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Review

Liquid biopsy: Circulating exosomal long noncoding RNAs in cancer

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ABSTRACT

Despite many advances in diagnostics and multimodal treatment (surgery, radiotherapy, chemotherapy), cancer still remains one of the most important public health challenges worldwide because of the associated morbidity and mortality. Liquid biopsy has been developed to detect cancer at an early stage based on minimally invasive and serial body fluid tests with the advantage of following tumor evolution in real time. Circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), circulating cell-free noncoding RNAs (cfRNAs) and circulating exosomes represent the major components of liquid biopsy analysis. Liquid biopsy already has been implemented in cancer management, and most studies thus far are mainly focused on CTCs and ctDNA. In fact, the circulating long noncoding RNAs (lncRNAs) in exosomes have been discovered and confirmed to be closely related to tumorigenesis, metastasis and therapy. Thus this review is mainly focused on the clinical potential of circulating exosomal lncRNAs as a source of liquid biopsy biomarkers in cancer diagnosis, prognosis, and response to treatment, offering novel insights into the precision medicine of oncology.

1. Introduction

Currently, cancer is still the world's major public health and safety issue, and the second leading cause of death in the United States [1]. Liquid biopsy, a technology to detect cancer very early—even before symptoms arise, has been widely used in noninvasive diagnosis and molecular phenotyping. In oncology, the clinical application of exosomes has become a hot spot in liquid biopsy for cancer precision medicine. Exosomes, composed of lipid bilayer-enclosed nanoscale extracellular vesicles, have been identified to carry the characteristic molecules of maternal cells, such as protein, lipid, DNA, messenger RNA (mRNA), microRNA (miRNA) and long noncoding RNA (lncRNA), etc. [2,3]. Exosomes are widely found in body fluids, including blood, tears, urine, saliva, milk, ascites, etc. [4]. It has been shown that cancer cells can secrete more exosomes than normal cells, and the biological information of cancer can be directly obtained by analyzing cancer-derived exosomes [5]. Exosomes are involved in critical processes of cancer development, including tumor growth, metastasis, drug resistance and tumor microenvironment [6–8]. Compared with the circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) currently used in human liquid biopsy, exosomes present in the human circulatory system have the advantages of real-time dynamic detection, are minimally invasive, and provide a large amount of information about cancer [9–11]. Therefore, exosomes have been recognized as not only promising biomarkers for cancer diagnosis, but also ideal prognostic biomarkers for cancer management.

Circulating exosomal lncRNAs are attracting researchers' attention in precision oncology. The lncRNAs are a class of noncoding RNAs (ncRNAs) greater than 200 nt in length and have been found to be closely related to the development of many types of cancers [12]. Large amounts of lncRNAs are identified to be stably present in exosomes and can be released into the human circulatory system [13]. However, compared with CTCs and ctDNA, the research and application of exosomal lncRNAs are still in their infancy [14]. In addition, the clinical significance of exosomal lncRNAs remains to be fully explored. Therefore, the current review mainly focuses on the application of exosomal lncRNAs in cancer liquid biopsy, including early diagnosis, prognosis, management of cancer, and the potential application value.

2. Exosomes and circulating exosomal lncRNAs

Exosomes are extracellular vesicles that are 40–100 nm in diameter

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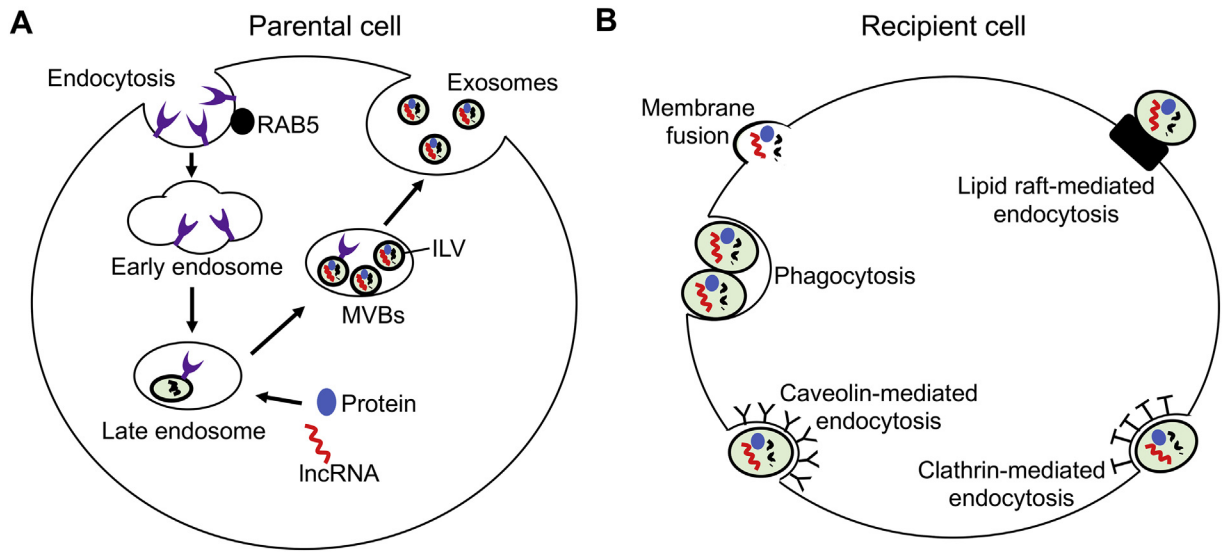

Fig. 1. The schematic diagram of exosome secretion from the parental cell and action on the receptor cell. (A) The formation process of exosomes is mainly reliant on the endocytosis of cell membrane to form endosomes. Briefly, the endosomal-limiting membrane occurs in multiple depressions and inwardly sprouts to form intraluminal vesicles (ILVs), which transform early endosomes into multivesicular bodies (MVBs). Then MVBs can fuse with the plasma membrane and secrete extracellularly to form exosomes. (B) Exosomes containing lncRNAs act on the recipient cell in different ways: binding to recipient cells by direct fusion with cell membrane, binding by clathrin-mediated endocytosis/caveolin-mediated endocytosis/ lipid raft-mediated endocytosis, and direct phagocytosis by recipient cells.

with a two-layer lipid structure. The formation process of exosomes is mainly relies on the endocytosis of cell membrane to form endosomes. In brief, the endosomal limiting membrane occurs in multiple depressions and inwardly sprouts to form intraluminal vesicles (ILVs), which transform early endosomes into multivesicular bodies (MVBs); that is, the late endosomes. MVBs can fuse with the plasma membrane and secrete extracellularly to form exosomes (Fig. 1A). Hence, exosomes contain large amounts of nucleic acids (DNA and ncRNA, including mRNA, miRNA, and lncRNA), proteins and lipids. They act on the recipient cells by carrying these substances [15–17]. The way in which exosomes bind to recipient cells depends on the size of the exosomes and the substances they carried. It is currently believed that the combination of exosomes is mainly in the following three ways: (1) binding to recipient cells by direct fusion with cell membrane; (2) binding by clathrin-mediated-, caveolin-mediated- or lipid raft-mediated- endocytosis; and (3) direct phagocytosis by recipient cell (Fig. 1B). It has also been shown that the binding of exosomes to recipient cells may be involved in micropinocytosis [18].

Exosomes contain a variety of ncRNAs. Exosomal miRNA, one major type of ncRNAs, has been found to be a novel biomarker in various cancers [19]. In addition to miRNA, lncRNA was originally thought to be a by-product of RNA polymerase II transcription, a “noise” of genomic transcription, with no biological function [20]. However, in recent years, lncRNA has been identified to regulate gene expression through various mechanisms [21]. It has been shown that lncRNA plays an important role in tumorigenesis and metastasis [22]. Interestingly, Dong et al. examined the RNA content in exosomes, apoptotic bodies, microvesicles in blood, and found that lncRNAs in blood are mainly distributed in exosomes [23], suggesting that lncRNAs could be secreted into the blood by the form of extracellular vesicles. The lncRNAs from exosomes also have been shown to be stable in other body fluids, such as urine, alveolar lavage fluid, saliva, etc.; hence, circulating exosomal lncRNAs are considered to be meaningful for cancer diagnosis, prognosis monitoring, and cancer treatment [24].

3. Analysis of tumor-derived exosomal lncRNAs

3.1. Methods for exosome isolation

Since Valadi et al. [25] discovered in 2007 that exosomes containing biologically active mRNAs and miRNAs were secreted by cells, the researchers' enthusiasm for exosomes has soared. However, as the first step in research, how to prepare high-purity exosomes has always been a challenge. Currently, the most commonly used method for exosome extraction is still ultracentrifugation. By using low-speed and high-speed centrifugation alternately, vesicle particles of similar size can be separated. Ultracentrifugation is the most commonly used approach because it has the advantages of simple operation, no contamination by separation reagents, and large amounts of exosomes. It is also considered to be the gold standard for exosome extraction and purification [26,27]. Ultracentrifugation is generally combined with sucrose density gradient centrifugation to separate low abundance exosomes. Exosomes in the samples are enriched in a sucrose density range of 1.13–1.19 g/mL by centrifugation at 100,000–200,000 g for 120 min. Because sucrose is non-toxic to cells, low in viscosity, and pH-neutral, the purity of exosomes obtained by sucrose is also high [28,29]. However, these traditional methods are complicated to operate and have lower acquisition efficiency [30]. Furthermore, ultra-filtration was used to obtain the high-quality and sufficient exosomes [31]. In fact, the ultrafiltration method is simple in operation and requires a low-volume sample, and low-speed centrifugation by greatly reducing the exudate rupture [26]. Therefore, it is considered that ultrafiltration is more effective than density gradient centrifugation and is the best alternative to ultracentrifugation [32].

In addition to using the conventional methods, some methods previously used to extract and separate other substances have been found to be useful for the isolation of exosomes, such as magnetic bead immunoassay and polyethylene glycol (PEG) -base precipitation. Exosomes with specific markers (such as CD63, CD9) can be adsorbed and separated by binding the magnetic beads coated with the anti-marker antibody to exosomal vesicles. This method has the advantages of high specificity and simple operation, not affecting the morphological integrity of the exosomes. However, the disadvantages are low efficiency and expensive beads [33]. Polyethylene glycol can be

Table 1
The main techniques for exosome isolation and their advantages/disadvantages.

Isolation techniques	Principle	Advantages	Disadvantages
Ultracentrifugation	Using low-speed and high-speed centrifugation alternately to separate vesicle particles with similar size.	High purity	Cumbersome, time consuming
Sucrose density gradient centrifugation	Distributing particles to certain specific positions in the sucrose density gradient by a certain centrifugal force.	High purity	Complicated preparation
Ultrafiltration	Using a microporous semipermeable membrane and a certain external pressure as a driving force to achieve selective separation and recovery of substances.	Simple, time-saving, non- affected biological activity of exosomes	Insufficient purity
Magnetic bead immunoassay	Using magnetic beads coated with the anti-marker antibody to bind with the exosomal vesicles.	Simple, high integrity, high specificity	Affected biological activity of exosomes
PEG -base precipitation	Using competitive binding of PEG to free water.	Complete precipitation, economical convenience	Low purity and recovery, uneven particle size

coprecipitated with hydrophobic proteins and lipid molecules, and now it is used to precipitate exosomes by competitively binding to free water molecules. However, low purity and recovery, heterozygous proteins (false positives), uneven particle size, polymers, and additives are disadvantages [34].

In recent years, various commercial kits have been developed to separate and purify exosomes. Some kits are commonly known to precipitate exosomes by water-excluding polymers [35], and by size exclusion chromatography [36]. The kits do not require special equipment, and they are continuously updated, and the extraction efficiency is gradually increased [37]. Hence, the appropriate method could be chosen to ensure the excretion of higher purity and integrity (Table 1). To yield high-quality exosomes, the traditional approaches (ultracentrifugation [38], reducing agent of dithiothreitol [39], etc.) could be adapted to combine with the kit method.

3.2. Strategies for exosomal lncRNA detection

With the development of biochemical techniques, the methods for lncRNA detection have been continuously innovated and improved (Table 2). In detail, the analysis of northern blot was used for detection and quantification of RNA in cancer cells and tissues [40]. As a non-radioactive method, it is also suitable for small RNA detection [41]. With the rapid development of next-generation sequencing (NGS) technology, the high-throughput sequencing has been widely used for discovery of candidate genes and lncRNAs [42,43]. Similarly, lncRNA microarray is applied to show the differentially expressed lncRNAs. However, the cost of NGS and microarray analysis is relatively high, and the amount of data are very large. Therefore, the complicated analysis of data is needed to improve the accuracy of lncRNA detection [44,45]. The quantitative reverse transcription polymerase chain reaction (qRT-PCR), is a common detection method for detecting lncRNA with a relatively simple procedure and low cost, and it is also the gold standard for verifying the accuracy of high throughput sequencing technology [46]. In addition, digital PCR (dPCR) has become a new strategy for highly sensitive absolute quantification of nucleic acids without the need for standard curves [47]. Compared to qRT-PCR, dPCR technology significantly improved the sensitivity (30-fold) and accuracy (10-fold) of quantitative abundance [48]. Therefore, in addition to the conventional biopsy tissue as a genetic test object, dPCR can be combined with liquid biopsy technology to achieve the purpose of genetic testing for samples with a very small portion of nucleic acid content [49]. It was determined that dPCR technique was used to improve the accuracy of miRNA and lncRNA detection [49,50]. Although dPCR still has some disadvantages, such as high cost and complicated operation, the development of microfluidic technology and emulsion chemistries makes a wider application of dPCR in field of absolute quantification of target sequences [51–53]. Hence, dPCR extends the range of nucleic acid analysis beyond the reach of other methods in cancer liquid biopsy.

4. Circulating exosomal lncRNAs in cancer liquid biopsy

4.1. Circulating exosomal lncRNAs in cancer diagnosis

The continuous development of diagnostic techniques provides a technical basis for liquid biopsy in patients with cancer and for quantitative detection of circulating exosomal lncRNAs in exosomes [54]. By comparing the expression of lncRNA in the normal population, the exosomal lncRNA-based liquid biopsy diagnostic technique can be established for patients with cancer.

Currently, most of the biomarkers are clinically tested in blood samples of patients. Hence, blood samples have become the preferred body fluid for cancer liquid biopsy-related diagnosis. Both serum and plasma can be obtained from blood and serve as a major source of body fluids for liquid biopsy. For example, Wang et al. [55] detected the expression of exosomal lncRNA H19 in serum and found that exosomal lncRNA H19 in patients with bladder cancer (BC) was significantly upregulated compared with that in healthy people, suggesting that lncRNA H19 in exosome is expected to become a novel minimally invasive diagnostic biomarker. The early diagnosis of BC has been greatly improved by establishing a diagnostic model based on the detection of serum exosomal lncRNA [56]. In addition, Peng et al. [57] found that lncRNA PCA3 and BCAR4 were upregulated in serum samples from patients with colorectal cancer (CRC) and a panel of two exosomal lncRNAs was established for combined diagnosis. The diagnostic value

Table 2
The main techniques for circulating exosomal lncRNA detection and their advantages/disadvantages.

Detection techniques	Principle	Advantages	Disadvantages
Microarray	Utilizing the molecular hybridization to compare the differences in gene expression levels of different specimens.	High throughput	High cost, numerous data, complicated operation
qRT-PCR	Total RNA was reversely transcribed into cDNA and then amplified by specific primers for interest genes.	Simple, low cost	Low flux
Northern blot	Transferring RNA from an agarose gel onto a nitrocellulose membrane and detecting it using an RNA probe.	Low cost	Complicated operation, low sensitivity, toxic supplies
RNA-seq	Reducing the abundance of rRNA and then performing library construction, sequencing and analysis on the enriched RNA.	High throughput	Not applicable for the diagnosis of single gene, high cost

of exosomal lncRNAs was also validated in hepatocellular carcinoma (HCC) by establishing a new liquid biopsy diagnostic model [58–60]. However, studies have shown that platelets may secrete large amounts of exosomes during coagulation, suggesting a decrease of exosome in serum [61]. Therefore, plasma has been used as a more important sample source to identify the relative expression of exosomal lncRNA [62,63]. For example, it was found that lincRNA 152 in plasma could be used for the diagnosis of early gastric cancer (GC) [64,65]. In addition, six exosomal lncRNAs (LNCV6_116109, LNCV6_98390, LNCV6_38772, LNCV_108266, LNCV6_84003, and LNCV6_98602) were found to be significantly upregulated in patients with CRC and these lncRNAs served as novel biomarker for CRC diagnosis [66]. Although there is no systematic study on the difference of circulating lncRNAs between serum-derived and plasma-derived exosomes, the method of blood sample testing is widely used in the clinic because of the wide material source, minimally invasive operation, and high stability.

In addition to blood samples, the use of other body fluids is constantly being discovered. In the urinary system cancers, urine has been always a popular experimental material. For example, Yazarlou et al. [67] detected the expression levels of four lncRNAs (LINC00355, UCA1–203, UCA1–201 and MALAT1) and found three of them were highly expressed in patients with BC. The combined diagnostic model of lncRNA showed a higher sensitivity (92%) and a higher specificity (91.7%) compared with the traditional biomarkers. Similarly, the exosomal lncRNA P-21 in urine also could be used to distinguish or diagnose prostate cancer and benign prostate disease [68]. Interestingly, lncRNA in saliva might be a new biomarker for the diagnosis of oral squamous cell carcinoma (SCC) [69]. Although studies on exosomal lncRNAs as candidate diagnostic markers have been accumulating in recent years (Table 3), circulating exosomal lncRNAs still have certain issues, such as extraction with high purity, high cost, and difficulty in popularization. With the future progress of biological sciences, it is believed that exosomal lncRNA is expected to become a novel potential diagnostic biomarker for early-stage cancer [70].

4.2. Circulating exosomal lncRNAs in cancer prognosis

LncRNAs in exosomes are involved not only in the early detection of cancer, but also in postoperative diagnosis and disease monitoring. A variety of exosomal lncRNAs have been identified to be new prognostic biomarkers for cancers (Table 4). It is known that lncRNA MALAT1 can promote the expansion and migration of various cancer cells [71]. The up-regulated exosomal lncRNA MALAT1 in serum of epithelial ovarian cancer (EOC) was found to be closely correlated with tumor metastasis and an independent predictor of overall survival for patients with EOC [72]. This correlation makes it possible to monitor the development of EOC by detecting exosomal lncRNA MALAT1. Furthermore, the 5-year survival for patients with BC with higher expression levels of lncRNA MALAT1 and PCAT-1 was significantly lower than that of patients with lower expressions of these two lncRNAs [73]. Similarly, the survival rate of patients with HCC with high expression of lncRNA ENSG00000258332.1 and LINC00635 was also lower than that of patients with low expression of the two lncRNAs [59]. These results would provide useful information for clinicians to make a more accurate judgment of the patient's condition. It is a powerful evaluation of therapeutic effect on cancer by comparing the expression levels of preoperative and postoperative blood markers. For example, Wang et al. collected 32 postoperative serum samples from patients with BC and found that lncRNA H19 in the exosomes of the postoperative patients was significantly decreased [55]. To observe the change of exosomal lncRNA in preoperative and postoperative serum beforehand can help clinicians track the patient's condition in real time.

With the deepening of research on exosomal lncRNA, the prognostic accuracy in patients with cancer will continue to increase in the future [74,75]. As the prognostic value of exosomal lncRNA is being continuously explored, the circulating exosomal lncRNA is expected to be a novel prognostic biomarker for patients with cancer.

4.3. Circulating exosomal lncRNAs in cancer therapeutics

In addition to being a biomarker for cancer early diagnosis and prognosis, exosomal lncRNAs have been shown their important roles in drug resistance of tumor cells, suggesting a clinical application in cancer-targeted therapy.

Gefitinib, a tyrosine kinase inhibitor, is currently used as a first-line treatment for patients with lung cancer [76], but it often comes with drug resistance and affects the therapeutic effect. Interestingly, exosomal lncRNA was found to have a significant correlation with gefitinib resistance. The tumor-released lncRNA H19 promoted the resistance of gefitinib to patients with non-small cell lung cancer (NSCLC) via

Table 3
Diagnostic values of circulating exosomal lncRNAs in cancers.

Biological specimen	Exosomal lncRNA	Expression change	Type of cancer	Reference
Serum	H19	Up	Bladder cancer	[55]
	PCA3	Up	Colorectal cancer	[57]
	BCAR4	Up	Colorectal cancer	[57]
	LINC00161	Up	Hepatocellular carcinoma	[58]
	ENSG00000258332.1	Up	Hepatocellular carcinoma	[59]
	LINC00635	Up	Hepatocellular carcinoma	[59]
	HEIH	Up	Hepatocellular carcinoma	[60]
	ZFAS1	Up	Gastric cancer	[65]
	MALAT1	Up	Non-small cell lung cancer	[71]
	CRNDE-h	Up	Colorectal cancer	[75]
Plasma	PTENP1	Down	Bladder cancer	[62]
	UEGC1	Up	Gastric cancer	[63]
	LNCV6_116109	Up	Colorectal cancer	[66]
	LNCV6_98390	Up	Colorectal cancer	[66]
	LNCV6_38772	Up	Colorectal cancer	[66]
	LNCV_108266	Up	Colorectal cancer	[66]
	LNCV6_84003	Up	Colorectal cancer	[66]
	LNCV6_98602	Up	Colorectal cancer	[66]
Urine	LINC00355	Up	Bladder cancer	[67]
	UCA1–203	Up	Bladder cancer	[67]
	UCA1–201	Down	Bladder cancer	[67]
	MALAT1	Up	Bladder cancer	[67]
	p-21	Up	Prostate cancer	[68]
Saliva	HOTAIR	Up	Oral squamous cell carcinoma	[69]

Table 4
Prognostic values of circulating exosomal lncRNAs in cancers.

Biological specimen	Exosomal lncRNA	Expression change	Type of cancer	Prognostic significance	Reference
Serum	H19	Up	Bladder cancer	OS($P = .006$)	[55]
Serum	MALAT1	Up	Epithelial ovarian cancer	OS($P < 0.01$)	[72]
Urine	PCAT-1	Up	Bladder cancer	OS($P < 0.001$)	[73]
Serum	CRNDE-h	Up	Colorectal cancer	OS($P < 0.001$)	[75]
Serum	ENSG00000258332.1	Up	Hepatocellular carcinoma	OS($P < 0.05$)	[59]
Serum	LINC00635	Up	Hepatocellular carcinoma	OS($P < 0.05$)	[59]

packaging into exosomes [77]. Therefore, oncologists could predict the response of gefitinib to patients with NSCLC by detecting the exosomal lncRNA H19 and prepare for the next targeted therapy. In addition, exosome-mediated transfer of lncRNA PART1 induced gefitinib resistance in esophageal squamous cell carcinoma (ESCC) via functioning as a competing endogenous RNA (ceRNA) [78]. These findings indicate that exosomal lncRNAs can be used as therapeutic targets for future precision oncology.

Sunitinib is an oral small molecule multitarget receptor tyrosine kinase inhibitor (TKI) with multiple effects of inhibiting tumor angiogenesis and antitumor cell growth. Sunitinib treatment was active in most patients with advanced renal cell carcinoma (RCC) and was associated with manageable toxicity [79]. It was determined that exosome-transmitted lncRNA ARSR promoted the resistance of sunitinib to RCC cell by acting as a ceRNA [80]. To improve the therapeutic effect of tumor chemotherapy, targeting the exosomal lncRNA may enhance the response of the current clinical first-line cancer drugs to a variety of tumors.

Cetuximab is a chimeric human mouse anti-epidermal growth factor receptor (EGFR) monoclonal antibody in the treatment of patients with CRC. LncRNA UCA1 expression was markedly higher in cetuximab-resistant CRC cells and their exosomes. The circulating UCA1-containing exosomes could predict the clinical outcome of cetuximab therapy in patients with CRC, and UCA1 expression was considerably higher in the patients with progressive disease or stable disease than in those with a partial response or complete response patients. Furthermore, exosomes derived from cetuximab-resistant cells could alter UCA1 expression and transmit cetuximab resistance to sensitive cells [81]. These results showed the capability of UCA1-containing exosomes of transmitting drug resistance and the potential clinical use in predicting cetuximab resistance. Thus, the liquid biopsy by detecting exosomal lncRNA provides a minimally invasive, real-time monitoring of drug response, and a more accurate information for clinicians to administer a reasonable medication.

5. Conclusion

With the continuous development of quantitative lncRNA detection techniques, exosomal lncRNA-derived liquid biopsy becomes a novel approach in precision oncology [82]. Patients with cancer are often unable to undergo highly invasive examinations because of their poor physical condition. A new type of minimally invasive, reproducible, real-time detection is extremely desirable. Therefore, exosomal lncRNA-based liquid biopsy will inevitably become a new way for cancer diagnosis and prognosis in the future. Most of exosomal lncRNAs can be stably present in human body fluids because of the protection of exosomes. In fact, lncRNAs have been confirmed to be closely related to the development of cancer. Therefore, exosomal lncRNAs have a wide range of applications in the diagnosis, prognosis, and treatment of cancer [83,84]. Although the studies on exosomal lncRNAs are accumulating, widespread clinical use has not occurred.

First, the clinical significance of exosomal lncRNAs in cancer diagnosis, prognosis and treatment needs fully to be fully explored. Because the dysregulated lncRNAs in exosomes may be caused by tumorigenesis, metastasis, or microenvironment, exosomal lncRNAs as ideal biomarkers are challenged. To distinguish patients with cancer from healthy people, patients with other diseases or cancer-related disease should be considered. To use exosomal lncRNAs as novel biomarkers, high purity and accuracy of lncRNAs in exosomes are required for detection. Currently, many methods have been developed for exosome extraction, as described in this review, each has many advantages and disadvantages [85]. The procedures for extraction and purification of exosomes need to be continuously optimized to improve the quality of exosomal lncRNAs [86]. In addition, the quantitative detection of lncRNA is currently carried out by qRT-PCR. However, the internal reference of lncRNA is still controversial, because there is no uniform standard.

Then, the precision mechanism of exosomal lncRNAs in cancer development needs to be fully understood. Furthermore, the drug-loading method via exosomes still needs to be improved. The potential of the nanoscale carriers requires long-term and deeper exploration. With the rapid development of high-throughput omics technology and nanotechnology, the clinical role of exosomal lncRNA in cancer science and the exact mechanism of exosomal lncRNA in the processes of cancer development will be determined. The circulating exosomal lncRNAs as biomarkers may provide great potential in cancer liquid biopsy and certainly benefit patients with cancer.

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Disclosure of potential conflicts of interest

No potential conflicts of interest are disclosed.

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