Utilization of Tumor-derived Extracellular Vesicles for Patient Stratification and Biomarker Discovery

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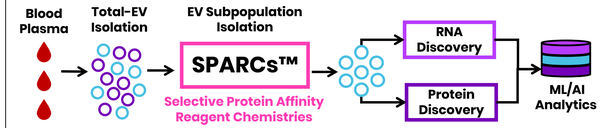
Introduction



- Liquid biopsy is poised to play an increasingly important role in clinical decision making by enabling earlier cancer detection, diagnosis, patient stratification, and treatment monitoring.
- Most approaches rely on detection of cell-free circulating tumor DNA or circulating tumor cells, but limitations exist, including miniscule abundance, unfavorable signal to noise ratios, and limited insight into mechanisms driving disease.
- Extracellular vesicles (EVs) are an alternative liquid biopsy analyte comprising lipid membrane encapsulated particles carrying diverse protein, nucleic acid, and metabolite cargos.
- We have developed a clinically applicable, multi-omic EV subpopulation interrogation pipeline that robustly profiles tumor and brain derived EVs (TDEVs and BDEVs) in biofluids utilizing FYR's novel EV enrichment technology called SPARCs.
- We have applied our EV-Omic (EVO) pipeline in combination with machine learning to distinguish adult high-grade gliomas and benign brain tumors from healthy patients, using blood-based EV proteomic and transcriptomic signatures.

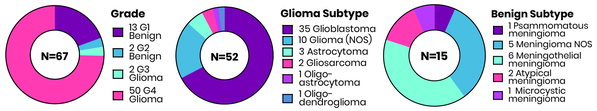
Methods

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. EV subpopulations were split for RNA and protein downstream processing. Small RNA was sequenced on an Element Biosciences AVITI (San Diego, CA). Protein was subjected to LC-MS/MS on an Orbitrap Astral (Thermo Scientific Fisher, Waltham, MA) at Cedars Sinai Precision Biomarker Laboratories (Beverly Hills, CA). Machine Learning model 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

	N	Specimen Providers	Age				Sex		Race/Ethnicity		
			<51	51-60	61-70	71-80	Male	Female	White	Asian	Black
Glioma	**52**	2	18	14	18	2	23	29	51	1	0
Benign	**15**	1	6	3	5	1	2	13	15	0	0
Healthy	**38**	2	13	12	11	2	21	17	34	0	4

Table 1. Patients included in the study.



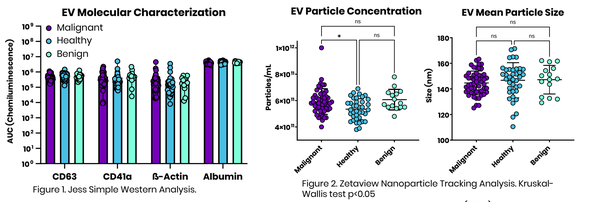


Figure 1. Jess Simple Western Analysis.

Figure 2. Zetaview Nanoparticle Tracking Analysis. Kruskal-Wallis test p<0.05

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) yielded an average of 5.7x10^{11} particles/mL plasma with a mean diameter of 145.7nm. There was a significant difference in concentration between Brain Cancer and Healthy donors where p=0.036 via Kruskal-Wallis test. This is consistent with literature citing elevated circulating EV content in brain cancer patient plasma.

Brain Tumor Plasma Proteomic Signatures

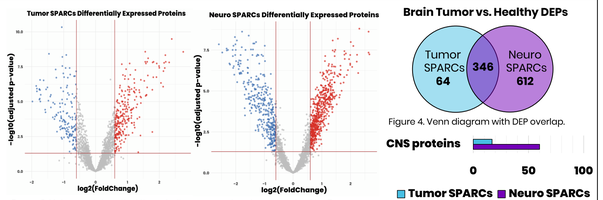


Figure 3. Volcano plots with differentially expressed proteins enriched by Tumor or Neuro SPARCs. padj<0.05 and $\log_2$ fold-change ±0.6.

Figure 4. Venn diagram with DEP overlap.

Figure 5. Count of CNS derived proteins identified by method.

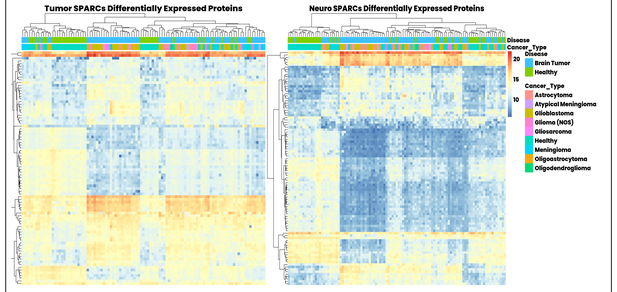


Figure 6. Heatmaps showing top DEPs from Tumor and Neuro SPARCs methods comparing brain tumor vs. healthy patients.

Differential expression analysis of brain tumor (malignant and benign) patients relative to healthy controls identified 256 significantly enriched proteins and 154 significantly depleted proteins using Tumor SPARCs and 598 significantly enriched proteins and 360 significantly depleted proteins using Neuro SPARCs (Fig.3). Comparison of identified DEPs from Tumor SPARCs vs Neuro SPARCs demonstrates that both panels provide unique DEP information (Fig.4). Neuro SPARCs enrichment demonstrated increased identification of CNS proteins (classified as brain elevated according to the Human Protein Atlas) (Fig.5). Heatmap hierarchical clustering by disease status of Tumor SPARCs DEPs and Neuro SPARCs DEPs (Fig.6).

Benign Tumor Discrimination

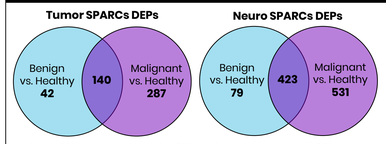


Figure 7. Venn diagrams showing DEP count comparisons at padj<0.05 and $\log_2$ fold-change ±0.6 of benign vs. healthy and malignant vs. healthy patient populations in both tumor and neuro SPARCs.

Differential expression analysis comparing benign vs. healthy and malignant vs. healthy shows that while malignant disease displayed more significant DEPS, benign disease also demonstrated a unique DEP signature in TDEVs and BDEVs (Fig.7). Identification of unique and biologically relevant GO terms between malignant and benign DEPs (Fig.8) supports creation of unique biomarker panels that can distinguish benign from malignant disease.

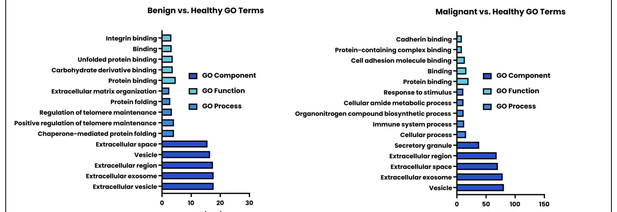


Figure 8. Gene ontology terms listing the top 5 most significant terms for each GO category (cellular component, molecular function, biological process) for DEPs identified in differential expression analysis.

Biomarker Discovery

Objective: Distinction of brain tumors (malignant and benign) from healthy patients.

	Protein Tumor SPARCs	Protein Neuro SPARCs	miRNA Tumor SPARCs	miRNA Neuro SPARCs
AUC	0.98	0.96	0.80	0.83
Accuracy	0.87	0.88	0.77	0.74
Sensitivity	0.82	0.82	0.47	0.56
Specificity	0.95	0.97	0.89	0.89
Features	16	17	13	17

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

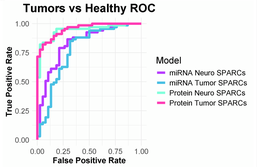


Figure 9. ROC curve comparison of leading models by method and analyte.

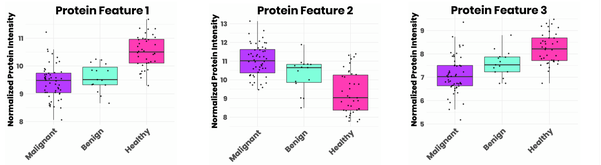


Figure 10. Box plots of candidate biomarker protein features identified by the Protein Tumor SPARCs model.

Objective: Distinction of glioblastomas from healthy patients.

	Protein Tumor SPARCs	Protein Neuro SPARCs	miRNA Tumor SPARCs	miRNA Neuro SPARCs
AUC	0.93	0.94	0.86	0.83
Accuracy	0.89	0.84	0.79	0.74
Sensitivity	0.75	0.72	0.78	0.56
Specificity	1	0.95	0.79	0.89
Features	11	16	17	18

Table 3. Performance metrics for leading models. Models optimized for maximum specificity.

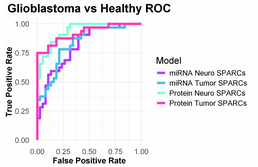


Figure 11. ROC curve comparison of leading models by method and analyte.

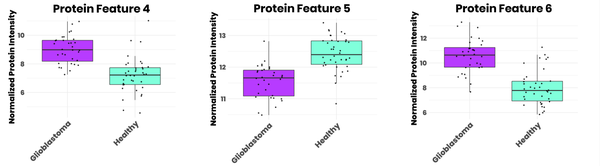


Figure 12. Box plots of candidate biomarker protein features identified by the Protein Tumor SPARCs model.

Subtyping Pilot: Tissue MGMT Status

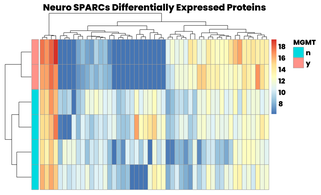


Figure 13. Hierarchical clustering heatmap comparing n=4 non-MGMT methylated GBMs, with n=2 MGMT methylated GBMs.

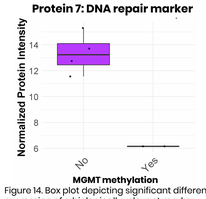
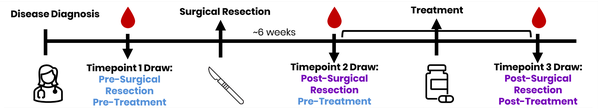


Figure 14. Box plot depicting significant differential expression of a biologically relevant marker.

For n=6 glioblastoma patient plasma specimens, matched tissue was assayed for MGMT promoter methylation status using PCR. Neuro SPARCs proteomic profiles provide preliminary evidence supporting the ability of BDEV subpopulations to identify novel liquid biomarkers for MGMT methylation status.

Longitudinal Pilot: Glioblastoma



	Age	Timepoints 1	2	3	Treatment	Response
Donor 127	46	✓	✓	-	NA	NA
Donor 134	58	✓	-	✓	Radiation	Stable
Donor 140	66	✓	✓	✓	Radiation + Temozolomide	Stable
Donor 198	46	✓	✓	✓	Radiation + Temozolomide	Progressing (leukopenia, anemia)
Donor 284	69	✓	✓	-	NA	NA

Table 4. Patients included in the longitudinal pilot study.

Longitudinal profiling of TDEV DEPs yields unique DEPs at each timepoint (Fig.15). Gene ontology of DEPs at each timepoint highlights changes in GO categories that reflect known tumor biology. Pre-surgical resection, EV and synaptic markers are enriched. Post-surgical resection GO terms highlight immune system activation. Post-treatment GO terms highlight changing lysosome dynamics, which have been associated with radioresistance and chemoresistance (Fig.16). Markers relevant in the GBM patient stratification model change post-treatment, as well as known glioma stem cell markers (Fig.17).

Timepoint 1: Gene Ontology EV and Synaptic Markers

Timepoint 2: Gene Ontology Immune Activation

Timepoint 3: Gene Ontology Lysosome Dynamics

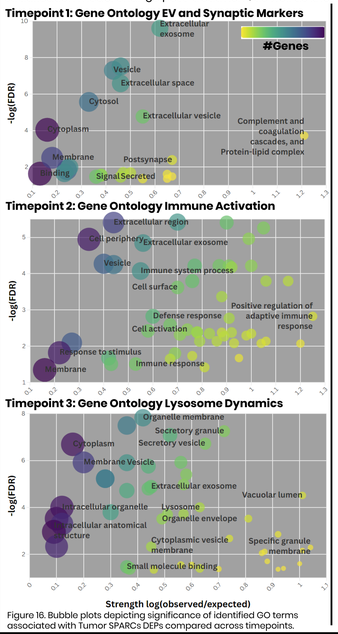


Figure 16. Bubble plots depicting significance of identified GO terms associated with Tumor SPARCs DEPs compared across timepoints.

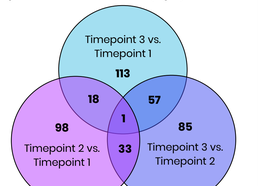


Figure 15. Venn diagram depicting Tumor SPARCs DEP overlaps across timepoint comparisons.

Example feature from Glioblastoma ML Model

Example Glioma Stem Cell Protein Marker

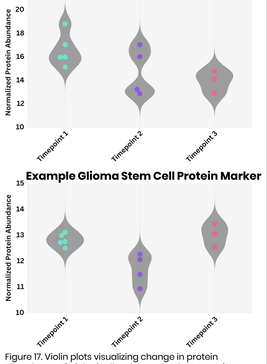


Figure 17. Violin plots visualizing change in protein abundance of biologically relevant markers over time.

Conclusions and Future Directions

- EV subpopulations in brain tumor patient plasma provide 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, but Tumor SPARCs information alone provided slightly improved performance scores. Combining scores and markers from both panel models is under investigation for ability to improve patient stratification and biomarker discovery.
- Preliminary data indicate that SPARCs will prove useful in blood-based tissue subtyping, as well as longitudinal monitoring of patients.