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Supporting Information

Phospho-proteome Analysis of Cerebrospinal Fluid Extracellular Vesicles in Primary Central Nervous System Lymphoma

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Table of Contents

Supporting Experimental Section

Ultracentrifugation for EV Isolation

The frozen CSF were thawed in 37℃ water bath and centrifuged for 10 min at 2500 g firstly at room temperature. The collected supernatant was then diluted two-fold with pre-cooled PBS (4℃) and centrifuged at 20,000 g for 1 hr. Afterward, the supernatant was centrifuged at 100,000 g for 70 min at 4℃. After removal of supernatant, the pre-cooled PBS was added to resuspend the pellet and samples were centrifuged again for 70 min at 100,000 g. Finally, the washed EV pellet was collected for further experiment.

TEM Imaging and NTA

EV solution of 10 μL was dropped onto 200-mesh formvar carbon-coated copper grids and incubated with the negatively stained solution (2% phosphotungstic acid solution, pH = 7.0) at room temperature for 2−3 min. Finally, transmission electron microscopy (TEM) imaging was carried out on a JEOL JEM-2100 electron microscope (JEOL, Tokyo, Japan) with an accelerating applied potential of 200kV.

EVs isolated from 0.5 mL CSF sample were eluted twice with 100 μL of 100 mM TEA and diluted five-fold with PBS. Then, the EV solution was used for nanoparticle tracking analysis (NTA) on a ZetaView (Particle Metrix, Meerbusch, Germany) after calibration with 100 nm polystyrene particles.

Western Blotting Analysis

Purified EV samples were first boiled in LDS loading buffer at 95°C for 5 min. The equivalent volumes of samples were then loaded onto a 15% SDS-PAGE gel for separation and transferred onto PVDF membranes (Millipore, Sigma). Subsequently, the PVDF membranes were blocked with 1% BSA in TBST for 1hr, followed by overnight incubation with primary antibody, rabbit anti-CD9 (D3H4P; Cell Signaling Technology), mouse anti-CD81 (ab79559; Abcam) and rabbit anti-TSG101 (ab125011; Abcam), at 1:2000 ratio in 1% BSA in TBST. After washing, the PVDF membranes were incubated with HRP-conjugated secondary antibody at 1:5000 ratio in 1% BSA in TBST and then scanned by an enhanced chemiluminescence imager (ImageQuant LAS500) for imaging and quantitation.

LC-MS Sample Preparation

EVs isolated from CSF were first lysed in the buffer with 12 mM sodium lauroyl sarcosinate, 12mM sodium deoxycholate, 40 mM CAA, 10 mM TCEP, and phosphatase inhibitor cocktail (Millipore-Sigma) and 50 mM Tris·HCl (pH 8.5) through incubation at 95 °C for 10 min. After five-fold dilution with 50mM triethylammonium bicarbonate (TEAB), the proteins were digested with Lys-C (Wako) with 1:100 w/w ratio for 3 hr at 37°C and subsequently incubated overnight with Trypsin with 1:50 w/w ratio at 37°C. After acidifying the samples by trifluoroacetic acid (TFA) with a final TFA concentration of 1%, ethyl acetate was then added to the samples at 1:1 v/v ratio and the mixed solution was vortexed for 2 min then centrifuged at 15,000 g for 3 min, afterward, the organic phase on the top was removed. Ethyl

acetate was added again and above steps were repeated once more. The aqueous phase on the bottom was further dried down in a vacuum freeze centrifuge (Laconco CentriVap) and desalted using an in-house-made C18 Stage-Tip (3M Empore 2240-SDB-XC).

For proteome analysis experiment of EVTRAP and UC comparison, whole of each sample was freeze-dried for further LC-MS/MS analysis. While for phosphoproteome analysis of clinical samples, each sample was split into 99 and 1% aliquots for phosphoproteomic and proteomic analysis, respectively, and then dried down and stored at -80℃. The 99% proportion of each sample was used to conduct phosphopeptides enrichment through PolyMAC Phosphopeptides Enrichment Kit (Tymora Analytical) following the manufacturer's instructions and were dried completely after elution.

LC−MS/MS Analysis

Dried peptides and phosphopeptides were dissolved in 10 μL 0.1% formic acid (FA). For proteome analysis of EVTRAP and UC processed samples, 3μL of each sample was injected into a nanoElute for proteomic analysis. For proteome and phosphoproteome analysis of clinical samples, 2 μL and 7 μL of each was injected, respectively.

The mobile phase buffer consisted of 0.1% FA in ultrapure water (buffer A) and in acetonitrile (buffer B). Peptides were separated on a 25cm in-house packed column (360μm OD × 75μm inner diameter (ID)) containing C18 resin (1.9μm, 100 Å; Michrom Bioresources) at a flow rate of 300 nL/min with a linear 120 min gradient

(2−22% solvent B for 90 min, 22−37% B for 10 min, 37−80% B for 10 min, and 80% B for 10 min) for proteome analysis and a linear 90 min gradient (2−22% solvent B for 67.5 min, 22−37% B for 7.5 min, 37−80% B for 7.5 min, and 80% B for 7.5 min) for phosphoproteome analysis.

The nanoElute was coupled online with a timsTOF Pro mass spectrometer (Bruker). The mass spectrometer was operated in the data-dependent mode for all the experiments described here, which consists of a full scan MS (from m/z 100 to 1700 with the intensity of 1.5 X 10^6 at the 'target value' resolution of 40,000) along with 10 PASEF MS/MS frames. The quadrupole isolation width was set to 2 Th for $m/z < 700$ and 3 Th for $m/z > 700$. Collision energy was set stepwise changing within a TIMS elution ramp: 52 eV for 0−19%, 47 eV for 19−38%, 42 eV for 38−57%, 37 eV for 57−76%, and 32 eV for the remainder.

Database Search and Label-free Quantitation Analysis

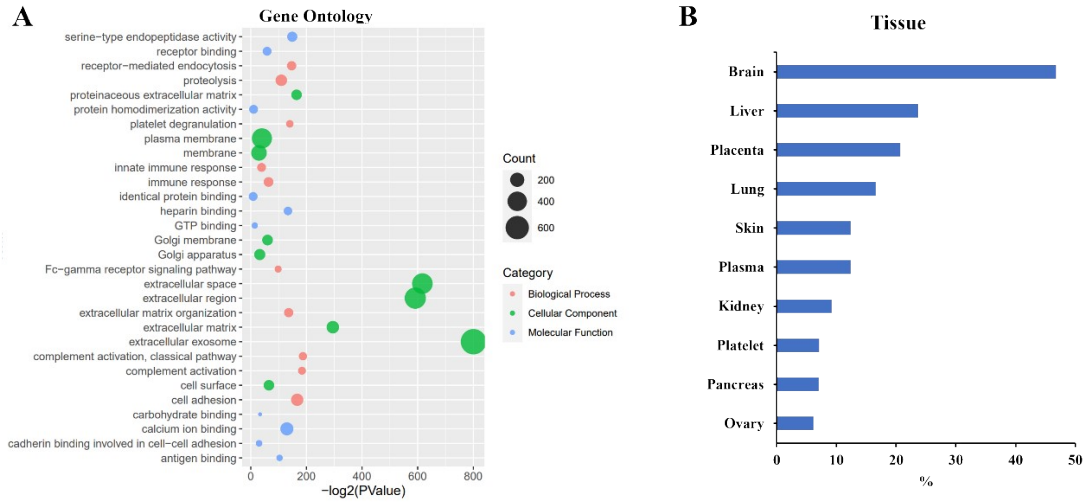
The raw files were searched directly against the Uniprot database downloaded in November, 2019 with no redundant entries, by PEAKS Studio X+ software (Bioinformatics Solutions Inc.). MS1 precursor mass tolerance was set at 15 ppm initially and the final was set at 6 ppm. MS2 tolerance was set at 0.6 Da. Modification criteria included a static modification of carbamidomethylation on cysteines (+57.0214 Da) and variable modifications of acetylation (+42.011 Da) at N terminus of proteins, oxidation (+15.9949 Da) on methionine residues and an extra modification of phosphorylation (+79.996 Da) on serine, threonine, or tyrosine residues for

phosphoproteome analysis. Search was performed with semi-specific trypsin/P digestion, allowing a maximum of three missed cleavages on the peptides. The false discovery rates (FDRs) for peptides, proteins, and phosphosites were all set at 1%. All protein and peptide identifications were grouped with no redundant entries. Only unique peptides/phosphopeptides/master proteins were reported.

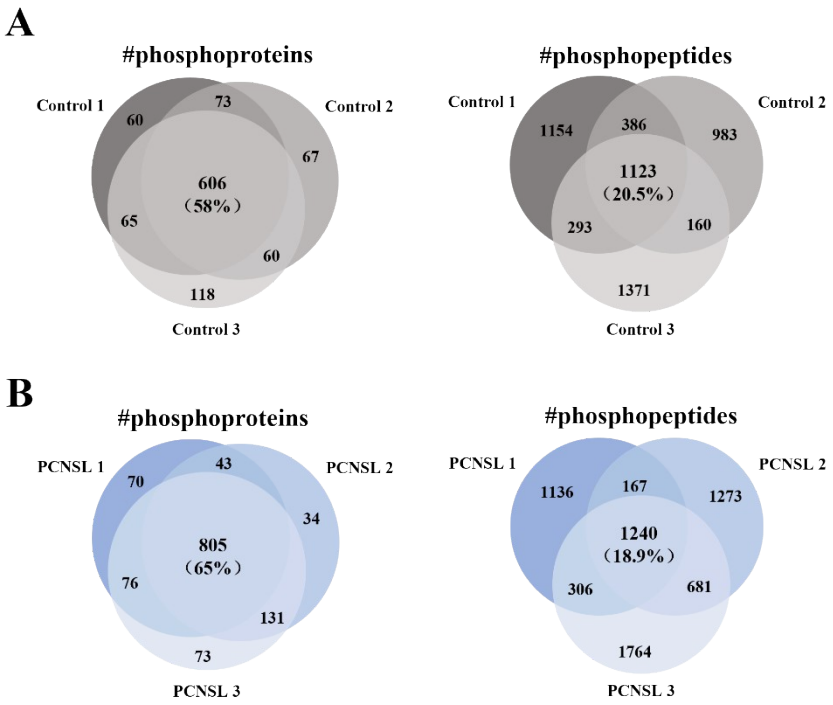
For the quantification of both proteome and phosphoproteome data, the intensities of peptides were extracted with initial precursor mass tolerance of 15 ppm, minimum number of isotope peaks of 2, maximum ΔRT for isotope pattern multiplets −0.2 min, and PSM confidence FDR at 1%. The abundance level of all peptides and proteins was normalized by Total Ion Current (TIC) signals.

For differential expression analysis of clinical samples, missing value replacement, normalization, z-score normalization, KW test and heatmap drawing were all carried out using R studio. The significantly changed proteins or phosphopeptides in any sample were identified by the P-value and change fold: P-value ≤ 0.05, $|log_2(\text{ratio})| \geq 2$.

Supplementary Figures



Supplementary Figure S1. Gene Ontology (GO) analysis of LC-MS proteome data from EV samples captured by EVtrap, using DAVID Bioinformatics Resources (2021 Update). (A) Bubble chart of Top10 results for 'Molecular function', 'Biological process', 'Cellular Component' with −log2 (p-value) and (B) Bar graph of Top10 results for 'Tissue' with %.



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136 **Supplementary Figure S2.** Repeatability among biological replicates of
137 phosphoproteome analysis. (A) Venn diagram of identified phosphoproteins (left) and
138 phosphopeptides (right) in three biological replicates of Control group. (B) Venn
139 diagram of identified phosphoproteins (left) and phosphopeptides (right) in three
140 biological replicates of PCNSL group.

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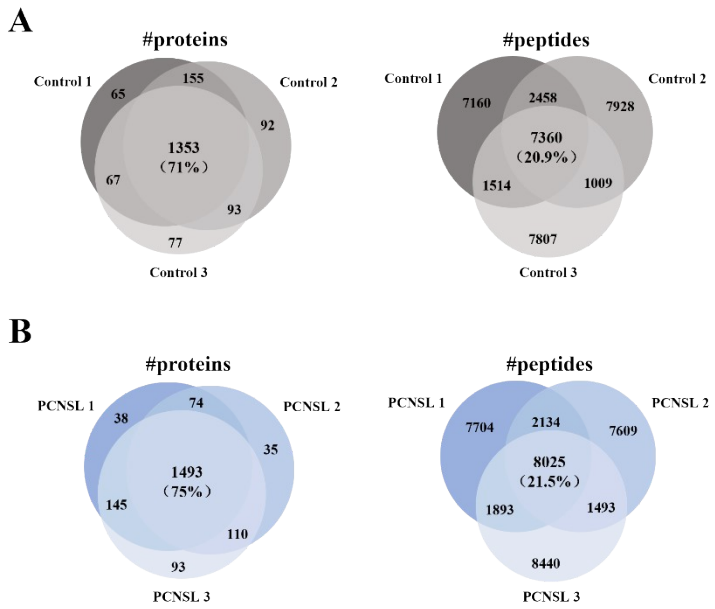

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143 **Supplementary Figure S3.** Number of identified peptides and proteins in CSF EV
144 from PCNSL group and non-PCNSL control, and repeatability among biological
145 replicates of proteome analysis. (A) Venn diagram of identified proteins (left) and
146 peptides (right) in three biological replicates of Control group. (B) Venn diagram of
147 identified proteins (left) and peptides (right) in three biological replicates of PCNSL
148 group.

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Supplementary Table S2. Sample Information.

	PCNSL 1	PCNSL 2	PCNSL 3	Control 1	Control 2	Control 3
Age, mean	63.7	64.3	63.7	50.7	48.7	43
Gender	2F, 1M	2F, 1M	3F	1F, 2M	1F, 2M	1F, 2M

M: Male, F: Female.