Sequential Method for Analysis of CTCs and Exosomes from the Same Sample of Patient Blood

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ABSTRACT: Circulating tumor cells (CTCs) and exosomes, both released from the primary tumor into peripheral blood, are a promising source of cancer biomarkers. They are detectable in the blood and carry a large diversity of biological molecules, which can be used for the diagnosis and monitoring of minimally invasive cancers. However, due to their intrinsic differences in counts, size, and molecular contents, studies have focused on only one type of vesicle. Herein, we have developed an integrated system to sequentially isolate CTCs and exosomes from a single patient blood sample for further profiling and analysis. The CTCs are isolated using a commercial filtration method and then the remaining blood is processed using multiple cycles of ultracentrifugation to isolate the exosomes. The method uses two available technologies where the eluent from CTC isolation is usually discarded and interfaces them, so that the eluent can be interfaced to exosome isolation methods. The CTCs are identified based on fluorescence staining of their surface markers, while the exosomes are analyzed using transmission electron microscopy, nanosight tracking analysis, and mass spec proteomic analysis. This analysis showed CTCs detected by their surface markers for metastatic hepatocellular carcinoma (HCC), while essentially none were detected for cirrhosis. The exosome analysis resulted in the identification of ~500−1000 exosome proteins per sample confirmed by detection of exosome surface markers CD9, CD63, CD81, and TSG101 in addition to proteins related to cancer progression. Proteins enriched in HCC exosomes were shown to be involved in the immune response, metastasis, and proliferation.
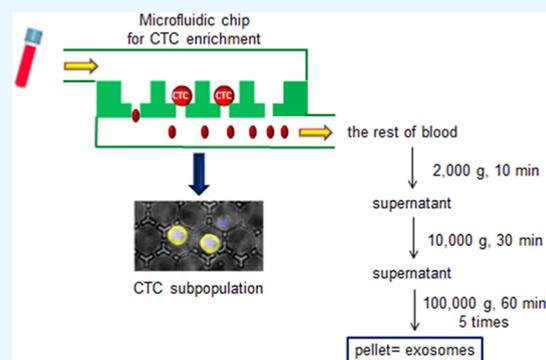
INTRODUCTION

An important area of research is developing biomarkers to monitor changes in patient status during the progression of cancer. This may involve the early detection, diagnosis, prognosis, or monitoring of anticancer therapies. Minimally invasive methods for monitoring patients in the clinic are highly desirable. These generally include analysis of biofluids such as blood or urine which can be readily obtained during routine clinic visits. Methods that could monitor more than one type of vesicle would be valuable in assessing patient response to therapy.

There are several methods that can serve to analyze markers in patient blood. One such method involves the detection of circulating tumor cells (CTCs). CTCs are released from the primary tumor and travel through the blood to distant organs where they may form metastases.[1] These cells are very heterogeneous with multiple sub-populations which may have different functions and levels of aggressiveness. The distribution of these sub-populations may be very different than that of the primary tumor reflecting different survival capabilities of each type of circulating cell. Characterization of these CTCs is thus critically important for understanding the progression of cancer and in turn may be valuable as

biomarkers for diagnosis, prognosis, and ultimately for treatment response.[2−12] CTCs can be isolated from blood by a number of techniques including affinity methods,[12] filtration,[13] and microfluidic devices.[14]

Another potential source of biomarkers involves the use of exosomes or other types of microvesicles secreted by cells into the bloodstream.[15−26] Their secretion has been observed from most types of normal cells and tumor cells where they carry various cargos including proteins, miRNAs, mRNAs, metabolites, and lipids throughout the body and act as a means of intercellular communication to transport signals to other organs. Tumor-derived exosomes usually contain tumor-related antigens and contain many of the diagnostic CD membrane proteins which are upregulated in cancer.[15] These exosomes have been identified as a potential method for early detection and to monitor response to cancer therapies in a

variety of patient fluids including serum/plasma,[26,34] urine,[27] saliva,[28] and fecal matter. Exosomes can be isolated from these biofluids by several techniques[29−32] including ultracentrifugation,[35,36,44] ultrafiltration,[33] size exclusion chromatography,[33,36−38] immunoaffinity methods,[39−43] polymer precipitation,[44,45] and chromatographic methods[46] among others.

An issue with the isolation of CTCs is that they must be isolated from fresh whole blood, as CTCs lose viability after a 24 h period. Also, most CTCs are present in peripheral blood at the level of several CTCs per milliliter, so that a significant amount of blood, usually 6−10 mL, is required for their analysis. When CTCs are isolated for further analysis, a large amount of remaining blood components are discarded. The discarded blood after CTC isolation is a missed opportunity, as the remaining fluid may be an important source of other biomarkers, such as exosomes. Exosomes could be isolated from the remaining fluid and further analyzed for their content, but the large amount of eluent from the CTC isolation is not compatible with exosome isolation methods. After CTC isolation, there may be a significant exosome content which can be used to obtain a second set of potential markers for cancer progression or therapeutic monitoring.

In this work, we present a dual liquid biopsy method for analysis of CTC and exosome content of blood from a single patient. The method involves a filtration method to first isolate CTCs from white blood cells (WBCs) and then an isolation of single cells for further staining and identification of sub-types of these cells. The remaining eluent from blood is then processed to reduce the volume, so that it is compatible with methods for analysis of exosomes and other microvesicles. We demonstrate that following CTC filtration, exosomes can be isolated from the remaining fluid and that they can be analyzed for their proteomic content. This method provides the possibility for multiple types of markers to be obtained from a patient sample for potential clinical applications.

■ MATERIALS AND METHODS

The workflow for this dual CTC/exosome enrichment platform is shown in Figure 1. We first used a chip-based
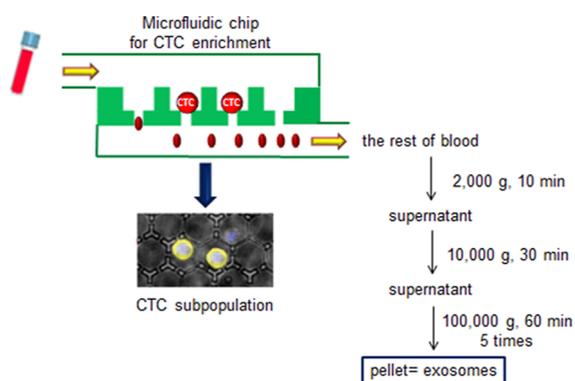

Figure 1. Dual CTC/exosome enrichment platform.

filtration platform to isolate CTCs from patient blood for marker identification and cell count, followed by isolation of the exosome content of the remaining fluid using ultracentrifugation (UC). The CTCs (12−25 μm) were isolated from patient's blood using a size-based filtration device with 8 μm filters (Celsee), where most red blood cells (RBCs) and WBCs pass through leaving behind the larger CTCs. The

CTCs were then flushed out into a single-cell chip, where they undergo staining and visualization. The residual fluid following CTC extraction can then be used to isolate exosomes (30−120 nm) using UC. The exosomes can then be characterized by several techniques to ascertain their size distribution, numbers, and purity.

Isolation of CTCs via a Microfluidic Chip. For isolation of CTCs, we used the Celsee (Ann Arbor, MI) microfluidic chip which was set up on the Celsee Prep100 instrument. A 4 mL blood sample was drawn and collected into an anticoagulant tube coated with K_3EDTA (BD Vacutainer, San Jose, CA). The collected blood samples were placed at room temperature before CTC isolation. The blood sample was diluted with an equal volume of PBS dilution buffer and loaded into the inlet funnel. After running the diluted blood sample through the microfluidic chip, which is shown in Supporting Information Figure S1, the flow through blood was collected for exosome isolation. The microfluidic chip has a hexagonal configuration, as shown in the figure, and CTCs are captured while WBCs and RBCs pass through. The cells were washed four times, and the fluid (containing CTCs) was removed from the inlet and placed into a separate tube. After centrifugation at 1000 rpm for 3 min, the supernatant was removed, and the potential CTCs in the tube bottom were suspended by blowing up with a pipet repetitively. The suspension was used for CTC number counting under a microscope (Nikon Ti, Japan) and then for CTC immunohistochemistry.

CTC Immunohistochemical Staining. For CTC immunohistochemistry, 1 mL of primary antibody cocktail containing 5 μL PanCK and 5 μL CD45 was prepared from the CTC immunohistochemistry kit (cat# 9701, Celsee, Ann Arbor, MI). A related secondary antibody was also prepared by adding 1.5 μL of a second antibody conjugated with AlexaFluor 488 and 1.5 μL of a second antibody conjugated with AlexaFluor 647 into 1 mL of dilution buffer. The potential air bubbles were removed from the microfluidic chip with priming buffer passing through the chip using the Primer Genie machine (Celsee, Ann Arbor, MI). The collected potential CTCs in suspension were sent through the microfluidic chip using a syringe. The CTCs then underwent cell fixation, permeabilization, and blocking with related reagents in the reagent cartridge. The CTCs were incubated for 45 min with 1 mL of PanCK (Clone C11, BioLegend, CA) and CD45 primary antibody (Clone F10-89-4, AbD Serotec, CA). After washing, 1 mL of secondary antibody [antibody cocktail of AlexaFluor 488 and AlexaFluor 594 (Life Technologies, CA)] was added and the entire system was incubated in the dark for 30 min. The cell nucleus was stained with 4′,6-diamidino-2-phenylindole (DAPI) solution from the reagent cartridge in the dark for 5 min. After washing with 3.5 mL of PBS solution, the microfluidic chip was observed under a fluorescence microscope (Nikon Ti, Japan).

The CTCs inside the chip can be detected by immunohistochemistry, but retrieval of individual CTCs from the chip requires additional work. Thus, we also conducted immunofluorescence with the CD90/CD45 (Abcam, MA) antibody pair using the single-cell chip (Celsee, Ann Arbor, MI) in the Celsee staining station. After setting the single-cell chip on the staining station, it was washed using 1 mL of 70% ethanol solution, following which 0.8 mL of alcohol solution was removed with a pipet from the single-cell chip. One mL of PBS was added into the single-cell chip and 0.7 mL

of solution was removed from the single-cell chip. The CTC suspension was added into the single-cell chip and incubated for 40 min for the CTCs to settle. After blocking with 0.2% BSA solution, 1 mL of CD90/CD45 primary antibody pair solution was added into the single-cell chip and incubated for 1 h. After washing with PBS solution three times, 1 mL of secondary antibody was added into the chip and incubated for 30 min. DAPI was added and incubated for 15 min. The single-cell chip was washed using PBS solution three times and was examined for CTCs under a fluorescence microscope.

Pretreatment of the Residual Fluid following CTC Isolation. The residual fluid following CTC isolation was collected (∼8 mL) and centrifuged at $1000g$ for 10 min at 4 °C to remove blood cells. The supernatant was about 4 mL and was transferred into a clean centrifuge tube for centrifugation at $2000g$ for 10 min at 4 °C to deplete platelets. The resulting supernatant was the plasma which was transferred into another tube and centrifuged at $10,000g$ for 30 min at 4 °C to remove cell debris and large extracellular vesicles (EVs). The supernatant (4 mL) was filtered through a 0.22 μm filter prior to UC.

Exosome Enrichment Using Five Cycles of UC. Exosome enrichment was performed using a Beckman Optima XL-70 ultracentrifuge. Each sample after the pre-clear step (4 mL) was transferred into an ultra-clear tube (Beckman Coulter, Indianapolis, IN) and centrifuged first at $110,000g$ for 120 min at 4 °C, followed by five cycles of UC for 70 min each at 4 °C. Between each UC step, the supernatant was carefully removed by a pipet without disturbing the pellet, retaining ∼2 mm of the supernatant at the bottom of the tube. The pellet was then suspended with 4 mL of PBS. Our previous studies have demonstrated that five cycles of UC are required to eliminate the blood proteins.[30,31] This procedure can avoid contamination with blood proteins for the analysis of the exosomal proteome. The final pellet was the purified exosomes and was kept in 100 μL of PBS for subsequent analyses.

Transmission Electron Microscopy. The exosomes were assessed by transmission electron microscopy (TEM) using negative staining. First, the 200 mesh Formvar-/carbon-coated grid (Electron Microscopy Sciences, Hatfield, PA) was processed by glow discharge to make the surface hydrophilic. Then, 5 μL of exosome preparation was loaded on the grid and incubated for 5 min. The liquid was removed using a filter paper. Next, the grid was negatively stained using 5 μL of 1% uranyl acetate for 1 min and dried using a filter paper. TEM images were obtained on a JEOL 1400-plus transmission electron microscope.

NanoSight Analysis. The concentration and size distribution of exosomes were measured using a NanoSight NS300 (Malvern, UK). Each exosome sample was diluted with PBS to 500 μL and automatically infused into the NanoSight at a flow rate of 10 μL/min. The exosomal motion was captured on video five times, 1 min each. After capture, the videos were analyzed by the built-in software NTA to generate the histogram of particle size distribution and concentration.

Exosomal Protein Extraction and Digestion. Exosomal protein extraction and tryptic digestion were performed using the filter-aid sample preparation method. After removing the supernatant, the exosome pellet was lysed with 30 μL of lysis buffer composed of 0.1 M Tris-HCl, 4% SDS, and 0.1 M 1,4-dithiothreitol at 99 °C for 5 min. The extract was cooled down to room temperature, mixed with 200 μL of 8 M urea,

transferred to a centrifugal spin YM-30 filter (Millipore, Billerica, MA), and centrifuged at $14,000g$ for 20 min. Then, 100 μL of 50 mM iodoacetamide in 8 M urea was added to the filter and incubated in the dark for 20 min. The filter was then subsequently washed with 8 M urea and 50 mM NH_4HCO_3, respectively, three times each. Then, 400 ng of trypsin (Promega, Madison, MI) in 75 μL of 50 mM NH_4HCO_3 was added to the filter and incubated at 37 °C overnight. The released peptides were collected by centrifugation and desalted with a ZipTip C18 tip (Millipore, Billerica, MA). The eluted peptides were dried down in a SpeedVac concentrator (Thermo) for the following mass spectrometry analysis.

Nano-LC-MS/MS. Exosomal peptides were dissolved in 0.1% formic acid (FA) and analyzed in duplicate on the Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo) coupled with a Dionex UPLC system. A binary solvent system was composed of H_2O containing 0.1% FA (A) and 80% CH_3CN containing 0.1% FA (B). Peptides were separated on a 75 μm × 50 cm column (Thermo, P/N 164942) under a 90 min linear gradient from 2 to 40% B at a flow rate of 300 nL/min.

The MS instrument was operated in positive ion mode and the data were acquired in a data-dependent mode. MS1 spectra (m/z 375−1700) were acquired in the Orbitrap (120k resolution, 2×10^5 AGC target). Up to 20 most intense MS1 peaks were selected for tandem MS2 analysis by collision-induced dissociation in the linear ion trap. The normalized collision energy was set at 34% for MS/MS. The isolation width was set at 1.6. Dynamic exclusion was enabled with an exclusion window of ±10 ppm and an exclusion duration of 40 s.

Data Analysis. All MS/MS spectra were searched against the human UniProt database using SEQUEST (Proteome Discoverer 1.4, Thermo Scientific). The search parameters were as follows: fixed modification, carbamidomethyl (C); variable modifications, oxidation (M); up to 2 missed cleavages allowed; MS1 mass tolerance 10 ppm; MS2 mass tolerance 0.6 Da. Identified peptides were filtered using a 1% false discovery rate.

The proteins identified in each sample were then subject to ingenuity pathway analysis (IPA) to obtain molecular information on the subcellular distribution, function, and canonical pathway annotations of these proteins.

■ RESULTS AND DISCUSSION

To demonstrate the method, we have collected blood from five patients with intermediate or advanced stage hepatocellular carcinoma (HCC) compared to three control patients with cirrhosis. The median age of the patients is 62 with three hepatitis C virus-related HCCs and two alcohol-related HCCs. Additional data on these patients is shown in Supporting Information Table S1. These blood samples were collected in K_3EDTA-coated tubes to prevent clotting, and the samples were processed within 6 h of collection. Four mL of blood was diluted 1:1 with PBS to decrease the viscosity and then CTCs were isolated by the Celsee PREP device as described above and were isolated in the single-cell device for immunofluorescence staining and visualization. This device also allows for subsequent cell picking where a variety of other experiments are possible.

The cells collected in the single-cell device were stained for CTC markers including EpCAM (see Figure 2), PanCK (see Figure 3), and CD90 (see Figure 4). They were also stained for
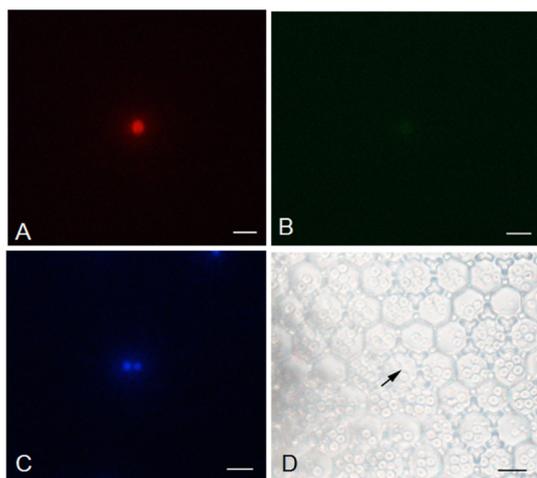
Figure 2. Immunofluorescence staining of enriched CTCs from HCC patient. Representative images of staining by antibody EpCAM (red, A) and antibody CD45 (green, B); the nucleus was stained with DAPI (blue, C), and the size and shape were confirmed in bright field (D). Scale bars, 20 μm.
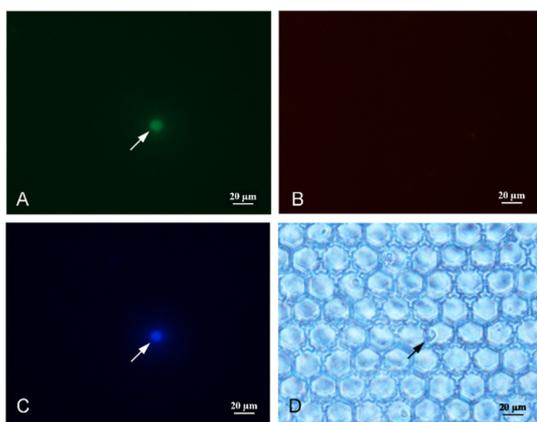

Figure 3. Representative images of CTC (white arrow) from the HCC patient. CTC was detected using immunofluorescence staining for PanCK (green, A) and CD45 (red, B); the nucleus was stained with DAPI (blue, C), and the size and shape were confirmed in the bright field (D). Scale bars, 20 μm.

Figure 4. Immunofluorescence image of CTC stained with anti-CD90 (green, A), anti-CD45 (red, B), and DAPI (blue, C). The bright-field image (D) shows the CTC size and shape. Scale bars, 20 μm.

Table 1. CTCs from HCC and Cirrhosis Patient Samples

sample	blood volume (mL)	immunofluorescence staining	CTC number
HCC1	4	PanCK+/CD45−	14
HCC2	4	PanCK+/CD45−	3
HCC3	4	EpCAM+/CD45−	4
HCC4	4	PanCK+/CD45−	1
HCC5	4	CD90+/CD45−	9
			average, 6
Cirr1	4	PanCK+/CD45−	1
Cirr2	4	PanCK+/CD45−	1
Cirr3	4	PanCK+/CD45−	0

Table 1 shows the number of CTCs detected in each sample for either PanCK or other markers. We could only stain for one marker in each sample, so we used PanCK for most cases where this is a general CTC marker. In general, even in 4 mL of blood, there are only a limited number of CTCs since these are rare cells. We observed an average of 6 CTCs per 4 mL sample in this set for the HCC samples, while there are significantly fewer CTCs, that is, less than 1 CTC in the cirrhosis samples as expected since this is a non-cancer state. The HCC samples are all late-stage samples (see Supporting Information Table S1) and non-curative, except for HCC4 which has a low number of CTCs and the patient was cured.

CTC/Exosome Interface. The flow through from the CTC experiment was then collected to isolate the exosome content of the fluid. One of the main issues is the large amount of liquid that results from additional dilution buffer where initially 8 mL of fluid (blood plus PBS) is processed and collected. This fluid contains large numbers of red and white blood cells which must be eliminated. Thus, an initial low-speed (2000g) centrifugation was used to eliminate these cells, where the exosomes remained in the fluid. After this step, the volume is reduced from 8 to 4 mL, where a large number of cells have been eliminated. Further centrifugation was used to eliminate other cell debris, large bioparticles, and some proteins dissolved in the blood. To isolate the exosomes, multiple cycles of UC were used as described in previous work.[35]

Exosome Characterization. The enriched exosomes were characterized by TEM and NanoSight Tracking Analysis

CD45 to check for the presence of WBCs and for DAPI to verify the presence of a nucleus and that we are dealing with the presence of a cell. There were only three fluorescence channels available where each sample needed to be stained for CD45 and DAPI leaving only one channel for a selected marker. In general, there was only enough material per sample to stain for one of the markers, where the samples stained for each are shown in Table 1. The staining of HCC CTCs is shown in Figures 3 and 4 for the markers PanCK and CD90, where PanCK is a marker for the presence of CTCs. Figure 4 shows a blown-up version of the single-cell chip and the presence of a CD90 CTC, where CD90 is an important cancer stem cell marker in HCC. The lack of staining for CD45 shows that these are not WBCs and they all stain for DAPI. We did not observe WBCs in the fields of view shown in Figures 2−4; however, there are some WBCs observed in the larger field. This is shown in supplemental Figure 2, where WBCs stain for CD45. There were not enough CTCs in the cirrhosis samples to stain where there was <1 CTC on average.
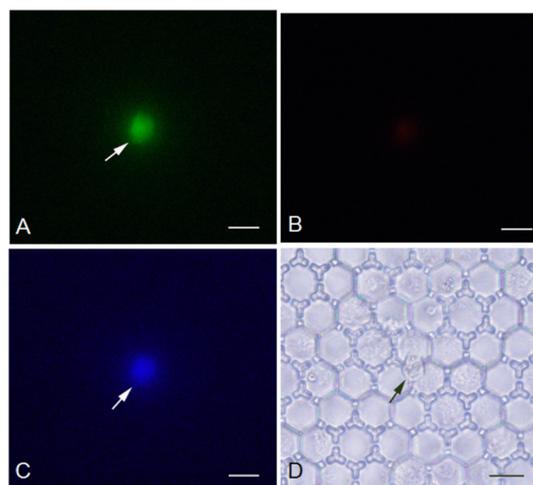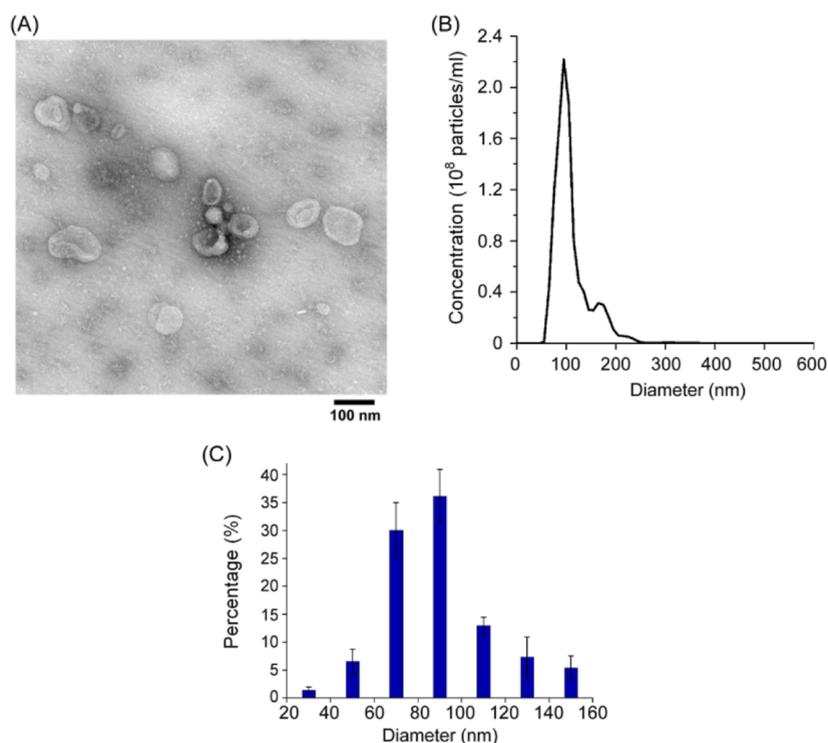
Figure 5. Characterization of exosomes enriched from the residual fluid following CTC isolation. (A) TEM image showing the morphology of exosomes. Scale bar = 100 nm. (B) NTA by NanoSight, demonstrating the size and concentration of exosomes, 90% of which are within the size range of 30−150 nm. (C) Size distribution of the exosomes across five samples. The mean diameter of exosomes was 95 nm. Error bars indicate standard derivations.

(NTA). The morphology of the exosomes was assessed by TEM through negative staining, which showed that the exosomes were spherical, membrane-encapsulated particles in the size range of 35−130 nm (Figure 5a).

The concentration and size distribution of exosomes were further evaluated by NanoSight, which can overcome some limitations of TEM in absolute quantification and rapid size determination of exosomes. The nanoparticle tracking analysis (NTA) provides a real-time visualization/measurement of individual nanoparticles in liquids by video recording to create a size distribution and particle numbers. Figure 5b shows the NTA result of five videos to determine the particle concentration and size distribution of exosomes. A major peak around 100 nm was observed for exosomes with a concentration of 2.2×10^8 particles/mL. The NTA result showed that 90% of exosomes were within the expected size range of 30−150 nm, confirming a high purity of exosomes enriched from the residual fluid after CTC isolation. Minor signals were observed between 150 and 250 nm, less than 10% of particles (Figure 5b), accounting for large EVs that were co-isolated with exosomes.

Figure 5c shows the histogram of size distribution of exosomes across the five HCC samples, based on the NTA result. The average diameter of the enriched exosomes was 95 nm. Over 65% of EVs were within the size range of 60−100 nm.

Exosome Proteome Profiling. Exosomal proteins were identified by mass spectrometry. Proteomic analysis showed that around 500−1000 proteins were identified in exosomes from the various samples collected after CTC analysis, which is consistent with the results from our prior studies[35] (see Supporting Information Tables S2−S6). The number is higher than previous studies though because of the large amount of starting material where 4 mL of blood was used for initial processing. Sample HCC1, for example, was found to contain over 1000 proteins from three replicates. Exosome markers CD9, CD63, CD81, and TSG101 were observed among these identified proteins. Also, CD proteins CD151 and CD44 which are associated with metastatic cancer were observed. Several proteins associated with immune evasion were also detected including CD47 and CD59. Syndecan-1 and syntenin which are markers of exosomes were also observed.[47] Several members of the ALDH family were observed which is associated with the cancer stem cell population (CSC), but we did not detect the CD90 CSC marker in the exosomes. Other cancer-related proteins such as Ras-related proteins and glycophorin were observed in the exosomes. Several Ras-related proteins were detected, where Ras is a key component of pancreatic and other GI cancers. Vimentin, which is a protein associated with the epithelial to mesenchymal transition in metastasis, was also observed.[48] Platelet factor 4, which has been shown to be an important marker for HCC and pancreatic cancer, was observed in most samples.[35,49] Platelet factor 4 and vimentin will be important markers of metastasis together with the CTC count in future studies. Several members of the annexin family of proteins were detected (see Table 2), where these are often important markers of cancer. Increased expression of ANXA2 protein has been correlated with invasion and metastasis in a variety of human cancers. In clinical studies, ANX A2 protein expression has been correlated with aggressive cancers and with resistance to anticancer drugs.[50] Hsp70 and Hsp90 were also detected, where HSP70 has been an important marker of EVs,[43] and both these proteins are often detected as upregulated in cancer.

Table 2. Lists of (a) Exosome-Related and (b) Cancer-Related Protein Markers Identified in This Study

category	description
(a) exosome-related protein markers detected	CD81
	CD63
	CD9
	TSG101
	syndecan-1
	syntenin
(b) cancer-related protein markers detected	CD44
	CD151
	glycophorin
	platelet factor 4
	Ras-related proteins
	annexin A1
	annexin A2
	annexin A3
	annexin A4
	annexin A5
	annexin A6
	annexin A7
	annexin 11
	ALDH A1

An IPA based on the proteins detected revealed signature proteins involved in the networks relevant to immune evasion, cellular movement, and cell-to-cell signaling and interaction.

CONCLUSIONS

In this study, we developed a dual CTCs/exosomes liquid biopsy by sequential isolation of CTCs and exosomes from the same patient blood for biomarker assessment. A filtration method was used to isolate CTCs, while multiple cycles of UC were used for isolation of EVs. An important advantage of this method is that it uses all commercially available equipment and reagents. The CTCs tested positive for the general marker of PanCK and stained for DAPI but negative for CD45, the WBC marker. Also, a much higher number of CTCs were observed in the HCC samples compared to the patients with cirrhosis where essentially no CTCs were observed. Proteomic analysis of exosomes enriched from the same blood sample revealed significant proteins related to cancer progression and metastasis and the characteristic protein surface markers associated with exosomes. The combination of CTC phenotype characterization and exosomal proteome profiling from the same blood sample may provide a potential complementary tool for discovery of cancer biomarkers in future work, especially for therapeutic monitoring in late stages in patients with a significant number of CTCs.

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c04428.

Additional clinical information on patient blood samples; list of proteins detected in mass spec analysis of EVs collected from sample HCC1; list of proteins detected in mass spec analysis of EVs collected from sample HCC2; list of proteins detected in mass spec analysis of EVs collected from sample HCC5; list of proteins detected in mass spec analysis of EVs collected from sample Cirr1; list of proteins detected in mass spec

analysis of EVs collected from sample Cirr2; Celsee filtration device for isolation of CTCs from other blood components; and staining of WBCs from patient sample in the Celsee device using a CD45 antibody (XLSX)

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Author Contributions

‖J.Z. and Z.T. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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