Detection of Circulating Tumor Cells from Glioblastoma patients' blood samples

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Introduction

Glioblastoma (GBM) is the most common form of primary brain tumor in adults and is often associated with poor prognosis. The application of liquid blood biopsy methods for GBM monitoring and diagnosis has been limited due to the low shedding of commonly utilized circulating biomarkers, such as cell-free DNA. Circulating tumor cells (CTCs) may present as a minimally invasive alternative for dynamic assessment of cancer progression through protein and molecular analysis as GBM patients undergo treatment (**Figure 1**). However, current challenges with CTC detection stem from the fact that GBM tumors arise from central nervous system tissue and thus present a mesenchymal phenotype which is undetectable by methodologies which rely on EpCAM for CTC isolation[1].

Additionally, extracellular vesicles (EVs) represent an additional potential liquid biopsy biomarker for GBM. EVs are released from the primary tumor and can contribute to the formation of the pre-metastatic niche[2]. They have the potential to be utilized as diagnostic and prognostic biomarkers based on their circulating concentration and molecular cargo[3].

This proof-of-concept study aimed to assess the feasibility of detecting CTCs in GBM patients' blood samples using a Parsortix® CTC Research Use Only (RUO) workflow developed by ANGLE Europe Limited.

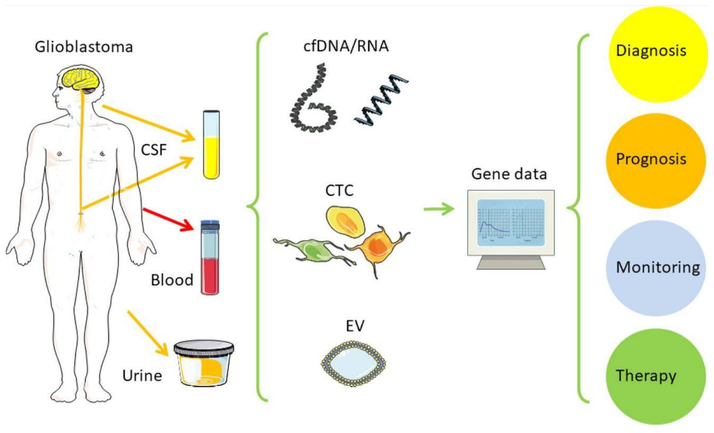


Figure 1. Schematic depicting utility of liquid biopsy for GBM patients. *Figure adapted from Eibl, R.H. and Schneemann, M. (2023). Liquid biopsy and glioblastoma. Exploration of Targeted Anti-tumor Therapy[1].*

Workflow

A total of 15 GBM patients were included in this study. All patients were newly diagnosed at grade IV (within 33 days prior to blood collection), treatment naïve, and ≥18 years of age.

Blood samples were collected into a Streck Cell-Free DNA (Streck) blood collection tube (BCT) for processing at approximately 72–144 hours post-draw. For each patient, 7.5 mL of blood were processed using Parsortix® instruments, a microfluidic technology capable of capturing CTCs from blood based on cell size and relative lack of deformability (as compared to red and white blood cells). The enriched samples were harvested and processed onto ANGLE's CellKeep™ slides for immunofluorescent (IF) staining with ANGLE's RUO CTC assay for the detection and characterization of CTCs (**Figure 2**). The assay included a nuclear marker for cellular identification, epithelial and mesenchymal markers for positive CTC identification, and hematopoietic exclusion markers.

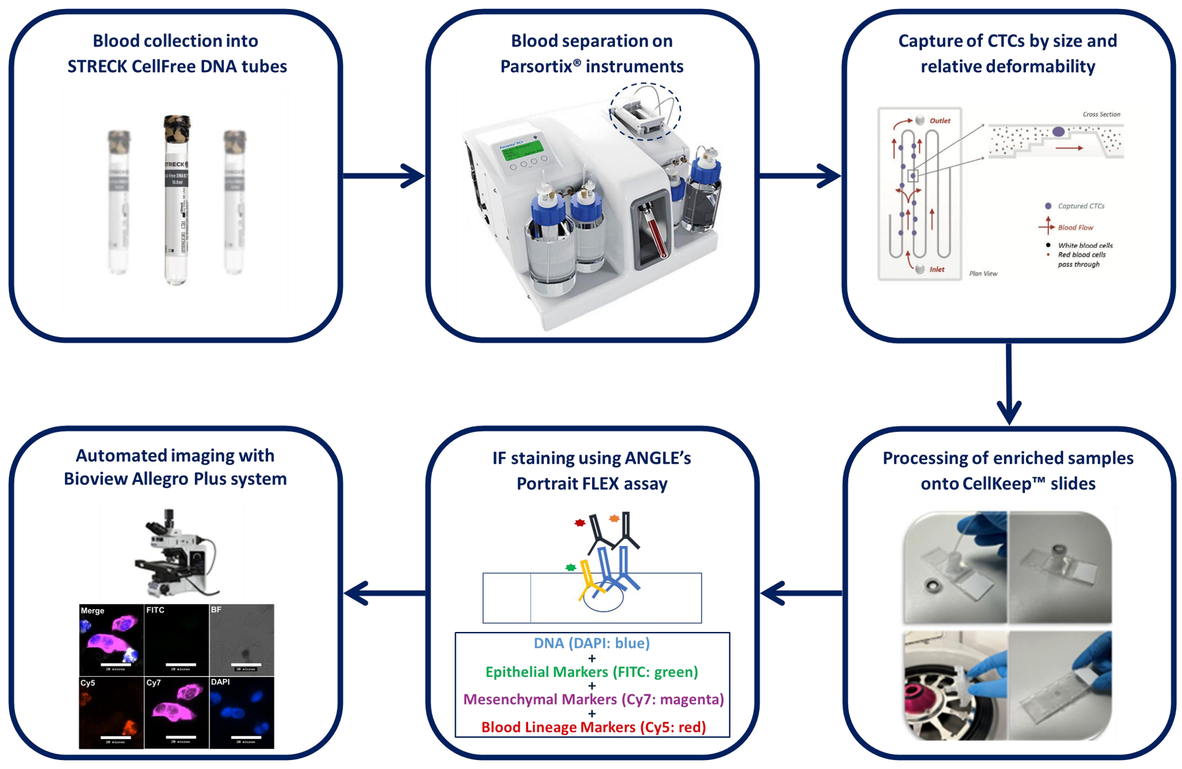


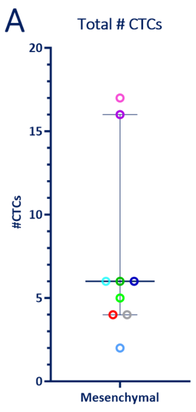
Figure 2. Schematic representation of the complete Parsortix® CTC Research Use Only (RUO) workflow. *The complete Parsortix® CTC Research Use Only (RUO) workflow is depicted. This includes blood sample collection, enrichment of CTCs with the Parsortix instrument, IF staining, imaging, and analysis. ANGLE's RUO CTC assay includes a nuclear dye conjugated for detection in the DAPI channel (visualized as blue), antibodies against epithelial markers conjugated for detection in the FITC channel (visualized as green), antibodies against mesenchymal markers conjugated for detection in the Cy7 channel (visualized as magenta), and antibodies against hematopoietic markers conjugated for detection in the Cy5 channel (visualized as red). A BioView Allegro Plus LED imaging system was used, wherein targets of interest identified at 10x magnification and revisited at 40x magnification.*

Results: Circulating Tumor Cells

Blood samples (7.5 mL) from all 15 patients were successfully processed using the Parsortix® CTC RUO workflow.

- One CTC or more was detected in 9/15 (60.0%) patients (**Figure 3A, 3C**).
  - The number of CTCs detected per patient ranged from 2 – 17 CTCs.
- Homotypic CTC clusters were observed in 7/9 (77.8%) patients determined to be CTC+ (≥1 CTC) (**Figure 3B, 3C**).
  - The number of CTC clusters observed per subject ranged from 1 – 2 clusters.
  - The number of CTCs per cluster ranged from 2 – 6 CTCs.
- The diameter of single CTCs ranged from 8.9 – 21.8 µm, with a mean value of 14.5 µm, determined from 28 single CTCs (**Figure 3C**).
- All CTCs detected presented a mesenchymal phenotype (**Figure 3A, 3D, 3E**).
  - No epithelial or epithelial-to-mesenchymal transition (EMT) CTCs were detected.
- CTCs commonly exhibited a large cytoplasmic area, far exceeding the nucleus as compared to that of red and white blood cells (**Figure 3E**).

A



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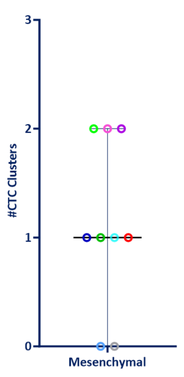
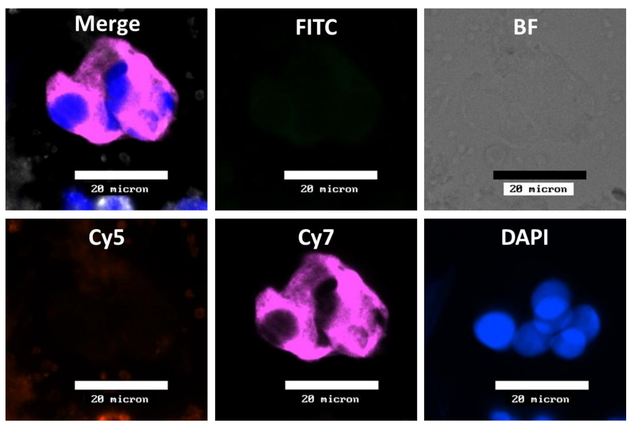


C

Patients			
#Total			15
#CTC+			60.0% (9 / 15)
#CTC+ with Clusters			77.8% (7 / 9)
CTCs			
All CTCs	#Total		66
	Count	Mean	7
		Median	6
		Range	2 – 17
CTC Clusters (Range)	#Clusters per patient		1 – 2
	#CTCs per cluster		2 – 6
Single CTCs (Size)	#Single CTCs		28
	Diameter (µm)	Mean	14.5
		Median	14.6
		Range	8.9 – 21.8
Phenotype	Epithelial		0.0% (0 / 66)
	EMT		0.0% (0 / 66)
	Mesenchymal		100.0% (66 / 66)

D



E

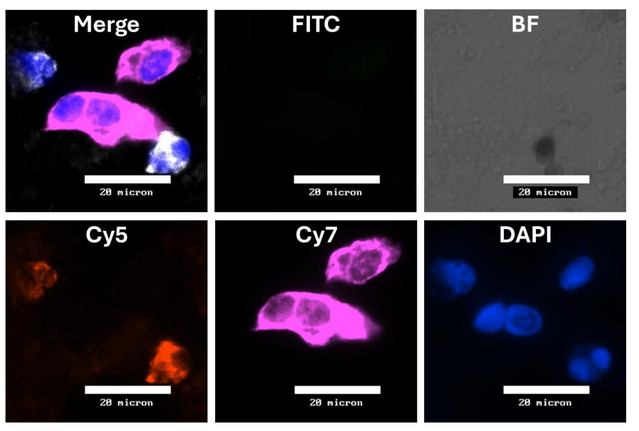


Figure 3. Assessment of CTCs detected in GBM patients' blood samples. *Blood samples (7.5 mL) from GBM patients (n patients =15) were assessed for the presence CTCs through IF staining and microscopy. CTC negative samples were not included in graphs or calculations.* ***(A)*** *Dot plot displays the total number (with median ± 95% confidence interval [CI]) of CTCs identified per CTC+ (≥1 CTC) patient (n patients =9).* ***(B)*** *Dot plot displays the total number (with median ± 95% CI) of CTC clusters identified in CTC+ patients.* ***(A, B)*** *Each patient is color coded.* ***(C)*** *Summary table showing first the total number of patients in the cohort, the number and percentage of patients with ≥1 CTC, and the number and percentage of CTC+ patients with ≥1 CTC cluster, followed by a summary of the CTC data with the total number, mean, median and range of CTCs captured across patients, the range of number of CTCs per cluster, the range of CTC clusters per subject, the total number of single CTCs and the diameter of those CTCs as a mean, median and range, and the phenotype of all CTCs detected. Representative images of* ***(D)*** *a cluster of six mesenchymal CTCs and* ***(E)*** *a cluster of two mesenchymal CTCs near one single mesenchymal CTC. Epithelial markers (FITC) in green, brightfield in grayscale, blood lineage markers (Cy5) in white (merge) or red, mesenchymal markers (Cy7) in magenta, and nuclear dye (DAPI) in blue.*

Results: Extracellular Vesicles

- EVs are non-nucleated (DAPI-) structures, positive for cytokeratin 18 (FITC+) and distinct in brightfield (**Figure 4B, 4C**).
  - No mesenchymal or blood lineage marker positivity was expected or observed (Cy7-/Cy5-) in EVs.
- EVs were observed in 73.3% (11/15) of subjects (**Figure 4A**).
  - The number of EVs detected per patient ranged from 1 – >300 EVs.
  - The size of EVs ranged from approximately 1.5 – 36 µm, with a few outliers observed up to approximately 80 µm.
- No statistically significant relationship was identified between CTC positivity and the observed presence of EVs.
  - Of the EV+ donors, 72.7% (8/11) were also CTC+.
  - Of the CTC+ donors, 88.9% (8/9) were also EV+.

A

Patient	Total #CTCs	#EVs
1	17	>300
2	5	222
3	0	218
4	2	97
5	16	75
6	0	20
7	0	6
8	4	5
9	4	3
10	6	2
11	6	1
12	6	0
13	0	0
14	0	0
15	0	0

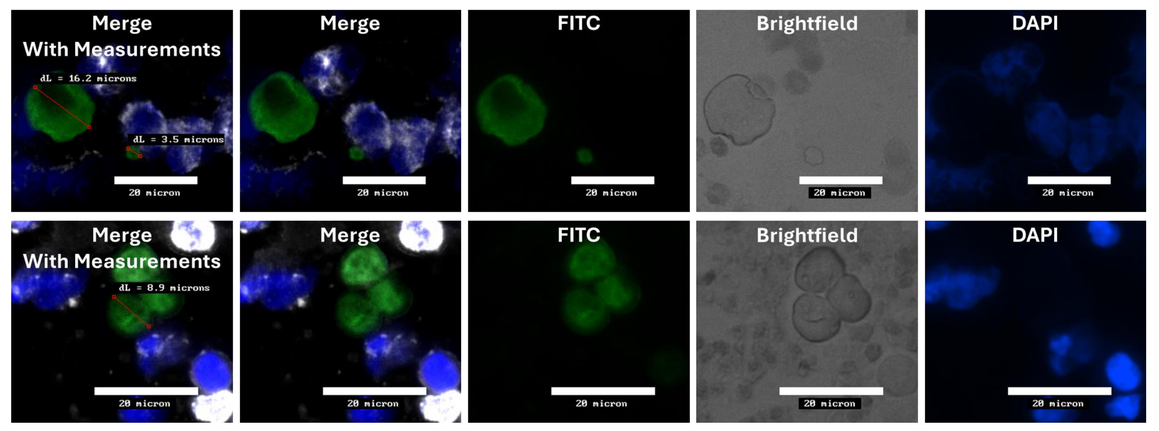


Figure 4. Assessment of EVs in GBM patients' blood samples. ***(A)*** *Table summarizing the CTC status and the EV status of patients, showing the total number of CTCs, and the number of EVs enumerated per patient. Representative images of* ***(B)*** *a larger EV (diameter of 16.2 µm) in proximity of a smaller EV (diameter of 3.5 µm) and* ***(C)*** *a cluster of three EVs of approximately the same size (diameter of 8.9 µm). Epithelial markers (FITC) in green, brightfield in greyscale, and nuclear dye (DAPI) in blue.*

Conclusions

This proof-of-concept study demonstrated the ability to harvest mesenchymal CTCs from GBM patients' blood samples, both as single CTCs and as homotypic clusters, allowing for visual analysis and enumeration. The presence of both CTCs and EVs within patients indicates the potential to develop dynamic protein and molecular testing for advancement of precision cancer treatments.

References

1. Eibl RH, Schneemann M. *Liquid biopsy and glioblastoma.* Explor Target Antitumor Ther. 2023;4:28–41. doi:10.37349/etat.2023.00121
2. Yang X, Zhang Y, Zhang Y, et al. *The Key Role of Exosomes on the Pre-metastatic Niche Formation in Tumors*. Front Mol Biosci. 2021;8:703640. Published 2021 Sep 14. doi:10.3389/fmolb.2021.703640
3. Del Bene M, Osti D, Faletti S, Beznoussenko GV, DiMeco F, Pelicci G. *Extracellular vesicles: The key for precision medicine in glioblastoma*. Neuro Oncol. 2022;24(2):184-196. doi:10.1093/neuonc/noab229