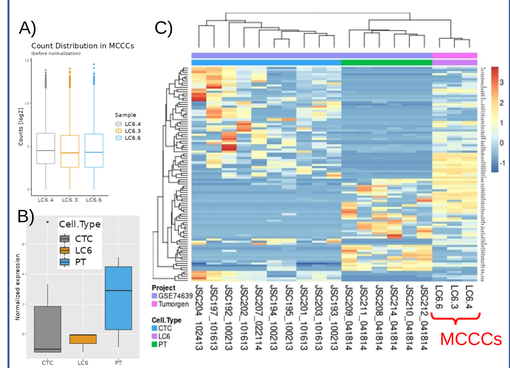

Figure 10: A) The overall count distribution was similar for the three replicates LC6.4, LC6.3, and LC6.6. **B)** INMC-44.6 is positive for the adenocarcinoma marker TFT-1. We confirmed expression of the gene "NKX2.1" which encodes the TFT-1 protein in MCCCs (together with CTCs and PTs). **C)** Heatmap showing the relationship of MCCCs to lung CTCs and primary lung tumor cells (PT), using genes that are differentially expressed between CTCs and PTs.

Detailed analysis of the RNA seq data obtained from capture MCCCs confirmed tissue of origin (not shown) as well as unique expression patterns specific to the patient (TFT-1). Furthermore, differentially expressed gene profiles unique to the MCCCs provides evidence that these vectors of metastasis require closer observation. Recent work by Aceto, *et al.*[13], has highlighted highly promising therapeutic value in targeting MCCCs directly in the fight against metastasis.

Acknowledgements

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