

Advancements and Clinical Significance of Breast Cancer Exosome Detection

Xinjie Zhou *

Jining Medical University, Jining, China

* Corresponding Author Email: zhouxinjie0615@163.com

Abstract. Exosomes, which are lipid bilayer vesicles actively secreted by cells with a diameter of 30-100 nm, play a crucial role in the occurrence, development, and formation of the tumor microenvironment in breast cancer. They have become an important component of liquid biopsies for breast cancer and ideal targets for early diagnosis, treatment assessment, and clinical therapy. Breast cancer is the most common malignancy among women. However, traditional exosome detection methods have several limitations, and there are still many difficulties and challenges in achieving widespread clinical application of exosomes. Nowadays, significant progress has been made in exosome detection and analysis techniques, including optical methods such as colorimetry, fluorescence, surface-enhanced Raman scattering, as well as various electrochemical and thermal signal methods. This review summarizes the current biomarkers and novel developments in detection techniques applied to liquid biopsy of exosomes in breast cancer.

Keywords: Exosomes; breast cancer; liquid biopsy; early diagnosis; colorimetry; fluorescence; surface-enhanced Raman scattering; aptamer.

1. Introduction:

According to the estimated data on global cancer incidence and mortality released by the International Agency for Research on Cancer in 2020, the incidence of breast cancer in women has surpassed lung cancer, becoming the most common cancer and the leading cause of cancer-related death among women [1]. Early detection and intervention in breast cancer often lead to favorable outcomes, with approximately 70-80% of early non-metastatic breast cancer patients being curable. However, more than half of the patients are diagnosed at an advanced stage, experiencing local or distant metastasis. The 5-year survival rate of clinical stage I-II breast cancer patients is approximately 50% higher than that of clinical stage III-IV breast cancer patients [2, 3], while the 5-year and 10-year survival rates for patients with metastatic breast cancer (MBC) are 27% and 13%, respectively [4].

Currently, the gold standard for clinical breast cancer diagnosis is histopathological examination [5, 6]. Invasive histopathological examination imposes psychological and physiological burdens on patients. Its scope is insufficient to capture the overall genomic profile of breast tumors and is not suitable for monitoring treatment efficacy and prognosis. Moreover, there may be errors in biopsies and an increased risk of tumor metastasis [7]. Imaging techniques, as an auxiliary diagnostic method, have limited sensitivity and specificity for early-stage tumors and cannot provide sufficient diagnostic evidence for tumors. A large number of false-positive results can also lead to unnecessary biopsies. Additionally, for metastatic breast cancer (MBC), repeated imaging is expensive and has limited effectiveness [8].

Exosomes are extracellular vesicles (EVs) with a diameter of 30-100nm, formed by the fusion of multivesicular bodies with the cell membrane and actively secreted into the extracellular environment [9-11]. Exosomes contain various small molecules such as nucleic acids and lipids, and they are characterized by the presence of different types of surface proteins. Widely distributed in body fluids, exosomes are involved in a variety of physiological and pathological activities [11, 12], playing important roles in tumor initiation, development, metastasis, recurrence, and drug resistance [10, 13]. For example, exosomal PD-L1 can directly bind to PD-1 on the surface of effector T cells, inducing immune suppression [14]. Exosomal miR-105 secreted by breast cancer cells can promote

tumor cell growth, proliferation, and survival, inhibit the formation of tight junctions between endothelial cells, and facilitate tumor cell extravasation and distant metastasis[15]. Exosomes can be non-invasively obtained from various body fluids (blood, urine, saliva, ascites, and pleural effusion) and carry characteristic information of tumor cells. Therefore, exosomes have great potential in cancer diagnosis and monitoring of prognosis [16, 17].

To isolate and enrich exosomes in samples, conventional methods such as ultracentrifugation, size exclusion chromatography, immunocapture, and commercial kits are commonly used. However, these methods suffer from issues such as time-consuming procedures, low purity, limited yield, susceptibility to contamination, cumbersome operations, and significant sample loss [18]. These challenges and difficulties hinder the wide clinical application of EVs. In this review, we summarize the latest discoveries and new developments in detection techniques for breast cancer-derived EVs, aiming to provide insights and references for the future development of liquid biopsy based on EVs.

2. Liquid Biopsy

Liquid biopsy refers to the isolation and genomic or proteomic evaluation of tumor-derived materials, such as circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and exosomes, from body fluids[7, 19].

Liquid biopsy offers advantages such as high sensitivity, strong specificity, large detection volume, and good reproducibility, which make it a promising approach for early diagnosis, precise treatment, and prognostic assessment of cancer. The use of exosomal liquid biopsy has high clinical value in achieving early diagnosis, staging, and prognosis monitoring for breast cancer. However, challenges such as low levels of exosomes in the body fluids of breast cancer patients, complex fluid environments, different stages and subtypes of the disease, individual variations among patients, and the selection of biomarkers can make the detection process more complex. Therefore, there is an urgent need for more sensitive and accurate detection methods to improve the clinical application of liquid biopsy [20].

2.1. Exosome Isolation

To date, various methods based on size, immunoprecipitation, and different principles have been developed for exosome isolation [21]. However, density gradient separation can disrupt the integrity of exosomes and requires high centrifugation times. Differential centrifugation is expensive in terms of equipment and can result in exosome damage and protein contamination. Immunoprecipitation is not suitable for large-volume samples[18]. Plasma and serum contain a significant amount of free proteins, such as chylomicrons (80-100 nm) and very low-density lipoproteins (30-80 nm), which are similar in size to exosomes and can cause interference. Currently available separation methods based on size and density gradient are challenging for the isolation of exosomes. In recent years, several novel exosome separation techniques have emerged. These technologies include integrated dual-filter microfluidic devices, nanoplasmonic-enhanced scattering (nPES), membrane-based exosome isolation, and on-chip separation. However, to eliminate the influence of normal cell-derived exosomes and achieve high-purity exosomes in large sample volumes, there is still a need for the development of standardized separation and characterization methods [22-24].

2.1.1. Antibody-Based Methods for Exosome Detection

1) Electrochemical-Based Detection Techniques

Electrochemical methods offer several advantages such as low cost, rapid turnaround time, high sensitivity, and minimal sample requirements, making them promising for the detection of cancer-derived exosomes [25, 26].

Yacao proposed a bio-sensing method utilizing electrochemical technology for accurate identification of breast cancer-derived PD-L1 exosomes. In this method, the capture probe attached to the surface of the exosomes was used as a primer for in-situ hyperbranched rolling circle amplification (HRCA).

The continuous release of hydrogen ions resulting from DNA amplification reaction caused a decrease in pH, subsequently triggering the release of pH-responsive metal-organic frameworks (MOFs) as an enzyme catalyst to induce amplified electrochemical response for identification. The method demonstrated a linear range of 1×10^3 to 1×10^{10} particles/mL, with a limit of detection (LOD) of 334 particles/mL [27].

DOLDÁN et al. employed an antibody-capture approach to isolate breast cancer (MCF-7) exosomes, followed by the formation of a complex structure incorporating horseradish peroxidase (HRP)-conjugated anti-IgG antibody. This complex structure allowed for the electrochemical reduction of 3,3',5,5'-tetramethylbenzidine (TMB), enabling the detection of the isolated exosomes. With a sample volume of only 1.5 μ L, this assay achieved a detection limit of 200 particles/ μ L [28].

In contrast, Krishna et al. immobilized exosomes onto a screen-printed electrode (SPE) using antibodies and then employed electric field-induced lysis and electrochemical impedance spectroscopy (EIS) to detect exosome-associated protein, HER2. They isolated 3.5×10^8 exosomes/mL from serum. The linear detection range of HER2 was found to be 0.1 ng - 1 μ g with a detection limit of 10 pg. In this technique, electric field-induced lysis released proteins from exosomes, thereby enhancing the sensitivity of the sensing device [29].

Silio Lima Moura et al. employed antibody-modified magnetic particles (MPs) decorated with common tetraspanins CD9, CD63, and CD81, as well as specific cancer receptors (CD24, CD44, CD54, CD326, and CD340), to capture exosomes. They detected exosomes from three breast cancer cell lines (MCF7, MDA-MB-231, and SKBR3) using a magnetically-driven electrochemical sensor. The limit of detection (LOD) reached as low as 81 exosomes/ μ L [30].

2) Fluorescence-Based Detection Techniques

Fluorescence-based detection techniques rely on the excitation of different fluorescence molecules or probes under an excitation light source, resulting in emission of fluorescent signals at specific wavelengths. By detecting the intensity of these fluorescent signals, quantitative analysis of fluorescently labeled biomarkers from various sample sources can be achieved. Due to its high sensitivity and rapid response time, fluorescence has been widely applied in the field of exosome detection.

Xuemei Wang et al. utilized zirconium-based metal-organic frameworks (Zr-MOFs) for the detection of EpCAM-positive exosomes in breast cancer. Zr-MOFs, exemplified by uiO-66, exhibit high affinity towards phosphate groups, allowing for selective enrichment of phosphate molecules and nucleic acids through the formation of Zr-O-P bonds. Additionally, uiO-66-NH₂ particles possess excellent fluorescence properties. Based on these principles, magnetic modification of uiO-66 (uiO-66@Fe₃O₄) was performed to serve as an adsorbent, enabling rapid capture and enrichment of exosomes by anchoring phosphate groups. Subsequently, a fluorescent probe was constructed using uiO-66-NH₂ decorated with anti-EpCAM antibodies (μ iO-66-NH₂@anti-EpCAM), which formed a "MOF-exosome-MOF" structure for the identification of extracted EpCAM-positive exosomes. The method demonstrated a detection limit (LOD) of 16.72 particles/ μ L [31].

Wang Lei et al. proposed a exosome-zirconium-liposome sandwich structure for the detection of exosomes using zirconium phosphate coordination chemistry. By leveraging the inherent properties of zirconium ions and phosphates present in exosome lipids, a "magnetic bead-exosome-Zr⁴⁺-liposome-calcein" composite was prepared, which ultimately generated fluorescence signals through calcein. The method achieved a limit of detection (LOD) of 7.6×10^3 particles/ μ L [32].

Chunchen Liu et al. proposed a droplet digital ExoELISA method, where after efficient magnetic separation and washing, a "magnetic bead-antibody-exosome-antibody-enzyme" complex was prepared and encapsulated within a droplet. This method achieved high sensitivity, specificity, and absolute quantification of exosomes with specific protein biomarkers. The detection limit reached as low as 10 particles/ μ L (10–17 M). The expression levels of GPCF1(+) exosomes in breast cancer

patients were quantified using this approach, demonstrating different levels before and after surgery, thus validating its clinical significance [33].

Furthermore, Vojtech Vinduska et al. reported the construction of anti-exosome surface protein CD81 immunomagnetic beads, followed by the use of a secondary antibody labeled with quantum dots QD655, which possess strong fluorescence characteristics and photostability, to bind to the primary antibody. Finally, the fluorescence spectrum was used to detect the quantum dots conjugated with the secondary antibody. This method enabled specific detection of different surface markers on exosomes from various cancer cell lines and differentiation between cancerous and normal exosomes. The detection limit of this method was 9.3×10^6 particles/mL [34].

3) Surface-enhanced Raman Scattering (SERS)-based Detection Technique

Surface-enhanced Raman scattering (SERS) is achieved by detecting the Raman signals of small molecules adsorbed on metal surfaces, which are enhanced through electromagnetic and chemical mechanisms. SERS exhibits unique spectral signals in complex and variable biological environments, with very low concentrations/volumes of analytes required. As such, it is an ideal method for the analysis of exosomes [35, 36].

Kwizera et al. reported a novel method for exosome detection by combining surface-enhanced Raman scattering (SERS) nanotags with a microarray-based platform for antibody capture. Exosomes were captured on an array structure chip surface by immobilized antibodies, and the surface proteins of exosomes from breast cancer cells and HER2-positive breast cancer patients were quantified through the electrostatic interaction between SERS nanotags (gold nanorods, AuNRs) and exosomes. The results were obtained within 2 hours. The detection limit of this method was 2×10^6 /mL (3.3 fM) [37].

Guohao Li et al. developed a magnetic SERS platform by sequentially conjugating gold nanodot-fixed magnetic nanoparticles (MNPs@Au) with anti-fouling component PEG and anti-CD9 antibodies. This platform was capable of distinguishing between exosomes derived from breast cancer cell lines MCF-7 and MDA-MB-231, achieving a sensitivity of 91.67% and specificity of 100% in fully identifying breast cancer patients from healthy individuals [38].

2.1.2. Aptamer-based Exosome Detection Method

Aptamers are short DNA or RNA oligonucleotides that are selected from nucleic acid libraries using a technique called Systematic Evolution of Ligands by Exponential Enrichment (SELEX), which allows for efficient and specific binding to a ligand of interest [41]. Aptamers have a smaller molecular weight (8-15 kDa) compared to antibodies, lower immunogenicity, and broader specificity for various targets, including peptides, proteins, and even whole cells [39, 40]. Aptamer-based sensors are increasingly being applied in liquid biopsy [41, 42].

1) Fluorescence-based Assay Techniques

Fluorescence-based assay techniques combine the high specificity of aptamers with the excellent fluorescence catalytic performance of G4-hemin to detect HER2 protein, enabling quantitative detection of exosomes through fluorescent signal output. The linear detection range for breast cancer cell-derived exosomes is 2.5×10^5 to 1.00×10^7 particles/mL, with a detection limit of 0.54×10^5 particles/mL [43]. Additionally, a fluorescence aptamer sensor based on aggregation-induced emission luminogens (AIEgens) and graphene oxide (GO) has been used to analyze three tumor-associated proteins (EpCAM, EGFR, and HER2) on exosomes from breast tumor plasma samples. The sensor achieved a limit of detection (LOD) of 3.43×10^5 particles/ μ L (0.57 pM) [44]. Furthermore, Junli Zhang et al. employed multiple DNA aptamers (CD63/EpCAM/MUC1) for specific recognition of exosomes captured from patient plasma samples. Subsequent rolling circle amplification generated local fluorescence signals, achieving a detection limit (LOD) of 10 EV/ μ L [45].

2) Surface Plasmon Resonance (SPR)-based Detection Technology

Surface plasmon resonance (SPR) is an optical biosensor technique that allows real-time monitoring of the interactions between biomolecules on a metal surface. The basic principle of SPR involves the excitation of electron resonance at the metal interface using incident light. The interactions between molecules cause slight changes in the surface refractive index, enabling the detection of exosomes [46].

Qing Wang constructed an SPR-based aptamer sensor with amplified signal using dual gold nanoparticles (AuNP), achieving a low detection limit (LOD) of 5×10^3 exosomes/mL [47]. Wenqin Chen proposed an unlabeled surface plasmon resonance (SPR) biosensor. Exosomes were captured on the chip surface by HER2 aptamers, and guanine-quadruplex DNA (G4 DNA) formed a peroxidase-like G4-hemin complex, catalyzing the deposition of a large amount of tyramine-coated gold nanoparticles (AuNPs-Ty) on the exosome membrane under the assistance of H₂O₂, resulting in significantly enhanced SPR signals. This developed SPR biosensor demonstrated a wide linear range from 1.0×10^4 to 1.0×10^7 particles/mL [48]. The developed SPR strategy achieved label-free, real-time, and highly specific detection of low abundance HER2-positive exosomes, replacing horseradish peroxidase (HRP) with G4-hemin to avoid the inherent limitations of natural enzymes. However, the limitation of this method lies in its cumbersome experimental steps.

3) Colorimetric-based Detection Technology

Colorimetric assay is a method for qualitative or quantitative determination of substances by visually or instrumentally comparing the color or intensity changes in a solution. This technique is simple and suitable for instant detection [49].

Based on the principle that the competitive binding of exosomes and organic frameworks (MOFs) aptamers can induce changes in the conformation and peroxidase-like activity of the MOFs, researchers have developed several colorimetric assays. The recognition of CD63 aptamer-exosomes alters the binding conformation of Cu/Co bimetallic metal-organic frameworks (CuCo₂O₄ nanorods), thereby restoring peroxidase-like activity and enabling exosome detection. The method exhibits a detection range of 5.6×10^4 to 8.9×10^5 particles/ μ L, with a detection limit of 4.5×10^3 particles/ μ L [50]. Similarly, the specific binding of exosomes to CD63 aptamer modifies the conformation of DNA ligands on iron-based metal-organic frameworks (Fe-MOF), enabling visual detection of exosomes. The Fe-MOF biosensor demonstrates excellent performance in the quantitative analysis of exosomes derived from breast cancer cells, with a detection range of 1.1×10^5 to 2.2×10^7 particles/ μ L and a detection limit of 5.2×10^4 particles/ μ L [51]. In another study, the binding of exosomes with aptamers causes their detachment from the surface of single-walled carbon nanotubes (s-SWCNTs), resulting in weakened peroxidase-like activity and reduced catalytic ability towards TMB, leading to a lighter color in the solution. Exosome detection is achieved through UV-visible spectrophotometry. The linear range for exosomes using this method is 1.84×10^6 - 2.21×10^7 particles/ μ L, with a detection limit of 5.2×10^5 particles/ μ L [52].

Furthermore, the detection and quantification of exosomes have been achieved by utilizing the signal changes resulting from the in-situ deposition of polydopamine (PDA) around exosome particles, catalyzed by horseradish peroxidase (HRP) and hydrogen peroxide (H₂O₂). The method has a limit of detection (LOD) of 7.7×10^3 particles/mL [49]. The advantages of this aptamer sensor include relative simplicity and rapidity of detection, along with excellent sensitivity. However, the entire measurement process, from sampling to final readout, takes approximately 4 hours.

3. Clinical Application

Exosomes can be released into various biological fluids such as blood, urine, and saliva, interacting with cells and transmitting signals. Due to their stability and high abundance, plasma-derived extracellular vesicle proteins and nucleic acids have emerged as potential biomarkers for cancer diagnosis, treatment monitoring, and prognosis [53, 54].

3.1. Clinical Diagnostic Applications of Breast Cancer-Derived Exosomes

The epithelial cell adhesion molecule (EpCAM) is widely expressed at the tight junctions of epithelial cells in normal epithelial tissues (including stomach, prostate, breast, ovary, etc.), playing a role in mediating calcium-independent homotypic cell adhesion, cell morphogenesis, promoting cell migration, and regulating cell proliferation and cycle. EpCAM, along with its protein cleavage fragments, interacts with tight junction proteins, epidermal growth factor receptor (EGFR), and intracellular signaling components of the WNT and Ras/Raf pathways, thereby contributing to tumor heterogeneity and partial epithelial-to-mesenchymal transition (EMT) within the tumor [55, 56].

Kwizera et al. discovered that levels of EpCAM-positive exosomes in the plasma of patients with HER2-positive breast cancer were significantly higher compared to healthy controls ($P \leq 0.01$), enabling the differentiation of breast cancer patients from normal controls (AUC= 1) [37]. Wenwen Chen [57], Xuemei Wang [31], and others have validated the statistically significant higher levels of EpCAM-positive exosomes in serum samples of breast cancer patients compared to healthy individuals ($p < 0.01$).

Claudin proteins are key components of tight junctions. As a member of the claudin family, abnormal expression of claudin-7 is closely associated with tumor initiation and progression [58-60]. In addition, abnormal expression of claudin-7 has been found to be associated with recurrence, metastasis, and overall survival in various malignant cancers, such as laryngeal squamous cell carcinoma, oral squamous cell carcinoma, and nasopharyngeal carcinoma [61, 62].

A recent study examined the expression levels of claudin-7 in blood-derived exosomes from 60 early-stage breast cancer (BC) patients and 20 healthy volunteers. The results demonstrated that the levels of claudin-7 in BC patients were significantly higher compared to healthy controls [area under the curve (AUC) = 0.8517 ± 0.06 SD; 95% confidence interval (CI) = 0.732-0.971; sensitivity at 95% specificity = 75.13%]. The accuracy of claudin-7 in BC diagnosis can be improved by utilizing the claudin-7/CD81 ratio (AUC = 0.8908 ± 0.048 SD). Additionally, the levels of claudin-7 and claudin-7/CD81 in exosomes showed no significant association with the ER, PR, and HER2 status of BC patients, suggesting that exosomes claudin-7 could be a universal biomarker for early-stage BC diagnosis [63].

Heat shock proteins (HSPs) are a highly conserved group of proteins that are expressed at low levels in cells under normal physiological conditions but significantly upregulated in response to various stimuli. HSP70 exhibits high levels of expression in various tumor cells, including cervical cancer, endometrial cancer, and gastric cancer[64]. In breast cancer, the expression of HSP70 in cancer cells of different differentiation grades correlates with pathological staging, with increased expression indicating a poor prognosis[65]. Moreover, the level of exosomes HSP70 is increased in patients with metastatic breast cancer compared to those without metastasis (AUC=0.8968). Breast cancer patients who experience contralateral recurrence or metastasis within the first 2 years after radiotherapy have significantly higher levels of exosomes HSP70 compared to those who remain disease-free. Taken together, these two studies suggest that exosomes HSP70 has the potential to serve as a predictive factor for tumor growth/spread and can be used to monitor HSP70 expression in cancer patients[66, 67].

PD-1 (CD279) is a type I transmembrane protein belonging to the CD28 family and is an inhibitory receptor induced by activation of the immunoglobulin superfamily[68]. PD-1 is expressed inducibly on the surface of human T cells, B cells, and NK cells. PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273) are two ligands of PD-1. PD-L1 is inducibly expressed on activated T cells, B cells, monocytes, and various types of tumor cells such as lung cancer, liver cancer, and breast cancer[69, 70].

It is currently believed that exosomes PD-L1 can directly bind to PD-1 on the surface of effector T cells, thereby inducing immune suppression and inhibiting the function of cytotoxic T cells, leading to tumor immune escape[14, 71]. Xiaoling Qin et al. demonstrated a significant difference in the

expression levels of exosomes PD-L1 between plasma from breast cancer patients and healthy donor plasma ($p < 0.001$) [72]. In a study by Mengjiao Huang et al., quantitative analysis showed that exosomes PD-L1 can provide accurate cancer diagnosis (AUC: 0.999). They also found that the concentration of exosomes PD-L1 varies with different malignancy degrees in breast cancer patients and is highly correlated with metastasis [73]. Additionally, Zhichao Fan discovered an elevated relative abundance of circulating PD-L1 exosomes in undiluted serum samples from metastatic breast cancer patients. This finding effectively distinguishes healthy volunteers from breast cancer patients, and the abundance of exosomes is strongly correlated with the stage of breast cancer [74]. Taken together, these findings indicate that exosomes PD-L1 holds great potential in breast cancer diagnosis, clinical staging, and monitoring of metastasis.

The protein expression profile of PD-L2 is much narrower compared to PD-L1 and is primarily expressed on activated macrophages, dendritic cells, stromal cells derived from bone marrow, and individual tumor cell lines [75]. Elevated levels of PD-L2 in cancer and stromal cells are associated with increased risk of progression (HR, 1.9; 95% confidence interval, 1.3 to 2.7; $P = 0.003$) [76]. Furthermore, plasma exosomes PD-L2 levels are significantly increased in triple-negative breast cancer (TNBC) patients ($p < 0.0001$). They are correlated with progression-free survival (PFS) (AUC: 0.698; sensitivity: 80%, specificity: 60%; $p = 0.021$) and overall survival (OS) (AUC=0.738; sensitivity: 100%, specificity: 59.2%; $p = 0.008$). Additionally, the reduction of exosomes PD-L2 after chemotherapy is more pronounced in patients achieving pathological complete response (pCR) [77].

In addition, several common proteins carried by exosomes can be used as markers, such as membrane transport proteins (Rabs, Annexins), proteins involved in the biogenesis of multivesicular bodies (MVBs) (Tsg101, Alix), and tetraspanins (CD81, CD82, CD37, and CD63). Among them, tetraspanins CD63, CD9, and CD81 play key roles in sorting, selective recruitment of biomolecules, target selection, cell-specific entry, capture, and angiogenesis [78]. CD63 is the most commonly used marker protein for exosomes and one of the targets for exosomes capture [79]. Yumin Wang et al. validated that the expression level of CD63 in exosomes derived from breast cancer cell line (MCF-7) was higher than that from the control cell line (MCF-10A), and a similar trend was observed in clinical patient samples, suggesting that the surface expression level of CD63 on exosomes secreted by tumor cells is higher than that of normal cells [80]. Furthermore, Yingzhi Zhang et al. quantitatively confirmed that the quantity of exosomes derived from tumor cells was higher than that from non-tumor cells by using CD63 aptamer to capture exosomes [50]. Additionally, Qing Wang's experiments demonstrated higher protein expression of CD63 in exosomes derived from breast cancer cells compared to those secreted by normal breast cells (MCF-10A) [47]. Yaokun Xia et al. quantified exosomes in the serum of breast cancer patients and healthy individuals using CD63 as a marker and found approximately a 1.5-fold increase in exosomes in the serum of breast cancer patients compared to healthy individuals [52]. However, CD63 is a marker protein on the surface of all exosomes and lacks specificity for breast cancer, which may be subject to interference from non-tumor sources or other types of tumor-derived exosomes in clinical applications. Therefore, a more specific marker for breast cancer-derived exosomes is needed in clinical settings.

Nucleic acids located within exosomes can also provide information for the diagnosis or prognosis of breast cancer. RNA within exosomes is protected by the lipid bilayer, which prevents degradation by ribonucleases (RNases), and its expression profile is associated with tumorigenesis and tumor progression [81, 82]. Clinical sample studies have found that the expression level of Exo-miR21 in the plasma of breast cancer patients is significantly higher than that in healthy individuals ($p < 0.001$, AUC=0.99) [83]. Furthermore, a study validated that exosomes levels of miR-222 detected in the TN subtype of breast cancer were higher than in other subtypes, while high levels of miR-200c were specifically detected in the HER2+ subtype of breast cancer, revealing its potential as a diagnostic tool for breast cancer subtyping [84]. Serum exosomes lncRNA H19 is associated with various biological processes such as cell proliferation, invasion, and apoptosis in breast cancer and other tumors. A study validated that exosomes lncRNA H19 in the serum of breast cancer patients was significantly upregulated compared to participants with benign breast disease (BBD) and healthy

individuals (AUC=0.870, sensitivity: 87.0%, specificity: 70.6%), suggesting its potential as a novel biomarker for breast cancer diagnosis [85].

3.2. Co-testing

Due to the lack of an ideal breast cancer-specific exosomes biomarker with sufficient specificity, co-testing provides a more precise approach. Yike Li proposed a DNA computing device mediated by strand displacement, which utilizes a CD63 aptamer-based logic gate and utilizes EpCAM and HER2 on individual exosomes as inputs and thermal annealing amplification to enhance the output signal, reliably distinguishing serum samples from breast cancer patients and healthy donors (AUC=0.99) [86]. Furthermore, Fei Tian et al. performed molecular profiling of exosomes and validated that CA15-3, CA125, CEA, HER2, EGFR, PSMA, EpCAM, VEGF (a weighted sum of 8 EV protein markers) had an accuracy of 91.1% in differentiating metastatic breast cancer (MBC), non-metastatic breast cancer (NMBC), and healthy donors (HD) [87]. Additionally, Junli Zhang et al. demonstrated that the percentage of CD63/EpCAM/MUC1 triple-positive exosomes in breast cancer patients was significantly higher than in healthy donors. In differentiating metastatic BC, non-metastatic BC, and healthy donors, the receiver operating characteristic curve showed an AUC of 1.0, with a specificity and sensitivity of 100% and an overall accuracy of 91% [46]. Moreover, Hogyong Gwak et al. utilized EpCAM and CD47f as combined biomarkers to detect tumor-derived exosomes, and validated an area under the curve (AUC) of 0.89 for the epithelial-mesenchymal transition (EMT) index in distinguishing patients from healthy controls, indicating excellent diagnostic value [88]. Boli et al. found that the combination of EGFR, EpCAM, and HER2 showed higher accuracy in differentiating benign and malignant breast tumor patients from healthy controls compared to single markers (AUC: 0.9845, 95% CI: 0.9505-1.000). The sensitivity was 90.70% (95% CI: 78.40-96.32%), and the specificity was 100.00% (95%, CI: 60.97-100.00%) [44].

4. Conclusion

Exosomes liquid biopsy has demonstrated excellent clinical value; however, there are still challenges and controversies in achieving large-scale clinical application. The detection of exosomes in different body fluids provides potential directions for future development. Regarding plasma collection, the choice of anticoagulants, storage temperature, time and transportation, centrifugation protocols, types of blood collection tubes, fasting status, and physical exercise may all influence the isolation of blood samples [89]. Compared to blood, urine can be easily obtained non-invasively and has lower protein interference, making it advantageous for the detection of low-abundance proteins. However, urine has poor buffering capacity and extremely low abundance of exosomes compared to blood [90]. Additionally, considering the variable nature of urine (pH, osmotic pressure, protein concentration, etc.), further research is needed to develop methods for isolating exosomes from the urine of patients with nephrotic syndrome, taking into account the urine's variable properties.

Table 1. Exosomal Biomarkers

Method	Bio-Receptor	biomarker	LOD	Reference
Electrochemical	antibody	CD63,PD-L1	334particles/ml	[27]
		CD9	200particles/ml	[28]
		HER2	10pg	[29]
		CD9,CD63,CD81	81particles/ μ L	[30]
Fluorescent	aptamer	MUC1,HER2,EpCAM,CEA	NA	[91]
	antibody	CD63	1.4×10^4 particles/ μ L	[92]
		EpCAM	16.72	[31]

		CD63	particles/ μ L 7.6×10^3 particles/ μ L	[32]
		GPCf1	10particles/ μ L	[33]
		CD81	9.3×10^6 particles/ μ L	[34]
			10particles/ μ L	
	aptamer	CD63, EpCAM, MUC1	0.54×10^5 particles/mL	[45]
		HER2	0.57 pM	[43]
		EpCAM, EGFR, HER2		[44]
			3.3fM	
SurfaceEnhancedRaman Scattering (SERS)	antibody	HER2	NA	[37]
		CD9		[38]
			5×10^3 particles/mL	
Surface plasmon Resonance (SPR) biosensors	aptamer	—	NA	[47]
Colorimetric	aptamer	HER2		[48]
			4.5×10^3 particles/ μ L	
			5.2×10^4 particles/ μ L	
		CD63	5.2×10^5 particles/ μ L	[50]
		CD63		[51]
			7.7×10^3 particles/mL	
		CD63		[52]
		CD63		[49]

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