Unlocking the potential of liquid biopsy in glioblastoma: an extensive evaluation of circulating biomarkers

Mariacristina Ciccioli, Morgan Spode, Megan Coates, Elisha Duhig,, Owais Siddiqi, Chloe Goodwin, Amina Mezni, Michele Giunta, Anne-Sophie Pailhes-Jimenez
ANGLE Europe Limited, 10 Nugent Road, Surrey Research Park, Guildford, Surrey GU2 7AF United Kingdom

Introduction

Liquid biopsy offers a minimally invasive approach to monitor cancer progression through blood-based biomarkers including circulating tumor DNA (ctDNA), circulating tumor cells (CTCs) and extracellular vesicles (EVs). While widely studied in systemic cancers, its application in glioblastoma (GBM) remains limited due to:

- **Blood–brain barrier**: Low ctDNA shedding and low tumour burden may result in undetectable biomarker levels.
- **Heterogeneity**: Genetic and cellular diversity in GBM can produce variable biomarker profiles.
- **Phenotype**: Lack of epithelial CTCs limits detection by standard CTC-enrichment platforms.
- **Validation**: Absence of standardized protocols hinders reliable biomarker validation.

These challenges highlight the need for GBM-specific strategies to fully harness the potential of liquid biopsy in neuro-oncology. In this study, we performed a comprehensive evaluation of circulating biomarkers present in GBM patients' blood.

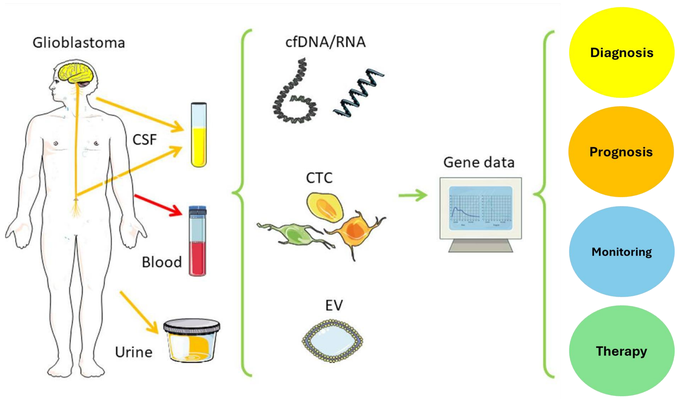

Figure 1. Schematic depicting utility of liquid biopsy for GBM patients. *Figure adapted from Eibl, R.H. and Schneemann, M. (2023). Liquid biopsy and glioblastoma. Exploration of Targeted Anti-tumor Therapy.*

Workflow

- Study included 15 Grade 4 GBM patients, newly diagnosed, treatment naïve and IDH Wild Type.
- Blood samples were collected into a Streck Cell-Free DNA blood collection tube (BCT) and PAXgene BCTs for processing at ~72–144 hours post-draw.
- For each tube, plasma was removed by centrifugation for ctDNA analysis and remaining blood was processed using Parsortix® instruments.
- The enriched samples were harvested and either:
  1) Processed onto ANGLE's CellKeep™ slides for immunofluorescent (IF) staining for the enumeration and characterization of CTCs.
  2) Processed for DNA extraction and NGS.

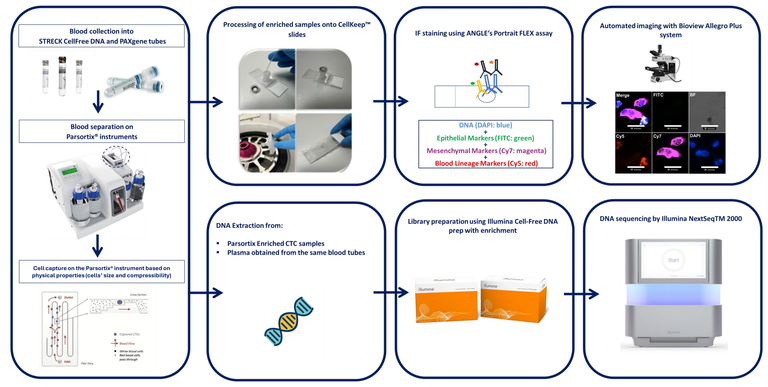

Figure 2. Schematic representation of the assay workflow. *Following plasma removal by centrifugation, blood samples are processed on the Parsortix® instrument, a microfluidic technology capable of capturing CTCs from blood based on cell size and lack of deformability. The Streck BCTs derived harvests were subjected to IF staining, imaging, and analysis using an IF assay comprising a nuclear dye, antibodies against epithelial, mesenchymal and hematopoietic markers. A BioView Allegro Plus imaging system was used for imaging. The PAXgene derived harvests, together with the plasma removed from the same tube, were subjected to DNA extraction, library prep using the Illumina Cell Free DNA prep kit and sequencing on the Illumina NextSeq™ 2000 for detection of low-frequency mutations such as IDH1, IDH2, BRAF, EGFR.*

Results: Circulating Tumor Cells

- One CTC or more was detected in 9/15 (60.0%) patients, with a range of 2 – 17.
- All CTCs detected presented a mesenchymal phenotype.
- CTC clusters were observed in 7/9 (77.8%) patients determined to be CTC+ (≥1 CTC), with a range of 2 – 6 CTCs per cluster and 1 – 2 clusters per patient.
- CTCs mean diameter was 14.5 µm with a range of 8.9 – 21.8 µm .
- CTCs commonly exhibited a large cytoplasm and larger size as compared to that of other blood cells.

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)

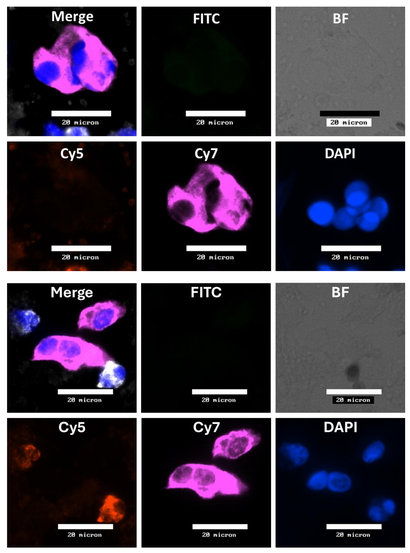

Figure 3. Assessment of CTCs detected in GBM patients' blood samples. *Summary table shows: the total number of patients in the cohort, the number and percentage of patients with ≥1 CTC, and the number and percentage of CTC+ patients with ≥1 CTC cluster, followed by a summary of the CTC data with the 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 show a cluster of six mesenchymal CTCs (top) and a cluster of two mesenchymal CTCs near one single mesenchymal CTC (bottom). 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: CTC DNA vs ctDNA

- 13/14 (93%) of the CTC samples were analysable and 12/14 (86%) had a variant detected in relevant genes.
- 7/14 (50%) of the cfDNA samples were analysable and had a variant detected in relevant genes.

A	1	2	3	4	5	6	7	8	9	10	11	12	13	15
CTC QC	Pass	Fail	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
# of variants (CTC)	61	NA	68	123	76	63	55	0	74	65	49	72	61	64
cfDNA QC	Fail	Fail	Pass	Pass	Fail	Fail	Fail	Pass	Pass	Fail	Fail	Pass	Pass	Pass
# of variants (cfDNA)	NA	NA	61	8	NA	NA	NA	37	53	NA	NA	62	49	35
Shared variants	NA	NA	7	4	NA	NA	NA	NA	7	NA	NA	5	3	1
Example detected variants (CTC / cfDNA / Both)	APC, EGFR, ERBB2, PDGFRA, PIK3CA, PTEN, TP53	NA	APC, EGFR, ERBB2, IDH2, MET, NF1, PDGFRA, PIK3CA, PTEN, TP53	APC, EGFR, ERBB2, IDH1, PDGFRA, PIK3CA, PTEN, TP53	EGFR, ERBB2, IDH2, MET, PDGFRA, PIK3CA, PTEN, TP53	APC, EGFR, ERBB2, MET, PDGFRA, PTEN, TP53	APC, EGFR, IDH1, PIK3CA, PTEN, TP53	APC, MET, PDGFRA, PIK3CA, PTEN, TP53	APC, EGFR, ERBB2, IDH1, IDH2, PDGFRA, PIK3CA, PTEN, TP53	APC	TP53, EGFR, APC.	TP53, NF1, EGFR, MET, APC, PDGFRA, ERBB2, PIK3CA, PTEN	TP53, NF1, EGFR, APC, PDGFRA, ERBB2, PIK3CA, PTEN	TP53, EGFR, MET, APC, PDGFRA, ERBB2, PIK3CA, PTEN

CASE STUDIES:

DONOR #4:

- **CTC detection**: A total of 16 CTCs were identified by immunofluorescence.
- **Variant analysis**: 123 variants were detected in CTCs, 8 in cfDNA samples and 4 shared.
- **Key findings**: Variants relevant to GBM pathogenesis were identified in the CTCs for APC, EGFR, ERBB2, IDH1, PDGFRA, PIK3CA, PTEN, and TP53.

DONOR #3:

- **CTC detection**: 2 CTCs were identified by immunofluorescence.
- **Variant analysis**: 68 variants were detected in CTCs, 61 in cfDNA, with 7 shared.
- **Key findings**: Variants relevant to GBM were identified in CTCs for ERBB2, IDH2, PDGFRA, and PIK3CA; in cfDNA only for MET; and shared between CTC and cfDNA for APC, EGFR, NF1, PTEN, and TP53.

Figure 5. Comparison of gene variants detection in CTC and corresponding ctDNA samples. *(**A**) Table shows number of CTC and ctDNA samples that passed QC for NGS analysis, number of variants of interest detected and genes for which variants were detected in either the CTC samples only (red), the cfDNA sample only (green) or both (purple). (**B**) Case study of subject #4 who had 16 CTCs identified by IF and a total of 131 variants detected with the vast majority (123 – 94%) only being detected in the CTC sample. (**C**) Case study of subject #3 who had 2 CTCs identified by IF and a total of 136 variants detected equally distributed between the CTC and the cfDNA sample.*

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 .
- The size of the EVs detected ranged from approximately 1.5 – 36 µm, with a few outliers observed up to approximately 80 µm.
- 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

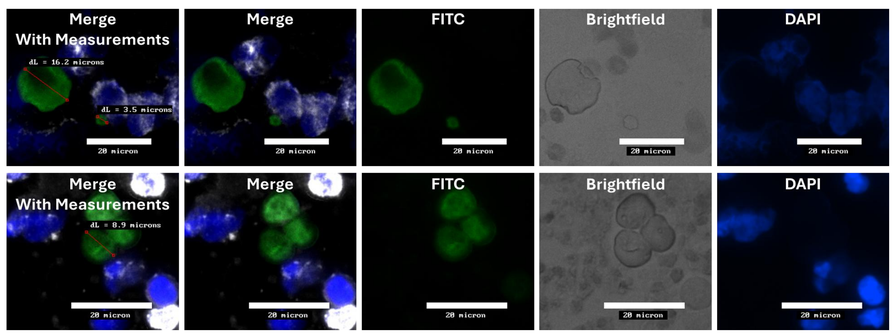

Figure 4. Assessment of EVs in GBM patients' blood samples. (A) *Table summarizing the enumeration of CTCs EVs in each patient. Representative images of **(B)** a larger EV (diameter of 16.2 µm) in proximity of a smaller EV (diameter of 3.5 µm) and **(C)** a cluster of three EVs of approximately the same size (diameter of 8.9 µm). Epithelial markers (FITC) in green, brightfield in grayscale, blood lineage markers (Cy5) in white, mesenchymal markers (Cy7) in magenta, and nuclear dye (DAPI) in blue.*

Results: CTC enumerations vs DNA mutations

- A strong correlation was observed between the number of oncogenic variants detected by NGS in CTC-derived DNA and the number of CTCs and CTC clusters identified in corresponding patients, whereas the counts of CTCs and CTC clusters showed no correlation with benign variants (**Figure 6A**).
- Patients with detectable variants in genes involved in the RAS, PI3K/AKT/mTOR, and other oncogenic signalling pathways exhibited a significantly higher number of CTCs and/or CTC clusters (**Figure 6B**). Moreover, the number of CTCs and CTC clusters showed a strong positive correlation with the number of variants detected in specific genes, including VHL, RET, ALK, PTEN, PIK3CA, FBXW7, HRAS, ERBB3, KIT, EGFR, and BRCA1 (**Figure 6C**).

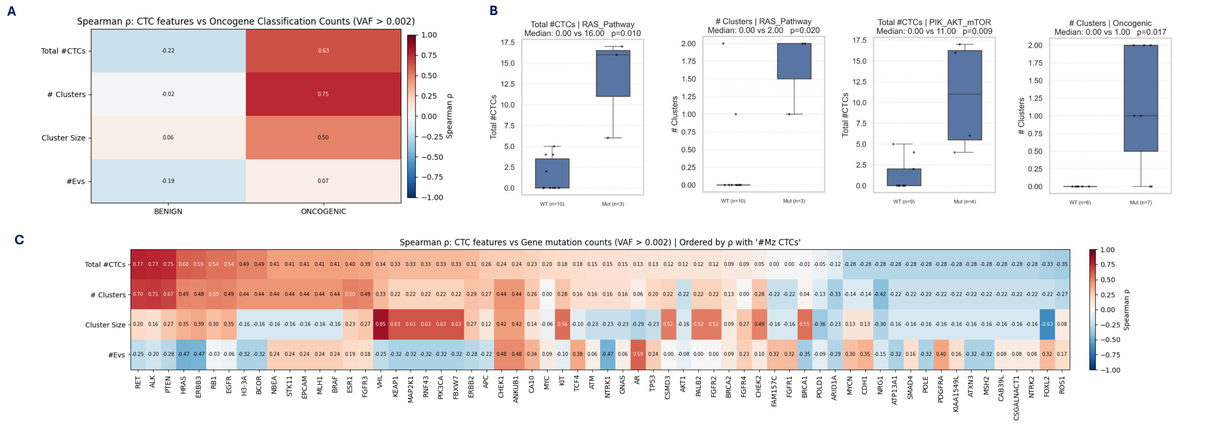

Figure 6. Correlation between CTC and CTC clusters presence and abundance with oncogenic variant profiles identified by NGS in CTC-derived DNA samples. (A) *Heatmap showing the correlation between the number of oncogenic and benign variants detected in CTC-derived DNA and the number of mesenchymal (Mz) CTCs, total CTCs, CTC clusters numbers and size and total CTCs events identified in the corresponding patients. **(B)** Box-and-whisker plots illustrating the distribution of CTC and/or CTC cluster counts in patients harbouring variants in genes involved in the RAS (NF1, KRAS, NRAS, HRAS, BRAF), PI3K/AKT/mTOR (PIK3CA, AKT1, PTEN, MTOR), and other oncogenic signalling pathways. Statistical significance was determined using the Mann–Whitney U test. **(C)** Heatmap depicting the correlation between the number of CTCs and CTC clusters and the number of variants detected in individual oncogenic genes, including VHL, RET, ALK, PTEN, FOXL2, PIK3CA, FBXW7, HRAS, ERBB3, KIT, AR, EGFR, and BRCA1.*

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 and the ability to detect and enumerate large EVs at high numbers in the same CTC sample using the Parsortix instrument. 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.

→CTC analysis by NGS demonstrated superior performance compared to cfDNA profiling. A higher proportion of CTC samples passed quality control (93% vs. 50%) and yielded detectable variants in relevant genes (86% vs. 50%), indicating that CTC-derived material provides more reliable and informative molecular data in this cohort. However, some unique variants were detected exclusively in cfDNA samples, suggesting that combining both CTC and cfDNA analyses offers complementary insights and maximizes variant detection.

→Presence of mesenchymal CTC and CTCs clusters positively correlated with the presence of oncogenic variants corroborating the malignant nature of the variants detected. The presence of oncogenic variants in the AKT and RAS pathways in CTCs of GBM could provide insights into tumor progression and therapeutic resistance, enabling more precise, targeted treatment strategies.