Review Article

Extracellular vesicles long RNA for cancer diagnosis and prognosis: a narrative review

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Background and Objective: Cancer is an aggressive disease and exhibits a low median survival rate. Accurate early diagnosis and prognosis prediction are essential to improve the patient's survival status. Extracellular vesicles (EVs), an emerging liquid biopsy, have shown promising performance in cancer diagnosis, tumor response prediction and treatment monitoring. The profiling of extracellular vesicle long RNAs (exLRs) was shown to be different in different physiological and pathological states. The review aims to provide a comprehensive overview of exLRs for cancer diagnosis and prognosis.

Methods: Full-length manuscripts published until August 2022 gathered from PubMed were used to inform this review. Non-English publications and studies not related to oncology were excluded.

Key Content and Findings: EVs are a heterogeneous group of double membrane structures that either originate from the endosomal system or are shed from the plasma membrane. ExLRs including mRNAs, lncRNAs and circRNAs, are abundant, stable and partially intact with the protection of membrane. They are also characterized as tissue-specific and show promise in cancer liquid biopsy. We highlight the current status and approaches of these exLRs in early diagnosis and treatment response assessment in oncology [e.g., breast cancer, lung cancer, colorectal cancer (CRC), hepatocellular carcinoma (HCC), prostate cancer (PCa), etc.]. In addition, the prospects and challenges of applying exLRs to precision medicine are discussed.

Conclusions: ExLRs are becoming a valuable tool for cancer diagnosis and prognosis. Efficient and stable methods for standardized EV isolating and exLR detection are needed to enhance their application in clinical applications.

Keywords: Extracellular vesicles long RNA (exLR); biomarkers; cancer; diagnosis; prognosis prediction

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Introduction

Cancer is an enormous socioeconomic burden on society worldwide, with an estimated 19.3 million new cancer cases diagnosed and almost 10.0 million cancer deaths in 2020 according to GLOBOCAN (1). In 2022, approximately 4,820,000 and 1,918,030 new cancer cases, 3,210,000 and 609,360 deaths are estimated in China and the United States, respectively (2,3). It usually takes years to develop invasive or metastatic cancer, and early detection is crucial to reducing cancer morbidity and mortality. However, most cancer patients are asymptomatic at the early stage. Additionally, treatment response assessment or prognosis prediction is valuable in determining treatment strategies and improving treatment effectiveness, thus enhancing survival rate (4). Traditional cancer detection methods (e.g.,

Table 1 Summary of the search strategy

Items	Specification
Date of search	July to August 2022
Databases and other sources searched	PubMed
Search terms used	Extracellular vesicle, or exosome, and cancer, and long RNA
Timeframe	From inception until August 2022
Inclusion and exclusion criteria	Inclusion criteria: studies published in English and including the search terms used
	Exclusion criteria: studies not related to tumors
Selection process	The selection process was conducted by the authors

serum biomarkers, imaging), are limited by challenges, such as low sensitivity or specificity, and high cost. Tissue biopsy and histopathological examinations are the gold standards for tumor diagnosis. However, due to invasiveness, they cannot be used for early detection and efficacy monitoring.

Liquid biopsy represents a minimally invasive approach to providing comprehensive information about tumors (5). Additionally, liquid biopsy can be repeatedly used to obtain samples, thus monitoring disease progression or recurrence. Circulating tumor cells, circulating tumor DNA and extracellular vesicles (EVs), are the three major resources of liquid biopsy (6). Among these, EVs, refer to a heterogeneous group of particles naturally released from the cells that are enclosed in a lipid bilayer and are composed of a series of nanoparticles with different cellular origins, sizes, and contents (7). They are present in various biological fluids, including blood-derived plasma/serum, urine, saliva, milk, pleural effusions, cerebrospinal fluid (CSF) and ascites (8,9). Studies showed that tumor cells secreted at least 10-fold more EVs than normal cells, and tumor-derived EVs could facilitate cell-to-cell communication through the transport of biomolecules (10,11). Specifically, the changes in EVs and their contents before and after therapy also show potential for anticancer treatment monitoring, promoting individual management in cancer patients (5,12). Thus, tumor-associated molecules found in EVs are potential biomarkers for the diagnosis, therapeutic monitoring, and prognosis of cancer patients. Massive studies underline that the variations of EV-derived microRNAs (miRNAs) are correlated with the progression of the disease (13,14). With the development of detection methods, such as high-throughput next-generation sequence (NGS), more than 10,000 extracellular vesicle long RNAs (exLRs), including messenger RNAs (mRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs), were detected from each human plasma (15). The unique properties of exLRs, such as tissue-specific expression, relative stability in circulation and partially intactness, widespread and abundant presence in a variety of biological fluids, have prompted studies of their function and their extensive clinical applications in diseases such as cardiovascular diseases (16), neurodegenerative diseases (17), osteoporosis (18), and various cancers.

With the growth of EV-related studies, novel diagnostic and prognostic biomarkers are emerging. Previous reviews have highlighted the characteristics and applications of EV-derived miRNAs or non-coding RNAs (including miRNAs, lncRNAs and circRNAs) (10,13). In this review, we attempt to comprehensively review the literature on EVs, in particular, exLRs, from their characteristics to their potential applications in the diagnosis and prognosis of cancer. This review summarizes the biogenesis and characteristics of EV and exLRs, and highlights the current status and approaches of these biomarkers that have the potential to be used for early diagnosis and accurate prediction of therapeutic approaches. In addition, the prospects and challenges of applying exLRs to precision medicine are discussed. We present this article in accordance with the Narrative Review reporting checklist (available at https://pcm.amegroups.com/article/view/10.21037/pcm-22-46/rc).

Methods

A systematic literature search was conducted in PubMed published up to August 2022 with the following keywords: extracellular vesicle, or exosome, and cancer, and long RNA. Only English language articles were included (*Table 1*). Abstracts were evaluated and manuscripts not focused on oncology were removed.

Biogenesis and characteristics of EVs

EVs were firstly described in 1967 by Wolf as platelet-dust and they were initially assumed to be released to discard unwanted molecules from cells (19). It was not until 1996 that EV research drastically changed when Raposo *et al.* revealed the role of B cell-derived EVs in immunoregulation (20). Since then, the EV-related research has gained more and more attention. Numerous studies have identified that EVs delivered biomolecules between cells and acted as a novel cell-to-cell communication form in both normal physiological processes and pathological progression (10,11).

Although the classification of EVs is continuously changing, they are generally divided into three major groups, exosomes, microvesicles, and apoptotic bodies on the basis of their biogenesis and size (11). Exosomes, with a majority size of 30–100 nm, originate from the inward budding of the endosomal membranes and the fusion of multivesicular bodies with the plasma membranes to secrete the membrane-encapsulated vesicles into the extracellular space (21,22). The Endosomal Sorting Complex Required for Transport (ESCRT) machinery is the most commonly described pathway for exosomal biogenesis (22,23). ESCRT-0 recognizes ubiquitinated proteins in the late endosomal membrane and initiates the process, ESCRT-I/II are then activated and drive the intraluminal membrane budding, and ESCRT-III is recruited via the programmed cell death 6-interacting protein and drives the shedding of vesicles. Finally, ESCRT system is disassembled by auxiliary proteins (specifically VPS4 ATPase). Microvesicles, ranging in size from 50 to 1,000 nm, their biogenesis is far less defined. In general, these vesicles are produced by the outward budding and fission of the plasma membranes, and then subsequently released into the extracellular environment (21,24). Unlike exosomes and microvesicles released by living cells, apoptotic bodies, with diameters of 0.5–2 μm, are released from blebbing of the plasma membrane of apoptotic cells or dying cells (25).

exLR

EVs are secreted from almost all cell types (26). They are involved in aspects such as intercellular communication as they deliver different cargos, including DNAs, RNAs, proteins, lipids and other bioactive molecules, to recipient cells. EV composition can vary due to the selective sorting of cargo into EVs (27). EV RNAs, mainly contain mRNAs and non-coding RNAs—such as miRNAs, lncRNAs, and circRNAs (27,28).

In the past decade, studies of EV-derived RNAs have placed emphasis on the miRNAs, and many of them have been identified as prognostic and predictive biomarkers of cancers, such as EV-miRNA panels for the early diagnosis of breast cancer (29) and ovarian cancer (30), EV-derived miR-17-92a to predict recurrence in colorectal cancer (CRC) patients (31). However, the clinical application of EV miRNAs is limited due to their low quantity (~2,500 EV miRNAs) and specificity (8,32). Meanwhile, a growing number of groups have reported the presence of exLRs and their function in oncology. In 2006, Baj-Krzyworzeka *et al.* firstly showed that EVs contained mRNAs of tumor cells, and these molecules can be delivered to recipient cells (33). And later in 2008, Skog *et al.* detected the mRNA mutant in the serum EV of glioblastoma patients, opening a new window to use EV-derived mRNA for cancer diagnosis and therapeutic decisions making (34). EV lncRNAs were initially reported in 2014, and their expressions might reflect cellular response to DNA damage (35). Our lab firstly demonstrated the presence of abundance circRNAs in EVs through RNA-seq analysis of cancer cells and cell-derived EVs in 2015 (36), and afterwards, we created a database containing mRNAs, lncRNAs and circRNAs in human blood EVs (named exoRBase) (37). Nowadays, the exoRBase database has been updated to version 2.0 by gathering about 1,000 RNA-seq data of EVs from four types of human body fluids (blood, urine, CSF and bile), covering 19,643 mRNAs, 15,645 lncRNAs and 79,084 circRNAs (38).

Besides the abundant level of tumor specific biomarkers, long RNAs, can provide additional opportunities to study other processes that may indicate disease development/progression. For example, fusion transcripts [e.g., echinoderm microtubule-associated protein-like 4/anaplastic lymphoma kinase (EML4-ALK) fusion variant (39)], splice variants [such as androgen receptor splice variant 7 (40)], represent alternative approaches that may not be detected using miRNAs (41), further promoting the research of exLRs. Thus in this review, we place our focus on the exLRs, including EV-mRNAs, lncRNAs and circRNAs.

EV-mRNA

mRNAs serve a crucial role in protein synthesis by delivering the message encoded in the DNA within genes to the cytoplasm (42). Three motifs ACCAGCCU,

CAGUGAGC and UAAUCCCA, are enriched in EV-mRNAs (43-45), and Y box binding protein 1 (YB-1) and NOP2/Sun RNA methyltransferase 2 (NSUN2) are involved in the process of the recognization of specific motifs and transfer of specific mRNAs into EVs (44).

EV-lncRNA

LncRNAs are transcripts with more than 200 nt and lack protein-coding ability (46). They interact with proteins, RNAs, and DNAs, thus playing key roles in regulating gene expression, cell growth, differentiation, and development, etc. EV-lncRNAs also participate in the onset and progression of disease. For example, a lncRNA, long intergenic non-protein coding RNA 858 (LINC00858), also termed lymph node metastasis-associated transcript 2 (LNMAT2), was enriched in bladder cancer and urine EV, EV-LNMAT2, promoted tumor lymphangiogenesis and lymph node metastasis (47).

EV-circRNA

CircRNAs are endogenous non-coding RNAs produced through the alternative splicing of a precursor RNA (pre-mRNA). Closed loop structures are formed by the covalent binding of 5'cap structures and 3'poly(A) tails, so circRNAs are resistant to nucleases and exhibit high stability, which ultimately leads to the high maintenance of its abundance (48). More than 79,000 circRNAs have been found in human EVs (38). EV-circRNAs contribute to the development and progression of many tumors through RNA-RNA competitive interactions. For example, circular RNA homeodomain interacting protein kinase 3 (circHIPK3) and taurine up-regulated 1 (TUG1) were upregulated, whereas lncRNA urothelial cancer associated 1 (UCA1) was downregulated in serum EVs (49,50).

Characteristics of exLR

ExLRs are ideal biomarkers because of their characteristics: (I) Abundance: long RNAs, including mRNAs, circRNAs, and lncRNAs, are identified for their enrichment in EVs (15,35-38). We have reliably detected more than 10,000 exLRs in each exLR-seq sample (15), and the abundance of exLRs allowed the detection of RNA extracted from the EVs. (II) Stability: due to encapsulation of long RNAs within bilayer lipid membranes, exLRs can resist degradation against RNases and remain stable in body fluids (51). (III) Intactness: several studies indicated that majority EV-derived long RNAs were fragments (52,53), while others demonstrated the presence of intact long RNAs in blood and urinary EVs (15,54), one study conducted by our lab revealed full-length genes spanning equally along the untranslated regions and coding regions accounted for 22.5% in blood EVs (15). Further research is needed to elucidate its intactness. (IV) Specificity: only specific RNA molecules are selectively sorted into these vesicles, for instance, the tumor-specific mRNA mutation, was also detected in EVs from serum of patients with glioblastoma (34), tumor-specific RNAs reflect the mutational status, thus long RNAs derived from EVs offer promise for their use as biomarkers for disease detection, prediction of prognosis or therapy response in cancer. (V) Dynamic: as EVs represent snapshots of parental cells, the molecular contents within them can vary depending on cell origins and pathophysiological conditions (5,12). By collecting samples at various times, dynamic changes of exLRs could be used as a potential biomarker for monitoring the efficacy of treatment. Finally, owing to the accessibility of EVs and the detection method for long RNAs [e.g., NGS, quantitative real-time polymerase chain reaction (qRT-PCR)], growing studies on exLRs have been applied in oncology.

Clinical applications of exLRs in cancer diagnosis and prognosis

EVs have gained great attention owing to their function in shuttling specific tumor markers in tumors. The profiling of exLRs was different in cancer patients compared with healthy controls and in different states. With the development of isolation, detection and analytical means for exLRs, recent studies have shown the application of exLRs in cancer liquid biopsy. Herein, we summarized the applications of exLRs for cancer diagnosis, patient monitoring, treatment decision-making and prognosis prediction (*Table 2*). Considering cancer morbidity, mortality and clinical accessibility, the applications of exLRs in breast cancer, lung cancer, CRC, liver cancer and prostate cancer (PCa) are detailed below.

Breast cancer

Breast cancer is the most commonly diagnosed cancer worldwide, with an estimated 2.3 million new cases (1). Su *et al.* characterized the plasma exLRs profiles in breast

Table 2 Researches of exLRs as biomarkers in oncology

Ref.	Cancer type	Biofluids	EV isolation	EV characterization	Study cohorts	Assay used	Biomarkers	exLRs type	Performance	Application
(55)	Bladder cancer	Plasma	Exoquick exosome precipitation solution (System Biosciences)	TEM, Western blot	50 patients and 60 controls	qRT-PCR	PTENP1	LncRNA	AUC =0.743	Diagnosis
(56)	Bladder cancer	Urine	–	TEM, NTA and Western blot	210 patients	Sequencing; qRT-PCR	BCYRN1	LncRNA	Poor prognostic factor	Prognosis prediction therapeutic target
(57)	Bladder cancer	Urine	A urinary exosome isolation solution (Hope Tech Biotechnology) based on precipitation	TEM, flow cytometry	168 patients and 90 controls	qRT-PCR	CA9	mRNA	AUC =0.837, sensitivity =85.18%, specificity =83.15%	Diagnosis
(58)	Bladder cancer	Urine	A commercial kit (Norgen)	TEM, NTA and Western blot	Training cohort: 10 patients and 10 controls; validation cohort: 80 patients and 80 controls	qRT-PCR	KLHDC7B, CASP14, PRSS1, MIR205HG and GAS5	mRNA + lncRNA	AUC =0.924 for cancer diagnosis, AUC =0.910 for early detection	Early diagnosis
(59)	Breast cancer	Plasma	exoRNeasy Serum/Plasma Maxi Kit (Qiagen)	–	Discovery cohort: 14 breast cancer patients and 6 breast benign patients; training cohort: 101 cancer patients and 81 controls; validation cohort: 43 breast cancer patients and 34 controls	RNA sequencing + qPCR	9 circRNAs	CircRNA	AUC =0.82	Early diagnosis
(60)	Breast cancer	Plasma	exoRNeasy Serum/Plasma Kit (Qiagen)	TEM, Flow cytometry, Western blot	112 breast cancer patients, 19 benign patients and 41 controls	exLR seq	11 exLRs (BEX2, AC104843.1, AL136981.2, KRT19, NPM1P25, CTSG, CBR3, HOXB7, AL691447.3, RNA5SP141, chr13_42953948_42970670_-)	mRNA + circRNA	AUC =0.90	Diagnosis and early detection
					58 NACT patients		MSMO1	mRNA	AUC =0.79	Treatment prediction
(61)	Colorectal cancer	Plasma	SeraMir™ Exosome RNA Amplification Kit (SBI)	TEM, Particle Metrix, Western blots	52 patients and 41 healthy donors	qRT-PCR	RPPH1	LncRNA	AUC =0.856	Diagnostic marker therapeutic target
(62)	Colorectal cancer	Plasma	exoRNeasy Serum/Plasma Kit (Qiagen)	TEM, Western blot, flow NanoAnalyzer	86 CRC, 56 colorectal adenomas, and 89 controls	exLR seq	17 exLRs	mRNA + lncRNA	Training AUC =0.938, validation AUC =0.943, independent cohort AUC =0.947	Early detection
(63)	Colorectal cancer	Serum	ExoQuick solution (System Biosciences)	NanoSight assay, flow cytometry	100 CRC patients	qRT-PCR	CCAT2	LncRNA	Upregulated in CRC EVs	Diagnosis
(64)	Colorectal cancer	Serum	0.22-μm pore polyvinylidene fluoride filter (Millipore) + ultracentrifugation	TEM, NTA, Western blot	130 patients and 130 healthy volunteers	qRT-PCR	ADAMTS9-AS1	LncRNA	AUC =0.835	Diagnosis
(65)	Colorectal cancer	Serum	ExoQuick™ solution (SBI)	TEM, NTA, Western blot	155 patients and 145 healthy controls	qRT-PCR	LINC02418	LncRNA	AUC =0.8978	Diagnosis
(66)	Esophageal cancer	Plasma	Total Exosome Isolation Kit (from plasma) (Invitrogen)	TEM, NTA, Western blot	317 ESCC patients, 64 esophagitis patients and 69 healthy controls	RNA-sequencing, RT-qPCR	5 lncRNAs (NR_039819, NR_036133, NR_003353, ENST00000442416.1, ENST00000416100.1)	LncRNA	AUC =0.9995	Diagnosis
(67)	Gastric cancer	Plasma	0.22-μm membrane filter (Millipore) + ultracentrifugation	TEM, NTA, Western blot	522 GC patients, 85 patients with gastric precancerous lesions, and 219 healthy controls	qRT-PCR	lncRNA-GC1	LncRNA	AUC =0.9033; increased with stage	Early diagnosis and disease progression monitor
(68)	Gastric cancer	Serum	Commercial exosome extraction kit	TEM, NTA, Western blot	43 GC patients and 27 healthy controls	qRT-PCR	Lnc-GNAQ-6:1	LncRNA	AUC =0.732	Diagnosis
(69)	Gastric cancer	Serum	–	–	126 GC patients and 120 healthy people	qRT-PCR	HOTTIP	LncRNA	AUC =0.827; correlation between increased HOTTIP levels and poor overall survival	Diagnosis; prognosis
(70)	Gastric cancer	Serum	Magnetic separation	TEM, NTA, Western blot	52 patients and 30 healthy controls	qRT-PCR	FRLnc1	LncRNA	AUC =0.863; sensitivity =80.6%; specificity =76.9%	Diagnosis and treatment prediction
(15)	Hepatocellular carcinoma	Plasma	exoRNeasy Serum/Plasma kit (Qiagen)	TEM, Western blot, size distribution measurement	71 HCC patients, 131 non-tumor samples	exLR seq	8 exLRs	–	Training AUC =0.9527, validation AUC =0.9825, testing AUC =0.9627	Diagnosis
(71)	Hepatocellular carcinoma	Plasma	Ribo Exosome Isolation Reagent	TEM, NanoSight, Western blot	112 cancer patients, 43 liver cirrhosis patients, and 52 healthy controls	RNA-sequencing	RP11-85G21.1 (lnc85)	LncRNA	AUC =0.873	Diagnosis
(72)	Hepatocellular carcinoma	Serum	ExoQuick (System Biosciences)	TEM, NTA, Western blot	72 patients and 67 healthy controls	qRT-PCR	4 lncRNAs (DLEU2, HOTTIP, MALAT1, SNHG1)	LncRNA	MALAT1 + SNHG1: AUC =0.899 for very early HCC; expression of MALAT1 was associated with poor overall survival	Early diagnosis and prognosis
(73)	Hepatocellular carcinoma	Serum	ExoQuick (System Biosciences)	TEM, NTA, Western blot	90 patients and 89 healthy controls	qRT-PCR	LINC00853	LncRNA	AUC =0.934	Diagnosis
(74)	Hepatocellular carcinoma	Serum	ExoQuick precipitation solution (System Biosciences)	Electron microscope, NTA, Western blot	183 hepatitis C virus-related HCC	Digital PCR	DANCR	LncRNA	Associated with HCC recurrence and mortality (HR: 7.0 and 2.7)	Prognosis

Table 2 *(continued)*

Table 2 *(continued)*

Ref.	Cancer type	Biofluids	EV isolation	EV characterization	Study cohorts	Assay used	Biomarkers	exLRs type	Performance	Application
(75)	Lung cancer	Plasma	Ultracentrifugation	NTA, TEM, Western Blot	64 patients with stage I lung adenocarcinoma, 24 patients with benign pulmonary nodule, and 22 healthy controls	exLR seq; RT-qPCR	8 exLRs (NFKBIA, NDUFB10, SLC7A7, ARPC5, SEPTIN9, HMGN1, H4C2, lnc-PLA2G1B-2:3)	mRNA + lncRNA	Training AUC =0.991; internal validation AUC =0.921; external validation AUC =0.9	Early detection and routine screening
(76)	Lung cancer	Plasma	ExoQuick Exosome Precipitation Solution kit (System Biosciences)	NanoSight, TEM, Western blot	10 healthy donors and 30 NSCLC patients with postoperative recurrence	qRT-PCR	circUSP7	CircRNA	Negative correlation	Therapeutic target
(77)	Lung cancer	Plasma	Differential centrifugation	TEM, Nanoparticle characterisation system, Western blot	142 NSCLC patients	RT-PCR	lncRNA-SOX2OT	LncRNA	Upregulated in EVs from patients with bone metastasis	Prognosis; treatment target
(78)	Lung cancer	Plasma	exoRNeasy Serum/Plasma Midi Kit (Qiagen)	–	32 lung cancer patients and 30 healthy controls	Digital PCR	SLC9A3-AS1, PCAT6	LncRNA	AUC =0.811	Detection
(79)	Lung cancer	Serum	–	TEM, NTA, Western blot	40 healthy donors, 42 pneumonia patients, and 54 NSCLC patients	qRT-PCR	UFC1	LncRNA	AUC =0.794, sensitivity =73.3%, specificity =74.1%	Diagnosis
(80)	Pancreas cancer	Plasma	exoRNeasy Serum/Plasma Kit (Qiagen)	TEM, flow NanoAnalyzer, Western blot	284 PDACs, 100 patients with chronic pancreatitis and 117 healthy subjects	exLR seq	8 exLRs (FGA, KRT19, HIST1H2BK, ITIH2, MARCH2, CLDN1, MAL2, TIMP1)	mRNA	Training AUC =0.960, internal validation AUC =0.950, external validation AUC =0.936	Diagnosis
(81)	Pancreas cancer	Serum	ExoQuick kits (System Biosciences)	TEM	20 PDACs, 22 intraductal papillary mucinous neoplasm patients, and 21 healthy controls	Digital PCR	HULC	LncRNA	AUC =0.92	Diagnosis
(82)	Prostate cancer	Serum	–	TEM, Nano track analysis, Western blot	31 cancer patients and 17 benign prostatic hyperplasia individuals	RNA-seq; RT-qPCR	CDC42, IL32, MAX, NCF2, PDGFA, SRSF2	mRNA	AUC =0.948	Detection
(83)	Prostate cancer	Urine	Exosome Diagnostics (St Paul, MN) EXOPRO Urine Clinical Sample Concentrator Kit	–	255 men in the training cohort, 519 patients in the validation set	RT-PCR	PCA3, SPDEF, ERG	LncRNA + mRNA	Training AUC =0.77, validation AUC =0.73	Diagnosis

exLRs, extracellular vesicle long RNAs; EV, extracellular vesicle; TEM, transmission electron microscopy; NTA, nanoparticle tracking analysis; NACT, neoadjuvant chemotherapy; CRC, colorectal cancer; ESCC, esophageal squamous cell cancer; GC, gastric cancer; HCC, hepatocellular carcinoma; NSCLC, non-small cell lung cancer; PDAC, pancreatic ductal adenocarcinoma; qRT-PCR, quantitative real-time polymerase chain reaction; PTENP1, phosphatase and tensin homolog pseudogene 1; BCYRN1, brain cytoplasmic RNA 1; CA9, carbonic anhydrase 9; KLHDC7B, kelch domain containing 7B; CASP14, caspase 14; PRSS1, serine protease 1; MIR205HG, MIR205 host gene; GAS5, growth arrest specific 5; BEX2, brain expressed X-linked 2; KRT19, keratin 19; NPM1P25, nucleophosmin 1 pseudogene 25; CTSG, cathepsin G; CBR3, carbonyl reductase 3; HOXB7, homeobox B7; RNA5SP141, RNA, 5S ribosomal pseudogene 141; MSMO1, methylsterol monooxygenase 1; RPPH1, ribonuclease P RNA component H1; CCAT2, colon cancer associated transcript 2; ADAMTS9-AS1, ADAMTS9 antisense RNA 1; LINC02418, long intergenic non-protein coding RNA 2418; lncRNA-GC1, gastric cancer associated long noncoding RNA1; HOTTIP, HOXA distal transcript antisense RNA; FRLnc1, Forkhead box protein M1 related long noncoding RNA; DLEU2, deleted in lymphocytic leukemia 2; MALAT1, metastasis associated lung adenocarcinoma transcript 1; SNHG1, small nucleolar RNA host gene 1; LINC00853, long intergenic non-protein coding RNA 853; DANCR, differentiation antagonizing non-protein coding RNA; NFKBIA, NFKB inhibitor alpha; NDUFB10, NADH:ubiquinone oxidoreductase subunit B10; SLC7A7, solute carrier family 7 member 7; ARPC5, actin related protein 2/3 complex subunit 5; SEPTIN9, septin 9; HMGN1, high mobility group nucleosome binding domain 1; H4C2, H4 clustered histone 2; circUSP7, circular RNA ubiquitin specific peptidase 7; SOX2OT, SOX2 overlapping transcript; SLC9A3-AS1, SLC9A3 antisense RNA 1; PCAT6, prostate cancer associated transcript 6; UFC1, ubiquitin-fold modifier conjugating enzyme 1; FGA, fibrinogen alpha chain; HIST1H2BK, H2B clustered histone 12; ITIH2, inter-alpha-trypsin inhibitor heavy chain 2; MARCH2, membrane associated ring-CH-type finger 2; CLDN1, claudin 1; MAL2, mal, T cell differentiation protein 2; TIMP1, TIMP metallopeptidase inhibitor 1; HULC, highly upregulated in liver cancer; CDC42, cell division cycle 42; IL32, interleukin 32; MAX, MYC associated factor X; NCF2, neutrophil cytosolic factor 2; PDGFA, platelet-derived growth factor subunit A; SRSF2, serine and arginine rich splicing factor 2; PCA3, prostate cancer associated 3; SPDEF, SAM pointed domain containing ETS transcription factor; ERG, V-ets erythroblastosis virus E26 oncogene homologs; AUC, area under the curve; HR, hazard ratio.

cancer patients, benign patients and healthy donors by RNA sequencing, and a breast cancer diagnostic signature comprising 11 exLRs [brain expressed X-linked 2 (BEX2), AC104843.1, AL136981.2, keratin 19 (KRT19), nucleophosmin 1 pseudogene 25 (NPM1P25), cathepsin G (CTSG), carbonyl reductase 3 (CBR3), homeobox B7 (HOXB7), AL691447.3, RNA, 5S ribosomal pseudogene 141 (RNA5SP141), and circRNA chr13_42953948_42970670_-] was constructed (60). The signature showed a high accuracy to distinguish breast cancer patients from controls both in the training and validation cohorts (92.5% and 92.3%, respectively). In addition, the signature identified early stage (stage I/II) breast cancer from controls with an area under the curve (AUC) of 0.94, which indicated the potential of exLR panel in the early diagnosis of breast cancer. Moreover, the level of EV methylsterol monooxygenase 1 (MSMO1) was higher in non-pathological complete response group compared with pathological complete response group, suggesting the potential of EV MSMO1 serving as a reliable biomarker for treatment effect prediction. In summary, the above evidences provide the proof of concept of exLRs as noninvasive diagnostic and prognostic markers for breast cancer. Despite great significance, more studies are needed to ensure the value of exLRs for future clinical use.

Lung cancer

Lung cancer is one of the leading causes of cancer-related morbidity and death worldwide (1). Molecular biomarkers and signatures of cancer-derived EVs may be used for diagnosis and therapeutic prediction. The expression of lncRNA ubiquitin-fold modifier conjugating enzyme 1 (UFC1) was found to be increased in serum EVs of non-small cell lung cancer (NSCLC) and promoted NSCLC progression (79). Chen *et al.* indicated that circular RNA ubiquitin specific peptidase 7 (circUSP7) was highly expressed in EVs of NSCLC patients, and mediated resistance to anti-PD1 therapy by inhibiting $CD8^+$ T cell function, suggesting the potential of circUSP7 as predictive biomarkers for immunotherapy in NSCLC (76). Guo *et al.* explored the biomarkers for the diagnosis of lung adenocarcinoma, and a panel of 8-exLR markers [NFKB inhibitor alpha (NFKBIA), NADH:ubiquinone oxidoreductase subunit B10 (NDUFB10), solute carrier family 7 member 7 (SLC7A7), actin related protein 2/3 complex subunit 5 (ARPC5), Septin 9 (SEPTIN9), high mobility group nucleosome binding domain 1 (HMGN1), H4 clustered histone 2 (H4C2), and lnc-PLA2G1B-2:3] was identified (75). The 8-exLR signature showed great potential to distinguish lung adenocarcinomas from controls with AUCs of 0.991, 0.921 and 0.9 in the training set, internal validation set and external validation set, respectively. More importantly, the panel could distinguish adenocarcinomas *in situ* and minimally invasive adenocarcinomas from the controls, with AUCs of 0.934 and 0.909, respectively, suggesting the potential of the panel for lung adenocarcinoma early detection.

The ExoDx Lung (ALK), which has been validated in Exosome Diagnostics' clinical laboratory improvement amendments certified laboratory, is the first laboratory-developed test (LDT) designed to isolate and analyze EV RNA from a blood sample to detect EML4-ALK fusion transcripts for the decision of druggability of ALK inhibitors (84). It was reported to detect the fusion with 88% sensitivity and 100% specificity by qRT-PCR. This represents a significant advancement in the use of EV-based biomarkers in precision medicine, particularly in the fields of companion diagnostics and tailored treatments. Overall, the value of exLR detection contains cancer diagnosis and medication guidance. However, clinical significance remains to be explored in larger patient cohorts and diverse population.

CRC

CRC is one of the most common malignancies worldwide and the second cause of cancer death worldwide (1,31). Liang *et al.* indicated that EV lncRNA ribonuclease P RNA component H1 (RPPH1) was upregulated in CRC patients compared with healthy donors (61). Besides, EV RPPH1 showed a better performance than traditional tumor markers carcinoembryonic antigen (CEA), carbohydrate antigen 199 and carbohydrate antigen 125 in CRC diagnosis (AUC was 0.856, 0.790, 0.544 and 0.654, respectively), which suggested the potential of EV RPPH1 in CRC diagnosis. In the work conducted by Guo *et al.*, the exLR expression profiles were characterized from CRC patients, colorectal adenomas (CRAs) and healthy donors, and a CRC diagnostic signature comprising 17 exLRs was identified (62). The signature could differentiate CRC from control efficiently (training AUC =0.938, validation AUC =0.943, independent cohort AUC =0.947), as well as differentiate early-stage (stage I–II) CRC and CEA-negative CRC from healthy individuals (AUC was 0.990 and 0.988 respectively), making the CRC screening of

high-risk patients efficiently. Furthermore, another exLR-based signature was also developed for CRA detection, with accuracies of 95.12% and 87.50% in the training and validation cohorts. These suggest that exLRs have a higher diagnostic value of early-stage CRC than traditional tumor markers. However, further validation is needed to ensure the reproducibility of the results.

Hepatocellular carcinoma (HCC)

HCC is the most common type of primary liver cancer in adults and among the leading causes of cancer mortality in the world, with more than 780,000 new cases and 740,000 deaths annually (85). The prognosis of HCC is poor as diagnosed at advanced stage and high rate of recurrence. Currently, only alpha fetal protein (AFP) is available for detection and surveillance of HCC; however, its clinical utility is limited by low sensitivity. One study identified the combined use of EV metastasis associated lung adenocarcinoma transcript 1 (MALAT1), deleted in lymphocytic leukemia 2 (DLEU2), HOXA distal transcript antisense RNA (HOTTIP), and small nucleolar RNA host gene 1 (SNHG1) as serum biomarkers for the very early diagnosis of HCC with low AFP levels, among them, a panel combining EV MALAT1 and SNHG1 achieved the best AUC of 0.899 for very early HCC, whereas a panel with EV DLEU2 and AFP reached high positivity in very early HCC (72). In a report by Wang *et al.*, EV lncRNA differentiation antagonizing non-protein coding RNA (DANCR) was positively associated with HCC recurrence and mortality in hepatitis C virus-related patients (hazard ratio: 7.0 and 2.7, respectively) (74). To conclude, multi-marker exLR panels, in addition to single exLR marker candidates, could detect HCC patients in very early stage and they could predict HCC recurrence. However, more research should be conducted to explore the potential application of exLRs in HCC diagnosis and treatment.

PCa

PCa is one of the most frequently diagnosed malignancies and also the second leading cause of cancer-related death in males in the United States (1,3). Although prostate-specific antigen (PSA) has been widely applied for PCa diagnosis, prognosis prediction and therapy monitoring, the specificity is low when the PSA is in the grey area. EVs in the blood and urine of PCa patients contain unique prostate-cancer-specific contents, which are biomarkers of PCa and cancer metastasis. Ji *et al.* identified 6 EV mRNAs upregulated in PCa, and constructed a screening signature that yielded an AUC of 0.948 in distinguishing PCa patients from healthy controls (82). A urine EV-based 3-gene test [the ExoDx Prostate IntelliScore (EPI) urine exosome assay] is another EV-based LDT product by Exosome Diagnostics, available since 2016. The test measures the expression of 3 RNAs, V-ets erythroblastosis virus E26 oncogene homologs (ERG), prostate cancer associated 3 (PCA3), and SAM pointed domain containing ETS transcription factor (SPDEF) from urine, for discriminating high-grade PCa from low-grade and benign disease. McKiernan *et al.* showed that the performance of the combination of EPI test and standard of care (AUC =0.73) was superior to the standard of care (AUC =0.63) in the validation set (83). Using a predefined cut point, 27% biopsies would have been avoided. The test has also been validated in multicenter trials. A meta-analysis from three independent prospective validation studies showed the EPI AUC (0.70) was superior to PSA (0.56), Prostate Cancer Prevention Trial Risk Calculator (0.62), and The European Randomized Study of Screening for Prostate Cancer (0.59), for risk management (86). To date, this urine EV-based test has been used by >50,000 patients and is included in the National Comprehensive Cancer Network guidelines for PCa early detection (87). Altogether, these studies suggest that exLRs in blood or urine samples might be effective biomarkers to detect patients with PCa, and urine-derived exLRs can be used for cancer discrimination from low-grade and benign disease population. However, the lack of standardized protocols for sample handling, which may affect reproducibility, is a limitation for clinical application.

Other tumors

ExLRs have also been suggested as novel diagnostic and prognostic indicators for others cancers. For example, Jiao *et al.* conducted a four-stage study to identify plasma EV lncRNAs with diagnostic potential in esophageal squamous cell carcinoma (ESCC) (66). Five lncRNAs, NR_039819, NR_036133, NR_003353, ENST00000442416.1, and ENST00000416100.1, were found to be significantly increased in the ESCC patients compared to esophagitis patients and healthy volunteers. The lncRNAs decreased in those patients after surgery patients, indicating that the plasma EV lncRNAs could be used as early detection and treatment prediction in ESCC. Yu *et al.* built an EV long RNA-based diagnostic signature [including fibrinogen

alpha chain (FGA), KRT19, H2B clustered histone 12 (HIST1H2BK), inter-alpha-trypsin inhibitor heavy chain 2 (ITIH2), membrane associated ring-CH-type finger 2 (MARCH2), claudin 1 (CLDN1), mal, T cell differentiation protein 2 (MAL2) and TIMP metallopeptidase inhibitor 1 (TIMP1)] for detecting pancreatic ductal adenocarcinoma, which showed a high accuracy, with an AUC of 0.960, 0.950 and 0.936 in the training, internal validation and external validation cohort, respectively (80). In summary, exLRs are of diagnostic value for multiple cancers as they may provide additional information. However, there is still a long way to go for clinical use.

Conclusions

From the discovery of RNA content in EVs, to the first studies published on exLRs as biomarkers, and finally, to the clinical application, exLRs have been the spotlight of liquid biopsy research. Studies have demonstrated that exLRs including mRNAs, lncRNAs, and circRNAs, could be potential tools for the detection of cancer, therapeutic monitoring and prognosis prediction, as they provide a comprehensive and dynamic genome landscape. Their accessibility, accuracy, and specificity made them attractive targets for researchers.

However, despite the improvements in exLR detection, there are some challenges facing EV research. The best practices in EV pipelines are currently still developing. For instance, the sample sources are widespread, such as plasma, serum, saliva, urine. The standardized method for pre-analytics process (e.g., sample collection, volume, preparation and storage), EV isolation methods, RNA detection (NGS, qRT-PCR) and data analysis has yet to be established, despite the International Society of Extracellular Vesicles (ISEV) has launched several standardization efforts to speed up the development, including, e.g., Minimal Information for Studies of Extracellular Vesicles guidelines, and targeted ISEV position papers (7). Differential ultracentrifugation is the gold standard for EV isolation, however it is unsuitable for clinical applications due to high requirement of equipment. EVs are also commonly isolated via precipitation or affinity-based membrane, which leads to contaminants (88) . Complete isolation of EVs from other entities to obtain pure EVs, is unrealistic. In addition, as samples collected from biofluids may include EVs released by cells other than tumor cells, it is challenging to ensure purity of tumor-derived EV samples. Improvements in developing new strategies to isolate EVs from body fluids and profile exLR efficiently and stably will facilitate the practical application of EV-based liquid biopsy for cancer precision medicine. Furthermore, most studies on EV as the liquid biopsy are based on the small cohorts of patients and lack further validation. Therefore, it is urgent to identify reliable EV biomarkers for early diagnosis and prognosis prediction in large scale samples, which can be adapted to clinical application.

In this review, we discussed exLRs in the hallmarks of cancer, as well as their clinical applications, as biomarkers for cancer diagnosis, prognosis and treatment prediction. The challenges were also pointed. Although great progress has made, exLRs are still in infancy and rarely used in the clinic. Advances in knowledge and technology will promote further clinical translation in the field of oncology.

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Footnote

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