Short Communication

Recovery of extracellular vesicles from liquid samples using polyamine solution

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Abstract Liquid biopsies mainly analyze nucleic acids and proteins in the free-state or extracellular vesicles (EVs) in non-solid biological samples, primarily blood. Collecting and processing liquid biopsy samples is challenging due to the large volume of samples and reagents and the need for special equipment. In a previous study, we reported a method for enriching free nucleic acids using a polyamine solution that is effective for liquid biopsy. We also investigated the reactivity of this method for EV recovery in cell culture supernatants using mass spectrometry. Samples were prepared from the cell line NCI-N87 supernatants after 48 h of culture in a serum-free medium. For comparison, samples were treated using a solution containing polyamines (PA method) or ultracentrifugation (UC method). The liquid chromatography-tandem mass spectrometry (LC-MS/MS) using the single-pot solid-phase-enhanced sample-preparation (SP3) method revealed differences between the two methods in the total ion chromatogram of the sample. However, the results of the Gene Ontology (GO) analysis showed that both methods achieved the best enrichment in GO terms related to EV. In addition, the volcano plot analysis revealed that proteins suggested to exist in EVs were distributed in areas consistent with both methods. These results indicated that the PA method can recover EV proteins in liquid samples, and their comprehensive analysis is possible using the SP3 method.

Key words: extracellular vesicles, liquid biopsy, LC-MS/MS, polyamine method, single-pot solid-phase-enhanced sample-preparation (SP3) method

Introduction

Blood samples from patients with cancer contain circulating tumor DNA (ctDNA), cell-free DNA (cfDNA), circulating tumor cells, and microRNAs (miRNAs) derived from cancer cells[1)]. Liquid biopsy is a method for the genetic and cytological analysis of these samples[1)]. It analyzed free-state nucleic acids and proteins in extracellular vesicles (EVs), including exosomes[2)]. Liquid biopsy can provide information on the nature of cancer without requiring invasive procedures associated with conventional tissue collection[3)].

EVs, including exosomes, contain proteins, mRNA, miRNA, and DNA on their surface or inside; they are stable in body fluids such as blood, urine, saliva, spinal fluid, and breast milk after being secreted from cells[4)]. EVs have attracted attention as critical biological factors that convey messages locally and remotely and are deeply involved in various diseases and biological phenomena, including cancer and immune and neurological diseases[5)]. They can be recovered using centrifugation, immunoprecipitation, particle size classification, column adsorption, or ultracentrifu-

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Received: February 13, 2024. Accepted: May 9, 2024.
Epub June 5, 2024.
DOI: 10.24508/mms.2024.06.006

gation[6]. Each method has different recovery rates, specificities, and the time and cost required for operation. Therefore, the method selected depends on its application. Although ultracentrifugation is considered the gold standard[7], it has limitations, such as poor recovery rates, and requires time and specialized equipment[8]. Moreover, we considered the possibility that the PA method coprecipitates EVs along with nucleic acids. In a previous study, we have devised a method for enriching free nucleic acids using polyamine-containing solutions[9].

Here, we investigated, using liquid chromatography-mass spectrometry (LC-MS/MS), whether this method can be used to recover EV proteins in cell culture supernatant.

Materials and Methods

Cell culture

For our LC-MS/MS analysis, we used a cell line, that is NCI-N87 (ATCC, Manassas, VA, USA).

The cells were cultured in RPMI-1640 with L-Glutamine and Phenol Red (Fuji film Wako, Tokyo, Japan) with 10% fetal bovine serum at 37˚C in a 5% CO^2 incubator. When the cells reached confluency, they were treated with Advanced RPMI 1640 Medium (Thermo Fisher Scientific, Waltham, MA, USA).

After 48 h of culture, the collected media were first centrifuged at 1,000×g for 10 min at room temperature (20−25˚C) to pellet and remove cells. All the centrifugation steps were performed at 4˚C. Next, the supernatant was centrifuged at 2,000×g for 20 min. The media supernatant was filtered through a 0.22-μm pore filter (HAWACH Scientific Co. LTD, Xian City, China). Each method was performed using a filtered culture medium.

Ultracentrifugation

Treated cell culture supernatant (50 mL) was used for ultracentrifugation to collect EVs. The ultracentrifugation was performed using an Optima L-90K ultracentrifuge (Beckman Colter, Fullerton, CA, USA) at 150,000×g for 100 min in a SW28.1 Roter Swinging Bucket rotor (Beckman Colter). After centrifugation, phosphate-buffered saline (PBS) was added to the top of the tube, and ultracentrifugation was performed again under the same conditions. Finally, the supernatant was removed, and the collected EVs were suspended in PBS.

Polyamine method

To begin with, 9.0 mL of the treated cell culture supernatant was transferred into a 15 mL conical centrifuge tube (Nunc, ThermoFischer Scientific, USA). Then, 1/10 volume of 5 M NaCl solution and 1/10 volume containing 0.01% Spermidine (191-13831, FUJIFILM Wako Chemicals, Osaka, Japan) were added to the tube. After the mixture was allowed to stand for 10 min at room temperature, it was centrifuged at 27,210×g (13,000 rpm) for 60 min using a micro-cooled centrifuge (Model 3700, KUBOTA CORPORATION, Tokyo, Japan). Then, the cell culture supernatant was removed and dissolved in PBS before analysis.

Mass spectrometry

Sample preparation was performed using the single-pot solid-phase-enhanced sample-preparation (SP3) technology. The protein in the sample was adjusted to 1−200 μg/mL, and dithiothreitol was added to a final concentration of 25 mM. After 30 min at room temperature, 1/10 volume of 500 mM 3-methyl-1-butanol was added and allowed to stand for 30 min at room temperature. Particles and SeraMag™ SpeedBead Carboxylate-Modified [E3] Magnetic Particles (Global Life Sciences Technologies Japan K.K., Tokyo, Japan). The bead mixture (10 μL) was added to the sample for 10 μg of protein. Then, three times the volume of ethanol was added, the sample was mixed at 1,000 rpm for 10 min at RT, and 500 μL of 80% ethanol was added, followed by washing three times using a magnetic stand. Then, 100 μL of lysyl endopeptidase plus 50 mM Tris buffer was added. After sonication, the mixture was incubated at 37˚C for 3 h. Trypsin solution was added, and the sample was mixed at 1,500 rpm at 37˚C. for 20 h. After mixing, 20% trifluoroacetic acid was added to the volume 1/20, the beads were attached to a magnetic stand, and the supernatant was collected. Then, the supernatant was desalted using GL-Tip SDB (GL Sciences Inc., Tokyo, Japan). The resulting solution was vacuum dried, and resuspended in 0.1% TFA in 2% acetonitrile to extract the peptides.

The LC-MS/MS analysis was performed using a PAL System autosampler (CTC Analytics AG, Zwingen, Switzerland) and Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific). The samples were concentrated on a C18 trap column (5-μm particle size, 300 μm inner diameter, 5 mm length; Chemical Evaluation and Research Institute, Tokyo, Japan) and separated on a

C18 column (3-μm particle size, 100-μm inner diameter, 125 mm length; Nikkyo Technos, Tokyo, Japan) at a flow rate of 0.5 μL/min with an injection volume of 7.5 μL. The mobile phases were solvent A (0.5% acetic acid) and solvent B (0.5% acetic acid in 80% acetonitrile). The elution gradient for solvent B was as follows: 5% to 40% B over 100 min, then 40% to 95% B for 1 min, holding at 95% B for 3 min, then back to 5% B over 1 min, and finally re-equilibrating at 5% B for 10 min. Electrospray ionization was performed in positive-ion mode.

The instrument was operated in the DDA mode. Xcalibur 4.1.50 (ThermoFisher Scientific) was used to record peptide spectra. The full scan was acquired from 350 to 1800 *m/z* with a resolution of 17,500, automatic gain control (AGC) as 3×10^6, and maximum injection time as 60 ms. MS/MS scans were performed with a resolution of 35,000, AGC target as 1×10^5, and maximum injection time as 60 ms. The 10 highest intensity precursor ions were isolated using the quadrupole analyzer in a window of 2.0 *m/z* and fragmented by higher energy collisional dissociation (HCD) fragmentation with normalized collision energy (NCE) of 27%. Multiply-charged peptides were chosen for MS/MS experiments. Dynamic exclusion time was set to 20 s.

Data analysis

UniProt Homo Sapiens (TaxID=9606) protein sequence database downloaded on 09/24/2022 and cRAP for contaminants (http://www.thegpm.org/crap/) were used. MS/MS spectra were interpreted, and peak lists were generated using Proteome Discoverer 2.5.0.400 (ThermoFisher Scientific). Searches were performed using SEQUEST (ThermoFisher Scientific). Search parameters were set as follows: enzyme selected with two maximum missing cleavage sites, a mass tolerance of 10 ppm for peptide tolerance, 0.02 Da for MS/MS tolerance, fixed modification of carbamidomethyl (C), and variable modification of oxidation (M) and *N*-terminal acetylation. Peptide identifications were based on significant Xcorr values (high confidence filter). Peptide identification and modification information returned from SEQUEST were filtered at a false discovery rate (FDR) of 1% using the Percolator node of Proteome Discoverer to obtain confirmed peptide identification and modification lists of HCD MS/MS. The LC-MS/MS data generated in this study have been deposited in jPOST repository database (project ID, JPST001807, accession ID, PXD035832)[10].

Furthermore, we used the proteins described in MISEV2018[6] and examined the validity of the proteins detected. Among the proteins listed, we identified peptide spectrum matches (PSM) for significant proteins. We also examined the correlation between the two methods using this data.

We also examined the properties of the PA and UC methods using total ion chromatogram, diagrams, and volcano plot, mainly to characterize the recovered peptides. The volcano plot was also used to examine both methods' agreement rate and characteristics of the proteins detected. Volcano plots were created with a fold change value (log2) greater than or equal to 1.5 and a p-value of 0.05>. The method's relative standard deviation (RSD) was determined and reproducibility was discussed. RSD was calculated by finding the mean and SD of the PSMs listed in Table 1.

We performed Go Ontology (GO) analysis on cellular component categories using proteins detected by both methods for PSMs with a mean of 4 or higher. The analysis used the DAVID open-source program, v2024q1[11,12].

Results

Table 1 shows the protein data obtained by both methods, using the detection data for the content described in MISEV2018 as protein content-EV characterization, and summarized for those with PSMs of 4 or higher. Using PSMs, a correlation coefficient of R=0.765 was obtained for both methods, resulting in an overall positive correlation in Fig. S1. In addition, results for all proteins listed in MISEV2018 are represented in Table S1.

Fig. 1A shows the total ion chromatograms. Although the same samples were treated using PA and UC methods, each method showed a different profile.

Fig. 1B show the results of the volcano plot for each method. Fig. 1B shows the markers CD9, CD63, CD81, and Flotillin-1, which were considered particularly important in EVs. The PA method identified common proteins believed to be contained in EVs near the origin. Table S2 presents the list of proteins with quantitative information used in the analysis.

Table S3 summarizes the number and percentage of proteins with different reproducibility based on the RSD values of PSMs in proteomics using PA and UC methods. About 80% of the proteins detected with the PA method have a smaller RSD value than 30%, whereas about 20% of the

Table 1. Protein content by LC-MS/MS

Description	Gene Symbol	PSMs			
		(a)		(b)	
		Mean	S.D.	Mean	S.D.
Integrin alpha-2	ITGA2	4.0	2.0	3.7	2.3
Integrin beta-3	ITGB3	6.7	1.2	4.5	0.7
HLA class I histocompatibility antigen, C alpha chain	HLA-C	9.0	1.7	8.7	6.1
Integrin alpha-6	ITGA6	10.3	0.6	9.0	8.0
Integrin beta-1	ITGB1	13.7	1.5	12.0	5.3
HLA class I histocompatibility antigen, A alpha chain	HLA-A	16.3	1.2	15.3	11.9
HLA class I histocompatibility antigen, B alpha chain	HLA-B	16.3	1.5	18.7	11.0
Integrin beta-4 OS=Homo sapiens	ITGB4	18.7	4.9	15.0	12.1
Syndecan-4 OS=Homo sapiens	SDC4	45.3	5.7	5.5	2.1
CD9 antigen OS=Homo sapiens	CD9	6.7	1.2	5.0	4.0
Receptor tyrosine-protein kinase erbB-2	ERBB2	22.0	1.7	24.3	16.0
Charged multivesicular body protein 1b	CHMP1B	4.0	1.0	6.3	4.0
Annexin A7 OS=Homo sapiens	ANXA7	1.3	0.6	4.0	1.4
ADP-ribosylation factor 6	ARF6	1.7	1.2	4.5	0.7
Flotillin-2	FLOT2	1.7	1.2	6.5	0.7
EH domain-containing protein 2	EHD2	3.7	1.2	5.5	3.5
Transforming protein RhoA	RHOA	4.0	0.0	6.7	3.8
Flotillin-1	FLOT1	4.0	1.0	7.0	5.3
Annexin A13	ANXA13	5.0	1.0	13.5	4.9
Charged multivesicular body protein 2b	CHMP2B	5.7	1.5	5.0	4.0
Arrestin domain-containing protein 1	ARRDC1	5.7	1.5	7.3	4.7
Charged multivesicular body protein 1a	CHMP1A	7.3	1.5	9.3	6.4
EH domain-containing protein 3	EHD3	8.0	0.0	8.3	6.7
Vacuolar protein sorting-associated protein 4A	VPS4A	8.0	3.0	12.5	4.9
Charged multivesicular body protein 3	CHMP3	8.3	3.8	12.7	9.3
Annexin A4	ANXA4	9.7	1.5	14.3	10.7
Tumor susceptibility gene 101 protein	TSG101	10.0	1.7	13.3	9.3
Charged multivesicular body protein 4b	CHMP4B	12.0	1.7	19.7	11.0
Vacuolar protein sorting-associated protein 4B	VPS4B	12.7	4.0	14.7	11.1
Charged multivesicular body protein 4c	CHMP4C	12.7	1.5	17.3	8.1
Annexin A3	ANXA3	16.3	0.6	12.3	6.4
EH domain-containing protein 4	EHD4	18.0	3.0	17.0	14.1
Annexin A1	ANXA1	21.3	1.2	18.0	8.5
Programmed cell death 6-interacting protein	PDCD6IP	23.7	1.5	34.7	23.2
Charged multivesicular body protein 2a	CHMP2A	26	2.6	45.7	28.7
EH domain-containing protein 1	EHD1	26.7	3.2	23.7	22.1
Putative annexin A2-like protein	ANXA2P2	50.3	3.2	42	18.1
Annexin A2	ANXA2	67.7	3.1	59.3	25
Syntenin-1	SDCBP	5.0	1.0	10.0	4.2
Annexin A11 OS=Homo sapiens	ANXA11	11.0	2.0	12.0	5.3
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	4.0	0.0	4.7	4.0
Actin, cytoplasmic 1	ACTB	61.0	2.6	49.0	32.8
Tubulin beta-3 chain	TUBB3	2.5	2.1	4.0	4.2
Tubulin beta-8 chain	TUBB8	2.7	1.5	4.5	4.9
Tubulin beta-2B chain	TUBB2B	2.7	1.2	7.5	6.4
Tubulin alpha-4A chain	TUBA4A	3.3	1.2	5.0	6.1
Tubulin alpha-3E chain	TUBA3E	4.0	1.0	5.0	5.7
Tubulin alpha-3D chain	TUBA3D	4.0	1.0	5.5	6.4
Tubulin beta chain	TUBB	4.3	0.6	9.0	5.7
Tubulin alpha-1A chain	TUBA1A	4.7	1.5	8.5	10.6
Tubulin alpha-1B chain	TUBA1B	4.7	1.5	9.5	12.0
Tubulin alpha-1C chain	TUBA1C	4.7	1.5	9.5	12.0
Tubulin beta-4B chain	TUBB4B	6.0	1.0	6.3	6.1
Tubulin beta-2A chain	TUBB2A	2.7	1.2	8.0	7.1
Heat shock protein HSP 90-beta	HSP90AB1	32.0	5.3	22.0	14.2
Heat shock cognate 71 kDa protein	HSPA8	39.3	4.7	43.0	27.1
Albumin	ALB	67.7	18.5	12.0	12.2

(a) PA method and (B) UC method. The analysis was performed with N=3, and the list was made up of PSMs obtained by any of the methods with a mean of 4 or higher (PSMs, peptide spectrum matches; SD, standard deviation).

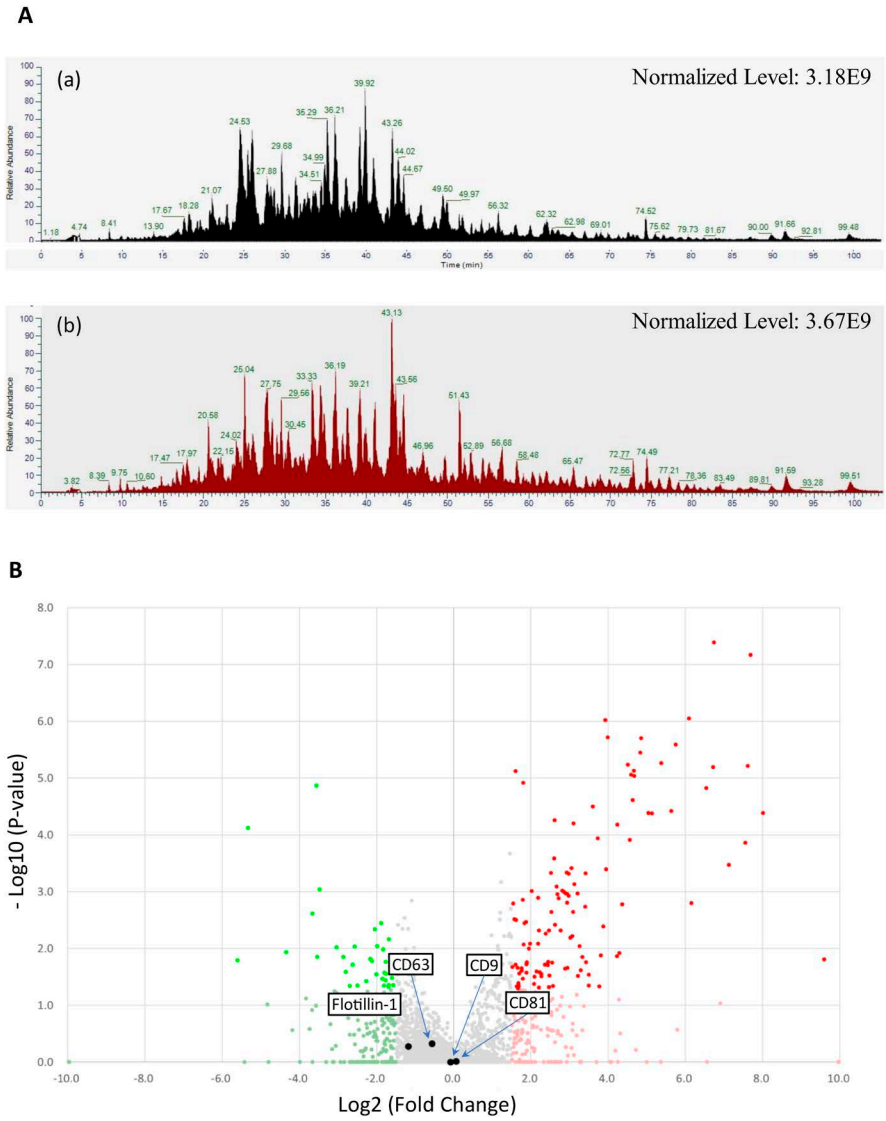

Fig. 1. Comparison of both methods using total ion chromatogram and volcano plot.

A. Total ion chromatogram for each method. (a) PA method, (b) UC method. B. Volcano plot for each method. Marked for proteins in EVs at NCI-N87. Created using abundance data obtained by LC-MS/MS. The x-axis indicates log2 fold change for each gene, and y-axis indicates -log10 p-value. Red dots indicate proteins with p-values <0.05, and green dots indicate proteins with p-values ≥ 0.05. The significance of differences was evaluated by t-test, with p-value <0.05 being significant.

proteins with the UC method.

In the GO analysis, EV markers, including exosomes, dominated the highest annotation cluster with enrichment. The enrichment scores for the PA and UC methods were 206.6 and 233.6, respectively (Table S4 A, B) .

Furthermore, information regarding cellular components obtained in this analysis is described in Fig. S2.

Discussion

Cancer is the leading cause of death worldwide; therefore, early cancer detection and appropriate treatment are crucial[13]. Usually, cancer is diagnosed by tissue biopsy; however, the invasive sampling and inability to accurately capture tumor dynamics owing to the heterogeneous distribution of tumor cells imposes challenges to cancer diagnosis[14]. Meanwhile, liquid biopsy is a promising method to address these challenges. In addition, liquid biopsy results have recently been used to administer companion diagnostic agents.

We compared the EVs obtained by PA and UC methods using the LC-MS/MS. Together with the UC method, LC-MS/MS detected proteins that indicate EVs according to the categories in the MISEV2018. The total ion chromatograms obtained using the PA method differed from those obtained using the UC method. In the analysis with the volcano plot, the common markers that were supposed

to be present in the EV appeared relatively close to the origin. However, their distribution showed different patterns.

Although the UC method is the standard method for EV recovery, it simultaneously recovers impurities such as vesicles and proteins other than EVs, resulting in lower purity of EVs[8]. These factors may have influenced the differences observed between the two methods in the present study. In addition, EVs contain exosomes, microvesicles, and apoptotic bodies, and the reactivity of each substance in each method[15], including PA method, can significantly affect the results.

The proteins detected by both methods are positively correlated and show a similar trend in detecting EV-related markers. Furthermore, we believe the PA method is less susceptible to contamination by dead cells. The results of the GO analysis also suggest that this is an efficient method of recovering EV-related substances. Although the PA method has shown recovery of proteins in EVs, its characteristics will need to be clarified through further studies.

Polyamines are involved in cell growth and survival, autophagy[16], and the onset and progression of neurological disease[17]. Additionally, a link between polyamine metabolic pathways and cancer has been reported[18]. Polyamines include streptomycin, spermidine, and spermine, which exist in cells at a level of several millimolar concentrations[19]. Moreover, polyamines are polycationic molecules[20], which is the characteristic the PA method focuses on.

This method can recover cell-free nucleic acids and EVs using polyamine-containing solutions[9], which we believe is due to the formation of ion complexes between polyamines and these substances with negative charges on the surface. Furthermore, under high salt concentration conditions, substances with small particle sizes and low charge density do not lead to the formation of coacervate[21], which may also affect not recovering a variety of free proteins.

The PA method does not require special equipment, is relatively easy to operate, and is low-cost. In addition, a pretreatment is possible when further downstream purification or nucleic acid extraction is performed on samples collected using the PA method. Regarding the reproducibility of the PA method, approximately 80% of the proteins detected in this study showed RSD within 30%. Maintaining and improving reproducibility is an essential issue in analysis. We believe that attention should be paid not only to technical proficiency but also to further simplifying the process.

Liquid biopsy has been reported in urine, saliva, cerebrospinal fluid, and stool other than blood[22], and is expected to be used in actual clinical practice. Free nucleic acids and proteins in the samples used in liquid biopsies are often present at low concentrations[23] and require a large sample volume. In addition, large amounts of reagents and special equipment are required. However, because the PA method can be enriched, it is possible to use ordinary processing reagents to handle enriched samples.

We have demonstrated the presence of protein markers in the characterization shown in MISEV2018 using the PA method; however, it is unclear to what extent the exosomes in EVs were recovered in this study. Furthermore, we believe that a comparative study of this point using methods other than the UC method should be conducted. Therefore, we are currently examining this point using various analytical methods.

The cargo of tumor-derived EVs is consistent with the genetic content of parent tumor cells[24]. Therefore, the cargo contained in the EVs has attracted attention as a new biomarker for cancer diagnosis and prognosis prediction[25]. Moreover, EVs are stable in body fluids, and the cargo in the EVs is protected from degradation[26,27]. Therefore, EVs are of interest as excellent biological components for liquid biopsy[1,28]. We are currently conducting various studies focusing on its application in liquid biopsy. We believe that the PA method can potentially demonstrate a broad utility by comparing its performance with that of other methods for EV recovery.

Mass spectrometry is being implemented in various ways in the medical field because of its ability for comprehensive analysis and its excellent sensitivity and specificity. We believe that this method will be an effective method in clinical testing of liquid biopsy samples.

Conclusions

EV analysis using LC-MS/MS with the SP3 method was also feasible. The LC-MS/MS results showed that the PA method can detect peptides with EV properties.

Conflict of Interest

The authors declare no competing interests.

Additional Information

The results of this study are organized in the text or in

supplementary materials.

References

1) Vaidyanathan R, Soon RH, Zhang P, Jiang L, Lim CT: Cancer diagnosis: From tumor to liquid biopsy and beyond. *Lab Chip* 19: 11−34, 2018.
2) Pantel K, Alix-Panabières C: Circulating tumour cells in cancer patients: Challenges and perspectives. *Trends Mol Med* 16: 398−406, 2010.
3) Jelski W, Mroczko B: Molecular and circulating biomarkers of gastric cancer. *Int J Mol Sci* 23: 2022.
4) Raposso G, Stoorovogel W: Extracellular vesicles; exosomes, microvesicles, and friends. *J Cell Biol* 200: 373−383, 2013.
5) Yáñez-Mó M, Siljander PR-M, Andreu Z, Zavec AB, Borràs FE, et al: Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles* v4: 27066, 2015.
6) Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, et al: Minimal information for studies of extracellular vesicles 2018 (MISEV2018): A position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles* 7: 1535750, 2018.
7) Royo F, Théry C, Falcón-Pérez JM, Nieuwland R, Witwer KW: Methods for separation and characterization of extracellular vesicles: Results of a worldwide survey performed by the ISEV rigor and standardization subcommittee. *Cells* 9: 1955, 2020.
8) Zhang H, Freitas D, Kim HS, Fabijanic K, Li Z, et al: Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation. *Nat Cell Biol* 20: 332−343, 2018.
9) Kikuchi A: Japan Patent Kokai 2022−86551.
10) Okuda S, Watanabe Y, Moriya Y, Kawano S, Yamamoto T, et al: jPOSTrepo: an international standard data repository for proteomes. *Nucleic Acid Res* 45(D1): D1107−D1111, 2017.
11) Sherman BT, Hao M, Qiu J, Jiao X, Baseler MW, et al: DAVID: A web server for functional enrichment analysis and functional annotation of gene lists (2021 update). *Nucleic Acid Res* 50(W1): W216−W221, 2022.
12) Dennis GJ, Sherman BT, Hosack DA, Yang J, Gao W, et al: DAVID: Database for annotation, visualization, and integrated discovery. *Genome Biol* 4: P3, 2003.
13) Hughes CS, Moggridge S, Müller T, Sorensen PH, Morin GB, et al: Single-pot, solid-phase-enhanced sample preparation for proteomics experiments. *Nat Protoc* 14: 68−85, 2019.
14) Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram, et al: Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 71: 209−249, 2021.
15) Sala M, Ros M, Saltel F: A complex and evolutive character: Two face aspects of ECM in tumor progression. *Front Oncol* 10:1620, 2020.
16) Zhou X, Xie F, Wang L, Zhang L, Zhang S, et al: The function and clinical application of extracellular vesicles in innate immune regulation. *Cell Mol Immunol* 17: 323−334, 2019.
17) Minarini A, Zini M, Milelli A, Tumiatti V, Marchetti C, et al: Synthetic polyamines activating autophagy: Effects on cancer cell death. *Eur J Med Chem* 67: 359−366, 2020.
18) Saiki S, Sasazawa Y, Fujimaki M, Kamagata K, Kaga N: A metabolic profile of polyamines in parkinson disease: A promising biomarker. *Ann Neurol* 86: 251−263, 2019.
19) Casero Jr RA, Stewart TM, Pegg TE: Polyamine metabolism and cancer: Treatments, challenges and opportunities. *Nat Rev Cancer* 18: 681−695, 2018.
20) Madeo F, Eisenberg T, Pietrocola F, Kroemer G: Spermidine in health and disease. *Science* 359: 6387, 2018.
21) Tsutsumiuchi K, Aoi K, Okada M: Ion complex formation between poly(amido amine) dendrimer HCl salt and poly(L-glutamic acid) sodium salt. *Polym J* 32: 107−112, 2000.
22) Firpo MR, Mounce BC: Diverse functions of polyamines in virus infection. *Biomolecules* 10: 628, 2020.
23) Peng M, Chen C, Hulbert A, Brock MV, Yu F: Non-blood circulating tumor DNA detection in cancer. *Onco Target* 8: 69162−69173, 2017.
24) Koukourakis IM, Xanthopoulou E, Koukourakis MI: Using liquid biopsy to predict relapse after radiotherapy in squamous cell head neck and esophageal cancer. *Cancer Diagn Progn* 3: 403−410, 2023.
25) Tovar‐Camargo OA, Toden S, Goel A: Exosomal microRNA biomarkers: Emerging frontiers in colorectal and other human cancers. *Expert Rev Mol Diagn* 16: 553−567, 2016.
26) Fu M, Gu J, Jiang P, Qian H, Xu W, Zhang X: Exosomes in gastric cancer: Roles, mechanisms, and applications. *Mol Cancer* 18: 41, 2019.

27) Baassiri A, Nassar F, Mukherji D, Shamseddine A, Nasr R, et al: Exosomal noncoding RNA in liquid biopsies as a promising bio marker for colorectal cancer. *Int J Mol Sci* 21: 1398, 2020.
28) Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, et al: Secretory mechanisms and intercellular transfer of microRNAs in living cells. *J Biol Chem* 285: 17442−17452, 2010.
29) Li S, Yi M, Dong B, Tan X, Luo S, et al: The role of exosomes in liquid biopsy for cancer diagnosis and prognosis prediction. *Int J Cancer* 148: 2640−2651, 2021.