



Circulating exosomes and their cargos in blood as novel biomarkers for cancer

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Abstract: Exosomes, the bilayer-lipid-membrane microvesicles are well known for intercellular communications. They are considered to be involved in tumorigenesis, progression and chemotherapy resistance via horizontal transfer of their functional biomolecules (i.e., proteins and nucleic acids) to neighboring or distant cells. Thus, circulating exosomes are becoming a new star among existing liquid biopsies [circulating tumor cells (CTCs); disseminated tumor cells (DTCs); cell-free DNAs; cell-free miRNAs]. The detection of circulating exosomes, in particular tumor-specific exosomes, along with the profiling of their contents are expected to facilitate early detection of tumor, prediction of prognosis and monitoring therapy response in cancer. In this review, the clinical relevance of circulating exosomes and their cargos in blood as novel diagnostic and prognostic biomarkers for cancer is summarized. Additionally, exosomes as therapeutic target and drug delivery vehicle in clinical applications are also highlighted.

Keywords: Exosomes; liquid biopsy; biomarker; proteins; miRNAs; lncRNAs; circRNAs; DNA mutation

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Introduction

Early diagnosis and real-time monitoring of therapeutic response could facilitate timely and efficient management of cancer patients. In this respect, many efforts have been made in developing minimally invasive, blood-based informative assays of liquid biopsies, such as circulating tumor cells (CTCs), circulating disseminated cells (CDCs), and circulating cell-free nucleic acids (RNAs and DNAs) (1-3). A decade ago, mRNAs and miRNAs were firstly identified in exosomes from the serum of patients with glioblastoma (GBM) (4) and ovarian cancer (5), which promotes the usage of exosomes and their contents as biomarkers for cancer. Since then, accumulating investigations started to

focus on this fast-evolving research field.

Exosomes are nano-sized extracellular vesicles with a diameter range of 30–100 nm. In 1981, they were initially described and confirmed as microvesicles released from neoplastic cells (6-8). During the initial step of exosome formation, early multivesicular endosomes (MVEs) produced from cell membrane bud inward to form small internal vesicles carrying genetic materials and proteins. Some of the late MVEs fuse with the cell membrane and are released as exosomes into extracellular space (*Figure 1*). The extracellular exosomes mediate cell to cell communication via discharging their cargos to recipient cells, and thus influence the physiological and pathological functions of recipient cells (9-11). The

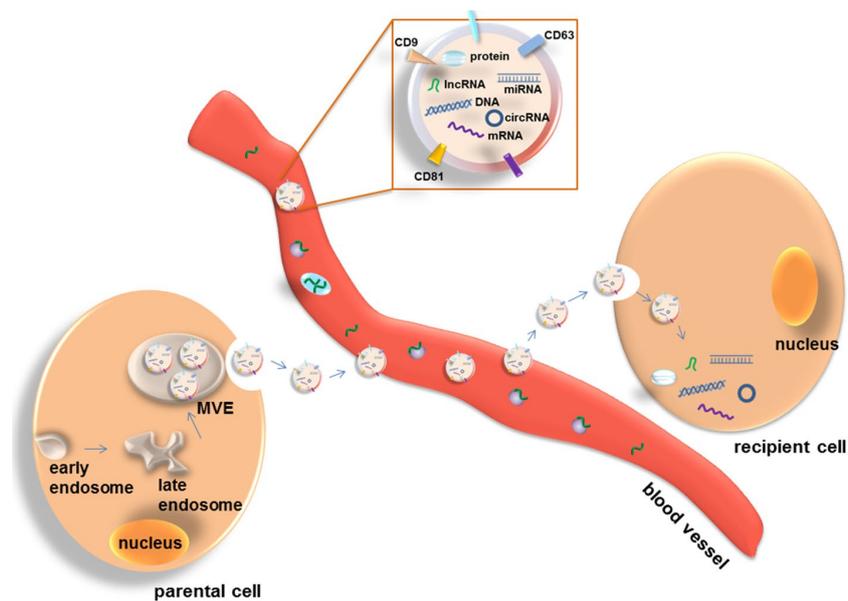


Figure 1 Circulating exosomes mediate cell to cell communication at distant site. The biogenesis of exosomes starts with the endocytosis of intracellular microvesicles that fuse to early endosomes, which further mature to late endosomes. A secondary invagination of the late endosome membranes forms multivesicular endosomes (MVEs). Following fusion of the MVEs with the plasma membrane, exosomes loaded with bioactive molecules (proteins, mRNAs, DNAs, miRNAs, lncRNAs and circRNAs) are released into extracellular area. Secreted exosomes are small enough to enter the blood circulation through the gaps between endothelial cells, and disseminate to distant organ, where they are uptaken by a recipient cell. The internalized exosomes discharge their cargos, including proteins, miRNAs, lncRNAs, circRNAs, mRNAs and DNAs, which are functional in the new environment, resulting in changed physiological or pathological conditions of the recipient cell.

functional investigation of exosomes originated from the description of exosome containing major histocompatibility complex class II (MHCII) secreted by antigen-presenting cells, implying their potential role in immune responses (12,13). Despite, all cell types release exosomes either under normal or pathological conditions, whereby tumor cells are the most avid producers (14). The characterization of exosomes carrying specific information dependent on their cell origin, and the relative facility to collect them from various biological fluids including blood, urine, tumor effusions, amniotic fluid or saliva (14), have suggested their use as diagnostic markers in pathology, in particular in cancer. Tumor-derived exosomes in blood are thought to promote tumor cell proliferation, angiogenesis and metastases by inhibiting antitumor immune responses, and thus, the depletion of these immune suppressive exosomes in blood circulation may be promising for the antitumor treatment (15,16). In addition, the cargos incorporated in exosomes are protected from degradation, which allow their quantification by various techniques. Thus, analyses of

circulating exosomes and their contents in blood could be promising strategies to advance cancer diagnosis with better sensitivity and specificity, and monitor patients' therapeutic response.

The property of exosomes in cancer

Considerable studies have suggested that tumor-derived exosomes influence multistep of tumor development, such as tumor initiation, growth, angiogenesis, hypoxia-driven epithelial-to-mesenchymal transition (EMT), metastasis, tumor immune escape, and drug resistance (17,18). For instance, GBM-derived exosomes which are enriched in angiogenic proteins have a capability to stimulate glioma cell proliferation as well as tubule formation (4). Exosomes derived from highly metastatic melanomas have been reported to increase the metastatic behavior of primary tumors by permanently "educating" bone marrow progenitors through the receptor tyrosine kinase MET. These melanoma-derived exosomes also induced vascular leakiness

at pre-metastatic sites and reprogrammed bone marrow progenitors toward a pro-vasculogenic phenotype (19). Another direct evidence verifying the impact of tumor cell-derived exosomes on tumor progression was provided by Dutta and coworkers. They isolated exosomes from cholangiocarcinoma (CCA) cells, namely KKU-M213 and KKU-100, and incubated them with normal human cholangiocyte (H69) cells. They found that these cancer cell-derived exosomes were internalized into H69 cells and induced migration and invasion of H69 cells (20). Consistently, *BRC11*-knockout human fibroblasts could be transformed into malignant cells after 2-week exposure to exosomes extracted from the sera of patients with breast, colorectal, pancreatic, and other cancer types (21). Recently, Nabet *et al.* reported that upon transfer to breast cancer cells, an endogenous RNA, unshielded RN7SL1 within stromal exosomes enhance tumor growth, metastasis and therapy resistance (22).

Therefore, these critical roles of tumor-derived exosomes in almost all aspects of cancer process provide opportunities for the development of exosome assays, and their functional cargos carrying tumor-specific signatures may be potential biomarker candidates in translational medicine. In this review, we refer the biofunctional cargos including proteins, miRNAs, lncRNAs, circRNAs, mRNAs and DNAs in exosomes to exo-proteins, exo-miRNAs, exo-lncRNAs, exo-circRNAs, exo-mRNAs and exo-DNAs, respectively.

Clinical relevance of circulating exosomes in cancer

Exosomes can be shed from various parental cells into blood, of which tumor cells are the most active producers. With concentrations as high as or higher than 10^9 vesicles/mL of blood, the number of exosomes secreted by tumor cells correlates with their malignant behavior. The relationship between circulating exosomes and tumor can be described as “seeds and plants”. Exosomes released by the parental cancer cells carrying multiple genetic materials are discharged into bloodstream. Some of circulating exosomes disseminate to distant organs and transform wild type cells to malignant ones, through transferring their bioactive contents into recipient cells (*Figure 1*). Thus, circulating exosomes may serve as a tool to detect tumor and predict treatment outcome, or as a therapeutic target.

In recent years, the clinical significance of circulating exosomes has been intensively investigated in blood of cancer patients. Tavoosidana and colleagues developed

an extremely sensitive and specific assay, the modified proximity ligation assay (PLA) to detect exosomes in plasma of prostate cancer patients. They successfully detected significantly increased prostasomes (prostate specific exosomes) in patients with prostate cancer before radical prostatectomy, compared with controls and patients with benign prostate diseases, and especially, with a capability to distinguish high/medium Gleason scores from low ones (23). Similarly, Turay *et al.* quantified exosomes in plasma of prostate cancer patients using the acetylcholinesterase enzymatic assay and found exosome levels significantly higher in the prostate cancer cohort than the healthy men (24). Excessive active secretion of exosomes detected by CD63-based ELISA was observed in the serum of ovarian cancer patients compared to healthy women and patients with benign ovarian diseases (25). Melo and colleagues demonstrated that the relative concentrations of circulating exosomes were significantly higher in the sera of patients with breast cancer or pancreatic ductal adenocarcinoma compared to healthy individuals (26). Using nanoparticle tracking analysis (NTA), Arbelaiz *et al.* found higher concentrations of serum extracellular vesicles (mainly exosomes) in the hepatocellular carcinoma (HCC) cohort than healthy, intrahepatic CCA and primary sclerosing cholangitis (PSC) cohorts (27). By immunocapture-based microfluidic chip analysis, Fang and colleagues detected more epithelial cell adhesion molecule (EpCAM)-positive exosomes in patients with breast cancer (28).

Apart from the increase of circulating exosomes in the blood circulation of cancer patients, amazingly, the size and morphology alterations of exosomes can be also found in cancer. For instance, exosome size distribution analysis by NanoSight[®] showed that the size of serum exosomes derived from pancreatic ductal adenocarcinoma was significantly smaller than those from healthy donors (26). Similarly, using atomic force microscopy, Zlotogorski-Hurvitz *et al.* observed that oral fluid-derived exosomes from oral cancer patients differ both morphologically and molecularly from exosomes present in healthy donors (29). However, the underlying mechanism of the morphological alterations of exosomes during tumor course is nubilous. Collectively, these investigations indicate that the amounts and morphological changes of exosomes in bloodstream are related to a specific course of the diseases, and may be used to estimate specific cancer status.

Compared to the detection of whole exosomes shed by all cell types, including malignant and wild type cells, into the blood circulation, it is more attractive and meaningful

to detect tumor-derived exosomes, since they may reflect pathological alterations of parental malignancy, and thus, enable the specific identification of their contents without the contamination of non-cancer exosomes. However, the isolation of tumor-specific exosomes from the blood of cancer patients remains a challenge due to the lack of specific markers that can be used to identify and distinguish cancer exosomes from non-cancer-derived ones.

Clinical relevance of circulating exosomal cargos in cancer

Circulating exosomal proteins (exo-proteins)

Considering their endosomal origin, exosomes released from different cell types are enriched of endosome-associated proteins, like Rab GTPase, annexins and flotillin (30). Tetraspanins, such as CD63, CD81, CD82 and CD9, are components of the exosomal membrane and frequently used as exosome markers. ExoCarta, an exosome database (<http://exocarta.org/>), has been developed to identify the exosomal contents. Up to now, approximately 10,000 different proteins have been characterized (31,32). The protein composition in total exosome fractions isolated from blood is significantly higher in cancer patients than in healthy controls. For example, the protein concentration in serum-derived exosomes from patients with acute myeloid leukemia (AML) showed a value of around 75 µg protein/mL, and was 60-fold higher than those isolated from the serum of normal controls (about 1.2 µg protein/mL) (33).

Recently, studies uncovered that, based on the property of proteins, either as a tumor promoter or suppressor, the export of proteins by exosomes impacts tumor environment and is also linked to chemotherapy response. In patients with AML undergoing chemotherapy, the protein levels in exosomes derived from plasma went down on day 14 after the initiation of chemotherapy, whereas during consolidation therapy with high-dose cytarabine, the exo-protein levels increased to a similar level at diagnosis, indicating that the dynamic changes of exo-protein levels after induction and during consolidation chemotherapy could be useful for evaluating patients' responses to chemotherapy (34). The study by Lv's group showed that anticancer drugs enhanced the release of heat shock proteins (HSPs)-enriched exosomes from HCC cells that elicited effective natural killer cell antitumor responses *in vitro* (35). Previously, Safaei and coworkers revealed that cisplatin-resistant ovarian cancer cells released exosomes that

contained an excess of proteins and also carried the cisplatin export transporters, MRP2 (mitochondrial 37S ribosomal protein), ATP7A (ATPase copper transporting alpha) and ATP7B (ATPase copper transporting beta). Following the exposure to cisplatin, the exosomes released from cisplatin-resistant cells contained 2.6-fold more platinum than those released from sensitive cells, indicating the active export of chemotherapy drug by exosomes (36). Consistently, the study by Szajnik *et al.* observed that patients with ovarian cancer whose exo-protein levels decreased after therapy were clinical responders to chemotherapy, suggesting that exo-proteins in blood could act as a potential biomarker to predict response to chemotherapy (37). In addition, exosomes released by the HER2-overexpressing breast cancer cell lines could discharge a full-length active HER2 molecule, resulting in resistance to Trastuzumab and implying the association of HER2-positive exosomes and sensitivity to Trastuzumab in breast cancer (38).

Besides their mediation of therapeutic response to anti-cancer drugs, exo-proteins also contribute to other actions during tumor development. Putz *et al.* showed that phosphatase and tensin homolog (PTEN), an important tumor suppressor protein, could be taken up via exosomes by recipient cells with resultant functional phosphatase activity, leading to the repression of cell proliferation in GBM (39). Annexin II, which was highly expressed in exosomes from malignant and premetastatic breast cancer cells was proved to promote tissue plasminogen activator (tPA)-dependent angiogenesis and facilitate the formation of favorable microenvironment for metastasis, via activating macrophage-mediated p38MAPK (p38 map kinase), NF-κB (nuclear factor kappa B), and STAT3 (signal transducer and activator of transcription 3) pathways and increasing secretion of IL6 (interleukin 6) and TNFα (tumor necrosis factor alpha) (40). While studying the role of Wnt5b in cancer progression, Harada and colleagues found that 55% of secreted endogenous Wnt5b was associated with exosomes. These exosomes containing activated Wnt5b proteins stimulated migration and proliferation of A549 lung adenocarcinoma cells (41). In summary, these findings open new and interesting avenues for the investigation of tumor-associated proteins not only at original tumor sites but also in distant metastatic organs where these proteins are trafficked by exosomes in a paracrine manner.

Concerning the vital roles of proteins within exosomes, proteomic analyses of whole exosomes, in particular tumor-derived exosomes have been performed to screen biomarkers for cancer (Table 1). For instance, by

Table 1 Proteins selectively encapsulated in blood-derived exosomes in cancer patients

Protein	Cancer type	Plasma/serum	Clinical value	Reference
CD44v6, Tspan8, EpCAM, CD104	Pancreatic cancer	Serum	Diagnostic	(42)
CD71, Lysozyme C, Obscurin-like protein 1, vimentin, PLF4, MARE2	Pancreatic cancer	Serum	Diagnostic	(43)
Galectin-9	Nasopharyngeal carcinoma	Serum	Diagnostic	(44)
CD63, caveolin-1	Melanoma	Plasma	Diagnostic	(45)
TYRP2, VLA-4, Hsp70, MET	Melanoma	Plasma	Diagnostic	(19)
Total proteins	Melanoma	Plasma	Prognostic	(19)
MDA-9, GRP78	Melanoma	Serum	Diagnostic	(46)
CD34	Acute myeloid leukemia (AML)	Plasma	Diagnostic	(47)
TGF- β 1, total proteins	AML	Serum	Diagnostic	(33)
TGF- β 1, total proteins	AML	Plasma	Diagnostic	(34)
CD9, CD147	Colorectal cancer	Serum	Diagnostic	(48)
FGFR1, IGF1, VTN, HSP90, complement factor H, complement C3, plasma protease C1 inhibitor, vitamin K-dependent protein C alpha-1-antitrypsin, alpha-2-antiplasmin, complement C9	Colorectal cancer	Serum	Diagnostic	(49)
Claudin 4	Ovarian cancer	Plasma	Diagnostic	(50)
PTEN	Prostate cancer	Plasma	Diagnostic	(51)
Survivin	Prostate cancer	Plasma	Diagnostic	(52)
Claudin 3	Prostate cancer	Plasma	Diagnostic	(53)
GGT(gamma-glutamyltransferase)	Prostate cancer	Serum	Diagnostic	(54)
Total proteins, MDR-1/3, PABP4	Prostate cancer	Serum	Diagnostic	(55)
CD81, PSA	Prostate cancer	Plasma	Diagnostic	(56)
AMPN, VNN1, PIGR	Cholangiocarcinoma (CCA)	Serum	Diagnostic	(27)
LG3BP, PIGR	Hepatocellular carcinoma	Serum	Diagnostic	(27)
Total proteins	Glioma	Plasma	Diagnostic	(57)
CD91	Lung cancer	Serum	Diagnostic	(58)

quantitative proteomics analysis, Chen *et al.* identified 36 up-regulated proteins involved in processes that modulate the protumorigenic microenvironment for metastasis, and 22 down-regulated proteins related to tumor growth and cell survival, in the serum-purified exosomes from patients with colorectal cancer. These findings suggest the utility of deregulated exo-proteins in the blood-based diagnosis of cancer (49). An and coworkers analyzed the exo-proteome in the serum of patients with pancreatic cancer undergoing chemoradiotherapy at different time points and identified 8 proteins that exhibited global treatment-specific changes,

implying their potential to monitor treatment resistance during the course of therapy (43). Database-augmented mass spectrometry analysis of exosomes isolated from plasma of patients with prostate cancer conducted by Worst *et al.* revealed that the amounts of the tight junction protein claudin 3 were higher in patients with Gleason ≥ 8 tumors than patients with benign prostatic hyperplasia (BPH) and Gleason 6–7 tumors. The levels of this exo-protein could predict a Gleason score ≥ 8 with an area under the curve (AUC) value of 0.705, suggesting the diagnostic power of exo-claudin 3 in prostate cancer (53). Arbelaz *et al.*

determined the protein composition of serum exosomes isolated from CCA, PSC, HCC and healthy individuals by mass spectrometry. In this profiling study, the researchers screened 95 exo-proteins differentially expressed in CCA *vs.* controls, 161 in PSC *vs.* controls, 50 in CCA *vs.* PSC, and 98 in HCC *vs.* controls, which are mostly related to response to wounding, defense to infections, inflammatory responses and immune activation. Specifically, among these differentially expressed exo-proteins, aminopeptidase N (AMPN), pantetheinase (VNN1) and polymeric immunoglobulin receptor (PIGR) showed the best diagnostic capacity to differ CCA and control with AUC values of 0.878, 0.876 and 0.844, respectively. Notably, galectin-3-binding protein (LG3BP) and PIGR present better diagnostic capability than alpha-fetoprotein (AFP), a non-specific serum tumor biomarker commonly employed in HCC, to discriminate HCC from healthy individuals (27). This study indicates that these multiple differentially expressed oncogenic proteins confined in exosomes can be utilized as diagnostic tools in cancer. Kawakami and coworkers observed higher gamma-glutamyltransferase activity in exosomes derived from the serum of patients with prostate cancer than patients with BPH (54). In the serum of patients with AML, exosome-associated transforming growth factor- β 1 (TGF- β 1) was significantly higher than that in normal controls (33). Following this discovery, Hong *et al.* firstly revealed the potential link between TGF- β 1 levels in plasma-derived exosomes and therapeutic response in patients with AML. Exo-TGF- β 1 levels at AML diagnosis were much higher than those of healthy donors, and then reduced drastically following induction of chemotherapy, but subsequently found to be elevated relative to post-induction levels in patients receiving consolidation (34). Furthermore, the researchers also observed 3 different forms of TGF- β 1 [the pro-peptide, latency-associated peptide (LAP) or active, mature form] in plasma exosomes from patients with AML before, during, or after chemotherapy. For instance, apart from the distinctly different levels of active TGF- β 1 in exosomes obtained before, during or after chemotherapy, the levels of exo-LAP expression were generally high at AML diagnosis and also in exosomes of patients undergoing consolidation chemotherapy, indicating that exo-TGF- β 1 levels and TGF- β 1 activation in exosomes might reflect response to chemotherapy (34). The same lab also found elevated total protein levels in plasma exosomes of patients with malignant glioma, and their positive correlation with WHO tumor grade (57). Moreover, exo-protein levels were measured in the plasma of patients who received phase I/II

dendritic cell/peptide-based vaccination treatment. The protein levels exhibited a rapid decrease from pre- to post-vaccination, and were related to immunological and clinical response, implying exo-proteins as a predictive marker of clinical response to vaccine (57). In patients with prostate cancer, the total protein levels contained in exosomes isolated from serum were abnormally increased in docetaxel resistant patient subset compared to the sensitive group. Specifically, western blot analysis showed that MDR-1/3 (multidrug transporter-1/3) and PABP4 (poly A binding protein 4) were exclusively overexpressed in the patients with docetaxel resistant prostate cancer, indicating their value to predict response to chemotherapy (55). Moreover, exosomes from the serum of patients with metastatic melanoma were observed to contain higher levels of MDA-9 and GRP78 proteins than those of patients without metastases, suggesting their utilities as biomarkers for early detection of metastasis (46). PTEN is frequently reduced or completely lost in aggressive and metastatic prostate cancer. However, PTEN has been detected in plasma-derived exosomes from patients with prostate cancer but not healthy subjects, suggesting that exo-PTEN may compensate for PTEN loss in advanced prostate cancer, and may have diagnostic value for prostate cancer (51). Khan and colleagues showed much higher levels of exo-Survivin in plasma of patients with prostate cancer, especially in relapsed patients on chemotherapy than patients with BPH and healthy individuals (52), indicating the association of exo-Survivin and sensitivity to chemotherapeutic agents in prostate cancer. Using anti-CD9 antibody-coupled highly porous monolithic silica microtips, Ueda *et al.* successfully captured exosomes from the serum of 46 patients with lung cancer. The subsequent mass spectrometric analysis identified CD91 as a lung adenocarcinoma specific antigen on exosomes to distinguish lung cancer from healthy individuals (58). In melanoma, the levels of 4 proteins, tyrosinase-related protein 2 (TYRP2), very late antigen-4 (VLA-4), heat shock protein 70 (HSP70) and MET oncoprotein were increased in plasma exosomes from stage III and IV patients compared to stage I patients as well as healthy controls (19). Moreover, the high protein content of plasma-derived exosomes correlated with poor survival (19). Prostate specific antigen (PSA) is the most important tumor marker in prostate cancer. Logozzi *et al.* detected that, compared to benign prostatic hypertrophy, patients with prostate cancer had high levels of exosomes expressing both CD81 and PSA in plasma, implying that PSA-exosomes, may represent a novel clinical tool for the screening of

prostate cancer (56).

To sum up, the identification of specific exo-proteins can not only facilitate diagnosis, prognosis and monitoring chemotherapeutic response, but also contribute to the screening of specific antigens characteristically expressed on tumor-derived exosomes, which can be used as makers to capture tumor specific exosomes.

Circulating exosomal miRNAs (exo-miRNAs)

miRNAs, the small non-coding RNAs of 18–25 nucleotides in length, are involved in the course of tumor development and progression via binding specifically to the 3' UTR of their target mRNAs, leading to posttranscriptional repression of tumor-associated mRNAs (59). miRNAs can be released into bloodstream as molecules incorporated in exosomes, or as cell-free miRNAs bound to argonaute (AGO) proteins or high-density lipoproteins (HDL) (2), which prevent them from the digestion of RNase and thus, exist stably in blood circulation. Owing to their important roles in cancer and their stability in blood, the utility of miRNAs as “liquid biopsy” for cancer detection has been intensively investigated. In bloodstream, miRNAs integrated in exosomes account for the largest proportion of circulating miRNAs discharged from different sources (60). Therefore, it is of great interest to investigate the patterns of circulating exo-miRNAs in cancer patients.

For example, Taylor *et al.* demonstrated that miR-21, miR-203, miR-205, miR-214 and miR-200 family (with the exception of miR-429) were selectively sorted into EpCAM-specific exosomes in the serum of ovarian cancer patients (5). In patients with breast cancer, serum exo-miR-373 seemed to have the capability to distinguish different molecular subtypes. Specifically, its levels were much higher in triple negative than luminal carcinomas. Also, estrogen and progesterone-negative patients harbored more serum exo-miR-373 than those with hormone-receptor positive tumors. These data suggest that serum exo-miR-373 correlates with receptor negative subtype and may contribute to the progression of breast cancer (61). Another study with a cohort of 163 patients with epithelial ovarian cancer (EOC) was conducted in the same lab. The patients with EOC displayed increased serum levels of exo-miR-373, which further verifies the prevalence of this oncogenic miRNAs in different cancers (25). Moreover, the study also showed that serum levels of exo-miR-200a, miR-200b and miR-200c differ between benign and malignant ovarian tumors, and the combination of the three exo-miRNAs

could improve the sensitivity to discriminate benign from malignant ovarian tumors (25).

Apart from breast cancer (61) and ovarian cancer (5,25), previous studies have reported that circulating exo-miRNAs in blood of various cancer types, including lung (62–66), thyroid (67), esophageal adenocarcinoma (68), esophagus adenocarcinoma (69,70), colon (71–77), hepatoblastoma (78), hepatocellular (79,80), pancreatic (81,82), prostate (83–85), multiple myeloma (86), leukemia (87,88), myeloma (89), melanoma (90), laryngeal squamous cell carcinoma (LSCC) (91) and osteosarcoma (92), also show distinct signatures, which may be used for cancer diagnosis, prognosis and monitoring response to chemotherapy (Table 2).

The above exo-miRNA profiling showed that during tumor procession, a subset of miRNAs was preferentially incorporated into exosomes, indicating a selective miRNA export mechanism by exosomes (94). However, whether the miRNA transport is rather selective or non-selective, is still arguable, and needs to be further investigated.

Still, there is no consensus on an endogenous control for the normalization of exo-miRNAs as well as cell-free miRNAs, which may lead to discrepant results among different studies, or even inaccurate results. For an accurate analysis of circulating miRNAs, a comprehensive screening of the relatively consistent miRNAs that can be used as endogenous normalizers is highly recommended. Moreover, the combination of endogenous and exogenous spike-in controls can minimize the bias caused by the quality of isolated miRNAs, and the sample preparations and experimental handling (95).

Circulating exosomal long non-coding RNAs (exo-lncRNAs)

Long non-coding RNAs are RNA molecules more than 200 nucleotides in length. They have been reported to have diverse functions in the regulation of gene expression, e.g., epigenetic regulation, chromatin modification, transcription or posttranscriptional processing (96). It is becoming increasingly clear lncRNAs are crucial regulators of processes involving the initiation and progression of tumors. For example, the lncRNA-activated by TGF- β (lncRNA-ATB) up-regulated ZEB1 and 2 (zinc finger E-box binding homeobox 1 and 2) through competitively binding with miR-200 family members to induce EMT and cell invasion. Moreover, lncRNA-ATB promoted organ colonization of disseminated tumor cells (DTCs) by binding with IL-11 mRNA to trigger STAT3 signaling (97). Ahadi and colleagues examined lncRNAs and miRNAs enriched in

Table 2 Deregulated miRNAs within blood-derived exosomes in cancer patients

miRNA	Cancer type	Plasma/serum	Clinical value	Reference
miR-23b-3p, -10b-5p, -21-5p	Non-small cell lung cancer	Plasma	Prognostic	(63)
miR-96	Lung cancer	Serum	Diagnostic	(64)
miR-30b, -30c, -103, -122, -195, -203, -221, -222	Lung cancer	Plasma	Diagnostic	(65)
miR-17-3p, -21, -146, -155, -191, -203, -205, -214,	Lung cancer	Plasma	Diagnostic	(66)
miR-4772-3p	Colorectal cancer	Serum	Prognostic	(73)
miR-21, -29a, -92a	Colorectal cancer	Serum	Diagnostic	(74)
miR-17-92a cluster, -19a	Colorectal cancer	Serum	Prognostic	(75)
let-7a, miR-1229, -1246, -150, -21, -223, -23a	Colorectal cancer	Serum	Diagnostic	(76)
miR-638, -5787, -8075, -6869-5p, -548c-5p, -486-5p, -3180-5p	Colorectal cancer	Serum	Diagnostic	(77)
miR-21, -181a	Thyroid	Plasma	Diagnostic	(67)
miR-16-5p, -15a-5p, -20a-5p, -17-5p	Multiple myeloma	Serum	Prognostic	(89)
let-7b, miR-18a	Multiple myeloma	Serum	Prognostic	(86)
miR-373, -200a, -200b, -200c,	Ovarian cancer	Serum	Diagnostic prognostic	(25)
miR-21, -141, -200a, -200c, -200b, -203, -205, -214	Ovarian cancer	Serum	Diagnostic	(5)
miR-373	Breast cancer	Plasma	Diagnostic	(61)
miR-141	Prostate cancer	Serum	Diagnostic	(83)
miR-375, -21, -574	Prostate cancer	Serum	Diagnostic	(84)
miR-1290, -375	Prostate cancer	Plasma	Prognostic	(85)
miR-17, -19a, -21, -126, -149	Melanoma	Plasma	Diagnostic prognostic	(90)
miR-18a, -221, -222, -224, -101, -106b, -122, -195	Hepatocellular carcinoma	Serum	Diagnostic	(79)
miR-21	Hepatocellular carcinoma	Serum	Diagnostic	(80)
miR-718	Hepatocellular carcinoma	Serum	Prognostic	(93)
miR-140-3p	Chronic myeloid leukemia (CML)	Whole blood	Diagnostic	(87)
miR-150, -221, -1246	AML	Serum	Diagnostic	(88)
RNU6-1, let-7e-5p, miR-16-5p, -25-3p, -320a, -15b-5p, -30a-5p, -324-5p, -17-5p, -194-5p	Esophageal adenocarcinoma	Serum	Diagnostic	(68)
miR-21	Esophageal adenocarcinoma	Serum	Diagnostic, prognostic	(70)
miR-223, -483-, -409, -196b, -192, -146a, -126-5p	Esophageal adenocarcinoma	Serum	Diagnostic	(69)
miR-21	Laryngeal squamous cell carcinoma	Serum	Diagnostic	(91)
miR-17-5p, -21, -155, -196a	Pancreatic cancer	Serum	Diagnostic	(82)
miR-1246, 4644, 3976, 4306	Pancreatic cancer	Serum	Diagnostic	(42)
miR-10b, -20a, -21, -30c, -106b, -181a	Pancreatic cancer	Serum	Diagnostic	(81)
miR-124, -133a, -199a-3p, -385, -135b, -148a, -27a, -9	Osteosarcoma	Serum	Diagnostic	(92)

Table 3 Deregulated lncRNAs confined in blood-derived exosomes in cancer patients

lncRNA	Cancer type	Plasma/serum	Clinical value	Reference
HOTAIR	Laryngeal squamous cell carcinoma	Serum	Diagnostic	(91)
Lnc ZFAS1	Gastric cancer	Serum	Diagnostic	(100)
LINC00152	Gastric cancer	Plasma	Diagnostic	(101)
BCAR4, MEG3, PCA3, PTENP1, TUC338, GAS5	Colorectal cancer	Serum	Diagnostic	(102)
Lnc CRNDE-h	Colorectal cancer	Serum	Diagnostic, prognostic	(103)
MALAT-1	Non-small cell lung cancer	Serum	Diagnostic	(104)
lncRNA-p21	Prostate cancer	Plasma	Diagnostic	(105)

exosomes that were released into the cell culture media by prostate cancer cell lines. They uncovered that 26 exo-lncRNAs are enriched for miRNA seeds with a preference for let-7 family members as well as miR-17, miR-18a, miR-20a, miR-93 and miR-106b, indicating the potential of exo-lncRNAs as miRNA sponges in tumor-related gene regulation (98). Accumulating evidence is revealing an important role of lncRNAs in tumor drug resistance. For instance, Wang's research group initially identified and named lncARSR (lncRNA activated in renal cell carcinoma with sunitinib resistance), which promoted sunitinib resistance via competitively binding with miR-34/miR-449 to facilitate the expressions of receptor tyrosine kinase AXL and c-MET in renal cancer cells. Intriguingly, the bioactive lncARSR could be incorporated into exosomes and transmitted to sensitive cells to transfer sunitinib resistance (99).

Currently, only a few reports regarding the utility of circulating exo-lncRNAs as biomarkers for cancer are available (Table 3). Dong and coworkers reported the presence of 21 cancer-related lncRNAs differentially expressed in serum exosomes of patients with colorectal cancer compared to healthy controls, indicating the potential of exo-lncRNAs to diagnose colorectal cancer (102). MALAT-1 (metastasis-associated lung adenocarcinoma transcript) is the first identified lncRNA that is related to lung cancer metastasis. Zhang *et al.* measured significantly higher levels of MALAT-1 in serum exosomes of patients with non-small cell lung cancer (NSCLC) than healthy donors. More importantly, the elevated levels of exo-MALAT-1 were positively associated with tumor stage and lymphatic metastasis (104). As a newly identified lncRNA, lncZFAS1 (zinc finger antisense 1) promoted cancer cell proliferation and migration by activating the expressions of *ZEB1*, *MMP14* and *MMP16*

(matrix metalloproteinase 14 and 16) in HCC (106). In the serum of patients with gastric cancer, the increased levels of exo-lncZFAS1 significantly correlated with lymphatic metastasis and TNM stage (100). In bloodstream of patients with gastric cancer, the lncRNA, LINC00152 was reported to be mainly incorporated in exosomes. The levels of exo-LINC00152 were significantly elevated in patients with gastric cancer compared with healthy controls (101). In the serum of patients with colorectal cancer, Liu *et al.* found that exo-CRNDE-h levels significantly correlated with regional lymph node metastasis and distant metastasis. In addition, high exo-CRNDE-h levels were associated with a shorter patients' overall survival (103). In the serum of LSCC, the amounts of exo-miR-21 and exo-lncRNA HOTAIR were significantly higher in patients with LSCC than those with vocal cord polyps. In addition, higher serum exo-miR-21 and exo-HOTAIR expressions were linked to tumor stages and status of lymph node metastasis. Interestingly, the combination of exo-miR-21 and exo-HOTAIR could improve the sensitivity and specificity to detect LSCC (91). To sum up, these tumor-associated exo-lncRNAs are promising cancer biomarkers.

Circulating exosomal circular RNAs (exo-circRNAs)

Circular RNAs (circRNAs) are a large class of non-coding RNAs that, unlike linear RNAs, form covalently closed continuous loops. They participate in cancer process, mainly through acting as miRNA sponges to indirectly influence miRNA targets, enhancing the expression of their parental mRNAs at transcriptional level and sequestering RNA-binding proteins or ribonucleoprotein complexes related to tumorigenesis and progression (107-110). The expression levels of circRNAs have been found to be deregulated

Table 4 Abnormal mRNAs confined in blood-derived exosomes in cancer patients

mRNA	Cancer type	Plasma/serum	Clinical value	Reference
<i>EGFRvIII</i>	Glioblastoma	Serum	Diagnostic	(4)
<i>TIMP-1, IL-8, TGF-β, ZAP70</i>	Glioma	Plasma	Diagnostic	(57)
<i>IL-8</i>	Glioma	Plasma	Prognostic	(57)
<i>KRTAP5-4, MAGEA3, BCAR4</i>	Colorectal cancer	Serum	Diagnostic	(102)
<i>BRAF, KRAS</i> (mutant)	Colorectal cancer	Serum	Diagnostic	(116)
<i>Annexin2, Smad2, MTAP, CIP4, PEDF, P27, Cdc5L, WWOX</i>	Osteosarcoma	Serum	Diagnostic	(92)
<i>C-MYC, BCL-XL, BCL-6, PTEN, AKT</i>	Non-Hodgkin lymphoma	Plasma	Diagnostic, prognostic	(117)

in all types of cancer cell lines, tumor tissues, as well as blood samples from patients, and linked to certain clinical characteristics (111,112), suggesting their involvement in tumor progression.

The presence of abundant circRNAs within exosomes was firstly reported by Li and colleagues (113). They reported that circRNAs are more abundant in exosomes compared to the cytoplasm of producer cells. Taking advantage of RNA-seq analyses and qRT-PCR, this research group identified 1,215 circRNAs in human serum, and pointed out that, these circulating circRNAs in bloodstream were predominantly located in serum-derived exosomes but not in exosome-depleted serum. Furthermore, they observed that abundance of tumor-derived exo-circRNAs in the serum of patients with colorectal cancer was correlated with tumor mass. They also found that the expression profile of exo-circRNAs in cancer serum was significantly different from that in normal serum. Specifically, the expression levels of circ-KLDHC10 were significantly increased in cancer serum than those in healthy individuals, indicating its diagnostic potential. Furthermore, concerning the sorting of specific circRNAs in exosomes, Li and colleague provided evidence that sorting of circRNAs in exosomes may be regulated by changes of associated miRNA levels in producer cells (113). Consistently, Zhang's research group also observed more abundant circRNAs in exosomes than in cells. Besides, they detected significantly down-regulated levels of circRNAs in *KRAS* mutant colon cancer cells compared to wild-type cells, indicating that circRNAs enclosed in exosomes may be associated with *KRAS* status in colon cancer (114). Apart from these both investigations, so far, no further reports concerning exo-circRNAs are available. However, the research interest is now shifting from miRNAs and lncRNAs to circRNAs.

Circulating exosomal mRNAs (exo-mRNAs)

The presence of mRNAs in exosomes and their horizontal transfer via exosome vehicle to recipient cells were firstly elucidated by Valadi and coworkers in 2007 (10). Bioactive mRNAs (oncogenes or tumor suppressor genes) incorporated in exosomes were demonstrated to be delivered to target cells and translated into functional proteins, to change the phenotype of targeted cells, and even, further transform normal to malignant cells (4,10,115). Consequently, the abnormal expression of these tumor-associated exo-mRNAs encapsulated in exosomes present a potential to be used as predictive markers in cancer (Table 4).

For instance, Skog *et al.* reported that serum exosomes from patients with GBM contained over 4,700 distinct mRNAs, of which tumor-specific *EGFRvIII* RNA was detected in 7 out of 25 patients with GBM (4). Selected RNAs, *TIMP-1* (tissue inhibitor of metalloproteinase 1), *IL-8*, *TGF- β* and *ZAP70* (zeta chain of T-cell receptor associated protein 70), which are related to angiogenesis and clinical outcome in gliomas were quantified in exosomes isolated from plasma of patients with glioma at baseline and 8 weeks after vaccination therapy by the Whiteside's group. The four mRNAs were highly expressed at baseline and strongly down-regulated after vaccination (57). Recently, Hao and colleagues detected three mutations in the *KRAS* (Kirsten rat sarcoma viral oncogene) mRNA (codons 12, 13 and 61) and a mutation in *BRAF* (B-Raf proto-oncogene, serine/threonine kinase) mRNA (codon 600) in the serum exosomes of patients with colorectal cancer of a Chinese population (116). Dong *et al.* quantified the expression levels of 3 exo-mRNAs, keratin associated protein 5-4 (*KRTAP5-4*), melanoma antigen family A3

Table 5 Mutant DNAs confined in blood-derived exosomes in cancer patients

DNA	Cancer type	Plasma/serum	Clinical value	Reference
<i>KRAS, p53</i>	Pancreatic cancer	Serum	Diagnosis	(121)
<i>KRAS, TP53</i>	Pancreatic cancer	Serum	Diagnostic	(122)
<i>KRAS</i>	Pancreatic cancer	Plasma	Diagnostic, prognostic	(123)

(*MAGEA3*) and breast cancer anti-estrogen resistance 4 (*BCAR4*), confined in the serum exosomes of patients with colorectal cancer. They found that the combination of the three exo-mRNAs were able to detect colorectal cancer with an AUC of 0.936 (102). While profiling the miRNA signature in serum exosomes from patients with osteosarcoma, Xu and coworkers observed that Annexin2, *Smad2*, methylthioadenosine phosphorylase (*MTAP*), Cdc42-interacting protein 4 (*CIP4*), pigment epithelium-derived factor (*PEDF*), WW domain-containing oxidoreductase (*WWOX*), cell division cycle 5-like (*Cdc5L*) and *p27* mRNAs were differentially expressed in exosomes, and associated with a different chemotherapeutic response (92). Additionally, a recent report demonstrated the diagnostic and prognostic relevance of exo-mRNAs of *c-MYC* (*c-MYC* proto-oncogene, bHLH transcription factor), *BCL-XL*, *BCL-6*, *PTEN* and *AKT* (*AKT* serine/threonine kinase) in the plasma of patients with non-Hodgkin lymphoma (117). Specifically, the presence of *AKT* mRNA was associated with a poor response to rituximab-based treatment. The lower levels of *PTEN* and *BCL-XL* mRNAs were associated with relapse or disease progression. The presence of *c-MYC* mRNA correlated with a short progression-free survival. *BCL-6* and *c-MYC* mRNAs were independent prognostic variables of overall survival.

Despite their less abundance than miRNAs, the tumor-related exo-mRNAs carry important information on expression patterns and presence of mutations and variants, indicating that they can be developed as surrogates of cancer diagnosis and targeted therapies.

Circulating exosomal DNAs (exo-DNAs)

Besides miRNAs, lncRNAs, circRNAs and mRNAs within exosomes, distinct types of DNAs (single-stranded DNAs, double-stranded DNAs and mitochondrial DNAs) are incorporated in exosomes, of which double-stranded DNAs account for the majority of DNA types (118). Genomic DNA (gDNA) fragments transferred from exosomes to cells have been shown not only to increase the gDNA-coding mRNA

and protein levels, but also to influence functions in recipient cells (119). It has been demonstrated by Thakur *et al.* that exo-DNAs represent the entire genome and reflect the mutational status of parental tumor cells (120). Thus, the investigation of molecular alterations of tumor-related exo-DNAs is an attractive strategy in the early detection of cancers and monitoring of treatment response (Table 5).

Using PCR-based DNA fragment analysis, Fricke *et al.* revealed frameshift mutation patterns in microsatellite stretches of microsatellite unstable (MSI) target genes, *MARCKS* (myristoylated alanine rich protein kinase C substrate), *TGFBR2* (transforming growth factor beta receptor 2) and *LMAN1* (lectin, mannose binding 1) in exosomes released by colorectal cancer cell lines harboring MSI, indicating that the coding MSI phenotype of DNAs is maintained in the exo-DNAs (124). By whole genome sequencing, Kahlert *et al.* demonstrated that serum exosomes from patients with pancreatic cancer contain gDNA spanning all chromosomes with mutated *KRAS* and *p53*, indicating that serum-derived exosomes can be used to determine gDNA mutations for cancer detection, treatment, and therapy resistance (121). Similarly, Yang and colleagues also detected the presence of mutant *KRAS* and *p53* in serum exosomes of patients with pancreatic cancer (122). Consistently, another study also reported a highly frequent mutation of *KRAS* in exo-DNAs in the plasma of patients with pancreatic cancer. These higher mutation allele frequencies of exo-*KRAS* DNA were associated with decreased disease-free survival in the patients with localized pancreatic cancer (123).

In summary, exo-DNAs in cancer may indeed represent a new treasure trove for monitoring changes in the mutational landscape to predict cancer presence, prognosis or response to chemotherapy. However, the issues associated with the diagnostic sensitivity and noise signal due to the contamination of tumor-specific DNAs with DNAs from normal cells are a challenge. Therefore, it is important to selectively enrich tumor-specific exosomes prior to the determination of translational value of exo-DNAs as liquid biopsy.

Conclusions

As the knowledge of biogenesis, biomolecule cargos and biological functions of exosomes, in particular tumor cell-derived exosomes, advances, the documents about exploiting exosomes and their cargos as “liquid biopsy” in real-time, to monitor cancer development and progression, are accumulating. However, a challenging aspect with respect to the exploration of exosomes-based biomarkers has turned out to be their effective and selective isolation. The plurality of developed isolation protocols including ultracentrifugation, chemical precipitation, affinity capturing, filtration techniques, and ultracentrifugation combined with iodixanol density gradient centrifugation or gel filtration results in qualitative and quantitative variability in terms of the extracted exosomes, which may lead to discrepant results of downstream analyses and make it difficult to compare or reproduce the results among different research groups. Tumor-specific exosomes, as a subpopulation of whole exosomes released by tumor cells, are distinct from exosomes secreted by normal cells, and may, of course, provide more information of tumor-related pathways. The identification of tumor-specific markers on exosomes with functional properties reflecting the characteristics of the parental tumor cells has been pressed ahead. Although remarkable progress in bringing exosomes, particularly tumor-specific exosomes to the attention of the scientific and clinical communities has been made, assays of exosomes in large patient cohorts with an exact description of the clinical pathological parameters (tumor stage, metastatic status, response to chemotherapy, long-term follow up) need to be conducted under standard criteria. All these hurdles have to be overcome before the clinical application of tumor-specific exosomes as a “liquid biopsy” in cancer patients.

Besides their potential value as biomarker, and based on their unique characteristics as stable, small molecules to cross the blood-brain barrier, exosomes can be created as carriers for the delivery of small interfering RNA (siRNA) or drugs, which have been proved to be effective in pre-clinical studies and clinical trials. In this regard, dendritic cell-derived exosomes as immunotherapeutic anticancer agents have entered clinical trials for colorectal cancer, metastatic melanoma and NSCLC. However, they have only achieved modest therapeutic effects (125,126). Moreover, due to the oncogenic property of tumor-associated exosomes to transform normal to malignant cells, the depletion of exosomes from blood of advanced cancer

patients is a novel strategy to treat cancer (127).

Since exosomes are small particles with big roles in cancer, they are promising biomarkers as well as target molecules for the clinical practice.

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Footnote

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