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Transcriptional profiling of extracellular vesicle subpopulations enables liquid biopsy-based brain tumor classification

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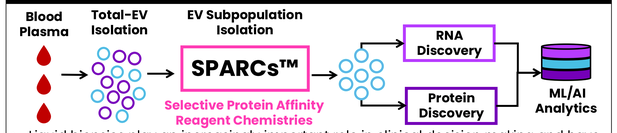
Introduction



- Liquid biopsies play an increasingly important role in clinical decision making and have the potential to facilitate cancer detection, diagnosis, patient stratification, and treatment monitoring.
- Liquid biopsy platforms based on circulating tumor cells or cell-free DNA suffer from suboptimal signal to noise ratios due to low target abundance.
- Extracellular vesicles (EVs) are cell-derived membrane encapsulated particles that carry diverse protein, nucleic acid and metabolite cargos. EVs provide a richer and more dynamic analyte base for liquid biopsy than cfDNA and can provide insight into mechanisms driving disease.
- Using FYR's novel SPARCs™ EV enrichment technology, we have developed a clinically applicable, multi-omic EV subpopulation interrogation pipeline that profiles tumor and brain derived EVs (TDEVs and BDEVs) from patient plasma.
- The EV-Omic (EVO) pipeline, in combination with a custom machine learning biomarker discover platform, can distinguish adult high-grade gliomas from healthy patients, using blood-based EV proteomic and transcriptomic signatures.

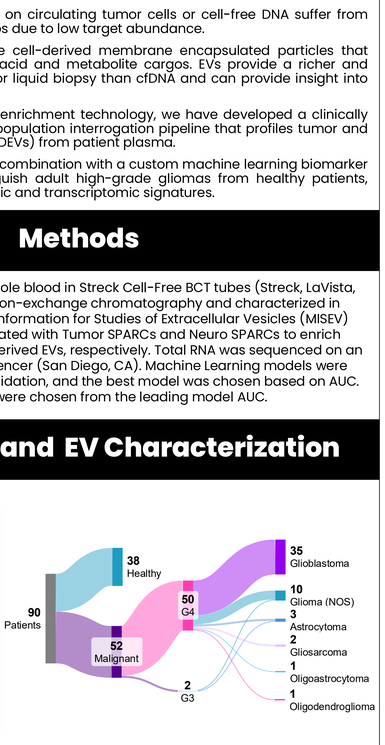
Methods

- Plasma was processed from whole blood in Streck Cell-Free BCT tubes (Streck, LaVista, NE). Total EVs were isolated via ion-exchange chromatography and characterized in concordance with the Minimal Information for Studies of Extracellular Vesicles (MISEV) 2023 guidelines. EVs were incubated with Tumor SPARCs and Neuro SPARCs to enrich tumor-derived EVs and brain-derived EVs, respectively. Total RNA was sequenced on an Element Biosciences AVITI sequencer (San Diego, CA). Machine Learning models were training utilized 10-fold cross validation, and the best model was chosen based on AUC. Candidate biomarker features were chosen from the leading model AUC.

Study Design and EV Characterization

Table 1. Study Cohort.

	Malignant	Healthy
N	52	38
Age		
<51	18	3
51-60	14	2
61-70	18	11
71-80	2	2
Sex		
Male	23	21
Female	29	17
Race/ethnicity		
Caucasian	51	34
Asian	1	0
Black	0	4



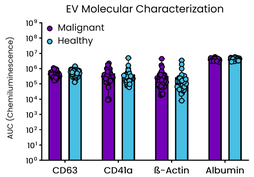


Figure 1. Jess Simple Western Analysis.

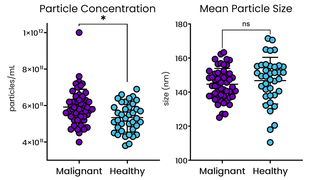
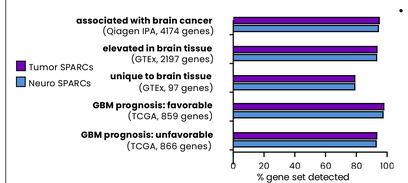


Figure 2. Zetaview Nanoparticle Tracking Analysis.

- EV marker protein composition was confirmed via Capillary Electrophoresis (CE) Western Blot.
- EV markers CD63 (MISEV Cat1a), β-Actin (MISEV Cat2b), CD41a (MISEV Cat1b), and Albumin (Cat3a), confirm the presence of EVs as well as the common plasma co-isolate Albumin.
- Nanoparticle Tracking Analysis (NTA) detected an average of $5.7x10^{11}$ particles/mL with a mean particle diameter of 145.7nm.
- There was a significant difference in particle concentration between Malignant and Healthy donors (Kruskal-Wallis test, p=0.036), consistent with literature citing elevated circulating EV content in brain cancer patient plasma.

Brain Tumor RNA Signatures from Plasma



- Transcriptome coverage of the Tumor SPARC and Neuro SPARC pipelines was between 79%-98% for brain-specific, brain cancer-specific and GBM prognostic genes from The Cancer Genome Atlas (TCGA), Qiagen IPA and The Genotype-Tissue Expression Portal (GTEx) (Fig. 3).

Figure 3. Tumor SPARCs and Neuro SPARCs coverage for gene sets associated with GBM prognosis, brain cancer and brain tissue

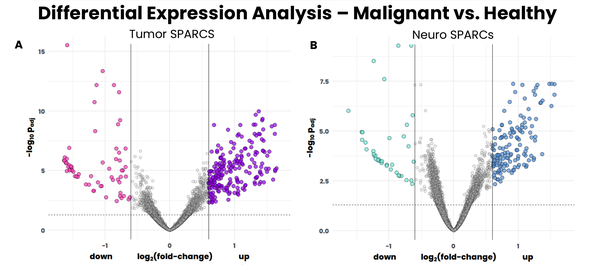


Figure 4. Volcano plots of differentially packaged (Malignant vs Healthy) transcripts. $p_{adj} < 0.05$ and $\log_2$ fold-change ≥ |0.6|.

- Differential expression analysis following EV subpopulation using Tumor SPARCs identified **212** enriched and **70** depleted transcripts in patients with malignant brain tumors relative to matched healthy controls (Fig. 4A), while Neuro SPARCs identified **138** enriched and **39** depleted transcripts (Fig.4B).

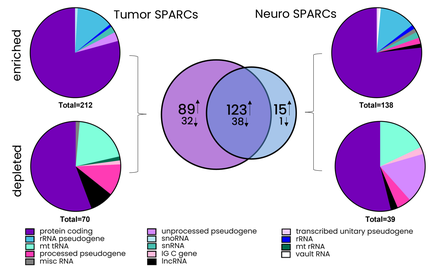


Figure 5. Distribution of differentially expressed genes (DEGs) across both panels for the Malignant Tumor vs Healthy comparison.

- Tumor SPARCs and Neuro SPARCs target different EVs pools that provide unique DEG information and contain distinct RNA biotypes (Fig. 5).
- Counter to the view that most EV disease markers are miRNAs, SPARC sub-populated EVs contain abundant protein-coding transcripts which can provide information about tumor physiology and/or be useful for monitoring targeted therapy.
- Both panels capture a variety of non-coding RNA classes (e.g. lncRNAs, pseudogenes and mtRNAs) increasingly recognized for their role in cancer-related changes in gene expression and disease progression.

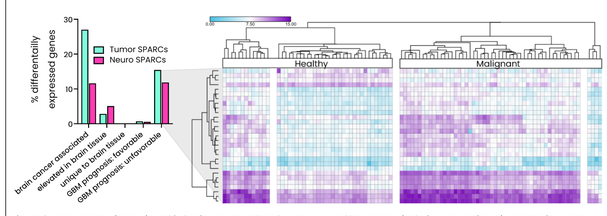


Figure 6. Percentage of DEGs found in brain-cancer related gene sets and heatmap of relative expression of prognostic genes across all samples.

- Many differentially expressed genes are brain-cancer associated, have enhanced expression in brain tissue or are GBM prognostic markers and have expression patterns that reflect disease status (Fig. 6).

EV Transcriptome Pathway Analysis

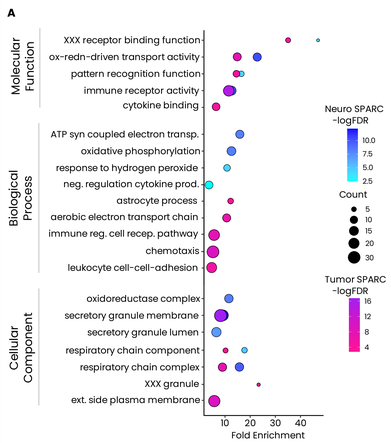


Figure 6. (A) GO term enrichment analysis for Tumor SPARCs and Neuro SPARCs of top 5-10 most significant driver terms for each GO category (molecular function, biological process, cellular component) (B) Reactome Gene Concept Network Plot showing nodes with the lowest FDR. Grey highlighted boxes denote categories that ate unique to Tumor SPARCs or NeuroSPARCs.

- Overrepresentation analysis of GO terms (Fig. 6A) and Reactomes (Fig. 6B) highlight the importance of changes in the inflammatory immune response, pattern recognition and respiration in distinguishing malignant brain cancer EV transcriptomes from healthy EV transcriptomes.
- Identification of biologically relevant terms and pathways supports the creation of targeted biomarker panels that can be used to identify malignant disease using EVs from blood plasma.

Tumor SPARCs Longitudinal Pilot

P= pre-treatment **S**= post-surgery **T**= post-therapy

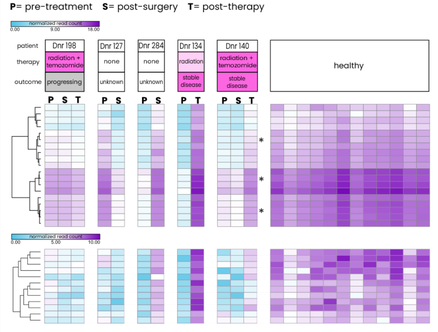


Figure 7. Heatmap of normalized read counts for differentially expressed genes that show longitudinal expression patterns in patients with malignant tumors.

- Expression profiles for a subset of patients for which longitudinal samples were available were examined for pre- and post-treatment changes in the EV transcriptome (Fig. 7).
- Several genes showed expression changes that suggest SPARCs sub-populated EVs may be useful for monitoring treatment response. Genes marked with an asterisk (*) are known therapeutic drug targets.

Biomarker Discovery

- Supervised machine-learning algorithms were used to detect differences between Malignant and Healthy samples.
- 10-fold cross-validation was used to calculate performance metrics (Table 2).
- The best model was selected based on AUC (Table 2).

Table 2. Performance metrics for leading models. Models optimized for maximum specificity.

	Tumor SPARCs	Neuro SPARCs
AUC	0.89	0.91
Accuracy	0.74	0.81
Sensitivity	0.61	0.73
Specificity	0.95	0.95

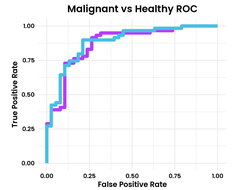


Figure 8. ROC curve comparison of leading models.

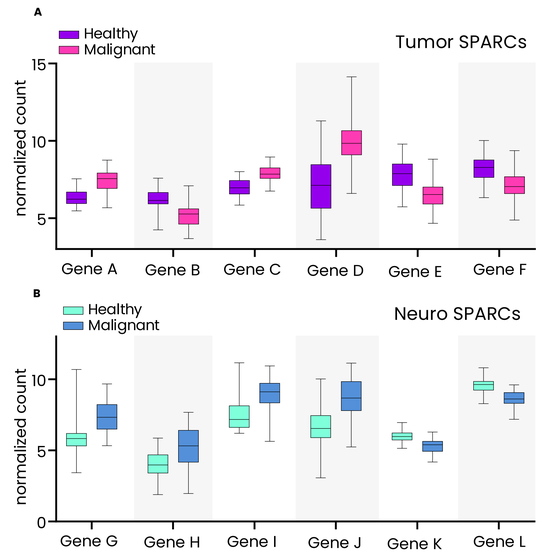


Figure 9. Box plots of candidate biomarker RNA features identified by the (A) the Tumor SPARCs model (B) the Neuro SPARCs model.

- Candidate biomarkers selected based on leading model AUC are cancer associated genes (Fig. 9).

Conclusions and Future Directions

- EV subpopulations from brain tumor patient plasma provide unique opportunities to identify disease via minimally invasive methods and provide a better understanding of tumor biology.
- Tumor SPARCs and Neuro SPARCs capture EV subpopulations with unique characteristics. Combining markers from both panels is under investigation for ability to improve patient stratification and biomarker discovery.
- Preliminary data indicate that SPARCs will prove useful in blood-based longitudinal monitoring of patients.
- Future work will include development of targeted panels for rapid detection of malignant brain cancers.