Original Article

JHSMR Journal of Health Science and Medical Research

Proteomic Analysis of Urinary Extracellular Vesicles Reveals Estrogen/Progesterone Status-Associated Biomarkers of Breast Cancer Patients: Potential Roles in Diagnosis and Prognosis

Rassanee Bissanum, Ph.D.[1,2], Nilobon Jeanmard, M.Sc.[1], Hutcha Sriplung, M.D., Ph.D.[3], Sawanya Charoenlappanit, M.Sc.[4], Sittiruk Roytrakul, Ph.D.[4], Raphatphorn Navakanitworakul, Ph.D.[1]

[1]Department of Biomedical Sciences and Biomedical Engineering, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand.

[2]Translational Medicine Research Center, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand.

[3]Department of Epidemiology, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand.

[4]National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Khlong Nueng, Khlong Luang, Pathum Thani 12120, Thailand.

Received 4 July 2025 ● Revised 11 November 2025 ● Accepted 8 December 2025 ● Published online 4 August 2026

Abstract:

Objective: This study aimed to identify potential diagnostic and prognostic biomarkers in urinary extracellular vesicles (uEVs).

Material and Methods: Urine samples (n=30) from patients representing four breast cancer (BC) subtypes—Luminal A, Luminal B, human epidermal growth factor receptor 2 (HER2)-enriched, and triple-negative breast cancer (TNBC)—were collected. uEVs were isolated using differential ultracentrifugation and characterized by transmission electron microscopy and western blotting, confirming a round morphology, size range of 50–150 nanometer (nm), and presence of extracellular vesicle (EV) markers.

Results: Proteomic profiling via liquid chromatography-tandem mass spectrometry identified 70 upregulated and 10 downregulated differentially expressed proteins (DEPs) between pre- and post-surgical samples. Upregulated DEPs

Contact: Sittiruk Roytrakul, Ph.D.
National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Khlong Nueng, Khlong Luang, Pathum Thani 12120, Thailand.
E-mail: sittiruk@biotec.or.th
Contact: Raphatphorn Navakanitworakul, Ph.D.
Department of Biomedical Sciences and Biomedical Engineering, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand.
E-mail: raphatphorn.n@psu.ac.th, nraphatp@medicine.psu.ac.th

J Health Sci Med Res
doi: 10.31584/jhsmr.20261398
www.jhsmr.org

were enriched in pathways related to chromosome segregation, inhibition of cell differentiation, and inflammation. Six BC-associated proteins were identified in uEVs: CTIP, ATAD2, TSP50, SCRIB, LRRC3, and POTEF. Notably, CTIP (fold change (FC)=9.54, adj-p-value=0.04) and ATAD2 (FC=8.02, adj-p-value=0.03) were significantly elevated in estrogen/progesterone receptor (ER/PR)-positive patients. Additionally, high expression of RANBP2 and LRRC16A/CARMIL1 correlated with poor overall survival (OS), while RRP9 and KIAA1967 were linked to favorable OS. One post-surgical sample from a patient with brain metastasis showed a unique protein profile.

Conclusion: This study highlights that uEVs contain BC-specific protein markers associated with molecular subtypes and survival outcomes. These findings support the potential of uEV-based proteomics as a noninvasive tool for BC diagnosis and prognosis prediction.

Keywords: breast cancer, ER/PR-negative breast cancer, ER/PR-positive breast cancer, proteomic analysis, urinary extracellular vesicles

Introduction

Breast cancer (BC) is the most prevalent cancer among women worldwide and is associated with high morbidity and mortality rates[1]. BC is a heterogeneous disease that can be categorized into four subtypes based on the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2)[2]. The subtypes include luminal A, luminal B, HER2-enriched, and triple-negative breast cancer (TNBC). ER/PR-positive BC patients generally exhibit better overall survival (OS) and a more favorable prognosis, which is mainly attributed to the availability of targeted therapies, such as tamoxifen and aromatase inhibitors. By contrast, ER/PR-negative BC often presents as a more aggressive type of cancer that lacks hormone receptors, rendering hormonal therapies ineffective. Consequently, OS and disease-free survival are worse for patients in this group[3,4]. Thus, the discovery of novel biomarkers specific to the prognosis of BC is urgently required.

Recently, liquid biopsy has been extensively studied for applications in cancer screening, early diagnosis, and monitoring. Liquid biopsy is minimally invasive; it involves sampling bodily fluids (such as urine, saliva, and blood) and the analysis of various biomarkers present in those biofluids[5]. Urine is a promising body fluid for liquid biopsy because it is non-invasive, easy to collect, and provides valuable information on various diseases. It contains components of circulating tumor cells, circulating tumor deoxyribonucleic acid (DNA), products of many metabolic pathways, and extracellular vesicles (EVs)[6]. EVs can be used as prognostic tools because they carry various molecules, such as DNA, ribonucleic acid (RNA), metabolites, and proteins, from both normal and cancer cells and play a crucial role in the extracellular environment[7,8].

Numerous studies have shown that EVs play a key role in promoting tumor growth, facilitating invasion, and enhancing metastasis of tumor cells[9,10].

BC research efforts have attempted to identify potential biomarkers in urine with the aim of developing noninvasive diagnosis and prognosis tools. Several studies have revealed that patients with BC exhibit higher numbers of EVs than controls[11,12]. However, the number of EVs alone is insufficient for cancer diagnosis[13]. Beretov et al. revealed 13 novel urinary proteins that were significantly upregulated in patients with BC compared to those in normal controls, which could thus be used to detect BC[14]. However, some biomarkers may be less stable in urine than in other biological samples, such as blood or tissue[15]. EVs

are small membrane-bound structures that protect their molecular cargo from instability. In a previous study, we identified thousands of urinary extracellular vesicle (uEV) proteins that could effectively differentiate patients with BC from healthy women. We conducted a comparative analysis of proteomic profiles between total urine proteins and uEV proteins; we observed that uEV proteins exhibited more distinct protein expression patterns than the broader urine proteome among patients with BC and healthy women[16].

Therefore, in this study, we aimed to evaluate the proteomes of uEVs derived from urine samples of patients pre- and post-surgery, with the goal of identifying potential biomarker candidates that could serve as noninvasive biomarkers for better predicting the prognosis of BC.

Material and Methods

Urine sample collection and processing

An overview of the study workflow is depicted schematically in Figure 1. This study included newly diagnosed BC patients (≥18 years) at Songklanagarind Hospital with no prior BC. Urine samples were obtained from patients who consented to biobank storage and retrieved under ethical approval (REC 67-120-38-2). Exclusion criteria included pregnancy, mental illness, or inability to communicate in Thai. Mid-stream urine (20–50 mL) was collected from 30 patients one day before and two weeks after surgery, processed within 2 hours by centrifugation, and stored at −80 °C without protease inhibitors or freeze-thaw cycles[17,18]. Samples were analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS). Subtypes included Luminal A (n=17), Luminal B (n=4), HER2-enriched (n=6), and TNBC (n=3) (See details in Supplementary Table 1).

Quantification of total particles and uEVs using nanoparticle tracking analysis (NTA)

The total particle concentration and size distribution of uEVs were assessed using the NanoSight NS300 system (Malvern Panalytical Ltd, Malvern, UK). Samples were diluted in sterile water using two separate dilution ratios to achieve a final concentration between 1×10^7 and 10^9 particles/mL, aligning with the optimal range of 20 to 100 particles per frame. The prepared samples were then introduced into the nanoparticle tracking analysis instrument via a syringe pump operating at 50 mL/min. For each dilution, five 30-second video recordings were captured at a stable temperature of 25 °C, using a camera level of 14 and a detection threshold of 6. Data analysis was performed using NanoSight NTA software version 3.0.

uEV isolation via differential ultracentrifugation

The uEV isolation protocol was adapted from Sequeiros et al.[19] and Jeanmard et al.[16]. Urine samples (9 mL) were centrifuged at 2,500×g for 15 min at 4 °C, followed by 20,000×g for 20 min to remove debris. The pellet was resuspended in the isolation buffer (10 mM triethanolamine, 250 mM sucrose), vortexed, and treated with dithiothreitol to remove Tamm–Horsfall protein (THP), then incubated at 37 °C for 10 min. After adding buffer, the samples were centrifuged again at 20,000×g. Supernatants were combined and ultracentrifuged at 120,000×g for 90 min at 4 °C using a Beckman Coulter Optima MAX-XP ultracentrifuge. The pellet was washed with Phosphate Buffered Saline (PBS), ultracentrifuged again, and resuspended in 150 µL PBS. uEVs were identified using western blotting and transmission electron microscopy.

Proteomic analysis

Proteins from pre- and post-surgery uEVs were extracted using Radioimmunoprecipitation assay buffer (RIPA) supplemented with protease inhibitors. Samples were vortexed on ice, sonicated, and centrifuged to remove debris. Protein concentrations were measured by the Lowry method. Five micrograms of protein were reduced with dithiothreitol, alkylated with iodoacetamide, and digested overnight with trypsin. Peptides were concentrated by vacuum centrifugation and reconstituted in 0.1% formic acid for LC–MS/MS analysis[20].

Tryptic peptides were analyzed on an Ultimate 3000 Nano LC system coupled to a Bruker Impact II Q-TOF mass spectrometer. Peptides were first trapped on a C18 precolumn, separated on an analytical C18 column at 60 °C, and eluted with a linear gradient of 5–55% acetonitrile over 30 min at 0.3 µL/min. Ionization was achieved by electrospray at 1.6 kV, and fragmentation by collision-induced dissociation (CID) using nitrogen gas. Tandem mass spectrometry (MS/MS) spectra were acquired in positive-ion mode (m/z 50–2200) at a scan rate of 2 Hz.

Protein identification and label-free quantification were performed using MaxQuant v2.1.0.0 with the Andromeda search engine against the UniProt Homo sapiens database. Search parameters included 0.6 Da mass tolerance, trypsin digestion, fixed carbamidomethylation, and variable methionine oxidation. False discovery rate (FDR) was set at 1%, and proteins were quantified using razor and unique peptides[21,22].

Data preprocessing and identification of differentially expressed proteins (DEPs)

DEPs were analyzed and compared between pre- and post-surgical samples. Proteins meeting the cutoff criteria, with a FDR <0.05 and a fold change (FC) >2 or <–2, were categorized as upregulated and downregulated DEPs, respectively. To visualize the overlap and uniqueness of the DEPs, volcano plots, heat maps, Venn diagrams, and Principal Component Analysis (PCA) were generated using MetaboAnalyst 5.0[23].

Functional and pathway enrichment analyses

Functional enrichment analysis of the upregulated DEPs was performed using Metascape (http://metascape.org/). This analysis covered three Gene Ontology categories: biological processes, molecular functions, and cellular components. Additionally, pathway enrichment analysis was carried out using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database[24].

Expression and survival analyses of candidate proteins

The expression levels of the candidate proteins were validated using clinical samples from both tumor and normal tissues. The UALCAN database (http://ualcan.path.uab.edu/index.html) is a valuable online tool for analyzing cancer transcriptome data, leveraging the data analysis of the Clinical Proteomic Consortium[25]. To validate the survival analysis of patients with BC, we utilized the Kaplan–Meier plotter (http://kmplot.com/analysis). OS was analyzed, and patients were stratified into high and low protein expression groups. The 'best cutoff' option in Kaplan–Meier Plotter was used to automatically determine the optimal expression threshold that yields the most significant difference between the groups. Hazard Ratios (HRs) with 95% confidence intervals were calculated, and a log-rank p-value <0.05 was used to identify statistically significant differences between the groups[26].

Results

uEV Protein content in pre- and post-surgical samples

Isolated uEVs from pre- and post-surgical samples displayed typical EV characteristics, including the presence of EV markers (CD9, Alix, and TSG101), round morphology, and sizes ranging from 50–150 nm. Cytochrome c, used as a negative control, was not detected in any samples. THP, the most abundant urinary protein known to form polymeric aggregates that co-isolate with uEVs, remained detectable in most samples (Figure 2A, 2B, and Supplementary Figure 1). NTA revealed a significant decrease in uEV concentration post-surgery in the ER/PR-positive group, whereas no significant change was observed in the ER/PR-negative group (Figure 2C, 2D).

Subsequently, uEV proteins were analyzed by LC–MS/MS, yielding 1,966 unique protein IDs shared between pre- and post-surgery samples. Additionally, 1,118 and 3,594 proteins were uniquely detected in pre-surgery and

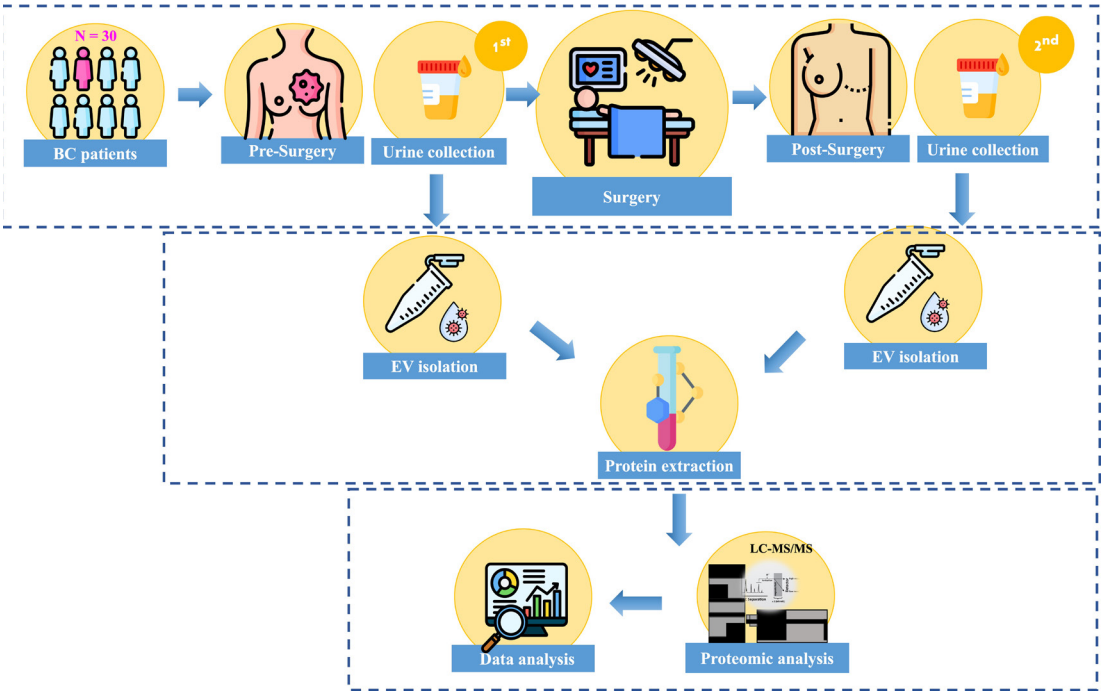
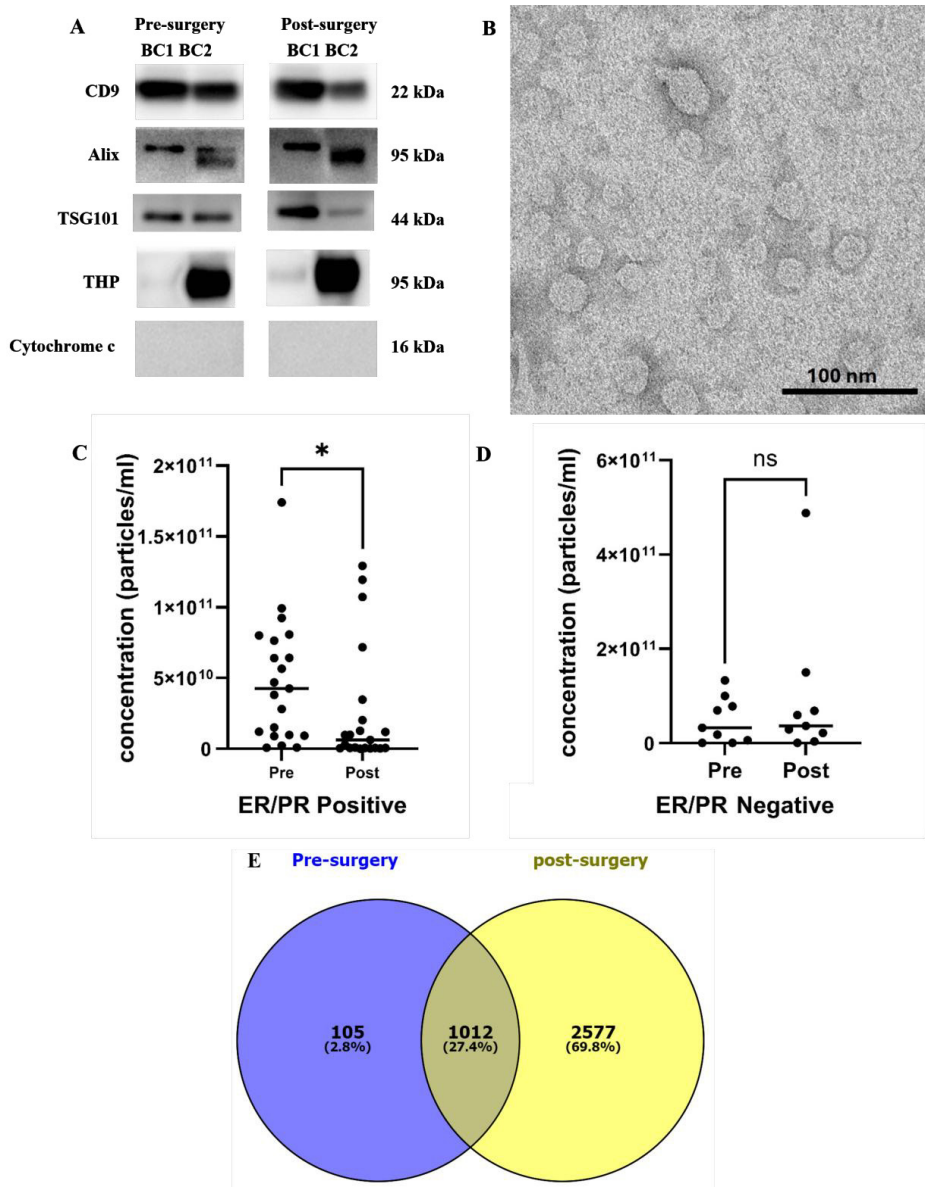


Figure 1 Study workflow. Mid-stream urine samples were randomly collected from breast cancer patients at two time points: the first before surgery and the second 1–2 weeks post-surgery. uEVs were then isolated via differential ultracentrifugation, and total protein from uEV samples, both pre- and post-surgery, was isolated and measured. Proteomic analysis was conducted using LC-MS/MS, and MaxQuant 2.1.0.0 was used to quantify the proteins in all samples.

post-surgery samples, respectively (Figure 2E). Distinct differences in protein expression profiles were evident between the two groups (Figure 3A). PCA demonstrated clear clustering between pre- and post-surgery samples, although a few samples showed partial overlap (Figure 3B). Differentially expressed proteins (DEPs) were identified using thresholds of FC >2 or <–2 and FDR <0.05, revealing 70 upregulated and 10 downregulated proteins (Figure 3C, Table 1, Supplementary Table 2). Several identified DEPs—such as CTIP, ATAD2, TSP50, SCRIB, LRRC3, and POTEF—have been previously associated with BC and were considered potential biomarker candidates. Functional enrichment analysis of upregulated DEPs using metascape indicated significant enrichment in biological processes, including regulation of chromosome segregation, cell polarity establishment, negative regulation of cell differentiation, and inflammatory response. Reactome pathway analysis revealed enrichment in solute carrier (SLC) transporter and transmembrane transporter disorders (Figure 4A and 4B, Supplementary Table 3).

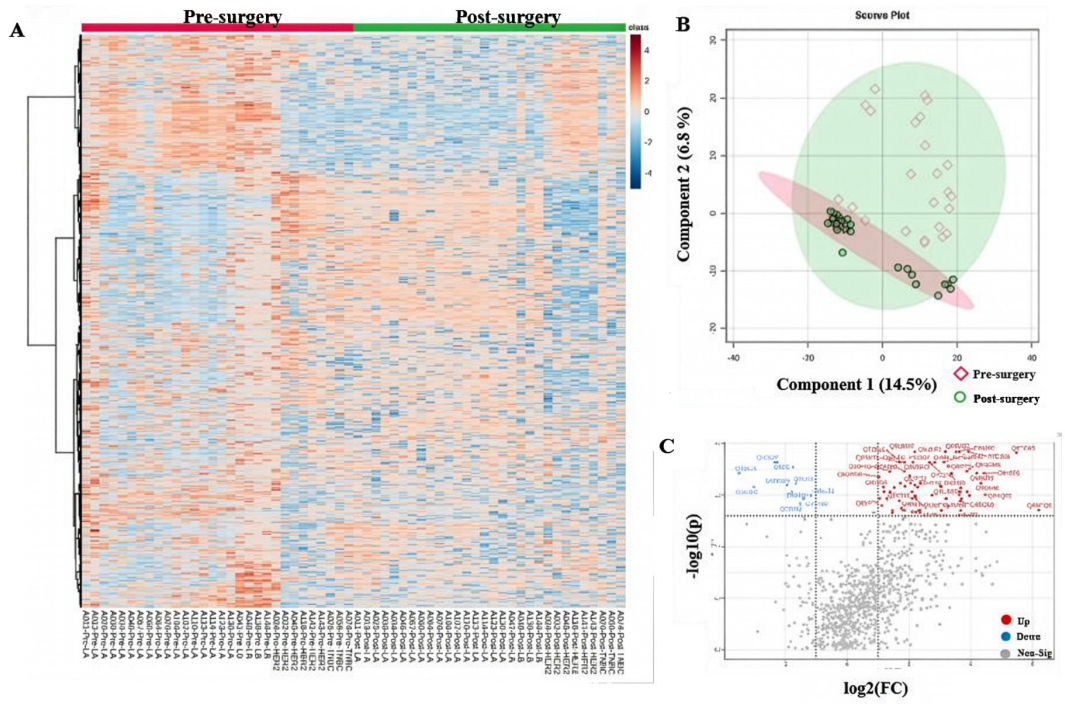
Identification of DEPs in ER/PR-positive and ER/PR-negative Groups

Heatmap and PCA analyses revealed distinct clustering for most pre- and post-surgical samples, although some showed overlapping protein expression patterns. Notably, hormone receptor–negative subtypes (TNBC and HER2-enriched) exhibited unique expression profiles. Previous studies reported upregulation of CTIP and ATAD2, DEPs associated with ER-positive BC[27-29].



*p-value<0.05, ns=not significant, ER/PR=Estrogen Receptor / Progesterone Receptor, IQR=Interquartile Range

Figure 2 Characterization of urinary extracellular vesicles (uEVs), (A) Western blotting analysis of isolated uEVs from pre- and post-surgical samples. (B) Transmission electron micrograph of uEVs isolated via ultracentrifugation (magnification: 100,000×). (C) uEV concentration (median±IQR) in pre- and post-surgical samples from the ER/PR-positive group. (D) uEV concentration (median±IQR) in pre- and post-surgical samples from the ER/PR-nagative group. (E) Venn diagram illustrating the distribution of all identified uEV proteins between pre-surgery and post-surgery samples.



DEPs=differentially expressed proteins, uEV=urinary extracellular vesicle, PCA=principal component analysis, FC=fold change

Figure 3 Proteomic profile and DEPs in uEVs from pre- and post-surgical samples, (A) Heat map of the uEV proteomic profiles of individual pre- and post-surgical samples (n=30). (B) PCA of the proteome of uEVs obtained from pre- and post-surgical samples. (C) Volcano plot showing differentially expressed proteins (DEPs) between pre- and post-surgical samples. Red and blue dots represent upregulated and downregulated DEPs, respectively. Gray dots represent proteins that are not significantly differentially expressed between groups. The horizontal red dashed line shows the adjusted p-value=0.05, and the vertical red dashed lines correspond to the $\log_2$ fold change (FC) value.

Stratifying samples into ER/PR-positive and ER/PR-negative groups, heatmap and PCA analyses demonstrated clear differences in sample distribution and cluster formation (Figure 5A–D). Differential expression analysis identified 128 upregulated and 63 downregulated DEPs in the ER/PR-positive group, and 16 upregulated and 35 downregulated DEPs in the ER/PR-negative group (Figure 5E, 5F). CTIP and ATAD2 were exclusively upregulated in the ER/PR-positive group (Supplementary Table 4 and 5).

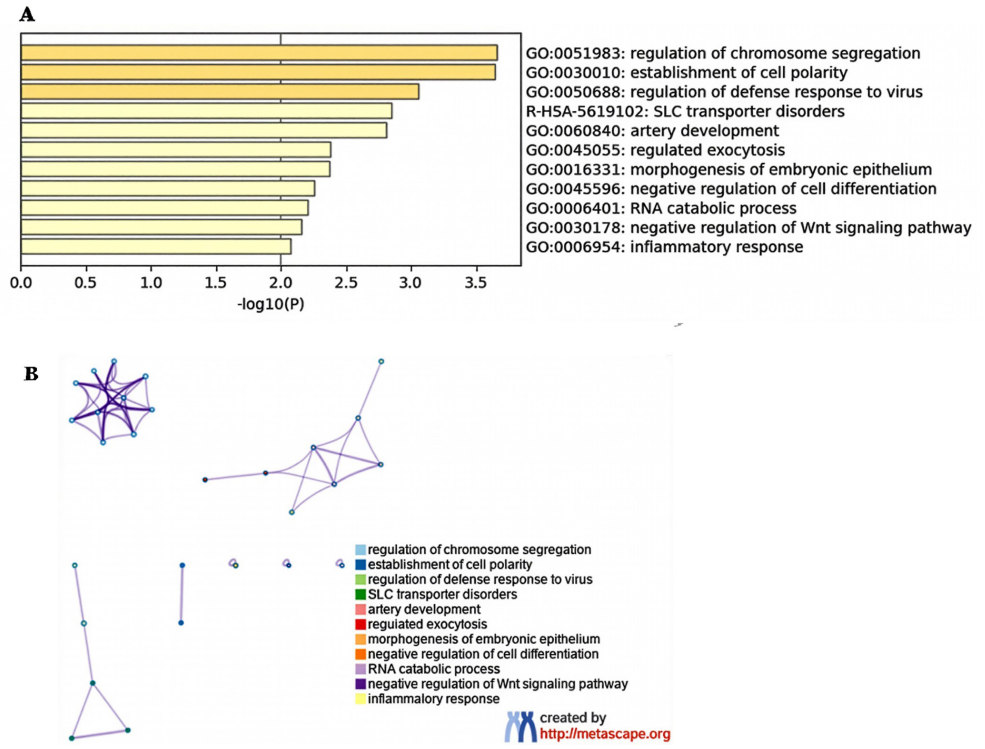
Survival analysis of candidate proteins in the ER/PR-positive and ER/PR-negative groups

To predict the prognostic value of candidate proteins, we performed survival analysis based on protein expression levels in tumor and healthy controls using the Kaplan–Meier plotter, a web-based tool. For the ER/PR-positive group, our results showed that high expression of RANBP2 was associated with worse OS of patients with BC, with a hazard ratio (HR) of 2.24 (log-rank p=0.027), whereas RRP9 was

Table 1 List of the top 10 upregulated and downregulated uEV proteins between pre- and post-surgical samples

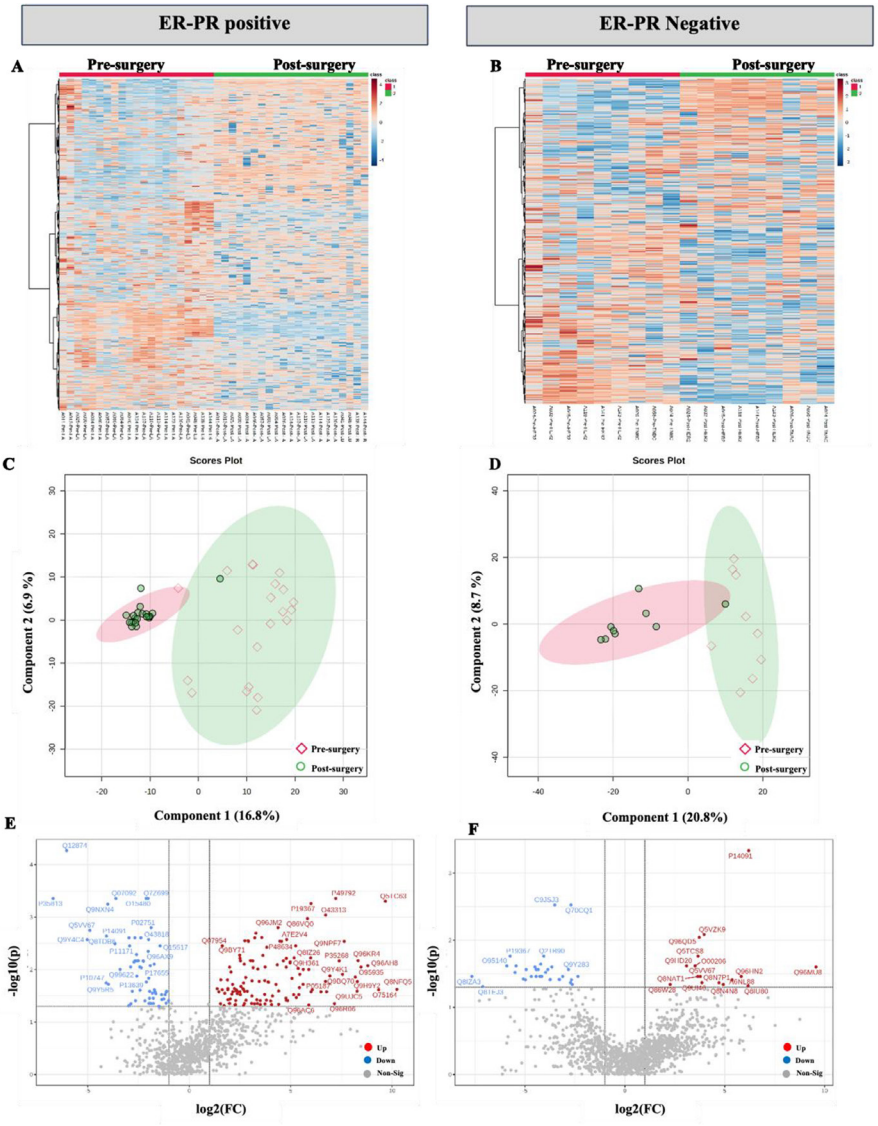
Upregulated DEPs				Downregulated DEPs			
Uniprot ID	Protein name	log_2(FC)	P-adjusted	Uniprot ID	Protein name	log_2(FC)	P-adjusted
Q8NFQ5	BPIB6	6.1982	0.044331	Q9UL12	POTEF	−1.1604	0.031791
Q5TC63	GRTP1	5.4712	0.012296	O75746	S2512	−1.3981	0.03429
Q9BQ70	TCF25	4.463	0.03136	Q07092	IST1	−1.534	0.038839
O94826	TOM70	4.4346	0.019381	Q96AX9	COGA1	−1.6481	0.024434
Q96AH8	RAB7B	4.3153	0.024244	P53990	SF3A3	−1.7959	0.029305
Q96KR4	LMLN	4.1908	0.019381	Q9NS39	MIB2	−1.929	0.025339
Q9H9Y2	RPF1	3.9526	0.031969	Q9BSI4	C10	−2.2487	0.015149
A7E2V4	ZSWM8	3.9263	0.012008	Q99622	TINF2	−2.341	0.015149
P05187	PPB1	3.8815	0.029305	A5A3E0	RED2	−3.0134	0.02648
Q8NGH6	O52L2	3.8704	0.018572	Q12874	SARDH	−3.4879	0.019381

DEP=differentially expressed protein, FC=fold change, uEV=urinary extracellular vesicle



DEPs=differentially expressed proteins, Cluster ID=Cluster Identifier

Figure 4 Functional enrichment results of the upregulated DEPs, (A) Bar graph of the top 20 cluster-enriched terms across input protein lists, colored by p-values. The smaller the p-value, the deeper the color. (B) Network of the top 20 clusters of enriched terms. Each node represents one enriched term colored by cluster ID, where nodes that share the same cluster ID are typically close to each other. Terms with a similarity score >0.3 are linked by an edge. The network is visualized using Cytoscape (v3.1.2) with "force-directed" layout and with edges bundled for clarity. One term from each cluster is shown as the label.



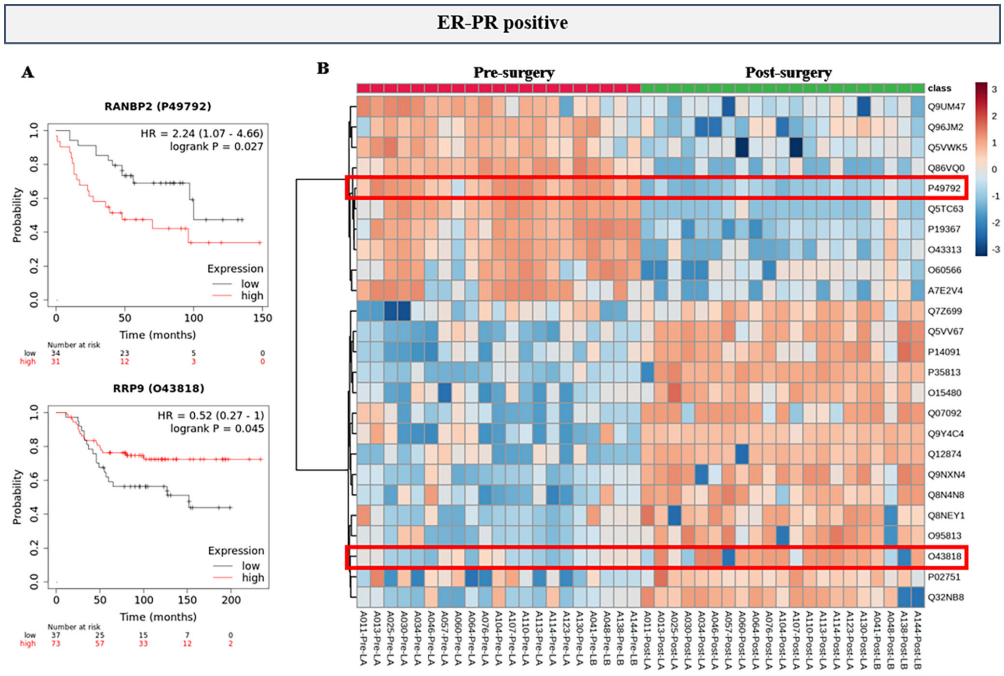
uEVs=urinary extracellular vesicles, ER/PR=Estrogen Receptor / Progesterone Receptor, DEPs=differentially expressed proteins

Figure 5 Proteomic profile of uEVs and DEPs in ER/PR-positive and ER/PR-negative groups. (A, B) Heat maps comparing the uEV proteomic profiles of pre- and post-surgical samples (n=30) between ER/PR-positive and ER/PR-negative groups. (C, D) Principal component analysis of uEV proteins derived from ER/PR-positive and ER/PR-negative groups. (E, F) Volcano plot illustrating the differentially expressed proteins (DEPs) between pre- and post-surgical samples of ER/PR-positive and ER/PR-negative groups. Red and blue dots represent the upregulated and downregulated DEPs, respectively. Gray dots represent proteins that are not significantly expressed between groups. The horizontal red dashed line shows adjusted p-value=0.05, and the vertical red dashed lines correspond to the log 2-fold change value.

associated with favorable OS of patients, with a HR of 0.52 (log-rank p=0.045) (Figure 6A). This finding is consistent with our protein expression results, as we observed high RANBP2 expression in the pre-surgical samples and low expression in the post-surgical samples. Conversely, the expression of RRP9 was high in post-surgical samples (Figure 6B).

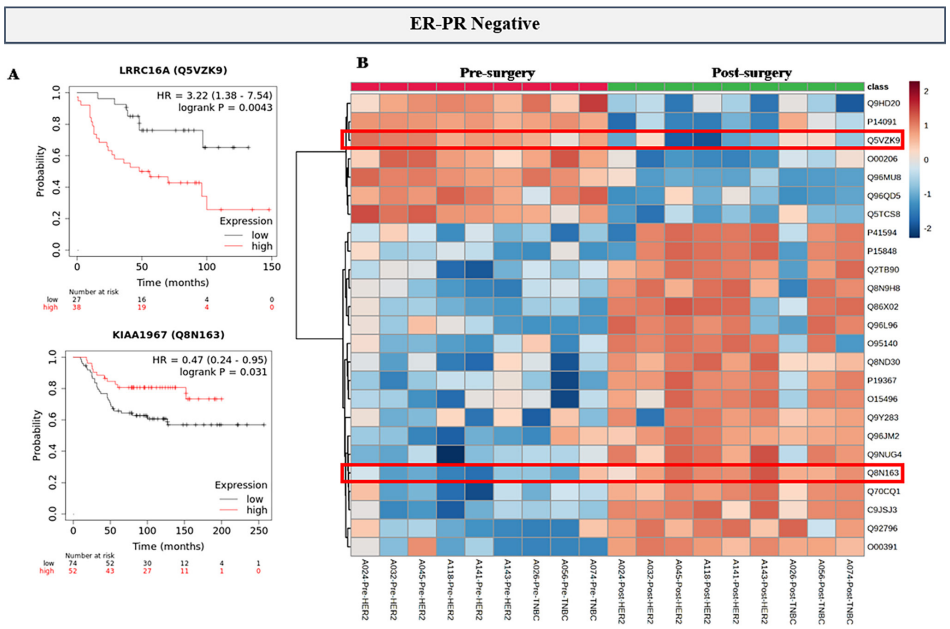
In the ER/PR-negative group, elevated expression of LRRC16A/CARMIL1 (HR 3.22, log-rank p=0.0043) was correlated with poorer OS in patients with BC, whereas KIAA1967 was associated with favorable OS, showing a HR of 0.47 (log-rank p=0.031) (Figure 7A). This observation was also consistent with the expression patterns observed in our patients' pre- and post-surgical samples (Figure 7B).

Notably, the expression profile of the post-surgical sample A026-TNBC exhibited a distinct pattern compared to that of the other samples (Figure 7B). Upon reviewing our clinical data, we found that this particular BC case was the only one associated with brain metastasis among the group (Supplementary Table 6). Based on our findings,



ER/PR=estrogen receptor / progesterone receptor, RANBP2=Ran-Binding Protein 2, RRP9=Ribosomal RNA-Processing 9
HR=hazard ratio, uEV=urinary extracellular vesicles

Figure 6 Survival analysis according to candidate proteins within the ER/PR-Positive, (A) Kaplan–Meier plot showing the association between RANBP2 and RRP9 expression levels and overall survival in breast cancer patients. The red line indicates samples with high gene expression, and the black line indicates samples with low protein expression. A log-rank p <0.05 was considered significant hazard ratio (HR). (B) Heat map of the uEV proteomic profiles of pre- and post-surgical samples in the ER/PR-positive breast cancer group.



ER/PR=Estrogen Receptor / Progesterone Receptor, LRRC16A=Leucine Rich Repeat Containing 16A, CARMIL1=Capping Protein, Arp 2/3, and Myosin-I Linker 1, HR=hazard ratio, uEV=urinary extracellular vesicles

Figure 7 Survival analysis according to candidate proteins within the ER/PR-Negative Groups, (A) Kaplan–Meier plot showing the association between LRRC16A/CARMIL1 and KIAA1967 expression levels and overall survival in breast cancer patients. The red line indicates samples with high gene expression, and the black line indicates samples with low protein expression. A log-rank p <0.05 was considered significant (HR, hazard ratio). (B) Heat map of the uEV proteomic profiles of pre- and post-surgical samples in the ER/PR-negative breast cancer group.

we hypothesized that these key proteins have a significant impact on the development and progression of BC. Consequently, these proteins have potential as prognostic biomarkers in patients with BC.

Discussion

BC is among the most common cancers affecting women worldwide. Through pairwise comparisons of pre- and post-surgical samples from the same patients, the heatmap and PCA results revealed distinct clusters for the majority of samples in both the pre- and post-surgical groups. However, certain samples did not exhibit clear separation in terms of protein expression patterns. In particular, samples from the hormone receptor-negative TNBC and HER2-enriched displayed distinct protein expression patterns. uEV quantification revealed a significant decrease in uEV concentration in post-surgery samples compared to pre-surgery samples within the ER/PR-positive group. Conversely, no significant difference was observed between pre-surgery and post-surgery samples within the ER/PR-negative group. Previous research has demonstrated that EVs play a role in promoting tumor

growth, facilitating tumor cell invasion, and enhancing tumor cell metastasis[9,10]. Morasso et al. reported that circulating EVs showed higher concentrations in BC patients compared to healthy controls, and the plasma levels of EVs significantly decreased in BC patients after surgery[27]. Our uEV results for the ER/PR-positive group are consistent with the findings of these studies. However, the concentration of EVs in BC can depend on several factors, including the stage, the aggressiveness, and BC subtypes. Presently, there are no studies on the concentration of EVs in the urine of BC patients. Further evaluation of larger cohorts is necessary to validate these findings.

We analyzed the functions of upregulated DEPs that were significantly enriched in various pathways, including the regulation of chromosome segregation, establishment of cell polarity, negative regulation of cell differentiation, and inflammatory response. Notably, we observed that upregulated DEPs from uEVs were functionally associated with cancer-related pathways, especially six proteins: CTIP, ATAD2, TSP50, SCRIB, LRRC3, and POTEF. All of these proteins have been reported to be associated with BC progression and poor prognosis. For example, ATAD2 plays an important role in BC development and is highly expressed in ER-positive BC[28]. Kalashnikova et al. demonstrated that ATAD2 is overexpressed in BC (>70%), and its high expression level is significantly correlated with tumor histologic grade and TNBC subtype, which represent highly aggressive diseases[29]. Moreover, ATAD2 is a proliferation marker that has been reported to predict poor outcomes in patients with ovarian cancer[30]. CTIP (also known as RBBP8) is a multifunctional protein that plays a crucial role in DNA repair and cell cycle regulation. According to Soria-Bretones et al., BC with high-grade lymph node metastasis is associated with low or no expression of CTIP. Additionally, low CTIP expression was predominantly observed in the ER/PR-negative, HER2-positive, and basal-like subtypes of BC by immunohistochemistry using paraffin-embedded BC biopsies[31]. Thus, based on previous reports and our results, it is evident that some of our DEPs were influenced by the presence or absence of breast hormone receptors. Consequently, we identified the protein profiles and DEPs that were specifically associated with ER, PR, and HER2 status. CTIP and ATAD2, known for their high expression exclusively in ER-positive BC, were both identified as upregulated DEPs within the ER/PR-positive group in our study. This result supports the heterogeneity of BC and suggests that conducting separate analyses for ER/PR-positive and ER/PR-negative groups would be more appropriate and informative. However, the sample sizes of the TNBC and HER2-enriched in this study were insufficient. Further research with larger sample sizes for each subtype is warranted to validate our findings.

Additionally, we found that elevated RANBP2 protein expression within the ER/PR-positive group was correlated with poor OS in patients with BC, whereas RRP9 was associated with good OS in patients with BC. Within the ER/PR-negative group, elevated expression of LRRC16A/CARMIL1 was correlated with poorer OS, whereas KIAA1967 was associated with favorable OS. DBC1, also known as KIAA1967 or CCAR2, is a potential tumor suppressor gene isolated from breast cancer samples. It plays a significant role in tumor suppression by regulating p53/TP53 function. Hiraike et al. found that the expression of DBC1 correlated with the nuclear grade and HER2-enriched[32]. In addition, we observed distinct protein expression patterns after surgical resection in brain metastasis cases within the ER/PR-negative group. Expression of these proteins may play a key role in BC progression. Hence, our findings indicate that uEVs carry BC-specific proteins that would have originated from BC cells and then secreted into the urine. Certain proteins were correlated with OS and progression in patients with BC, suggesting their potential as promising noninvasive prognostic biomarkers.

uEVs have gained increasing interest as robust cancer biomarkers[33]. Previous studies have suggested that urine may contain biomarkers with potential applications in

cancer prognosis. Sequeiros et al. identified biomarkers using targeted proteomics in uEVs for the diagnosis and prognosis of prostate cancer (PCa). They discovered a two-protein combination (ADSV, TGM4) in urinary EVs that distinguishes between benign and PCa patients, as well as a five-protein combination (CD63, GLPK5, SPHM, PSA, PAPP) that significantly differentiates high- and low-grade PCa cases. Additionally, they found that ADSV and TGM4 abundance demonstrated high diagnostic potential in tissue samples, with TGM4 showing promising prognostic value. Their findings suggest that urinary EVs could enhance the diagnosis and prognosis of suspected PCa patients[19]. In urinary bladder cancer (UBC) patients, bladder urine EVs exhibited a distinct molecular signature associated with muscle invasiveness. A trained classifier achieved 92% accuracy in predicting UBC. Bead-based flow cytometry analysis of differentially expressed proteins, including HO-1 and MMP7, confirmed HO-1's presence on the EV surface. These findings further support the potential of EVs as non-invasive biomarkers and prognostic tools for UBC[34].

Thus, our findings align with those of previous studies[16] , indicating that the identified proteins are likely to play a pivotal role in the development and progression of BC. Consequently, these proteins show great promise as potential biomarkers for clinical application of BC prognosis. However, this study has several limitations. First, the small sample size highlights the need for further research with a larger cohort. Second, we randomly collected mid-stream urine samples from BC patients, opting not to use morning urine samples to better reflect a typical clinical setting, where patients are not required to provide morning urine. Third, due to its retrospective biobank design, only one pre-surgical and one post-surgical urine sample per patient were available; therefore, day-to-day intra-individual variability could not be assessed. We mitigated this by strict pre-analytical standardization, technical triplicates for LC–MS/MS, replicate NTA acquisitions, run randomization, and robustness analyses, which collectively supported the stability of the principal findings. Finally, we used the default algorithm, which is single peptide per protein. In this case, the software had already been adjusted for any potential false protein discovery based on peptide -decoy posterior error probabilities. However, we are aware of a potential one-hit wonder. Future in vitro and in vivo studies are necessary to provide a deeper understanding of the functions of these proteins, particularly their roles in BC tumorigenesis and prognosis. A prospective validation study with triplicate urine collections over three consecutive days pre- and post-surgery is underway to quantify the within-person variance and refine the clinical thresholds.

Conclusion

In BC research, uEVs offer a noninvasive source of biomarkers for BC detection, prognosis, and treatment monitoring. Urine contains biomolecules from both the urinary system and distant organs. Candidate proteins previously linked to BC were elevated in pre-surgical samples and correlated with poorer overall survival, highlighting their potential as prognostic biomarkers. Urine-based EV analysis can thus provide clinically valuable insights into disease status, progression, treatment response, and recurrence.

Acknowledgement

We express our gratitude to all the patients who provided urine samples for this study.

Funding sources

This research was supported by the National Science, Research and Innovation Fund (NSRF) and Prince of Songkla University (Grant No. MED6701099S) to RN.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

R.B. and R.N.; data curation, formal analysis, investigation, methodology, and validation: R.B., N.J., H.S.,

S.C., S.R., and R.N.; data analysis and interpretation and writing the manuscript: R.B. and R.N.; supervision: R.N.; funding acquisition: R.N. All authors have read and agreed to the published version of the manuscript.

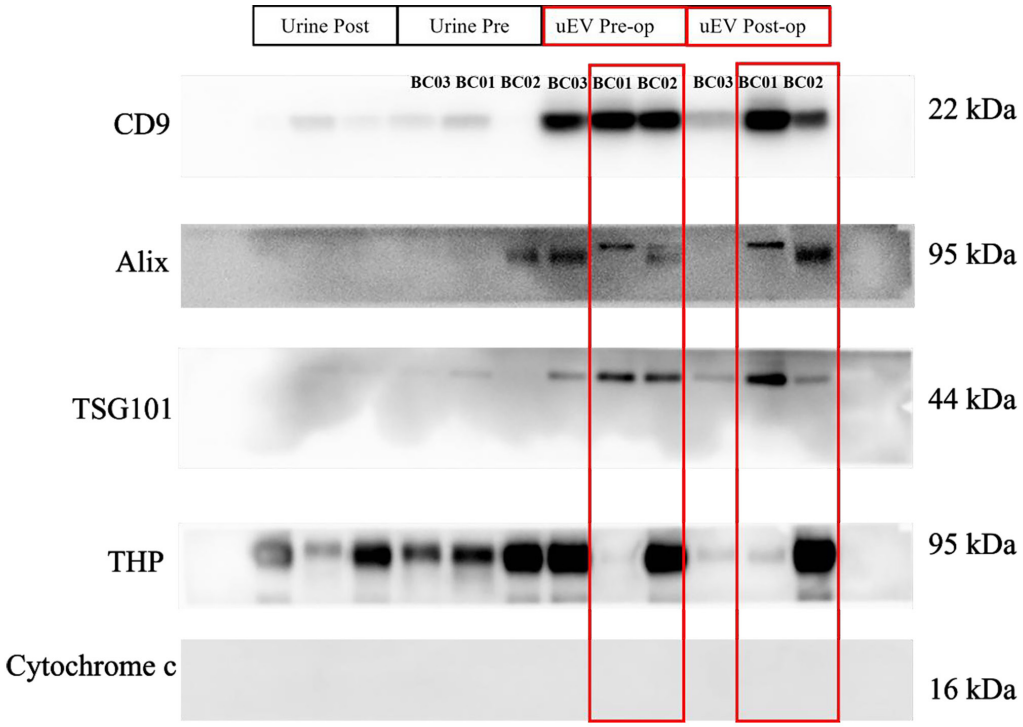
Data availability statement

The data supporting the reported results can be found in the manuscript. The MS/MS raw data is available at the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) with the data set identifier JPST001972 and PXD039122.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: globocan estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2021;71:209–49.
2. Yersal O, Barutca S. Biological subtypes of breast cancer: prognostic and therapeutic implications. World J Clin Oncol 2014;5:1–15.
3. Onitilo AA, Engel JM, Greenlee RT, Mukesh BN. Breast cancer subtypes based on ER/PR and her2 expression: comparison of clinicopathologic features and survival. Clin Med Res 2009;7:4–13.
4. Wei S. Hormone receptors in breast cancer: an update on the uncommon subtypes. Pathol Res Pract 2023;250:1–6.
5. Tellez-Gabriel M, Knutsen E, Perander M. Current status of circulating tumor cells, circulating tumor dna, and exosomes in breast cancer liquid biopsies. Int J Mol Sci 2020;21:1–23.
6. Piombino C, Mastrolia I, Omarini C, Candini O, Dominici M, Piacentini F, et al. The role of exosomes in breast cancer diagnosis. Biomedicines 2021;9:1–14.
7. Liu SY, Liao Y, Hosseinifard H, Imani S, Wen QL. Diagnostic role of extracellular vesicles in cancer: a comprehensive systematic review and meta-analysis. Front Cell Dev Biol 2021;9:1–17.
8. Petersen EV, Chudakova DA, Skorova EY, Anikin V, Reshetov IV, Mynbaev OA. The extracellular matrix-derived biomarkers for diagnosis, prognosis, and personalized therapy of malignant tumors. Front Oncol 2020;10:1–9.
9. Chan YK, Zhang H, Liu P, Tsao SW, Lung ML, Mak NK, et al. Proteomic analysis of exosomes from nasopharyngeal carcinoma cell identifies intercellular transfer of angiogenic proteins. Int J Cancer 2015;137:1830–41.
10. He M, Qin H, Poon TC, Sze SC, Ding X, Co NN, et al. Hepatocellular carcinoma-derived exosomes promote motility of immortalized hepatocyte through transfer of oncogenic proteins and RNAs. Carcinogenesis 2015;36:1008–18.
11. Zhong Z, Rosenow M, Xiao N, Spetzler D. Profiling plasma extracellular vesicle by pluronic block-copolymer based enrichment method unveils features associated with breast cancer aggression, metastasis and invasion. J Extracell Vesicles 2018;7:1–23.
12. Yu DD, Wu Y, Shen HY, Lv MM, Chen WX, Zhang XH, et al. Exosomes in development, metastasis and drug resistance of breast cancer. Cancer Sci 2015;106:959–64.
13. Sadovska L, Eglītis J, Linē A. Extracellular vesicles as biomarkers and therapeutic targets in breast cancer. Anticancer Res 2015;35:6379–90.
14. Beretov J, Wasinger VC, Millar EKA, Schwartz P, Graham PH, Li Y. Proteomic analysis of urine to identify breast cancer biomarker candidates using a label-free LC-MS/MS approach. PLoS One 2015;10:e0141876.
15. Bax C, Lotesoriere BJ, Sironi S, Capelli L. Review and comparison of cancer biomarker trends in urine as a basis for new diagnostic pathways. Cancers 2019;11:1–41.
16. Jeanmard N, Bissanum R, Sriplung H, Charoenlappanit S, Roytrakul S, Navakanitworakul R. Proteomic profiling of urinary extracellular vesicles differentiates breast cancer patients from healthy women. PLoS One 2023;18:e0291574.
17. O'Brien K, Ughetto S, Mahjoum S, Nair AV, Breakefield XO. Uptake, functionality, and re-release of extracellular vesicle-encapsulated cargo. Cell Rep 2022;39:1–31.
18. Kodam SP, Ullah M. Diagnostic and therapeutic potential of extracellular vesicles. Technol Cancer Res Treat 2021;20:1–10.
19. Sequeiros T, Rigau M, Chiva C, Montes M, Garcia-Grau I, Garcia M, et al. Targeted proteomics in urinary extracellular vesicles identifies biomarkers for diagnosis and prognosis of prostate cancer. Oncotarget 2016;8:4960–76.
20. Lowry O, Rosebrough N, Farr AL, Randall R. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
21. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M. Andromeda: A Peptide Search Engine Integrated into the MaxQuant Environment. J Proteome Res 2011;10:1794–805.

22. Tyanova S, Temu T, Cox J. The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. Nat Protoc 2016;11:2301-19.
23. Pang Z, Chong J, Zhou G, de Lima Morais DA, Chang L, Barrette M, et al. Metaboanalyst 5.0: narrowing the gap between raw spectra and functional insights. Nucleic Acids Res 2021;49:W388-96.
24. Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. Nat Commun 2019;10:1-10.
25. Chandrashekar DS, Bashel B, Balasubramanya SAH, Creighton CJ, Ponce-Rodriguez I, Chakravarthi BV, et al. UALCAN: a portal for facilitating tumor subgroup gene expression and survival analyses. Neoplasia 2017;19:649-58.
26. Ősz Á, Lánczky A, Győrffy B. Survival analysis in breast cancer using proteomic data from four independent datasets. Sci Rep 2021;11:1-15.
27. Morasso C, Ricciardi A, Sproviero D, Truffi M, Albasini S, Piccotti F, et al. Fast quantification of extracellular vesicles levels in early breast cancer patients by Single Molecule Detection Array (SiMoA). Breast Cancer Res Treat 2022;192:65-74.
28. Caron C, Lestrat C, Marsal S, Escoffier E, Curtet S, Virolle V, et al. Functional characterization of ATAD2 as a new cancer/testis factor and a predictor of poor prognosis in breast and lung cancers. Oncogene 2010;29:5171-81.
29. Kalashnikova EV, Revenko AS, Gemo AT, Andrews NP, Tepper CG, Zou JX, et al. ANCCA/ATAD2 overexpression identifies breast cancer patients with poor prognosis, acting to drive proliferation and survival of triple-negative cells through control of B-MLB and EZH2. Cancer Res 2010;70:9402-12.
30. Liu Q, Liu H, Li L, Dong X, Ru X, Fan X, et al. ATAD2 predicts poor outcomes in patients with ovarian cancer and is a marker of proliferation. Int J Oncol 2020;56:219-31.
31. Soria-Bretones I, Sáez C, Ruíz-Borrego M, Japón MA, Huertas P. Prognostic value of CtIP/RBBP8 expression in breast cancer. Cancer Med 2013;2:774-83.
32. Hiraike H, Wada-Hiraike O, Nakagawa S, Saji S, Maeda D, Miyamoto Y, et al. Expression of DBC1 is associated with nuclear grade and HER2 expression in breast cancer. Exp Ther Med 2011;2:1105-9.
33. Pisitkun T, Shen R-F, Knepper MA. Identification and proteomic profiling of exosomes in human urine. Physiology 2004;101:13368-73.
34. Steiner L, Eldh M, Offens A, Veerman RE, Johansson M, Hemdan T, et al. Protein profile in urinary extracellular vesicles is a marker of malignancy and correlates with muscle invasiveness in urinary bladder cancer. Cancer Lett 2025;609:1-10.



uEV=urinary extracellular vesicles, CD9=cluster of differentiation 9, TSG101=tumor susceptibility gene 101, THP=tamm-horsfall protein

Supplementary Figure 1 Western blotting analysis of isolated uEVs from pre- and post-surgical samples for characterization of urinary extracellular vesicles (uEVs); EV protein markers, including CD9, Alix, TSG101, and THP, were expressed in the urine samples and were enriched in the isolated uEV samples. In contrast, cytochrome c, used as a negative control, was not detected in any of our samples.

Supplementary Table 1-6 File: https://drive.google.com/file/d/1KiKtL4b_-HnhsEnGTbjMrQhCqW1mFw2q/view?usp=sharing (PDF)