Emerging function and clinical significance of extracellular vesicle noncoding RNAs in lung cancer

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Lung cancer (LC) is a commonly diagnosed cancer with an unsatisfactory prognosis. Extracellular vesicles (EVs) are lipid bilayer-delimited particles that mediate cell-cell communication by transporting various biomacromolecules, such as nucleic acids, proteins, and lipids. Noncoding RNAs (ncRNAs), including microRNAs, circular RNAs, and long noncoding RNAs, are important noncoding transcripts that play critical roles in a variety of physiological and pathological processes, especially in cancer. ncRNAs have been verified to be packaged into EVs and transported between LC cells and stromal cells, regulating multiple LC malignant phenotypes, such as proliferation, migration, invasion, epithelial-mesenchymal transition, metastasis, and treatment resistance. Additionally, EVs can be detected in various body fluids and are associated with the stage, grade, and metastasis of LC. Herein, we summarize the biological characteristics and functions of EV ncRNAs in the biological processes of LC, focusing on their potential to serve as diagnostic and prognostic biomarkers of LC as well as their probable role in the clinical treatment of LC. EV ncRNAs provide a new perspective for understanding the mechanism underlying LC pathogenesis and development, which might benefit numerous LC patients in the future.

INTRODUCTION

Lung cancer (LC), a commonly diagnosed and lethal cancer, accounts for 11.4% of the total cases and 18% of the total cancer-related deaths worldwide.[1] LC is a highly aggressive, rapidly metastatic, and highly heterogeneous cancer that can occur in many different parts of the bronchial tree, therefore exhibiting highly different symptoms and signs.[2] Even worse, LC metastasizes via blood vessels as well as the lymphatic system, which usually results in an increased rate of recurrence and distant metastasis, as well as shortened survival of patients.[3] Despite advances in treatments such as surgery, radiotherapy, chemotherapy, and targeted therapy, 70% of LC patients are already at an advanced stage when diagnosed.[2] The 5-year survival of patients with LC remains poor.[4] The poor prognosis of LC patients also stems from insufficient understanding of its molecular pathogenesis, lack of a timely diagnosis, and a lack of sensitive monitoring tools. Therefore,

it is crucial to clarify the molecular mechanism of LC occurrence and development and to identify reliable biomarkers.

The tumor microenvironment (TME) has been considered to be associated with cancer progression and high mortality.[5,6] The LC TME relies on complex interactions between tumor cells and adjacent noncancerous cells, such as fibroblasts, endothelial cells, and immune cells.[7] Cancer cells can directly interact with neighboring cells through membrane receptors and their ligands or interact with distant cells by releasing cytokines, chemokines, and metabolites into the circulatory system.[8] In recent decades, extracellular vesicles (EVs) have been considered a new type of intercellular communication and have received much attention.[9,10] EVs can realize the molecular exchange between cancer cells and stromal cells, reshape the local TME, and stimulate the occurrence, development, and distant metastasis of cancer.[11–13] Among the EV cargoes, noncoding RNAs (ncRNAs), such as microRNAs (miRNAs), circular RNAs (circRNAs), and long noncoding RNAs (lncRNAs), have become crucial regulators due to their roles in the initiation and progression of various cancers.[14,15] In this review, we summarize the functions of EV ncRNAs in LC, with a focus on their potential as biomarkers and therapeutic targets for LC.

BIOGENESIS AND CHARACTERISTICS OF EVs

EVs, with a diameter of 30–150 nm, are small EVs secreted by cells that exist in almost all body fluids, such as the blood, cerebrospinal fluid, saliva, urine, breast milk, amniotic fluid, and ascites.[16–18] The biosynthesis of EVs begins with early endosomes formed by endocytosis of the plasma membrane (Figure 1A). Then, the early endosomes become late endosomes, which then mature to form multivesicular bodies (MVBs) that package EVs and their cargoes. Finally, the MVBs can be phagocytosed and degraded by lysosomes or released outside the cell.[19]
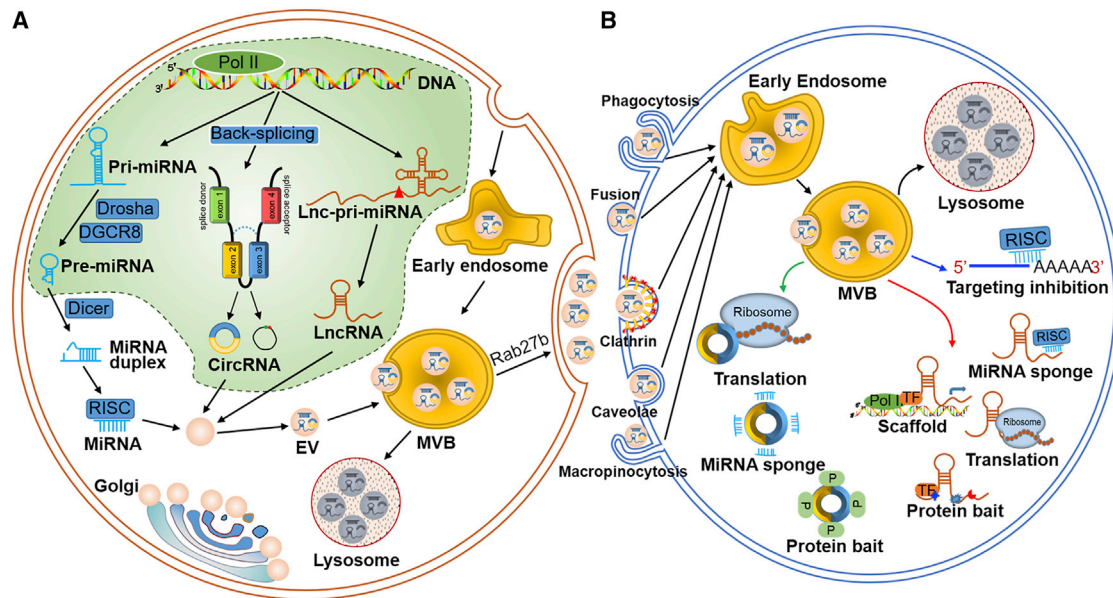
Figure 1. Biology of EV ncRNAs

(A) The biogenesis of ncRNAs and EVs. miRNA genes are transcribed into pri-miRNAs by RNA polymerase II (Pol II), and further cleaved by Drosha and DGCR8 to generate pre-miRNAs. After exporting into cytoplasm, pre-miRNAs are cleaved by Dicer to produce a miRNA duplex. Then the miRNA duplex loads on AGO. One strand of the duplex is selectively anchored into the AGO to form the RISC complex, thereby regulating the expression of target mRNA. circRNAs get their closed loop structures through back-splicing in nucleus. circRNAs are composed of extrons and/or introns depending on the methods of lariat-driven circularization, intron-pairing-driven circularization, and intron cyclization. lncRNA genes are transcribed through the mediation of Pol I/II to form lnc-pri-miRNAs, and are processed by RNase P/H to generate mature lncRNAs and pre-miRNAs. Mature ncRNAs are then sorted into MVBs. The MVBs are finally released as EVs in a Rab27b-dependent manner, or transported into lysosomes for degradation. (B) EVs can be internalized by recipient cells in different ways, such as phagocytosis, membrane fusion, clathrin-dependent endocytosis, caveolae-mediated endocytosis, and micropinocytosis. Once entering the recipient cells, EV ncRNAs exert their biological functions by acting as miRNA sponges, protein baits, protein scaffolds, and encoding proteins.

Accumulating evidence has revealed that both the endosomal sorting complex required for transport (ESCRT)-dependent and ESCRT-independent pathways are involved in the process of membrane shaping and scission of EVs, depending on the types of EV cargoes and the cellular context, and can be affected by signals and stimuli that the cells received. The ESCRT machinery consists of four sub-complexes as ESCRT-0, I, II, and III.[20] It was reported that these sub-complexes act in a stepwise manner. ESCRT-0 assembles on membranes and is responsible for capturing polyubiquitylated cargoes. Then ESCRT-0 recruits ESCRT-I and hands over ubiquitylated cargoes. ESCRT-I recruits ESCRT-II and co-mediates endosome membrane invagination, into which cargoes are sorted. ESCRT-III takes charge of fission and scission of vesicles. Moreover, the ESCRT-dependent pathway could intersect with the syndecan-syntenin-ALIX pathway, which supports cargo sequestration, the budding process, and the biogenesis of EVs.[21] However, EVs could still form once the ESCRTs were exhausted, suggesting the existence of an ESCRT-independent pathway.[22] Neural sphingomyelinase 2 (nSMase 2) and proteins of the tetraspanin family (like CD63, CD81, CD82, and CD9) are involved in the ESCRT-independent EV cargo sorting processes.[23–26] In contrast, EV cargoes' sortation could also modulate EV biogenesis. The abnormal expression of certain cargoes, such as the major histocompatibility complex (MHC) class II, might recruit

sorting machineries, thereby promoting MVB formation and the subsequent secretion of EVs.[26]

During EV biogenesis, multiple biomolecules, such as proteins, nucleic acids, and lipids, are packaged into EVs. Among the numerous EV cargoes, the sorting mechanism of EV miRNAs is relatively well studied. Increasing evidence has confirmed that miRNAs can be selectively sorted into EVs rather than a passive process. According to the current knowledge, there are four mechanistic hypotheses for the selective sorting of miRNAs into EVs. (1) The nSMase 2-dependent pathway. nSMase 2 is a rate-limiting enzyme in ceramide biosynthesis, while ceramide is involved in EV biogenesis.[27] nSMase 2 is the first regulatory proteins that is related to EV miRNAs secretion.[28] Kosaka et al.[27] found that the inhibition of nSMase 2 reduces the sorting of miR-16 and miR-146a into EVs, while nSMase 2 overexpression enhanced EV miR-16 and miR-146a levels with no changes in cellular miRNAs. (2) The heterogeneous nuclear ribonucleoproteins (hnRNPs) family-dependent pathway. The hnRNPA2B1 protein was found to be sumoylated and to bind with miR-198 and miR-601 through their GGAG/UGCA motifs, thereby controlling their loading into EVs.[29] In separate research, Lee et al.[30,31] demonstrated that hnRNPA2B1 mediates the sorting into EVs of miR-17 and miR-93, which possess AGG/UAG motifs. HnRNPA1 is another member of the hnRNP family, which could be

sumoylated and regulate the loading process of miR-198 into EVs.[32] In addition, hnRNP-Q was confirmed to bind with the GGCU sequences in miR-3470a and miR-194-2-3p, which are highly enriched in EVs.[33] The N-terminal unit for the RNA recognition domain of hnRNP-Q is critical for EV loading.[34] (3) The 3′ end of miRNA-dependent pathway. A study by Koppers-Lalic et al.[35] revealed that post-transcriptional modifications, especially 3′ end adenylation and uridylation of miRNAs, could affect miRNA sorting into EVs. Notably, 3′-end-adenylated miRNAs tended to be enriched in cells, whereas 3′-end-uridylated forms were more likely to be detected in EVs isolated from human urine samples. (4) The RNA-induced silencing complex (RISC)-dependent pathway. The RISC, which consists of miRNA, Argonaute protein (AGO), and GW182, is responsible for the translational repression of the mRNAs.[36] It was demonstrated that the core components of RISC were co-localized with MVB proteins.[37] Meanwhile, blocking lysosomal degradation of MVB results in RISC accumulation.[38] Besides, the expression level of the target gene can also affect the number of miRNAs sorted into EVs.[39] In sum, specific miRNA sequences may guide their sorting into EVs, and certain RBPs also participate in the regulation of EV miRNA sorting.

Once the MVBs move to and fuse with the plasma membrane, the internal vesicles are then released to the extracellular substance as EVs. Small GTPases of the Ral and Rab families are crucial factors in EV secretion. Rab27a was found to affect the size of MVBs, while Rab27b affects the distribution of MVBs in cells.[40] After secretion, EVs participate in the regulation of cell behavior and intercellular communication through various mechanisms. On the one hand, the EV cargoes directly interact with the surface molecules of the recipient cell to achieve signal transduction through ligand-receptor interactions.[41] On the other hand, EV cargoes can fuse with the plasma membrane and directly enter the recipient cell (Figure 1B).[42]

Several decades ago, Johnstone et al.[43,44] considered EVs as a mechanism of shedding surface membrane components, and the formation of EVs might be a major route for externalization of superfluous membrane proteins during reticulocyte maturation. Currently, extensive research has found that EVs are crucial regulators of various physiological and pathological processes, including cancer.[45] EVs might affect cancer initiation and progression in various ways. Cancer-derived EVs can be internalized by stromal cells, thus creating a suitable environment for cancer occurrence and development.[46] Meanwhile, EVs secreted by cancer-associated fibroblasts (CAFs) contain complete amino acids and lipids, which can meet the requirements of tumor growth under conditions of nutrient stress.[47] Increasing evidence shows that EVs play a role in regulating tumorigenesis by regulating angiogenesis, immunity, metastasis, and treatment resistance.[48] The potential of tumor EVs as biomarkers and novel anti-tumor targets has attracted great attention.

CHARACTERISTICS AND BIOLOGICAL FUNCTIONS OF EV ncRNAs

Various kinds of biomacromolecules, such as proteins, lipids, and nucleic acids, have been assessed in EVs where they can exist stably and exert their important biological functions.[49] In recent years, the identification of EV ncRNAs has substantially advanced due to high-throughput sequencing and other technologies used to detect low-abundance RNA samples. These ncRNA populations include miRNAs, circRNAs, lncRNAs, transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), Y RNAs, and piwi-interacting RNAs (piRNAs), which can be transferred from parent cells to recipient cells or communicate in the TME, thus regulating various normal physiological and pathological processes, especially in cancer.[50–54] Herein, we focus on the characteristics and biological roles of EV ncRNAs (Figure 1).

miRNAs in EVs

miRNAs are endogenous small RNAs consisting of approximately 20–24 nucleotides that recognize target genes through complementary base pairing to mRNA 3′ untranslated regions (UTRs), thus disrupting their stability and reducing the expression of those genes.[55] Mechanistically, the generation of miRNAs starts from the formation of pri-miRNAs in the nucleus, and they are further cleaved by Drosha and DGCR8 to generate pre-miRNAs. Then, the RNase III enzyme Dicer cleaves the terminal loop of the pre-miRNA to generate a 20–22-nucleotide miRNA duplex, which is then loaded on AGO. After that, the passenger strand is degraded, while the mature miRNA is loaded into AGO to form the RISC, and guides RISC to the target mRNA to perform post-transcriptional silencing functions. The miRNA may originate from the 5′ side of the pre-miRNA (referred to as the 5p strand) or the 3′ side of the pre-miRNA (referred to as the 3p strand).[56] Usually only one miRNA strand dominates. However, in some cases, both strands of the duplex generate the functional mature miRNA to regulate gene expression (Figure 1A).[57,58] To date, approximately 2,000 miRNAs have been identified in the human genome, and the number is still growing.[59] In the past few decades, identification of the crucial roles of miRNAs in cancer initiation, progression, and anticancer therapy has gained momentum.[60,61] Functionally, miRNAs might be oncomiRNAs as well as tumor-suppressive miRNAs in different cellular contexts, considering that a single miRNA might have several target mRNAs.[62]

Among various types of ncRNAs, miRNAs were first identified in EVs.[63] Peng et al.[64] identified 706 miRNAs from non-small cell lung cancer (NSCLC) patients' plasma EVs, among which 155 EV miRNAs were differently expressed between NSCLC patients and healthy persons. In another study, Jin's group[65] classified the EV miRNA profiles in early-stage NSCLC patients' plasma using next-generation sequencing, and identified several differently expressed EV miRNAs as highly sensitive, noninvasive biomarkers for early NSCLC diagnosis. Accumulating evidence has verified that EV miRNAs regulate numerous cancer-related life events through the participation of cell-cell communication.[50] Importantly, the high abundance of EVs in body fluids, as well as the relevance of miRNAs to cancer progression and prognosis, have made EV miRNAs potential biomarkers and ideal therapeutic targets for cancer.[66]

circRNAs in EVs

circRNAs are new types of ncRNAs with covalently linked circular structures.[62,67] In contrast to linear RNAs, circRNAs are thought to be the result of exon-skipping events through mechanisms of intron-pairing-driven circularization and lariat-driven circularization (Figure 1A).[68,69] Existing studies have described the characteristics of circRNAs: diversity and high abundance in various tissues, stability and conservation, and tissue and spatial-temporal specificity.[62] circRNAs might possess various functions in vital pathophysiological processes, such as serving as miRNA sponges, binding to functional proteins, encoding proteins, regulating pre-RNA splicing, and managing parental gene expression. Importantly, increasing evidence has verified that circRNAs participate in the occurrence and development of many kinds of diseases, especially cancer.[70]

EV circRNAs were first identified in 2015, and related studies have flourished since then.[71] Yang et al.[72] investigated the expression profile of EV circRNAs in NSCLC patients' serum by using a high-throughput circRNA microarray, and found that circRNA_102481 is highly expressed in serum EVs of EGFR-TKI-resistant patients. Additionally, Wang and colleagues[73] analyzed the circRNAs expression profile in plasma EVs of lung squamous cell carcinoma patients, and 252 differently expressed EV circRNAs were identified, among which hsa_circ_0014235 and hsa_circ_0025580 were significantly upregulated. Compared with producer cells, circRNAs are more stable and enriched in EVs, and can be transferred into recipient cells and the TME, thus regulating the behavior of recipient cells and promoting cancer progression (Figure 1B).[45] Specifically, EVs circRNAs have been recognized as crucial modulators of numerous physiological and pathological processes, and might become readily noninvasive biomarkers for the prediction, diagnosis, and prognostic evaluation of diseases, especially cancers.

lncRNAs in EVs

lncRNAs are a class of endogenous ncRNAs >200 nucleotides in length that were identified approximately three decades ago.[74] In mammalian genomes, 4%–9% of sequences produce transcripts that are lncRNAs (1%–2% for mRNAs).[75] With the development of high-throughput technologies, the number of lncRNAs in humans has been certified to be more than 160,000.[76] Increasing evidence has suggested that lncRNAs are not transcriptional "noise" but instead have various physiological functions, such as X chromosome inactivation, genomic imprinting, chromatin modification, transcriptional regulation, mRNA turnover, and protein translation.[77,78] Importantly, lncRNAs are abundantly expressed and are widely relevant to various kinds of cancers, and their aberrant expression and mutations are associated with tumorigenesis, tumor progression, and metastasis.[79–81]

Likewise, lncRNAs can be sorted into EVs and act as mediators in cell-cell communication (Figure 1).[82] Recently, EV lncRNAs have been reported to be involved in the regulation of proliferation, angiogenesis, metastasis, and drug resistance in LC.[83–85] Moreover, the dysregulation of EV lncRNAs has been found to affect cellular behavior and cell-cell communication in the LC TME.[86] Furthermore, EV lncRNAs have been demonstrated to be closely related to clinicopathological characteristics and treatment outcomes, indicating the possibility of EV lncRNAs serving as diagnostic and prognostic biomarkers for LC patients.[87,88] lncRNAs have gained increasing attention in EV investigations.

Other ncRNAs in EVs

tRNAs, rRNAs, Y RNAs, and piRNAs are also members of the ncRNA family. Increasing evidence shows that these ncRNAs can be encapsulated into EVs, and they participate in the initiation and development of many diseases, including cancer.

tRNAs are small nucleic molecules consisting of 73–95 nucleotides characterized by their evolutionarily conserved structures terminate with the CCA trinucleotide at the 3′ end and the three hairpins.[89,90] The main and well-known function of tRNAs is carrying amino acids to the ribosome for protein synthesis. In recent years, tRNAs have been thought to act as primers for reverse transcription in viruses, inhibit protein translation, or regulate cell proliferation and DNA damage.[91–93] Likewise, full-length mature tRNAs and tRNA-derived small RNAs (tsRNAs) have been identified in EVs. Moreover, the abundance of tRNAs in EVs varies in different cell types. Approximately 50% of total small RNAs in adipose mesenchymal stem cell-derived EVs are tRNAs, while in bone marrow mesenchymal stem cell (BMSC)-derived EVs, only 23%–35% are tRNAs.[94] Surprisingly, in the newest study by Zhu's group,[53] they demonstrated the ubiquitous presence of tsRNAs in EVs extract from liver cancer cell culture medium and liver patients' plasma. Compared with healthy controls, tRNA-ValTAC-3, tRNA-GlyTCC-5, tRNA-ValAAC-5, and tRNA-GluCTC-5 were verified to be upregulated in liver cancer patients' plasma EVs, suggesting the biomarker potential of EV tsRNAs in liver cancer. The clinical application value of EV tRNAs in cancer is worthy of further study.

rRNA is the major component of the ribosomes and is answerable for peptide bond formation during protein biosynthesis.[95,96] rRNAs are single stranded, but a wide double-stranded region is formed by spatial folding.[97] In recent years, research has suggested that the processing of rRNA is the prominent function of nuclear RNA EVs.[98] Schuller et al.[99] investigated the structure of the ribosomal subunit within RNA EVs, and found that the rRNA spacer sequences internal transcribed spacer 2 is removed from rRNA precursors by a complex of 10 proteins in EVs, thereby forming the matured 60S rRNA. Specifically, several rRNAs have been detected in EVs of cancer cells. A high-throughput sequencing of breast cancer cells revealed that about 80% of EV RNA sequencing (RNA-seq) reads match to rRNAs.[100] In another separate study, Fiskaa et al.[101] found that about 43% of breast cancer cell EV-derived small RNA reads map to rRNAs, and present a high abundance of 5′ 28S rRNAs in EVs compared with parent cells. In contrast, endothelial and immune cell-derived EVs did not include these 5′ 28S rRNA fragments. In total, these results suggest that rRNAs are specifically secreted into EVs under pathological conditions, and might be associated with cancer progression.

Y RNAs are a class of transcripts by RNA polymerase III (Pol III) that consist of 83–112 nucleotides.[102] The biological function of Y RNAs is not clear, although several hypotheses are proposed. Ro60 is an RNA-binding protein that binds with misfolded and aberrant noncoding RNAs and channels them into a decay pathway for unfolding and degradation.[103] Specifically, since Y RNA and rRNA possess overlapped binding sites to Ro60, a possible function of Y RNAs is to spatially prevent misfolded rRNAs from entering the Ro60 active pocket, thus participating in rRNA quality control.[103,104] Existing data from bacterial and animal systems support the function of Ro60-Y RNA interaction in reducing cellular stress for survival.[105] Moreover, Y RNAs and Y RNA-derived fragments have been verified to play a role in carcinogenesis due to their effects in regulating cell proliferation and inflammation, and have shown potential as diagnostic and prognostic biomarkers in cancer.[106] In addition, deep RNA sequencing demonstrated that Y RNA is a highly abundant ncRNA species enriched in EVs in cancer. In chronic lymphocytic leukemia (CLL), Y RNAs are identified as the most abundant significantly enriched RNA in EVs from patients' plasma, which is notably different from the cellular Y RNA distribution.[107] Transference of CLL-derived EV Y RNA conferred monocytes CLL-associated phenotypes, suggesting the establishment of tumor-supportive microenvironment in CLL. Likewise, Y RNA fragments were found to be massively packaged into the EVs of anaplastic large cell lymphoma patients that are more aggressive and advanced.[108] Y RNA fragments were also identified in EVs secreted by melanoma cells.[109] Research on the correlation between EV Y RNA and cancer progression may provide clues for the development of new anticancer strategies.

piRNAs constitute a type of small ncRNAs containing 24–31 nucleotides with a 10th adenosine or 5′-terminal uridine bias, lacking characteristic secondary structures.[110] The biosynthesis of piRNA is to first produce piRNA intermediates from the piRNA cluster, and then generate mature piRNA through the cleavage of Zucchini riboendonuclease.[111] Importantly, piRNAs interact with piwi protein to form a piRNA/piwi complex, which participates in the regulation of spermiogenesis, germline stem cell maintenance, genomic rearrangement, transposon silencing, and epigenetic modification.[112] So far, piRNAs have been identified in germinal tissues as well as somatic tissues, such as brain tissue, heart tissue, and plasma.[113,114] Several studies have dissected EV piRNA profiling in a number of diseases, such as heart failure, Alzheimer disease, and cancers, which contribute to the distinguishment of health volunteers from patients, revealing the potential of EV piRNAs as diagnostic biomarkers.[54,115,116] Dysregulation of piRNAs and piwi proteins is also found in cancers, and some of them are involved in tumorigenesis, cancer diagnosis, and prognosis.[112,117] Recently, piRNAs were identified in EVs of human semen, plasma, and cultured human cancer cell lines.[54,116,118] It has been confirmed that piRNAs could be delivered by EVs and affect the behavior of recipient cells. For example, De Luca et al.[110] reported that umbilical cord blood CD34+ stem (UCB-CD34+) cells become less differentiated and more viable after exposure to BMSC-derived EVs. Further analysis revealed that it is the miRNAs and piRNAs in EVs that regulate the differentiation

and apoptosis of UCB-CD34+ cells. However, functions of piRNAs in EVs have not been thoroughly examined. Thus, further experimental and clinical studies are required.

THE INVOLVEMENT OF EV ncRNAs IN LC BIOLOGY AND THERAPY

LC is the most common and deadly cancer, with the characteristics of rapid proliferation, high malignancy, late detection, aggressive metastasis, strong drug resistance, and a poor prognosis.[119] It is urgent to clarify the underlying mechanism of LC occurrence and development and to bring new insight to clinical therapy for LC. Recently, a growing number of studies have focused on EV cargoes and their usage in LC genesis and progression.[120] In this section, we pay attention to the roles of EV ncRNAs in LC proliferation and survival, angiogenesis, invasion, metastasis, epithelial-mesenchymal transition (EMT), and therapy resistance (Table 1; Figure 2).

EV ncRNAs and cell proliferation, invasion, and migration in LC

Uncontrolled proliferation and enhanced invasion and migration ability are typical malignant phenotypes of LC cells, which ultimately lead to poor prognosis of LC patients. Therefore, discovering the molecular mechanisms and key regulatory factors of LC cellular proliferation, invasion, and migration is vital for understanding the pathogenesis of LC and for selecting appropriate treatment options. Mounting evidence has revealed that EV ncRNAs might be important mediators in the regulation of LC cell proliferation, invasion, and migration (Figure 2A).

MiR-96 is upregulated in various cancers and is regarded as an oncomiRNA.[159] In LC-derived EVs, miR-96 is found at significantly higher levels than in the EVs of healthy donors.[127] Importantly, EV miR-96 is positively associated with the LC grade. Higher EV miR-96 levels can be detected in LC cells with higher invasiveness. Functionally, miR-96 promotes cell proliferation, migration, and cisplatin (DDP) resistance in A549 cells by directly inhibiting LMO7 expression. In another separate study, Hu and colleagues[143] demonstrated that EV miR-486-5p regulates the cell cycle and proliferation by targeting NEK2 in lung adenocarcinoma (LAC). EV miR-486-5p might be a potential biomarker for LAC diagnosis and a target for LAC treatment. Moreover, miR-let-7e was found to be downregulated in NSCLC tissues and serum-derived EVs, which is relevant to the low survival rate of NSCLC patients.[160] Functionally, serum EV miR-let-7e suppresses NSCLC cellular viability, invasion, and migration *in vitro* and *in vivo* by targeting SUV39H2.

In addition to miRNAs, EV circRNAs are involved in the regulation of proliferation in LC. Zhang et al.[148] reported that circSATB2 could be packaged in EVs and promote the proliferation, migration, and invasion of NSCLC cells, as well as induce abnormal proliferation of bronchial epithelial cells. Importantly, circSATB2 is highly expressed in the serum EVs of LC patients compared with healthy donors, suggesting its possible use as a noninvasive diagnostic biomarker of NSCLC. In another study, circARHGAP10 was reported to be upregulated in EVs of NSCLC cells and tissues, as well as in patients'

Table 1. The biological functions of EV ncRNAs in LC

ncRNAs	Dysregulation	Target	Experiment type (in vitro/*in vivo*)	Biological functions	Reference
miR-9	–	SOCS5	*in vitro* (NSCLC cells → endothelial cells)	promote endothelial cell migration and angiogenesis	Zhuang et al.[121]
miR-21	upregulated	PDCD4	*in vitro* (LAC cells → osteoclasts)	promote tumor osteolytic metastasis	Xu et al.[122]
miR-21/miR-29a	upregulated	TLR8	*in vitro* (LLCs → macrophages); *in vivo* (mouse LC model, intravenously injected with LLC-secreted EVs)	trigger pre-metastatic inflammatory response	Fabbri et al.[123]
miR-21a	upregulated	PDCD4	*in vitro* (LLCs → MDSCs); *in vivo* (mouse LC model, intravenously injected with LLC-secreted EVs)	promote MDSCs expansion and accumulation, promote cancer cell growth	Zhang et al.[124]
miR-23a	upregulated	ZO-1	*in vitro* (hypoxic LC cells → endothelial cells); *in vivo* (human, serum EVs)	promote angiogenesis, increase migration	Hsu et al.[125]
miR-31-5p	upregulated	SATB2	*in vitro* (hypoxic LC cells → endothelial cells); *in vivo* (human, plasma EVs)	promote migration, invasion, and metastasis	Yu et al.[126]
miR-96	upregulated	LMO7	*in vitro* (NSCLC cells → NSCLC cells); *in vivo* (human, serum EVs)	promote cell proliferation, migration, and DDP resistance	Wu et al.[127]
miR-100-5p	Downregulated	–	*in vitro* (A549/DDP cells → A549 cells); *in vivo* (mouse LC model, intratumorally injected with A549/DDP cell-secreted EVs)	alter other LC cells' sensitivity to DDP	Qin et al.[128]
miR-103a	upregulated	PTEN	*in vitro* (hypoxic LC cells → M2 macrophages)	increase M2 macrophage polarization	Hsu et al.[129]
miR-130a	–	PUM2	*in vitro* (CAFs → DDP-sensitive NSCLC cells)	promote survival of DDP-sensitive NSCLC cells	Zhang et al.[130]
miR-142-3p	upregulated	TGFbetaR1	*in vitro* (LAC cells → endothelial and fibroblast cells)	promote angiogenesis and cancer-associated fibroblast phenotype	Lawson et al.[131]
miR-143-3p	upregulated	ITM2B	*in vitro* (MDSCs → LC cells); *in vivo* (mouse LC model, intratumorally injected with MDSC-secreted EVs)	promote proliferation	Zhou et al.[132]
miR-144	Downregulated	CCNE1/2	*in vitro* (BMSCs → NSCLC cells); *in vivo* (mouse LC model, intravenously injected with BMSC-secreted EVs)	inhibit proliferation	Liang et al.[133]
miR-192	Downregulated	–	*in vitro* (NSCLC cells → osteoclasts); *in vivo* (mouse LC model, intravenously injected with NSCLC cell-secreted EVs)	inhibit angiogenesis and osteoclastogenesis	Valencia et al.[134]
miR-193a	–	LRRC1	*in vitro* (BMSCs → NSCLC cells); *in vivo* (mouse LC model, intravenously injected with BMSC-secreted EVs)	promote proliferation, migration, invasion, and DDP-resistant cell apoptosis	Wu et al.[135]
miR-193a-3p, miR-210-3p, and miR-5100	upregulated	–	*in vitro* (hypoxic BMSCs → Epithelial cancer cells); *in vivo* (human, plasma EVs; mouse LC model, intravenously injected with hypoxic BMSC-secreted EVs)	promote invasion and EMT	Zhang et al.[136]
miR-210	upregulated	TET2	*in vitro* (LC cells → fibroblasts); *in vivo* (human, plasma EVs; mouse angiogenesis model, fibroblasts mixed with LC cell-secreted EVs)	promote fibroblasts transfer into CAFs and angiogenesis	Fan et al.[137]
miR-222-3p	–	SOCS3	*in vitro* (A549/GR cells → A549 cells); *in vivo* (human, plasma EVs; mouse LC model, injected with A549/GR cell-secreted EVs)	promote proliferation, migration, invasion, and gemcitabine resistance	Wei et al.[138]
miR-214	upregulated	–	*in vitro* (PC-9/GR cells → PC-9 cells); *in vivo* (mouse LC model, hypodermic injected with PC-9/GR cell-secreted EVs)	promote gefitinib resistance	Zhang et al.[139]
miR-302b	upregulated	TGFbetaRII	*in vitro* (lowly metastatic LC cells → highly metastatic LAC cells)	suppress LC cell proliferation and migration	Li et al.[140]
miR-369	upregulated	NF1		promote proliferation, migration, and invasion	Guo et al.[141]

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Table 1. Continued

ncRNAs	Dysregulation	Target	Experiment type (in vitro/*in vivo*)	Biological functions	Reference
			in vitro (CAFs → LAC cells); *in vivo* (mouse LC model, hypodermic injected with CAF-secreted EVs)		
miR-425-3p	upregulated	AKT	*in vitro* (DDP-resistant NSCLC cells → DDP-sensitive NSCLC cells); *in vivo* (human, serum EVs)	facilitated autophagic activation and chemoresistance	Ma et al.[142]
miR-486-5p	Downregulated	NEK2	*in vitro* (NSCLC cells → NSCLC cells); *in vivo* (human, serum EVs)	induce cell-cycle arrest, inhibit cell proliferation, and metastasis	Hu et al.[143]
miR-499a-5p	upregulated	mTOR	*in vitro* (highly metastatic LAC cells → LAC cells)	promote cell proliferation, migration, and EMT	He et al.[144]
miR-564 and miR-658	upregulated	–	*in vitro* (PC-9/gefitinib cells → PC-9 cells)	induce drug resistance in sensitive cells	Azuma et al.[145]
miR-619-5p	upregulated	RCAN1.4	*in vitro* (NSCLC cells → endothelial cells); *in vivo* (human, plasma EVs)	promote proliferation, metastasis, and angiogenesis	Kim et al.[146]
miR-5787, miR-4532, and miR-4488	upregulated	–	*in vitro* (mesenchymal cells → epithelial cells)	promote migration, invasion, and EMT	Tang et al.[147]
circSATB2	upregulated	miR-326	*in vitro* (NSCLC cells → NSCLC cells); *in vivo* (human, serum EVs)	promote cell proliferation, migration, and invasion	Zhang et al.[148]
circARHGAP10	upregulated	miR-638	*in vitro* (NSCLC cells → NSCLC cells); *in vivo* (human, serum EVs)	promote proliferation, migration, invasion, and glycolysis	Fang et al.[149]
circUSP7	upregulated	miR-934	*in vitro* (NSCLC cells → CD8+ T cells); *in vivo* (human, plasma EVs)	inhibits CD8+ T cell function, promote resistance to immunotherapy	Chen et al.[150]
lncHOTAIR	upregulated	miR-203	*in vitro* (NSCLC cells → NSCLC cells); *in vivo* (human, serum EVs)	promote proliferation, migration, and invasion	Zhang et al.[83]
lncMALAT-1	upregulated	–	*in vitro* (NSCLC cells → NSCLC cells); *in vivo* (human, serum EVs)	promote cell growth and migration, inhibit apoptosis	Zhang et al.[151]
lncUFC1	upregulated	EZH2	*in vitro* (NSCLC cells → NSCLC cells); *in vivo* (human, serum EVs)	promote proliferation, migration, and invasion	Zang et al.[152]
lncRNA-p21	upregulated	–	*in vitro* (LC cells → HUVECs); *in vivo* (human, plasma EVs)	promote tube formation and enhance tumor cell adhesion to endothelial cells	Castellano et al.[153]
lncGAS5	Downregulated	miR-29-3p	*in vitro* (LC cells →HUVECs); *in vivo* (mouse LC model, serum EVs)	promote proliferation and tube formation, inhibit apoptosis	Cheng et al.[154]
lncAGAP2-AS1	upregulated	miR-296	*in vitro* (M2 macrophage → NSCLC cells)	promote the radiotherapy immunity	Zhang et al.[155]
lncPCAT-1	upregulated	miR-182/miR-217	*in vivo* (human, serum EVs)	promote pre-metastatic niche formation and tumor metastasis; induce G0/G1 cell-cycle arrest; promote chemoresistance and tumor growth	Domvri et al.[156]
lncRNA MSTRG.292666.16	upregulated	–	*in vitro* (osimertinib-resistant cells → osimertinib-sensitive cells); *in vivo* (human, plasma EVs)	associated with asimertinib resistance	Deng et al.[86]
lncRNA RP11838N2.4	upregulated	–	*in vitro* (erlotinib-resistant cells → erlotinib-sensitive cells); *in vivo* (human, serum EVs)	promote erlotinib resistance	Zhang et al.[157]
lncRNA H19	upregulated	–	*in vitro* (gefitinib-resistant NSCLC cells → gefitinib-sensitive NSCLC cells)	promote gefitinib resistance	Lei et al.[158]

NSCLC, non-small cell lung cancer; LAC, lung adenocarcinoma; LLC, Lewis LAC; LC, lung cancer; EV, extracellular vesicle; MDSC, myeloid-derived suppressor cell; DDP, cisplatin; CAF, cancer-associated fibroblast; BMSC, bone marrow mesenchymal stem cell; GR, gemcitabine resistance; EMT, epithelial-mesenchymal transition; HUVEC, human umbilical vein endothelial cell.

serum-derived EVs.[149] Functionally, circARHGAP10 promotes the proliferation, invasion, and migration of NSCLC cells and facilitates glycolysis. MiR-638 is the direct target of circARHGAP10, which inhibits the downstream oncogene FAM83F to repress the progression of NSCLC. The circARHGAP10-miR-638-FAM83F axis might be a potential target for the investigation of novel anticancer strategies in NSCLC.

In addition, some lncRNAs have been clarified to be selectively sorted in EVs and associated with the regulation of proliferation of LC.
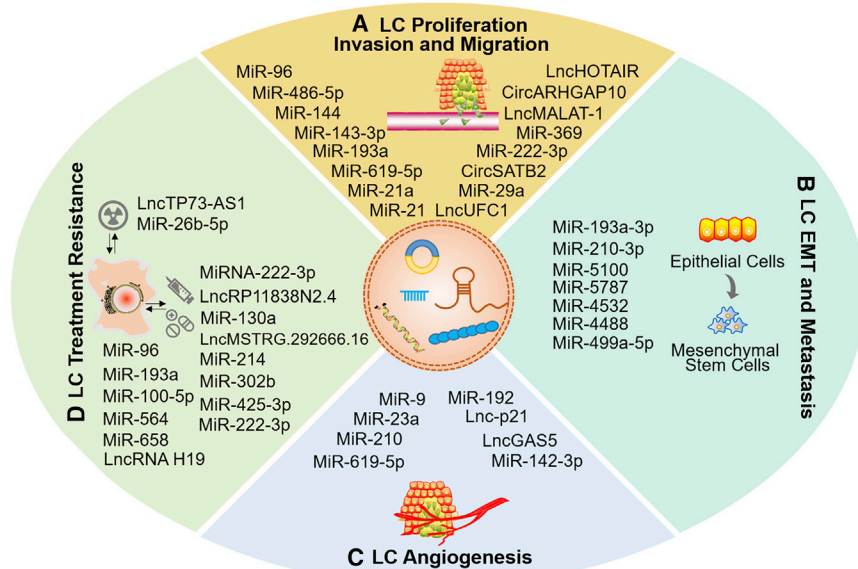
Figure 2. The participation of EV ncRNAs in lung cancer biology

EV ncRNAs play important roles in lung cancer progression, such as cell proliferation, invasion, and migration (A), EMT and metastasis (B), angiogenesis (C), and treatment resistance (D).

Zhang et al.[83] demonstrated that the expression of lncHOTAIR is increased in the serum EVs of LC patients compared with healthy donors. Additionally, EV lncHOTAIR enhanced the proliferation, migration, and invasion of A549 and H1299 cells, which might be mediated by sponging miR-203. In another separate study, Zhang's group[151] identified a highly expressed EV lncRNA, lncMALAT-1, from the serum of LC patients. More importantly, the expression of EV lncMALAT-1 is positively correlated with the tumor stage. Accumulation of serum-derived EV lncMALAT-1 could promote cell growth and migration and prevent apoptosis in LC cell lines. Furthermore, Zang and colleagues[152] demonstrated that the expression of lncUFC1 is elevated in tissues, serum, and serum EVs in NSCLC patients. NSCLC cell-derived EV promotes proliferation, migration, and invasion through transferring lncUFC1 in A549 cells. Specifically, lncUFC1 could downregulate PTEN expression and activate the AKT pathway by binding to EZH2, thus promoting the tumorigenesis of NSCLC. In brief, ncRNAs can be selectively packaged into EVs and participate in the regulation of proliferation, invasion, and migration in LC.

EV ncRNAs stimulate EMT and metastasis in LC

EMT is a process by which epithelial cells lose epithelial polarity and cell-cell adhesion to transform into mesenchymal stem cells (MSCs), granting the cells the ability to invade and migrate, therefore participating in multiple physiological and pathological processes, such as individual development, tissue healing, organ fibrosis, and cancer.[161] Importantly, cancer cells in the primary site lose their adhesion ability during the EMT process; at the same time, the expression of E-cadherin decreases, the expression of mesenchymal markers and fibronectin rises, and the cell motility and invasion ability are enhanced to invade the extracellular matrix.[162] In recent years, a growing body of research has revealed that EVs largely contribute to EMT and the subsequent metastasis in various cancers.[163] More impor-

tantly, EV cargoes, such as ncRNAs, could be transferred and internalized by certain recipient cells, thus regulating the behavior of these cells and the process of EMT, subsequently affecting cancer development and metastasis (Figure 2B).

Zhang et al.[136] reported that BMSC-delivered miR-193a-3p, miR-210-3p, and miR-5100 can be transferred to epithelial cancer cells, promoting the metastasis of LC cells through activating STAT3-dependent EMT. Moreover, Tang's group[147] recently found that EV miR-5787, miR-4532, and miR-4488 secreted by MSCs contribute to signal transduction in the EMT process and the stimulation of mesenchymal marker expression in epithelial cells, therefore promoting the metastasis of LC. In another separate study, He and colleagues[144] clarified the effect of miR-499a-5p on promoting the migration, EMT, and metastasis of LC cells. MiR-499a-5p-containing EVs derived from highly metastatic cells activate the mammalian target of rapamycin (mTOR) signaling pathway to facilitate cellular proliferation, migration, and EMT, which provides new insight into modulating LC metastasis. In total, these results demonstrated that EV ncRNAs could affect LC progression and metastasis through regulating the EMT process.

EV ncRNAs induce LC angiogenesis

Angiogenesis, namely the formation of new blood vessels from pre-existing vessels, is pivotal for tumor growth and hematogenous metastasis of various solid tumors, including LC.[164] Increasing evidence has demonstrated that ncRNAs play important roles in tumor angiogenesis. Vascular endothelial cells capture EVs released by stromal cells, tumor cells, and MSCs, regulating the formation of new blood vessels. Extensive studies have revealed that EV ncRNAs participate in the regulation of tumor angiogenesis (Figure 2C). Zhuang et al.[121] reported that miR-9 could be secreted from NSCLC cells via EVs and then be internalized by endothelial cells. MiR-9 reduced SOCS5 levels to activate the JAK-STAT pathway to promote angiogenesis, further promoting tumor cell growth. In addition, miR-23a was demonstrated to be upregulated in EVs in LC under hypoxic conditions.[125] EV miR-23a causes the accumulation of HIF-1α in endothelial cells by suppressing PHD1/2, thus leading to neovascularization in LC. Specifically, EV miR-23a inhibits ZO-1 to increase vascular permeability. Therefore, EV miR-23a could be an intercellular communication regulator involved in LC angiogenesis. Other studies have found that CAFs possess the ability to promote tumor angiogenesis. Fan's group[137] proved that miR-210 activates

the JAK2/STAT3 pathway by targeting TET2 in CAFs. Specifically, EVs containing overexpressed miR-210 could stimulate the transferring of fibroblasts into CAFs, resulting in the promotion of angiogenesis in LC. In another study, miR-619-5p was shown to be upregulated in NSCLC patients' plasma-released EVs, and it has dual functions in tumor progression: promoting angiogenesis in endothelial cells and increasing proliferation and metastasis in NSCLC cells.[146]

For EV lncRNAs, EV lncRNA-p21 was demonstrated to be upregulated in NSCLC patient plasma.[153] Functionally, tumor-derived EVs containing lncRNA-p21 promote tube formation and tumor cell adhesion to endothelial cells by targeting miRNAs related to angiogenesis and metastasis. In addition, Cheng's group[154] reported that the low expression of EV lncGAS5 derived from LC tissues is positively correlated with human umbilical vein endothelial cell (HUVEC) proliferation and tube formation. Lentivirus-mediated lncGAS5 overexpression reversed the effects of LC cell-derived EVs on HUVECs. EV lncGAS5 might be a novel target for inhibiting LC angiogenesis. Taken together, these results suggest that ncRNAs selectively sorted in EVs play important roles in regulating LC angiogenesis.

EV ncRNAs remodel the TME of LC

The TME is a complex scaffold that consists of diverse cell populations, structural molecules, and signaling factors that interact with tumor cells and support tumorigenesis and development.[165] In the TME, EV ncRNAs can transfer between cancer cells and stromal cells, thus regulating the behaviors of recipient cells and participating in various prominent biological processes involved in the TME, such as immune escape, metabolism alternation, hypoxia, and chronic inflammation.

Organisms are monitored by the immune system, which recognizes and eliminates the incipient cancer cells so as to restrain nascent tumors.[166] However, most solid tumors are able to avoid the monitoring and elimination of the immune system, leading to the occurrence and development of cancer. Accumulating evidence has revealed that the secretion of tumor-associated immunosuppressive factors and the development of tumor-antagonizing immune cells could be influenced by EV ncRNAs. For instance, transforming growth factor (TGF) is the immunosuppressive central to the TME.[167] In the recent study by Li et al.,[140] they reported that miR-302b was significantly upregulated in LC cell-derived EVs. Overexpression of miR-302b inhibits LC cell proliferation and migration accompanied by the decreased expression of TGFβRII. In addition, the dysfunction of tumor-antagonizing immune cells, CD8+ T cells, has been verified in NSCLC. Chen's group[150] demonstrated the existence of circUSP7 in LC patients' plasma EVs. CircUSP7 increased SHP2 expression by sponging miR-934, thereby inhibiting IFN-γ, TNF-α, granzyme-B, and Perforin secretion by CD8+ T cells. Moreover, Domvri and colleagues[156] verified that EV lncPCAT-1 was highly expressed KRAS-mutant LC patients' serum. LncPCAT-1 increased the expression of miR-182/miR-217, thus promoting pre-metastatic niche formation

and LC immunosuppressive metastasis. LncPCAT-1 could also regulate fibroblast differentiation and CAF-mediated stromal activation, thereby affecting chemoresistance and tumor growth.

Cancer cells undergo dramatic changes in the metabolism of glucose, amino acids, and fatty acids to survive in the stressful TME.[168] Aerobic glycolysis, also known as Warburg effect, is the major way through which cancer cells get energy and generate biomolecules under diminished nutrient supply conditions.[169] A growing body of evidence has demonstrated that EV ncRNAs could modulate the glycolysis metabolism of LC. For example, EV circRNAs, such as circ-MEMO1, circ_0008928, hsa_circRNA_0002130, and circARHGAP10 were found to facilitate glycolysis metabolism and pathological process of NSCLC through acting as miRNA sponges, which providing a perspective for further understanding the pathogenesis of NSCLC.[149,170–172]

As the rapid and uncontrolled proliferation of cancer cells exceeds the oxygen supply, hypoxia is one of the characteristics of the TME in almost all solid tumors.[173] Cancer cells, once they acclimate to the hypoxic conditions, lead to changes in gene expression and proteomics, resulting in a more aggressive and therapy-resistant phenotype. It was demonstrated that hypoxia enhanced the release of EVs into the TME.[174] EV ncRNAs derived from hypoxic LC cells or stromal cells participate in the regulation of biological processes such as the growth of new vasculatures, EMT, and metastasis, so as to guarantee the survival of cancer cells and promote LC development. MiR-193a-3p, miR-210-3p, and miR-5100 carried by hypoxic BMSCs EVs were confirmed to promote LC metastasis by directly targeting STAT3 in vitro and in vivo.[136] Especially, EV miR-193a-3p was able to distinguish LC patients from healthy controls, or metastatic LC patients from non-metastatic LC patients, revealing the diagnostic accuracy of EV miR-193a. In other research, hypoxic LC cell-secreted EV miR-23a, miR-103a, and miR-31-5p were reported to promote angiogenesis and affect LC malignant phenotypes, respectively.[125,126,129]

Chronic inflammation is one of the characteristics of cancer.[166] Inflammatory cells and inflammatory factors within the TME promote the activation of inflammatory pathways, thereby stimulating the malignant transformation, and the occurrence and development of cancer. Emerging evidence suggests that EV ncRNAs could target and modulate the key components and processes of chronic inflammation. Macrophages are the most studied inflammatory cells in LC and participate in inflammation by secreting inflammatory factors and chemokines, thereby regulating LC progression.[175] It was demonstrated that M2 macrophage-derived EV ncRNAs, such as miR-501-3p, miR-942, miR-155, miR-196-5p, and lncAGAP2-AS1, were able to promote LC cell growth, migration, invasion, EMT, and angiogenesis.[155,176–178] Moreover, inflammation-related signal pathways in LC could be affected by EV ncRNAs. For example, Ni et al.[85] found that LC cell-derived EV lncRNA-SOX2OT promotes cell invasion, migration, and bone metastasis by targeting the TGF-β/pTHrP/RANKL pathway. In the studies by Fan's and Du's groups, EV miR-3473b and miR-210 were verified to promote intrapulmonary colonization

and angiogenesis by modulating nuclear factor (NF)-κB and STAT3 signaling, respectively.[137,179]

In general, the TME is a complex environment composed of a variety of cellular and acellular components. EV ncRNAs can be secreted and transmitted between donor cells and recipient cells and regulate the behavior of recipient cells in the TME, thereby affecting the occurrence, development, and metastasis of LC.

EV ncRNAs interfere with LC therapy

Approximately 60% of LC patients need radiotherapy during the course of their cancer treatment. EV ncRNAs interfere with the resistance and sensitivity of LC cells to radiotherapy, as well as the effect of treatment (Figure 2D). Zheng's group[180] reported that EV miR-96 is highly expressed in NSCLC patients' plasma. Especially, EV miR-96 was correlated with radioresistance, vascular invasion, and poor overall survival (OS) rate in LC, suggesting the potential of EV miR-96 as a diagnostic and prognostic biomarker for LC radioresistance. In addition, Han et al.[181] demonstrated that LC cell-derived EVs containing miR-26b-5p could be internalized by radioresistant LC cells, promoting DNA damage, apoptosis, and radiosensitivity by inhibiting ATF2. Specifically, miR-26b-5p can be detected in serum EVs, revealing the potential of EV miR-26b-5p as a noninvasive biomarker for LC radiotherapy monitoring.

Chemotherapy is one of the most important approaches in LC therapy. However, chemotherapy resistance often appears in clinical practice and becomes an insurmountable obstacle in the treatment of LC. Therefore, an in-depth study of the molecular mechanism of chemotherapy resistance is crucial to find more effective treatment strategies for LC. Recent studies have shown that EV ncRNAs secreted by stromal cells regulate chemotherapy resistance in LC (Figure 2D). Zhang et al.[130] found that NSCLC-derived CAFs are innately resistant to DDP. EV miR-130a transferred from CAFs significantly improves the survival rate of NSCLC cells during DDP treatment. Furthermore, Wu's group[135] demonstrated that human BMSC-derived EVs that are transfected with miR-193a suppress cellular viability, invasion, and migration and promote the apoptosis of DDP-resistant NSCLC cells by targeting and downregulating LRRC1. These results suggest that stromal cell-derived EV ncRNAs might be potential therapeutic targets for DDP resistance in NSCLC.

Importantly, EVs originated from drug-resistant LC cells can be transferred to and internalized by chemotherapy-sensitive LC cells, thus spreading drug resistance, revealing the potential participation of EV ncRNAs in these processes. For instance, gefitinib-resistant LC cell-derived EVs containing miR-214, miR-302b, miR-564, and miR-658 could confer gefitinib resistance to normal LC cells.[139,140,145] Moreover, DDP-resistant LC cell-secreted EVs carrying miR-425-3p and miR-100-5p could be internalized by DDP-sensitive LC cells, providing chemotherapy resistance to recipient cells in c-myc/AKT1- and mTOR-dependent manners, respectively[128,142]. Furthermore, there are other EV ncRNAs that are transmitted from drug-resistant cells to drug-sensitive cells and affect chemotherapy resistance, such as lncMSTRG.292666.16 to osimertinib, lncRP11838N2.4 to erlotinib, and miRNA-222-3p to gemcitabine.[86,138,157] Overall, EV ncRNAs, as important molecules in the regulation of radioresistance and chemoresistance in LC, are of great significance for improving the clinical therapeutic effect.

DIAGNOSTIC, PROGNOSTIC, AND THERAPEUTIC APPLICATIONS OF EV ncRNAs IN LC

EV ncRNAs serve as promising biomarkers in LC

EVs contain a variety of functional molecules, such as ncRNAs, that reflect the complexity and heterogeneity of tumors.[101] Increasing research has implicated the important roles of EV ncRNAs in the diagnosis and prognosis of LC, as well as anti-LC therapeutic applications.[153,182–184] Importantly, EV ncRNAs are readily available from almost all types of human fluids, providing a liquid tool for noninvasive clinical detection (Table 2).[9]

Kim et al.[190] investigated the expression level of miRNAs in EVs isolated from bronchoalveolar lavage (BAL) fluid, comparing early-stage LAC patients and healthy controls. MiR-126 was upregulated in the BAL fluid of LAC patients compared with healthy people, which makes it a potential diagnostic biomarker for early-stage LAC. Specifically, miR-126 could also be detected in serum EVs.[191] Downregulation of miR-126 was detected in the serum of advanced-stage NSCLC patients compared with healthy controls. EV miR-126 might serve as a potential diagnostic biomarker and personalized therapeutic target in LC. Moreover, Roman-Canal et al.[185] performed pleural lavage on LC patients and analyzed the expression of EV miRNAs in the lavage fluid. Fourteen miRNAs were found to be dysregulated between cancer and control cases, among which miR-1-3p, miR-144-5p, and miR-150-5p were the three most significantly dysregulated with a high area under the curve (AUC) value, sensitivity, and specificity. These results suggest that EV miR-1-3p, miR-144-5p, and miR-150-5p are promising noninvasive biomarkers for LC diagnosis.

The expression of circRNAs and lncRNAs in EVs has also been investigated. In the work of Xian et al.,[200] three EV circRNAs (circ_0047921, circ_0056285, and circ_0007761) showed diagnostic value in NSCLC patients (AUC = 0.926). Additionally, circ_0047921 could distinguish NSCLC patients from chronic obstructive pulmonary disease controls (AUC = 0.890). However, circ_0056285-circ_0007761 combinations could distinguish NSCLC patients from tuberculosis controls (AUC = 0.820). The expression level of circ_0056285 was related to clinical stages and lymph node metastasis of NSCLC. These three EV circRNAs might serve as diagnostic and prognostic biomarkers of NSCLC in the Chinese population. According to the research of Castellano et al.,[153] patients with high expression levels of EV lncRNA-p21 have a shorter time to relapse and a shorter OS. In addition, they generated receiver operating characteristic (ROC) curves according to the survival time and lncRNA-p21 expression. The AUCs for 6-, 12-, 24-, and 36-month survival were 0.639, 0.737, 0.686, and 0.632, respectively.

Table 2. EV ncRNAs as diagnosis and prognostic biomarkers in LC

NcRNAs	Source	Cases and samples	Biomarker potential	AUC, sensitivity, and specificity	Reference
miR-1-3p, miR-144-5p, and miR-150-5p	pleural lavage	46 LC patients and 25 healthy controls	promising biomarkers of LC diagnosis	AUC = 0.899; sensitivity = 80.25%; specificity = 92.31%	Roman-Canal et al.[185]
miR-17-5p	serum	100 NSCLC patients and 90 healthy controls	potential diagnostic biomarker for NSCLC patients	AUC = 0.86; sensitivity = 63%; specificity = 93.3%	Zhang et al.[186]
miR-20b-5p and miR-3187-5p	serum	276 NSCLC patients and 282 healthy controls	diagnosis biomarkers for early-stage NSCLC	AUC = 0.848	Zhang et al.[187]
miR-21	pleural lavage	144 primary LAC patients and 55 healthy controls	prognostic biomarker in primary LAC	–	Watabe et al.[188]
miR-21 and miR-4257	plasma	195 NSCLC patients	predictive biomarker for recurrence in NSCLC patients	–	Dejima et al.[189]
miR-126	BAL fluid	13 LAC patients and 15 healthy controls	diagnostic biomarkers in early-stage LAC	–	Kim et al.[190]
miR-126	serum	45 NSCLC patients	diagnostics biomarker and therapeutic target for NSCLC	AUC = 0.875 and 0.835 for stage I/II and III/IV NSCLC, respectively	Grimolizzi et al.[191]
miR-146-5p	serum	six DDP-resistant NSCLC patients and six DDP-sensitive NSCLC patients	biomarker predicting DDP efficacy and real-time monitoring DDP resistance for NSCLC	–	Yuwen et al.[192]
miR-193a-3p, miR-210-3p, and miR-5100	plasma	21 metastasis LC patients and 20 non-metastasis LC patients	biomarkers for LC metastasis	AUC = 0.8600, 0.8369 and 0.8016, respectively	Zhang et al.[136]
miR-1290	serum	70 LAC patients and 40 healthy controls	potential biomarker for the diagnosis and prognosis of LAC	AUC = 0.937; sensitivity = 80%; specificity = 96.7%	Wu et al.[193]
miR-216b	serum	105 NSCLC patients and 60 healthy controls	potential diagnostic and prognostic biomarker for NSCLC	AUC = 0.84; sensitivity = 86.7%; specificity = 75%	Liu et al.[194]
miR-342-5p and miR-574-5p	plasma	seven early-stage LAC patients and seven healthy controls	diagnostic biomarkers for early-stage LAC	AUC = 0.813; sensitivity = 80%; specificity = 73.2%	Han et al.[195]
miR-378a, miR-379, miR-139-5p, and miR-200b-5p	plasma	30 LC patients	biomarkers for screening and diagnose LC	AUC = 0.76; sensitivity = 96%; specificity = 60%	Cazzoli et al.[196]
miR-1269a	serum	147 NSCLC patients and 149 healthy controls	diagnostic biomarker for NSCLC	AUC = 0.915; sensitivity = 77%; specificity = 89%	Wang et al.[197]
miR-5864 and miR-125b-5p	serum	330 NSCLC patients and 312 healthy controls	diagnostic and prognostic biomarkers for NSCLC	AUC = 0.733; sensitivity = 62.4%; specificity = 70%	Zhang et al.[183]
miR-7977	serum	65 LAC patients	LC biomarker	AUC = 0.787	Chen et al.[182]
circRNA-002178	plasma	120 LAC patients and 30 healthy controls	biomarkers for LAC early diagnosis	AUC = 0.9956	Wang et al.[198]
circRNA_0056616	plasma	42 LAC patients with lymph node metastasis and 48 without lymph node metastasis	potential biomarker for lymph node metastasis in LAC	AUC = 0.812; sensitivity = 79.2%; specificity = 81%	He et al.[199]
circ_0047921, circ_0056285 and circ_0007761	serum	30 NSCLC patients and 45 healthy controls	biomarkers for NSCLC diagnosis in Chinese population	AUC = 0.926	Xian et al.[200]
hsa_circ_0,002,130	serum	28 osimertinib-resistant NSCLC and 32 osimertinib-sensitive NSCLC patients	biomarker for osimertinib resistance in LC	AUC = 0.792	Ma et al.[172]
hsa_circ_0014235 and hsa_circ_0025580	plasma	30 pairs of LUSC patients and healthy controls	diagnostic biomarkers for LUSC	AUC = 0.8254 and 0.8003, respectively	Wang et al.[73]
circ-MEMO1	serum	52 pairs of NSCLC tissue sample and adjacent normal tissues	biomarker for the early diagnosis for NSCLC	AUC = 0.76; sensitivity = 56.67%; specificity = 96%	Ding et al.[170]
lncGAS5	serum	64 NSCLC patients	single diagnostic biomarker for NSCLC	AUC = 0.929; sensitivity = 85.94%; specificity = 70%	Li et al.[201]
lncTBILA and lncAGAP2-AS1	serum	150 NSCLC patients	potential diagnostic biomarkers for NSCLC	AUC = 0.853	Tao et al.[202]
lncSOX2-OT	plasma	75 LSCC patients and 79 healthy controls	noninvasive biomarker for LSCC	AUC = 0.815; sensitivity = 76%; specificity = 73.17%	Teng et al.[88]

Table 2. Continued

NcRNAs	Source	Cases and samples	Biomarker potential	AUC, sensitivity, and specificity	Reference
lncMALAT-1	serum	77 NSCLC patients	biomarker and therapeutic target for NSCLC	AUC = 0.73; sensitivity = 60.1%; specificity = 80.9%	Zhang et al.[151]
lncRNA-p21	plasma	56 NSCLC patients	prognostic biomarker for NSCLC	AUC = 0.639	Castellano et al.[153]

AUC, area under the curve; BAL, bronchoalveolar lavage; LUSC, lung squamous cell carcinoma; LSCC, lung squamous cell carcinoma.

EV ncRNAs might also be potential biomarkers for monitoring the outcomes of treatment. Yuwen's group[192] found that advanced NSCLC patients with low serum EV miR-146a-5p possessed a higher recurrence risk than those with high serum EV miR-146a-5p levels. Additionally, in the process of DDP resistance, miR-146a-5p expression is decreased in either serum EVs or NSCLC cells, suggesting that overexpression of miR-146a-5p might reverse DDP resistance in NSCLC. Moreover, Ma et al.[172] demonstrated that hsa_circ_0002130 is upregulated in osimertinib-resistant NSCLC cells and regulates glycolysis by targeting miR-498. Importantly, hsa_circ_0002130 was highly expressed in osimertinib-resistant NSCLC patient serum EVs compared with osimertinib-sensitive NSCLC patients (AUC = 0.792). Serum EV hsa_circ_0002130 might be a noninvasive biomarker monitoring osimertinib resistance in NSCLC. In summary, EVs regulate the progression of LC by mediating the communication between neighboring cells and distant cells. EV ncRNAs in body fluids possess pivotal clinical value as biomarkers for LC diagnosis, prognosis, and treatment monitoring, and their clinical application deserves further investigation.

Potential applications of EV ncRNAs in anti-LC therapy

Currently, an increasing number of studies have demonstrated that EVs are ideal delivery vehicles. Lai et al.[203] created a sensitive reporter for EV imaging and expression monitoring in organs and body fluids. Importantly, they found that injected EVs can be delivered to tumor tissues 1 h after injection. Additionally, due to the peptides and ligands on the surface, EVs are able to deliver their cargoes to recipient cells directly. Moreover, many candidate ligands (such as drugs and biomacromolecules) are unstable *in vivo*, which is a huge challenge for their clinical applications. Since the lipid bilayer membrane structure of EVs can sequester drug enzymes, nucleases, and proteases, encapsulation of these ligands into EVs can enhance their stability and delivery efficiency. Besides, since EVs are intracellular components, they possess low immunogenicity and high biocompatibility *in vivo*. Therefore, EV cargoes might provide a novel nanobiomedical therapeutic approach for cancers.

Increasing evidence has confirmed the anti-LC potency of exosomal ncRNAs. Nie and colleagues[184] demonstrated that EVs derived from breast cancer cells could be internalized by NSCLC cells through an interaction between integrin β4 on EVs and surfactant protein C on A549 cells. Especially, systemic administration of miR-126-loaded EVs significantly suppressed proliferation and migration in A549 cells by interrupting the PTEN/PI3K/AKT pathway. In a lung metastasis mouse model, miR-126-loaded EVs also had an efficacious anticancer effect. In another study, Jeong et al.[204] investigated the anti-angiogenic and anti-growth effects of miR-497-loaded engineered EVs on NSCLC cells cultured in 2D and 3D microfluidic devices. MiR-497-loaded EVs effectively inhibit the expression of YAP1, VEGF-A, and CCNE1 and further suppress the angiogenesis and migration of tumors compared with the control. Altogether, these studies demonstrated that EVs could be vehicles for ncRNA delivery, and EV ncRNAs can be used as predictive, cost-efficient tools for the development of targeted LC therapy.

In addition to ncRNAs, other kinds of biomacromolecules, such as peptides, lipids, and even exogenous DNAs and drugs, can be loaded into EVs and transferred to recipient cells to exert anti-tumor effects, providing broad application prospects for EV-based delivery systems.[205] However, many basic studies are required before the extensive application of EV ncRNAs in the clinical practice of LC treatment.

ISOLATION AND IDENTIFICATION OF EVs
Methods for EV isolation

EV ncRNAs have been proved to have great application potential. Therefore, it is imperative to isolate and identify EVs from wide range of interfering substances and cellular fragments. With the development of the biomedical industry, several kinds of techniques for EVs isolation have been widely adopted, such as centrifugation techniques, polymer precipitation, size-based isolation techniques, immunoaffinity capture techniques, and microfluidic techniques.[206]

The centrifugation technique is the most commonly used method and is the gold standard for EV separation.[207] This method usually requires a series of centrifugal forces with different rotation speeds and a certain amount of time. Although the technique is simple to operate and the amount of EVs obtained is large, the recovery rate is unstable and the purity is challenged. In addition, polyethylene glycol (PEG) is often used in polymer precipitation techniques to combine with water molecules, resulting in the decrease of solubility and precipitation of EVs.[208] In recent years, several PEG-based commercial EV extraction kits, such as ExoQuick, Pure-Exo, and Total EV Isolation, have appeared. Nevertheless, lipoproteins and polymers may be co-precipitated and affect the purity of EVs.

Moreover, there are two types of size-based EV isolation techniques: size exclusion chromatography (SEC) and ultrafiltration.[209,210] In SEC, the components with small hydrodynamic radius, such as proteins and oligonucleotides, can pass through the pore of the stationary

phase, resulting in a longer elution time, while EVs possess relatively large hydrodynamic radius, leading to an early elution. SEC has been shown to be superior to other techniques in the purity of isolated EVs due to the amelioration of protein contamination. However, it is worth noting that SEC cannot distinguish microvesicles with the same size.[208] If different EV subtypes need to be identified, a combination of SEC and immunocapture methods can be used. Ultrafiltration relies on the use of membranes with specified pore diameters to isolate particles of a pre-determined size range.[211] Since the volume of EVs is larger than biological macromolecules such as proteins and nucleic acids, ultrafiltration membranes with different cutoff molecular weight can be used for EV separation. This method is simple to operate and does not require special equipment and reagents.

Immunoaffinity capture techniques is a method that is based on the interaction between the antibodies fixed on the chromatographic stationary phase and magnetic beads and EV surface biomarkers, through which the specific enrichment of EVs can be achieved. Membrane proteins expressed on the EV surface, such as CD9, EpCAM, CD63, and RAB5, can be used as specific markers for EV separation.[212] Immunoaffinity capture can be used not only for the isolation of EVs with same surface markers but also for the qualitative and quantitative identification of EVs.[213] However, this method is not suitable for separating EVs from a large quantity of samples, and non-specific adsorption might cause impurities mixed into obtained EVs.

The microfluidic technique is a promising attempt at EV isolation in recent years.[214] Based on the biological, physical, and chemical properties of EVs (such as size, density, and immunoaffinity), the microfluidic technique can achieve precise separation of EVs. Additionally, innovative EV separation methods that combine electrophoresis, acoustics, and electromagnetics are also under rapid development.[215,216] Nevertheless, because of the lack of standardization and large-scale clinical sample testing, as well as the lack of relevant method verification, the microfluidic technique currently is mainly used in disease diagnosis. In general, efficient and economical separation and purification of a sufficient number of pure EVs is the main bottleneck of current research; therefore, it is urgent to develop novel techniques for EV isolation.

Methods for EV identification

Basic research and clinical applications require pure and uniform EVs. Therefore, it is urgent to identify and control the quality of the isolated EVs. Some molecular, biochemical, and biophysical properties of EVs, such as size, morphology, concentration, membrane potential, and surface markers, as well as the inside cargoes, can be utilized for EVs identification.[217] In this section, we summarize the current methods available for EV characterization.

Electron microscopy (EM), dynamic light scattering (DLS), and nanoparticle tracking analysis (NTA) are effective methods for the determination of EV size. EM is a technique that is widely used to visualize and characterize biological samples.[218] Three types of EM,

transmission electron microscopy (TEM), scanning electron microscope (SEM), and cryoelectron microscopy (cryo-EM), are commonly employed to represent the microstructures of EVs.[217] However, the requirement of complex and multistep sample preparations before EM detection, like dehydration or fixation, might induce alteration of EV morphology. In addition, the electron beam may also damage biological samples in some cases.[219] EVs possess obvious membrane boundaries under the EM, and form cup-shaped structures with varying sizes of 30–150 nm. DLS, also known as photon correlation spectroscopy, is a method that uses a monochromatic coherent laser beam to pass through suspended particles, and the size of the particles in the solution can be estimated by observing the time-dependent fluctuations in scattering intensity.[220] This method is simple and convenient to operate, and can be used to measure particles ranging from 1 nm to 6 μm in size. The effectiveness of DLS in measuring EVs' size and distribution has been confirmed in many studies.[221,222] However, the measurement of DLS is easily disturbed by the physical and chemical properties of the samples, such as color, electrical properties, and magnetic properties, and is sensitive to dust and impurities.[220] Therefore, DLS requires higher sample purity. In recent years, NTA has been used as one of the methods for characterization and identification of EVs.[223] Compared with other characterization methods, the NTA technique can maintain the original state of EVs through simple sample processing, and can quickly detect the average size, modal value, and size distribution of EVs. Specifically, fluorescence intensity can be detected by the NTA system.[217] Therefore, NTA can be used in combination with immunological methods to detect antigens on EVs by using fluorophore-labelled antibodies, which can be utilized for real-time monitoring of diseases.[224]

In addition, zeta potential is a physical property of EVs that affects the stability of EVs.[225] Zeta potential can be assessed by measuring the magnitude of electrostatic or charge repulsion or attraction between EVs. The ZetaView and Zetasizer Nano series are newly launched instruments that are capable of characterizing size and zeta potential of particles. Studies have employed them for the identification and characterization of EVs. For example, Jeong et al.[226] found that EVs from murine embryonic stem cells are 60–120 nm in diameter, with a zeta potential of -14.54 ± 1.31 mV. Danilo's group[225] measured the size and zeta potential of neuroblastoma cell-derived vesicles by using the Zetasizer Nano instrument. The diameter of EVs was about 70 nm, and the zeta potential ranged from -14.8 ± 1.55 to -12 ± 0.15 mV. Commonly, particles stably exist at absolute values of the zeta potential less than -30 mV or more than 30 mV.[227] Therefore, EVs are unstable, and tend to settle and aggregate automatically.

Moreover, western blot and flow cytometry are traditional identification and screening techniques that are based on EV surface markers (CD9, CD63, CD81, HSP90, etc.).[228] High-quality monoclonal antibodies are key factors in improving the reliability of detection. In a recent study by Zhou et al.,[229] they identified the EVs isolated from LC cells through detecting the surface markers CD9 and CD63 by western blot assays. In comparison, flow cytometry, with faster detection speed, is suitable for high-throughput screening, at the same time

analyzing the size and volume of EV particles. The widely used enzyme-linked immunosorbent assays can be also utilized for EV identification. The quantitative detection of EV surface markers can be performed using specific antibodies.[230] In addition to these methods, some new technologies for EV identification have emerged based on EV surface markers. Yoshioka et al.[231] performed the ExoScreen method for detecting EVs isolated from colorectal cancer patients' serum. This method was described as an amplified luminescent proximity homogeneous assay that need streptavidin-coated donor beads and photosensitizer acceptor beads. The emitted fluorescent signal will appear only if the acceptor beads are within about 200 nm from donor beads, thereby avoiding the interference of proteins and large vesicles.

For characterization of the constituents and functional properties of EV cargoes, multi-omics analysis is needed. The obtainment of EV hallmarks requires evaluation at several levels, including transcriptome, proteome, epigenome, and metabolome. In the past few decades, researchers have made many attempts to reveal the structures, compositions, and biological functions of EVs through single-omics analysis, such as searching the abnormal expression of EV ncRNAs and proteins through transcriptomics and proteomics analysis, respectively.[232–234] However, these single-omics analyses often have limitations. It is difficult to establish a dynamic relationship between the expression changes of EV cargoes and the disease phenotypes. In contrast, multi-omics analysis obtains large-scale omics data from different molecular levels. The integrative analysis of multiple omics information is conducive to in-depth understanding of the regulation and causality between EV cargoes, thereby resolving the molecular mechanism and genetic basis of biological and pathological processes. Multi-omics analysis of EVs has been widely employed in cancer research. For instance, Luo et al.[235] reported the transcriptomics and proteomics landscape of EVs derived from human LAC stem-like cells. They obtained a variety of mRNAs, circRNAs, and lncRNAs enriched in EVs through transcriptomics analysis. Importantly, integrative analysis of transcriptomics and proteomics data suggested the association of EV RNAs and proteins to tumorigenesis, revealing the capacity to serve as diagnosis biomarkers for LC.

In total, as an important basis for investigating the biological characteristics and clinical application of EVs, the isolation and identification technology of EVs still needs to be continuously improved.

CONCLUSIONS AND PROSPECTS

According to the Cancer Report 2020, LC is the most common malignancy in humans worldwide.[1] Due to the complexity of LC occurrence and development, as well as the great heterogeneity among patients, there are few universal treatment strategies for LC. In recent years, the morbidity and mortality of LC have been on the rise, and therapeutic resistance often occurs. In view of this, it is urgent to investigate the underlying mechanisms of LC and to discover effective strategies for LC precision therapy. ncRNAs, including miRNAs, circRNAs, and lncRNAs, provide a treasure house for discovering pathogenic mechanisms and finding therapeutic targets for LC. EVs are a novel method

of intercellular communication and are enriched with cargoes, including ncRNAs.[15] Increasing evidence suggests that ncRNAs can be selectively taken up into EVs, secreted, and transferred between specific donor and recipient cells, regulating numerous malignant phenotypes of LC, such as proliferation, migration, EMT, metastasis, angiogenesis, and treatment resistance.[139,144,148,154] Many studies have confirmed that EV ncRNAs have the potential to be used as diagnostic and prognostic biomarkers in LC, and they are promising therapeutic targets and reoccurrence risk monitoring tools.[87,236]

However, some challenges should not be ignored before EV ncRNAs can be used in the clinic. EVs in body fluids are often derived from a combination of complex sources, and it is necessary to explore their sources to confirm the direct correlation between certain EV ncRNAs and LC. In addition, due to the low content of EVs in body fluids, it is necessary to optimize the separation and detection methods of EVs and to establish a standardized and quantitative evaluation system. Current EV extraction methods, such as ultracentrifugation, precipitation, ultrafiltration, and immunoaffinity capture, are time consuming, expensive, low yield, and easy to contaminate, bringing inconvenience to follow-up studies.[237] A more convenient and accurate extraction method for EVs needs to be discovered. Moreover, EVs mediate cell-cell communication by transferring biomolecules between cells. Understanding how cargoes are selectively packaged into EVs is an urgent research question. Furthermore, it has been reported that engineered EVs can be used as delivery vehicles for ncRNAs, peptides, lipids, exogenous DNAs, and even drugs.[184,205] However, how EVs mediate the recognition of target cells and the release of their cargoes remains unknown.

In summary, EV ncRNAs are promising biomarkers and potential therapeutic targets, as well as prospective drug delivery vehicles for LC. Further study is required to elucidate the properties and regulatory mechanisms underlying EV ncRNAs, benefiting additional LC patients in the near future.

AUTHOR CONTRIBUTIONS
C.S. and Y.L. contributed equally to this work. C.S. and Y.L. drafted and wrote the manuscript. H.J.C. and F.W. collected the related paper. X.Z.C. and Q.K.Y. revised the manuscript. K.W. and Y.W. participated in the design of the review and helped to draft and revise the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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