

Isolation and Detection Technologies of Extracellular Vesicles and Application on Cancer Diagnostic

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Abstract

The vast majority of cancers are treatable when diagnosed early. However, due to the elusive trace and the limitation of traditional biopsies, most cancers have already spread widely and are at advanced stages when they are first diagnosed, causing ever-increasing mortality in the past decades. Hence, developing reliable methods for early detection and diagnosis of cancer is indispensable. Recently, extracellular vesicles (EVs), as circulating phospholipid vesicles secreted by cells, are found to play significant roles in the intercellular communication as well as the setup of tumor microenvironments and have been identified as one of the key factors in the next-generation technique for cancer diagnosis. However, EVs present in complex biofluids that contain various contaminations such as nonvesicle proteins and nonspecific EVs, resulting in the interference of screening for desired biomarkers. Therefore, applicable isolation and enrichment methods that guarantee scale-up of sample volume, purity, speed, yield, and tumor specificity are necessary. In this review, we introduce current technologies for EV separation and summarize biomarkers toward EV-based cancer liquid biopsy. In conclusion, a novel systematic isolation method that guarantees high purity, recovery rate, and tumor specificity is still missing. Besides that, a dual-model EV-based clinical trial system includes isolation and detection is a hot trend in the future due to efficient point-of-care needs. In addition, cancer-related biomarkers discovery and biomarker database establishment are essential objectives in the research field for diagnostic settings.

Keywords

extracellular vesicles, isolation, cancer diagnostic

Introduction

Extracellular vesicles (EVs), as phospholipid vesicles secreted by cells,¹ can be classified into 3 categories: apoptotic bodies, microvesicles (MVs), and exosomes.¹⁻⁵ Among them, MVs originate from the buddings of the plasma membranes of tumors, neutrophils, and platelets¹ with the diameter larger than 200 nm,⁶ while exosomes are derived from the inward buddings of the plasma membranes⁷ with the size between 30 and 200 nm.⁸ Firstly, the inward buddings of the membrane inside the endosome produce the multivesicular bodies (MVBs),⁹ then exosomes are released because MVBs fuse with the plasma membranes of various cells.¹⁰⁻¹² In virtue of appropriate isolation technologies, EVs can be easily extracted from blood,¹³ urine,¹⁴ breast milk,^{15,16} saliva,¹⁷ and cerebral spinal fluid body fluids, and contain proteins,¹⁸⁻²⁰ messenger RNAs (mRNAs), microRNAs (miRNAs),²¹⁻²³ and DNA²⁴ with similar genetic characteristics of their parenting cells.^{25,26} Hence, EVs can be used for diagnostic settings, especially for cancers.²⁷⁻³⁰ In

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addition to EVs, circulating tumor cells (CTCs) and circulating mRNA can be also used for liquid biopsy.^{31,32} However, their undesirable maneuverability and high minimum amount for detection limit further application. In this review, we will highlight the EV-based liquid biopsy.

Comparing with traditional tissue biopsies that require complex sampling procedures and are invasive,³¹ EV-based liquid biopsy shows advantages of monitoring tumors with time evolution,³³⁻³⁶ long-term treatment response of the tumors, repeated sampling,^{37,38} and easy sampling managements.^{39,40} Moreover, traditional biopsies based on symptoms may fail to detect early-stage cancer because some symptoms only show at late-stage cancers; on the other hand, EV-based liquid biopsy, on genetic level, can waive the symptoms limitations and detect early-stage cancer. Extracellular vesicle-based liquid biopsy is also important for liver diseases,^{41,42} immune diseases,^{43,44} neuro diseases,⁴⁵ and Parkinson disease.⁴⁶ We only focus on cancer diagnostic in this review paper.

Extracellular vesicles show potential cancer diagnostic functions; however, EV-based liquid biopsy is limited in identifying EVs since they have size heterogeneities (30 nm to 1 μm)²⁵ and present in various human biofluids that contain outside vesicular small molecules (eg, RNAs and proteins).⁴⁷ Hence, efficient EV isolation methods are necessary before clinical analysis. In addition, normal EVs secreted by host cells influence the specificity and sensitivity of tumor EVs,⁴⁸ which can be possibly resolved through tumor biomarker immunofluorescence, so defining reliable cancer biomarkers is essential.

This review article presents recent EV isolation and characterization techniques and highlights the diagnosis progresses among various cancers. We compare the isolation methods by purity, recovery rate, processing time, and tumor specificity and evaluate the characterization techniques. Even though some reviews have published, we believe this review is helpful because of compiling latest diagnostic progresses for various cancers.

Extracellular Vesicle Isolation and Enrichment

Various isolation methods have been developed in the past decades and have been evaluated by recovery rate, purity, integrity, tumor specificity, and processing time.⁴⁹ In this review, we present bulk isolations that rely on size, density, coprecipitation, and affinity binding,⁴⁹ and subpopulation isolation methods that isolate and enrich subpopulation by targeting antibodies against EV surface biomarkers include common biomarkers (eg, CD9, CD63, and CD81)^{50,51} and tumor-specific biomarkers (eg, HER2, EpCAM, EGFR, EGFRvIII, and GPC1).^{22,52-54}

Size-Based Isolation

Ultracentrifugation. Ultracentrifugation (UC) is the gold standard protocol for isolating EVs.⁵⁵ Ultracentrifugation can scale-up sample volume and isolate EVs with high centrifugal force

(100 000g),⁵⁶ while expensive equipment, skill dependent, time consuming, low purity, and yield are the drawbacks. **Tangential flow filtration (TFF):** Comparing with traditional dead-end filtration, TFF is a cross-flow filtration that avoids the filter cake and fractionates EVs through a module contains hollow fiber filters.⁵⁷ When the sample flows through the module, small molecules (permeate) pass through the hollow fiber filters, while EVs (retentate) are remained inside the module. Tangential flow filtration has been applied to isolate EVs from cell culture medium with 500 kDa hollow fiber filters, and enriched EVs from scalable sample volume at the meantime purified EVs with high recovery rate (5 times higher than UC) in a rapid, sterilized manner.⁵⁷ **Size-exclusion chromatography:** Size-exclusion chromatography (SEC) isolates EVs based on gel column with specific size pores.⁵⁸⁻⁶⁰ When the sample goes through the gel, smaller molecules are trapped into the pores, while EVs flow through directly that come faster than smaller molecules. Commercial columns (qEV; iZON, Boston, MA) have been applied to separate EVs from various biofluids such as plasma, serum, and cell culture media. Takov et al recently compared UC and qEV and showed qEV contained high EV number, EV protein, and stronger marker signal than UC; nevertheless, the qEV failed to remove all non-EV proteins.⁶¹ Based on SEC principle, there is a balance between purity and recovery rate. Moreover, several factors such as pore size, structure, length of column, and loading volume can contribute to the yield and purity to some extent.

Density-Based Isolation

Sucrose density gradient UC separates EVs in a continuous size-based UC mode; serial ultracentrifugations are applied to remove living cells and cell debris, then the EV and proteins are separated based on different flotation densities under UC.⁶² Despite the high purity, sucrose has lengthy problem due to multiple centrifugations, and the recovery rate is low due to multiple centrifugations.

Coprecipitation Isolation

Coprecipitation reduces the solubility of EVs by adding polymer or reagent (eg, ExoQuick) and causes precipitation, and EVs are isolated from precipitation with lower centrifugal forces later.^{63,64} Comparing with UC, coprecipitation saves processing time but lacks scale-up and EV specificity due to additives.

Affinity Binding-Based Isolation

Membrane affinity binding. Recently, commercial kits (eg, exoEasy) use membrane-based affinity binding to isolate EVs,⁶⁵ which are selected based on generic, biochemical feature of vesicles and fixed on the affinity spin column that can be washed and eluted with buffers. The advantages of kits are easy handling, high purity, and extremely fast (25 minutes); however, low throughput and recovery rate are the

Table 1. Comparison of EV Enrichment, Separation, and Purification Methods.

Platform	Principle	Advantages	Limitations
Ultracentrifuge	Size	Large scale, gold standard	Lengthy, low yield, low purity, expensive equipment, lack tumor specificity
Tangential flow filtration	Size	Large scale, high purity, yield, rapid, integrity	Lack tumor specificity
Size-exclusion chromatography	Size	User-friendly, relative high yield	Low purity, small scale, lack tumor specificity
Sucrose density gradient centrifuge	Density	User-friendly, gold standard, purity	Lengthy, expensive equipment, low yield, no tumor specificity
Coprecipitation	Charge	User-friendly	Lack tumor specificity, small scale
Membrane affinity binding	Surface	High yield, integrity	Lack tumor specificity, small scale
TiO ₂ and lipid bilayer binding	Surface	High yield, integrity	Lack tumor specificity, small scale
Immunoaffinity	Surface	Tumor specificity	Small scale, low recovery rate, low purity

shortcomings. Macías et al tested the kit and demonstrated it had low EV number and weak CD63 and CD9 Western blotting signal.⁶⁶ **TiO₂ and lipid bilayer of EV binding:** Gao et al utilized the advantage of the specific interaction between titanium oxide and EV phosphate lipid bilayer to separate EVs from serum.⁶⁷ Micron-sized TiO₂ particles enriched EVs through the bidentate binding with high recovery rate (93.4%) in a simple manner. They also tested the platform with patients with pancreatic cancer (PC) and found 29 novel proteins, which showed high potential diagnostic function. However, this method is limited by low throughput.

Immunoaffinity

One of the advantages of immunoaffinity compared to bulk isolation is the EV subpopulation isolation with high recovery rate and specificity. The immunoaffinity is the specific affinity between an antibody and antigen, which normally means the bindings between a capture antibody, a EV surface antigen, and a detection antibody.^{68,69} Shao et al applied immunomagnetic beads to extract glioblastoma multiforme (GBM) EVs with 93% specificity, and they detected EPHA2, EGFR, and PDPN mRNA from patients with GBM successfully.⁷⁰ Recently, Sharma et al used the monoclonal antibody (mAb) with magnetic beads to capture tumor EVs from patients with melanoma that expresses CSPG4 epitope.⁷¹ Masud et al applied gold-loaded ferric oxide nanocubes functionalized with antibodies that work as “dispersible nanocarriers” on separating population of EVs.⁷² Bai et al have captured lung cancer EVs through queued beads functionalized with antibodies and combined with quantum dots in a microarray, which showed distinctive lung cancer marker detection level.⁷³ Immunoaffinity can isolate EV subpopulation, especially for tumor EVs, while limited sample processing volume is a big challenge for the immunoaffinity.

Comparison

Different isolation techniques are compared in Table 1 based on processing time, purity, recovery rate, sample scale, and

tumor specificity. High recovery rate and purity are important because of clinical sensitivity and accuracy.⁴⁷ Flexible sample scale is another key point for clinical settings since various biofluids require different pretreatment, for example, urine must be in large scale to obtain enough EV number, while serum and plasma must be in small scale due to collecting limitation. It is worth to note that immunoaffinity isolation methods can be directly used for point-of-care diagnostics since tumor subpopulation capture.⁷⁴ However, immunoaffinity is highly dependent on the quality of antibodies. Until then, a platform that can guarantee high yield, purity, rapid time, and tumor specificity is still missing.

Extracellular Vesicle Detection and Characterization

Microscopy Quality Characterization

Scanning electron microscopy. Scanning electron microscopy (SEM) is a high-resolution technique in the EV field.^{22,75,76} Extracellular vesicles are fixed by chemicals such as glutaraldehyde and dehydrated by critical point dry in ethanol; osmium tetroxide can be used to increase contrasts. Scanning electron microscopy scans EV surface with a focused beam of electrons, normally a thin sputter gold coating is required for focusing, and generates EV topography image due to the interaction between electrons and atoms in the EV sample. Majorities of EVs present spherical-shaped or cup-shaped morphologies under the SEM.⁷⁷ **Transmission electron microscopy (TEM):** TEM has higher resolution compared with SEM⁷⁷ and has similar procedures for fixation and contrast enhancement. The focused beam of electrons are transmitted through samples to generate images, and molecular contents on the EV surface can be characterized through TEM with immunolabeling.⁷⁸ **Atomic force microscopy (AFM):** AFM is another high-resolution microscopy in EV studies.^{79,80} Extracellular vesicles are fixed on a mica substrate coated with 3-aminopropyltriethoxysilane and are air dried or nitrogen dried after extensive Deionized (DI) water washing steps. A metal probe is used for scanning EV surface to provide surface

topography information under the amplitude modulation and local stiffness and adhesion information under phase modulation.⁸⁰ All 3 microscopies show outstanding images of EVs. Among them, SEM and TEM show higher resolution than AFM but require complex sample preparations. Moreover, TEM is able to characterize molecular content level, while AFM is professional in acquiring stiffness information.

Quantitative Characterization

Dynamic light scattering. Dynamic light scattering (DLS) shows EV size distribution based on the intensity of the scattered light.⁸¹ The suspended EVs are illuminated by a laser, and the intensity of the light fluctuates over time since EV goes through Brownian motion.⁸¹⁻⁸³ The effective size of EVs is calculated by Stokes-Einstein equation based on the transformation between fluctuation rates and diffusivities of the EVs. However, the intensity of the scattered light is also associated with the size of the EV; larger EV size reflects higher intensity, which may influence accuracy. **Nanoparticle tracking analyzer (NTA):** Similar to DLS, a laser beam is used to illuminate EVs in the sample.^{76,84} The path of every single EV under Brownian motion is recorded by a camera,³⁷ then a software such as NanoSight calculates the concentration and size distribution mathematically based on Stokes-Einstein equation that converts velocity and diffusivity of the EV. Unlike DLS that deals with bulk scattering intensity, NTA is capable of tracking a single EV that overcomes polydisperse problems. Both methods provide accurate and sensitive size distributions, while NTA shows concentration quantification. **Tunable resistive pulse sensing (TRPS):** TRPS is capable of measuring both EV concentration and size distribution.⁸⁵⁻⁸⁷ Suspended EV in electrolytes generates different voltage pulse when it passes through a nanopore, and the voltage pulse information can be transferred into size and concentration based on standard calibration sets. Comparing with NTA, TRPS represents higher sensitivity and accuracy since it can measure the narrow size distribution of EVs based on the nanopores, which cover specific size ranges (eg, NP 80, ranges from 40 to 225 nm). Dynamic light scattering, NTA, and TRPS are popular in the EV field for characterizing. Comparing with NTA and TRPS, DLS lacks quantification even though it can detect very small EVs. Both NTA and TRPS provide quantification information. Compared with NTA, TRPS overcomes the detection limit caused by low EV concentration and is more precise in size distribution. But notably, TRPS may need to take effort on nanopore maintenance to avoid EV clogging and instability issues.

Protein Analysis

Pierce bicinchoninic acid protein assay kit. Bicinchoninic acid is used to quantify total protein concentration including membrane proteins and intravesicular proteins.⁸⁸⁻⁹⁰ It is a highly sensitive and rapid method based on colorimetric solution but cannot characterize EV protein.⁹¹ **SDS-PAGE and Western blotting:** sodium dodecyl sulfate (SDS)-PAGE is a qualitative

method to detect EV proteins⁹² that includes protein lysis, denature, and separation based on molecular weight through gel electrophoresis; protein with less mass moves faster than with greater mass. Once proteins are separated by electrophoresis, they can either use for downstream proteomics or can be transferred onto a membrane for Western blotting after staining. Western blotting is also a qualitative technique for specific protein analysis.⁹³ Primary antibody, secondary antibody, and detection reagents are used in sequence after membrane transferring. Even though Western blotting is time-consuming, it is a powerful technique to demonstrate target proteins that are associated with EVs, and it can process multiple proteins at the same time. **Enzyme-linked immunosorbent assay (ELISA):** ELISA is a plate assay for membrane protein detection.⁹⁴ Enzyme-linked immunosorbent assay applies a “sandwich” format, EVs with specific surface biomarkers are fixed between the support that is pretreated with antibodies that can bond with EV surface biomarkers and another detection antibody that is linked to an enzyme (horseradish peroxidase), which is incubated with a substrate to produce measurable products. Enzyme-linked immunosorbent assay is commercially available and relies on antibody-antigen interaction that can improve the specificity of detection, while it cannot quantify multiple proteins simultaneously. **Flow cytometry:** Flow cytometry is another technique that can quantify and characterize EV surface biomarker based on fluorescence intensity of the detection antibody.^{95,96} A laser beam illuminate EVs, and the scattered light is converted to an intensity-associated voltage pulse that can be quantified later.⁹⁷ The advantage of flow cytometry is parallel multiple surface biomarker detection with different fluorescent antibodies; however, EV is too small for flow cytometry to capture the fluorescence signal since it is originally designed for cell surface expression analysis, so immunoconjugated beads are required to increase fluorescence signals since EVs can be mounted on the beads.

Nucleic Acids Analysis

Precipitation and spin columns. Both precipitation and spin columns complete total RNA extraction and are commercially available.^{22,98} Precipitation relies on phase separation; RNA is suspended into the aqueous phase and is recovered through ethanol precipitation.⁹⁸ The spin column is based on solid-phase extraction that relied on silica and RNA binding with chaotropic agents. **Amplification and sequencing:** Amplification is used for detecting a target sequence by end-point electrophoresis or real-time fluorescence measurements through polymerase chain reaction.^{22,52,99} Sequencing RNA profiling is able to generate a wider and deeper RNA characterization of the whole transcriptome, which is able to detect unknown RNAs.⁴⁷

Cancer Clinical Applications of EV

Conventional biopsies, such as tissue biopsy, are not only invasive¹⁰⁰ but have limitations to profile tumors due to tumor heterogeneous characteristics, and they cannot reflect the

Table 2. Potential Biomarkers of EVs for Cancer Diagnostic Application.

Type	Protein Biomarker	Nucleic Acids	Long Noncoding RNA
Breast	HER2, CD82, ER, CD24, Ki67, TGF- β	miR-10b, miR-21, miR-145, miR-1246, miR-105, miR-222, and miR-200c	N/A
Lung (non-small cell lung cancer)	EGFR, MET, PIK2CA, ALK, KRAS, MAP2K1, HER2, BRAF, AKT1, CD151, CD171, and tetraspanin 8, CD91, CD317, ECM1, LRG1	7b-5p, let-7e-5p, miR-23a-3p, miR-486-5p	lncRNA GAS5
Ovarian	HER2, TrKB, CD24, EpCAM	miR-375, miR-1307, miR-21, miR-200b, miR-100, miR-320, miR-141, miR-125b, miR-1246, and miR-93, miR-30a-5p, miR-145, miR25, miR148a, miR-101	N/A
Prostate	N/A	miR-1246, GATA2, miR-141, miR-375	SAP30L-AS1, SchLAPI
Pancreatic	GPC1, MIF, EGFR, Glypican-1, ZIP4, PD-L1, CD104, Epcam	miR-122-5p, miR-125b-5p, miR-1192-5p, miR-193b-3p, miR-221-3p and miR-27b-3p	N/A
Bladder	N/A	miR-148b-3p, miR-141, miR-27a-3p, miR-100, miR-92a, miR-99a, miR-93, miR-940, miR-375, miR-146a-5p	PCAT-1, SPRY4-IT1, UBC1 and SNHG16
Melanoma	PD-L1, CSPG4+	N/A	N/A
Brain (glioblastoma multiforme)	EGFR, EFGRvIII	miR-301a, miR-182-5p, miR-328-3p, miR-339-5p, miR-340-5p, miR-485-3p, miR-486-5p and miR-543, miR-22, miR-222	lncRNAs HOTAIR

Abbreviation: N/A, not applied.

whole tumor information.^{101,102} Hence, liquid biopsy shows advantages since it is noninvasive and on genetic level that can provide comprehensive information. Extracellular vesicles play an important role in the cancer liquid biopsy since they carry all types of genetic information from original tumors,^{103,104} and they also obtain attention because of preinvasive and early state diagnosis.¹⁰⁵ Extracellular vesicle-based diagnostic relies on tumor exosomal biomarkers, so defining and discovering reliable biomarkers is vital. In this review, we will present the latest EV diagnostic based on exosomal biomarkers among different cancers (Table 2).

Breast Cancer

Breast cancer (BC) plays the second position of cancer mortality in women.^{106,107} Common breast X-ray screening is invasive and radioactive, thus EV, diagnostic setting is important. Both blood samples and breast milk contain reliable EV resources, while collecting milk requires specific time point even though it is simpler than serum.¹⁰⁸ There are several BC biomarkers; proteins such as HER2, ER, and Ki67 are highly expressed.^{106,109-111} Yang et al said the expression of TGF- β in the breast milk increased the risk of BC.¹¹² van't Veer et al claimed that CD24 was abundant in patients with late-stage BC,¹¹³ and Wang et al demonstrated CD82 expression level was negatively associated with patients with BC.¹¹⁴ As for miRNA, miR-10b and miR-145 are abundant in patients with BC.¹¹⁵ Hannafon et al demonstrated both miR-21 and miR-1246 expressed in patients were higher than healthy donors.¹⁰⁸ Recently, Zhai et al have used Au nanoflare probe to detect miR-1246 in plasma samples successfully (Figure 1A).¹¹⁶ Alba

et al claimed miR-105 was higher in patients with metastasis BC than in healthy donors.¹¹⁷ Jong et al detected miR-21, miR-222, and miR-200c with high sensitivity with their surface-enhanced Raman scattering sensor.¹¹⁸

Lung Cancer

Lung cancers (LCs) are the most common and high death leading type of cancer because the majority of LCs are at late stage and go through metastasis that cannot be cured when they are first found.¹¹⁹ Non-small cell lung cancer (NSCLC) is the most common type of LC and only shows symptoms at the late stage¹²⁰; hence, early-stage detection is essential. EGFR, MET, PIK2CA, ALK, KRAS, MAP2K1, HER2, BRAF, AKT1, CD151, CD171, and tetraspanin 8 were revealed to be highly associated with LC.¹²¹ Ueda et al found CD91 was a powerful surface biomarker in advanced stage LCs.⁹⁴ Jakobsen et al showed CD317 was able to distinguish patients with LC with 75% accuracy.¹²² Niu et al found patients with NSCLC expressed a high level of α -2-HS-glycoprotein (AHSG), the extracellular matrix protein 1 in the serum compared to healthy donors.¹²³ Li et al found α -2-glycoprotein (LRG1) was strongly expressed in urinary EVs from patients with NSCLC (Figure 1B).¹²⁴ Recently, Castellanos-Rizaldos et al improved the detection sensitivity and specificity of EGFR T790M from the plasma of the patients with NSCLC by combining exoRNA/DNA and circulating free tumor DNA.¹²⁵ Jin et al found let-7b-5p, let-7e-5p, miR-23a-3p, and miR-486-5p were related to early-stage NSCLC.¹²⁶ Xu et al demonstrated miR-21 and miR-155 were higher in patients with NSCLC with recurrence than without recurrence and healthy donors.¹²⁷ Moreover, Li

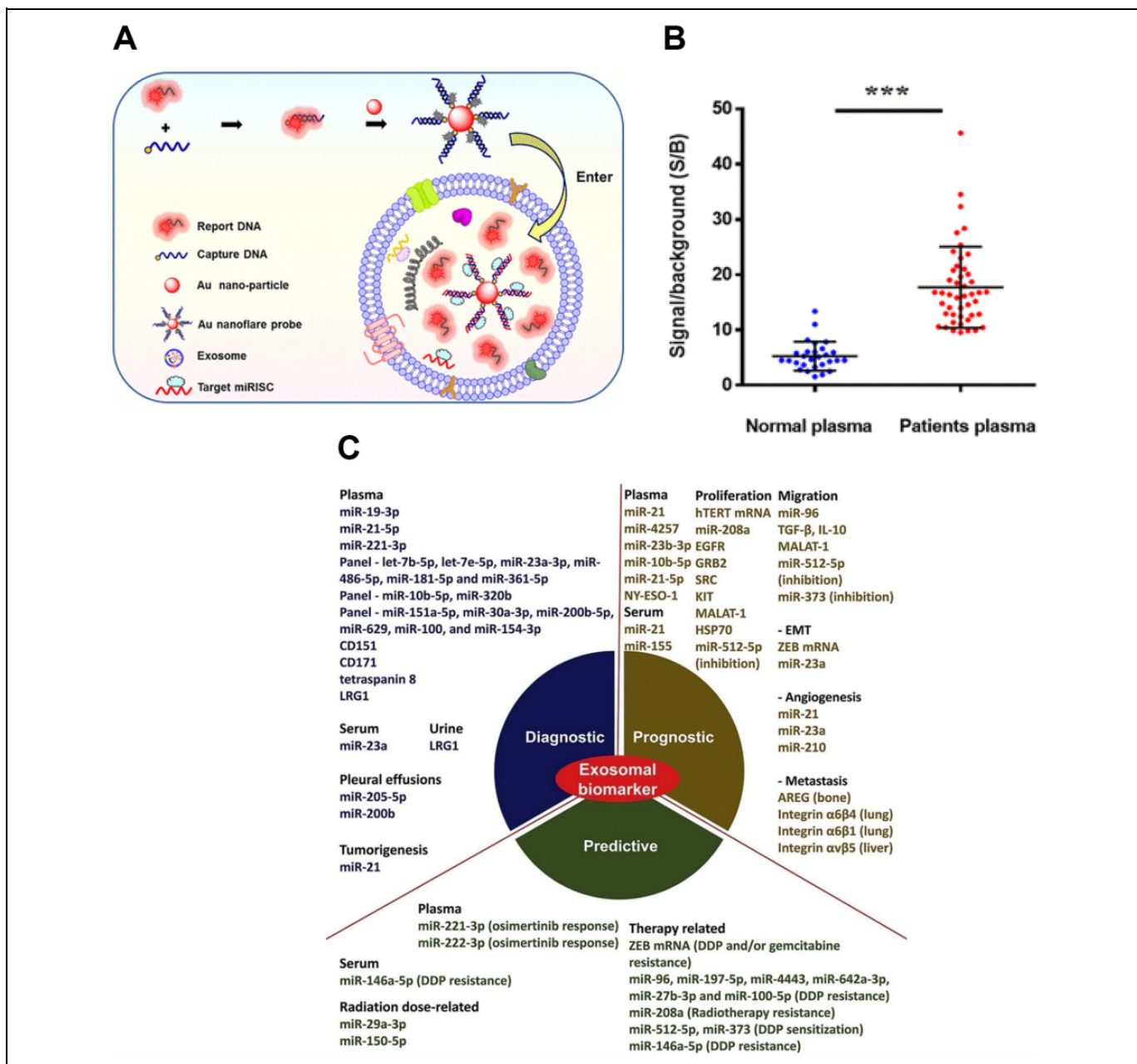


Figure 1. A, Schematic of Au nanoflare probe to detect miR-1246. Fluorescence-treated probes enter the exosomes and bind to the targets after incubation with exosomes from breast cancer cells. B, Comparison of miR-1246 expression level in patients with breast cancer ($n = 46$) and healthy controls ($n = 28$). Patients with breast cancer showed higher exosomal miR-1246. $P < .0001$. Reprinted with permission from Zhai et al.¹¹⁶ Copyright © 2019 American Chemical Society. C, Lung cancer liquid biopsy-related exosomal biomarkers. Reprinted with permission from Cui et al.¹²⁵ Copyright © 2019 from Elsevier Ltd.

et al found lncRNA GAS5 was downregulated in early-stage patients with NSCLC compared with healthy donors.¹²⁸

Ovarian Cancer

Ovarian cancer (OC) is difficult to be detected until it has spread within the pelvis and abdomen at the late stage, so early-stage detection is necessary.¹²⁹ Symptoms of early-stage OC are rare and nonspecific even in the advanced stage,

so EV diagnostic setting on genetic levels has advantages. Protein biomarkers such as claudin-4, HSP70, HER2, and TrkB derived from exosomes from patients showed different expression compared with healthy controls (Figure 2A).¹³⁰⁻¹³³ CD24 and EpCAM are also possible biomarkers for OC.¹³⁷ Exosomal miRNAs are much more powerful for OC diagnosis. Overexpressed level of miR-21, miR-200b, miR-100, miR-320 miR-141, miR-125b, miR-1246, miR-375, and miR-93 differed between OC patients and healthy donors.¹³⁸ The miR-1290 also

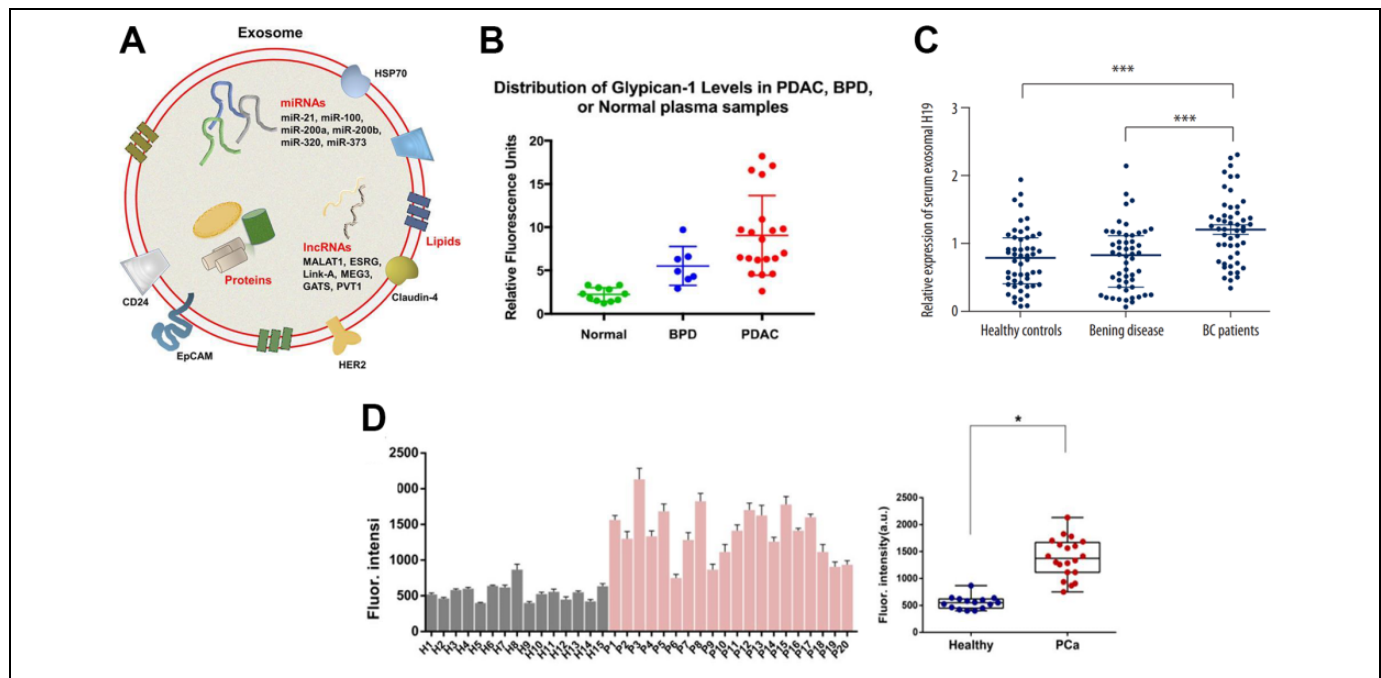


Figure 2. Molecular components (long noncoding RNAs, microRNAs, and membrane proteins) in exosomes from patients with ovarian cancer. Reprinted with permission from Yang et al.¹³⁰ Copyright © 1999-2019 John Wiley & Sons, Inc. B, Histogram and boxplot of fluorescence intensity of exosomes (Target: Prostate-specific membrane antigen (PSMA) positive) from patients with prostate cancer and healthy donors detected by superparamagnetic conjunctions and molecular beacons (SMC-MB) platform. Reprinted with permission from Li et al.¹³⁴ Copyright © 2019 American Chemical Society. C, Comparison of glypican-1 expression level in patients with pancreatic cancer ($n = 20$), benign pancreatic disease ($n = 7$), and healthy controls ($n = 11$). Glypican-1 in patients with pancreatic cancer were elevated. Reprinted with permission from Lewis et al.¹³⁵ Copyright © 2019 American Chemical Society. D, Quantitative reverse transcription polymerase chain reaction analysis of exosomal H19 from patients with bladder cancer, benign disease, and healthy controls. $P < .001$. Reprinted with permission from Wang et al.¹³⁶ Copyright © International Scientific Information.

showed the possibility of diagnosis on high-grade OC.¹³⁹ The miR-30a-5p was highly expressed in the urine samples of patients with OC,¹⁴⁰ while miR-145, miR25, and miR148a were under expressed.¹⁴¹ Xu et al found miR-101 was expressed lesser in patients with OC than in healthy donors.¹³² Qiu et al found metastasis-associated lung adenocarcinoma transcript 1 (MA-LAT1) was positively associated with OC.¹⁴²

Prostate Cancer

Prostate cancer (PCa) is one of the most common types of cancer in men.¹⁴³ Some types of PCa grow slowly with minimum harmful effects, while some types are aggressive.¹⁴⁴ The early stage of the PCa that may be defined as the prostate gland is easy to cure, but they show no signs or symptoms. So the liquid biopsy based on exosomal molecular contents is important. Bhagirath et al used the nCounter technology and found miR-1246 was a promising aggressive PCa biomarker.¹⁴⁵ Donovan found PCA3 and ERG mRNAs predicted high-grade PCa.¹⁴⁶ Li et al used an ultrasensitive and reversible nanoplatform to detect PSA, PCA3, and mRNA successfully in urinary exosomes from patients with PCa (Figure 2B).¹³⁴ However, PSA has limitation because it may not differ cancer and benign prostatic hyperplasia (BPH).¹⁴⁶ Some miRNAs, such as miR-141 and miR-375, from serum EVs were

associated with metastatic PCa, and the miR-19b distinguished PCa with 100% specificity and 93% sensitivity.¹⁴⁷ As for long noncoding RNAs, Wang et al found SAP30L-AS1 was related to tumor invasion, and SChLAP1 was expressed higher in PCa compared with BPH and healthy controls.¹⁴⁸

Pancreatic Cancer

Early-stage PC can only be detected in people with pancreatic cysts or family history of PCs,¹⁴⁹ but it can seldom be detected in other conditions.¹⁵⁰ Serum cancer antigen 19-9 (CA19-9) is a possible biomarker for PC¹⁵¹; however, it fails to show sensitivity and specificity of early-stage PC. Zhou et al compared 216 patients with PC with 220 healthy controls and found miR-122-5p, miR-125b-5p, miR-1192-5p, miR-193b-3p, miR-221-3p, and miR-27b-3p were significantly higher in patients with PC.¹⁵² Goto also found higher expression levels of miR-191, miR-21, miR-451a in PC.¹⁵³ Lewis et al developed an AC electrokinetic microarray chip that was capable of differing 20 patients with PC from healthy donors based on glypican-1 and CD63 expression levels with 99% sensitivity (Figure 2C).¹³⁵ Li et al designed an ultrasensitive polydopamine bifunctionalized Surface-Enhanced Raman Scattering (SERS) immunoassay with a detection limit of one exosome in 2 mL, and they discriminated patients with PC based on GPC1, MIF,

and EGFR surface biomarkers successfully.¹⁵⁴ Jin et al claimed ZIP4 promoted PC growth and could be a novel diagnostic biomarker for PC.¹⁵⁵ Recently, Lux claimed the combination of CA19-9 and c-Met improved sensitivity test of patients with PC.¹⁵⁶ They found PD-L1-positive patients showed shorter postoperative survival time that can be used as a negative prognostic factor. Moreover, the combination of CD104, Epcam, Tspan8, and some miRNAs such as miRNA-1246 improved diagnostic sensitivity and specificity of patients with PC.¹⁵⁷

Bladder Cancer

Bladder cancer is usually in the bladder but can show in other parts that belong to the urinary tract drainage system.¹⁵⁸ Around 50% of the patients with muscle-invasive bladder cancer will go through metastasis and die in 2 to 3 years, even patients with non-muscle-invasive BC usually have recurrence rate.¹⁵⁹ The symptoms of bladder cancer include hematuria, pelvic pain, and urination pain,¹⁶⁰ while these symptoms normally show at the middle or late stage; hence, the early diagnosis of bladder cancer is very important. Cystoscopy is a gold standard diagnostic tool for non-muscle invasive bladder cancer,¹⁶¹ but it is expensive and time-consuming since subsequent cystoscopy is necessary once the result is negative,¹⁶¹ and it fails to provide sensitive surveillance information. Hence, EV diagnosis with novel biomarkers is essential for patients with bladder cancer and suspected individuals. Both blood and urine provide reliable EV source for bladder cancer diagnosis; miR-148b-3p, miR-141 were increased, but miR-27a-3p, miR-100, miR-92a, and miR-99a were decreased in serum and plasma.¹⁶² Compared with blood, urine shows advantages because urine contacts with bladder directly and can be collected with various time point easily to reflect different stages of the diseases, and EVs are able to cross the basement membrane into the urine with miRNAs. The miR-375 was decreased in high-grade bladder tumors, while miR-146a-5p was increased, especially in low-grade tumors.¹⁶² Wang et al also found serum exosomal H19 expression level was higher compared to healthy controls and benign disease patients (Figure 2D).¹³⁶ Zhan et al found PCAT-1 and SPRY4-IT1 were capable of the bladder cancer diagnosis.¹⁶³ Zhang et al claimed UBC1 and SNHG16 identified by multivariate logistic regression model also provided high diagnostic accuracy. Moreover, the high UBC1 expression level was associated with low recurrence-free survival.¹⁶⁴

Melanoma Cancer

Melanoma is the most serious type of skin cancers, but it can be treated successfully in the early stages.¹⁶⁵ Melanomas can occur in any areas of the skin that are exposed to excess UV light.¹⁶⁶ Sharma et al isolated melanoma tumor-derived exosomes successfully with an mAb 763.74 that captured the chondroitin sulfate peptidoglycan 4 (CSPG4+) that are expressed on the surface of exosomes.⁷¹ Chen et al claimed that exosomal PD-L1 was associated with anti-PD-1, which has shown promise in treating melanoma tumors. Hence, detection of PD-L1 on the exosomes is essential.¹⁶⁶

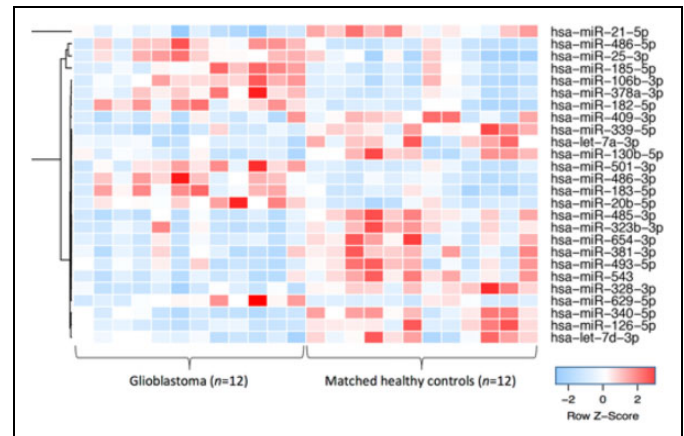


Figure 3. Hierarchical clustering of 26 microRNAs shows differences in GBM and healthy control exosomal profiles (fold change ≥ 2 or ≤ 0.5). Reprinted with permission from Ebrahimkhani et al.¹⁷⁰ Copyright Springer Nature Publishing AG.

Glioblastoma Multiforme

Glioblastoma multiforme is an aggressive type of cancer that occurs in the brain or spinal cord, and it tends to occur in older adults.^{167,168} Curing GBM is seldom possible but effective treatment can slow the progression of cancer and relief the symptoms.¹⁸ However, treating early-stage GBM guarantees minimum dissatisfactory effects. Normal diagnosis methods include the neurological examination, imaging tests (magnetic resonance imaging), and tissue tests. Nevertheless, compared with EV liquid biopsy, they are expensive and invasive. EGFR-vIII was a well-known GBM-related biomarker, and it was highly expressed on the surface of EVs from GBM patients.²² Lan et al found miR-301a was a potential biomarker for GBM.¹⁶⁹ Tan et al also found serum lncRNAs HOTAIR was significantly higher than in the healthy controls.¹⁷⁰ Ebrahimkhani et al found miR-182-5p, miR-328-3p, miR-339-5p, miR-340-5p, miR-485-3p, miR-486-5p, and miR-543 were most likely stable for GBM classification after modeling and data comparisons among 26 relative microRNAs (Figure 3).¹⁷¹ Santangelo also claimed miR-22 and miR-222 were expressed higher in high-grade GBM patients.¹⁷²

Conclusion and Perspective

Extracellular vesicles play an important role in tumor micro-environment; besides carrying parenting genetic information, the specific tumor-related genetic information can be used for diagnosis and immunotherapy, while size and biomolecular heterogeneities of EVs bring problems on isolating EVs that challenge the EV diagnostic setting, which requires EV isolation in a pure and rapid manner. Hence, standard sample collection, storage procedures, and efficient isolation mechanisms are important. In this review, we summarized updated isolation methods, and majorities of them enriched the bulk EVs, and immunoaffinity can isolate tumor subpopulation EV based on tumor surface biomarkers. Successful isolation is the first step of the cancer diagnosis; characterizing EVs in qualitative and

quantitative way, such as microscopy, DLS, NTA, and TRPS, is also necessary. The most important thing is the molecular content characterization such as protein classification and RNA sequencing, which is the fundamental of EV-based cancer liquid biopsy because clinical trials make decision based on expression level of reliable cancer-associated RNA and protein biomarkers. However, protein biomarkers lack tumor precision and show no superiority since the same type of biomarkers can be present in multiple cancers. For example, HER2 has high expression level in breast and LCs, while CD24 is abundant in both ovarian and BCs. In that, combination of different protein biomarkers to define a specific type of cancers is necessary. Comparing with proteins, RNAs may show high specificity toward single-type cancer, but it is relatively expensive. Overall, defining and exploring specific and reliable biomarkers and compiling a cancer biomarker database are big breakthroughs for cancer diagnosis. In this review paper, we introduce EV isolation and detection separately, and we believe in the future the combination of isolation and detection methods with high efficiency is desirable and plays key role since they fulfill the point-of-care cancer diagnostic need. Moreover, even though EV-based liquid biopsy is advantageous in many aspects such as noninvasive collection, early-stage detection compared with traditional biopsy, until now, gold standard operating procedures for collecting, isolating, and detecting EVs are still missing. Also, the universality and pricing are other concerns about liquid biopsy. Up to now, only HER2 and EGFR are qualified as biomarkers tested in clinical trials. Nevertheless, EV-based liquid biopsy is still a long road ahead, and we believe it will be more reliable and standard in the future since outstanding researchers make efforts on it.

Author Contribution

Chunyan Ma and Fan Jiang has contributed equally to this work.

Declaration of Conflicting Interests


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