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Exosomes: a new horizon in lung cancer

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Circulating exosomes are the major mediators of cell–cell communication. They have been found in various body fluids of healthy individuals and patients with malignancies as cargos of several molecules including miRNAs. Several studies have underlined the role of exosome miRNAs in different tumor types, including lung cancer, suggesting their potential use as biomarkers and therapeutic agents. An overview of the biology and function of exosomes and exosome miRNAs as indicators of diagnosis and treatment response in lung cancer is presented. In addition, preliminary data on exosomes as potential therapeutic agents are reported.

Introduction

Lung cancer is one of the main cancer killers worldwide [1]. Despite the increase of biological knowledge, the clinical outcome of patients diagnosed with advanced disease is still disappointing [2]. Indeed, the survival rate falls dramatically from early- to advanced-stage cancer. Diagnostic procedures are at times inconclusive owing to problematic tumor tissue accessibility and poor performance status of some patients [3,4].

Liquid biopsy is a minimally invasive test that can detect circulating tumor cells and tumor-derived nucleic acids (e.g., cell-free DNA and miRNAs) in the blood of cancer patients. Recently, this definition has also been extended to the evaluation of microvesicles and tumor-educated platelets as alternative sources of tumor-derived genetic material [5]. In particular, the early identification of extracellular vesicles (EVs), named exosomes, has a great potential in cancer diagnosis and for monitoring treatment efficacy. Exosomes are EVs of endocytic origin containing various molecules, such as nucleic acids (DNA, mRNA, microRNA and other small RNAs), lipids and proteins [6]. Increasing evidence has shown that exosomes can transfer DNA, RNA and protein from one cell to another, playing a key part in a multitude of physiological and/or pathological processes including cancer. The molecules transferred by exosomes are protected from degradation by bilayered lipids, and pure tumor-derived exosomes can be found in all body fluids (Fig. 1a). These peculiar features make the exosomes ideal biomarkers for clinical applications and open new perspectives in the nanomedicine field as therapeutic drug carriers [7].

Here, we report a comprehensive overview on the role of tumor-derived exosomes as potential biomarkers in lung cancer. In particular, we first describe the most recent approaches of exosome isolation focusing on the advantages and limitations of each methodology. We will then encompass the most recent studies dealing with the role of exosomes in the intercellular communication during lung cancerogenesis. Finally, the latest data on exosomes as cargos of miRNAs, drug delivery vehicles and vaccines will be also discussed.

Exosomes

Living cells release different types of EVs into the extracellular environment that are mainly involved in intercell communication. The EVs can be categorized into three main classes according to their biogenesis: microvesicles, apoptotic bodies and exosomes. Microvesicles are formed by a direct outward budding of the plasma membrane; apoptotic bodies are released by the outward bleb and fragmentation of the apoptotic cell membrane; exosomes are originally formed by an endocytic process [8] (Fig. 1b).

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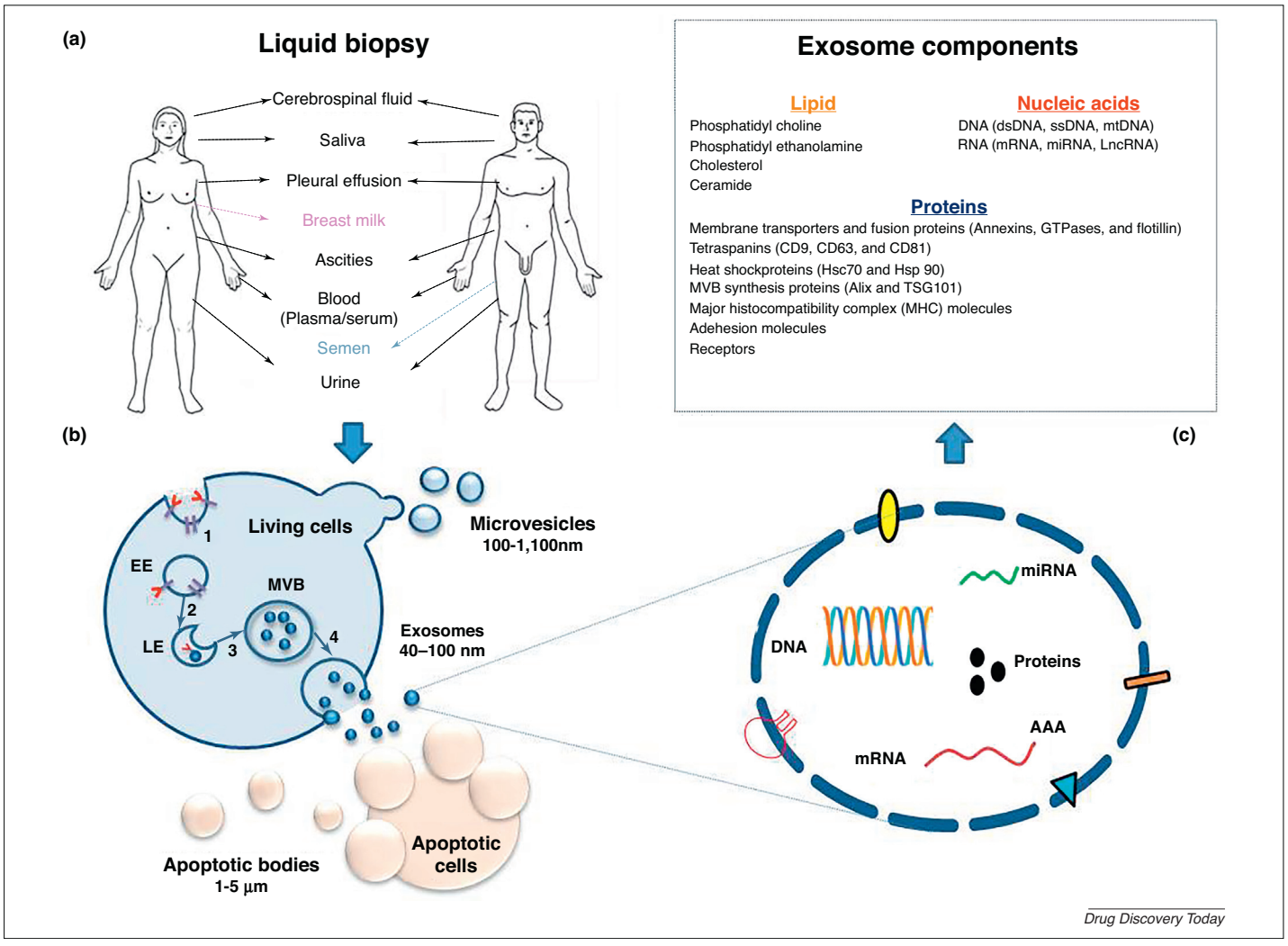

FIGURE 1

Circulating exosomes. **(a)** Body fluids as a potential source for extracellular vesicle isolation. **(b)** Biogenesis of the main extracellular vesicles: microvesicles, apoptotic bodies and exosomes. Microvesicles are released by a direct outward budding of the plasma membrane and apoptotic bodies are formed by outward bleb and fragmentation of the apoptotic cell membrane. Exosomes are generated by an endocytic process as follows: (1) cell membrane internalization producing an early endosome (EE); (2) incorporation of protein to early endosome inner side with maturation to late endosome (LE); (3) inward budding process of the endosome membrane forming intraluminal vesicles (ILVs) trapped inside multivesicular body (MVB); (4) fusion of MVB with cell membrane and release of ILVs (known as exosomes) into the extracellular space. **(c)** Structure and composition of exosomes. Exosomes are bilayered lipid membranes containing proteins and genetic materials. The genetic materials include: single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), mitochondrial DNA (mtDNA), mRNA, miRNA and long noncoding RNA (LncRNA) which are all functionally active.

One of the first descriptions of the exosomes dated back to the 1980s, when two research groups observed the presence of nanovesicles of endocytic origin by studying the reticulocyte differentiation. These vesicles were involved in the removal of transferrin receptor from the reticulocyte surface and the authors suggested that the vesicles might have a potential role in the maturation of red blood cells [9,10]. Two years later the term 'exosomes' was coined to define these vesicles [11]. Other than reticulocytes, the secretion of exosomes has been reported in a wide range of mammalian cells, including immune system cells [12], epithelial cells [13] and endothelial cells [14]. In addition, exosomes have been detected in physiological fluids such as plasma or serum, saliva, urine, amniotic fluid, breast milk, semen, nasal secretion, cerebrospinal fluid, as well as in pathological fluids as ascites (Fig. 1a) [15].

Following their release, the exosomes can be captured by the neighboring cells (paracrine) or alternatively enter the bloodstream reaching distant organs. The transfer of exosome contents (proteins and nucleic acids) to neighboring or distant recipient cells produces physiological or pathological effects [6]. Although the physiological or pathological status of the cell origin mainly contributes to exosome constituents, a specific pattern of conserved proteins has been observed (Fig. 1c). A number of databases such as ExoCarta (http://www.exocarta.org) [16], Vesiclepedia (http://www.microvesicles.org/) [17] and EVpedia (http://student4.postech.ac.kr/evpedia2_xe/xe/index.php?mid=Home)

[18] have been generated to provide a comprehensive depiction of exosome constituents.

Exosome isolation techniques

Owing to the increasing interest in the exosome field, several studies have been performed to identify the most efficient isolation protocol to isolate high-yield, pure exosomes from cell culture supernatants and biological fluids. At present, the methodologies of isolation are mainly based on the physical [differential centrifugation (DC), ultrafiltration, size-exclusion chromatography], chemical [polymeric-based precipitation (PBP)] or biological (immune-affinity) properties of exosomes. In particular, the gold standard to purify exosomes would be an ultracentrifugation-based tool (e.g., DC). This method generally involves four steps of centrifugation. The first one, at low speed, removes intact cells. The following two steps, at increasing speeds, eliminate dead cells and apoptotic bodies, and microvesicles plus cell debris, respectively. The final ultracentrifugation precipitates the expected exosomes. Although DC is one of the most common approaches to purify exosomes, it is time-consuming, labor intensive and the quality and quantity of the exosomes can be altered by the duration and relative force of centrifugation as well as the temperature [19]. Moreover, the viscosity of the sample source (plasma > serum > cell culture) can be an additional limiting factor. Alternative isolation methods have been proposed to reduce these restricting factors although each technique shows specific advantages as well as potential drawbacks [15,19–29] (Table 1). One of the most appropriate methods in cancer research is the immune-affinity isolation approach. This technique can select the exosome population by binding to antibodies directed to specific markers present on the exosome surface (usually anti-EpCAM: epithelial cell adhesion molecule) [25,29]. However, the recent evidence that serum exosomes from epithelial tumors might lose the EpCAM antigen could be a matter of concern [30]. To reduce labor-time, costs and increase reproducibility among the laboratories, several companies have released various kits. Specifically, several systems were developed to enrich exosomes efficiently from blood (e.g., plasma and serum) as well as from bronchoalveolar lavage (BAL), pleural effusions, saliva, among others, in the perspective of their application in cancer diagnosis (Table 2).

Exosomes in lung cancer

It is well known that cancer cells can communicate with the surrounding and distant cells via exosomes and several data support their potential role in proliferation, invasion and metastasis of various cancers including lung [31]. One of the first studies that analyzed the exosomes in lung cancers dated back to 2004, when Bard and colleagues screened the proteomic profile of exosomes derived from the pleural effusions of nine patients with different malignant neoplasms (four out of nine were mesotheliomas and two were lung adenocarcinomas). The proteomic analysis identified proteins already described (e.g., MHC class I and II, heat shock) as well as proteins never detected in exosomes, such as pigment epithelium-derived factor (PEDF), B cell translocation gene 1 (BTG1) and sorting-nexin protein (SNX). In particular, PEDF and BTG1 were found to be related to cell growth, whereas SNX was linked to epidermal growth factor receptor (EGFR) internalization. These findings suggested a potential implication in lung cancer development [32]. Thereafter, several research groups better elucidated the role of exosomes in lung cancer by profiling the structures of exosomes from different body fluids.

In vitro *studies on the role of exosomes in lung cancer*

Different *in vitro* studies in lung cancer have shown that tumor-derived exosomes can represent a noninvasive surrogate of the parental cancer cells. In this regard, a pioneering study was conducted by Thakur et al. who demonstrated, for the first time, that tumor-derived exosomes carry a prevalence of double-stranded DNA (dsDNA). By examining the exosome dsDNA isolated from lung cancer cell lines harboring EGFR genetic alterations (H292, EGFR wild type; H1975, EGFR L858R/T790 M; H1650 and PC-9, EGFR exon 19 deletion), the authors observed that the exosome dsDNA reflected the mutational status of the parental cell lines, supporting the feasibility of molecular profiling in lung cancer patients in absence of tumor biopsy [33]. EGFR and its signaling network proteins were found frequently expressed in lung cancer exosomes from different sources [34–36]. In particular, an intriguing *in vitro* study demonstrated that EGFR could be transferred via exosomes from human carcinoma cell lines with activated EGFR (A431, epidermoid carcinoma; A549, lung carcinoma; DLD-1, colorectal adenocarcinoma) to endothelial cells leading to activation of mitogen-activated protein kinase (MAPK) and AKT pathways as well as to vascular endothelial growth factor (VEGF) expression [34].

Additional data supported the role of exosomes in the metastatic dissemination of lung cancer. Indeed, Rahman et al. demonstrated that the exosomes derived from the culture medium of a highly metastatic human lung cancer cell line (PC14HM, lung adenocarcinoma) and from the sera of lung cancer patients could drive the epithelial mesenchymal transition of human bronchial epithelial cells inducing their migration, invasion and proliferation [37]. Moreover, exosomes also emerged as mediators of resistance to chemotherapy [38] and target therapy [39,40]. A recent study proposed that modification of the exosome phospholipid composition might predict resistance to tyrosine kinase inhibitors. In detail, a phospholipid profiling of exosomes derived from a human lung cell line resistant to gefitinib (PC9R, lung adenocarcinoma) was performed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). A distinct signature in the PC9R compared with the parental cell line was reported, suggesting that the lipid components of exosomes could also function as predictive biomarkers of drug resistance [41].

Exosomes as candidate biomarkers in lung cancer

The late diagnosis of lung cancer is widely recognized as a crucial factor in the outcome of patients; therefore, the identification of biomarkers, by minimally invasive procedures, within lung cancer screenings is strongly pursued although challenging. In this context, different models of exosome protein or lipid profiles in lung cancer have been proposed. In 2015, Jakobsen et al. profiled the exosome proteins from the plasma of 219 suspected lung cancer patients (109 diagnosed with lung adenocarcinoma) using a custom array containing 37 antibodies targeting lung-cancer-related proteins. Multivariate analysis produced a 30-marker model classifying correctly 75% of patients (sensitivity of 0.75 and specificity of 0.76) and suggesting that plasma exosomes

TABLE 1

Exosome isolation techniques

Isolation technique	Principle	Protocol	Advantage	Potential drawback	Refs
Differential centrifugation	Physical (based on density)	Isolation by four steps of centrifugation at increasing speeds; each step eliminates a component (intact cell, dead cells, apoptotic bodies, microvesicles and cell debris); exosomes are collected in the final ultracentrifugation (100.000 g).	Large sample volume; separation of different size-based EVs.	Time-consuming; not high-throughput processing; centrifugation time, relative force, and temperature can alter the exosome structure; sample viscosity can reduce the exosome yield; co-isolation of proteins (albumin) starting from plasma samples (not suitable for proteomics or RNA analyses); required specific device (ultracentrifugation).	[19–21]
Density gradient separation	Physical (based on density)	Isolation by a combination of a sucrose density gradients or sucrose cushions with ultracentrifugation.	High EVs yield	Time-consuming; co-isolation of high density lipoproteins (HDL carries miRNAs and proteins that could contaminate the downstream analyses); centrifugation time can alter the exosome structure; not suited for high-throughput applications; required specific device (ultracentrifugation).	[22–24]
Ultrafiltration	Physical (based on size)	Isolation using micropillar pore silicon ciliate structure; exosomes are isolated by trapping through pores.	High reproducible protocol (single step of purification); short processing time and easy procedure.	Co-isolation of proteins and other smaller contaminants; low exosome yield due to their snared in the pores; small sample volumes; force of filtration through membrane could alter the exosome structure.	[25–26]
Size-exclusion chromatography	Physical (based on size)	Isolation using columns packed with porous polymeric beads; molecules pass through the beads, depending on their diameter whereby larger particles are eluted faster than the smaller ones; exosomes are eluted by a buffer in the final step.	Relative low cost; high exosome purity; no significant albumin contamination from plasma; commercial kit available	Small sample volumes; low exosome yield.	[20,27–28]
Polymeric-based precipitation	Chemical (based on solubility)	Isolation by incubation with a polymer (polyethylene glycol); exosomes are collected by a final low speed centrifugation.	High exosome yield; short processing time; commercial kit available	Co-isolation of contaminants (e.g. lipoproteins and ribonucleic proteins); presence of polymers could interfere with down-stream analyses.	[15,19]
Immunoaffinity	Biological (based on specific markers)	Isolation by capture using specific antibodies coated with beads or other matrices; pure exosome population are separated by low-speed centrifugation or magnetic device.	High exosome purity; commercial kit available; easy procedure.	Small sample volume; low exosome yield, depending on the marker expression.	[25,29]

might be valuable diagnostic indicators in lung cancer [42]. Similarly, Sandfeld-Paulsen et al. analyzed the plasma exosomes from 581patients (431 with lung cancer and 150 control individuals) using a custom array (49 antibodies). The authors demonstrated that CDC151, CDC171 and tetraspanin 8 were the strongest discriminators of malignancy, compared with healthy controls, and proposed a 10-marker model as a diagnostic tool [43].

TABLE 2

List of some commercially available kits to isolate exosomes starting from different sample sources

Company	Kit name	Sample source	Input sample	Protocol time
ThermoFisher Scientific	Total Exosome Isolation (source specific)	Cell culture media, plasma, serum, urine	1–10 ml cell culture media; 0.1–1 ml plasma or serum; 0.8–5 ml urine	~14 h cell culture media; ~2 h plasma; ~1.5 h serum; ~2.5 h urine
	Total Exosome Isolation (Bio Fluids)	Amniotic fluid, ascites, cerebrospinal fluid, milk, saliva	0.2–1 ml	~4 h
Qiagen	exoEasy Maxi	Cell culture media, plasma, serum	16 ml for cell culture media; 4 ml plasma or serum	~30 min
Exiqon	miRCURY™ Exosome Isolation	Cell culture media, cerebrospinal fluid, plasma, serum, urine	1–10 ml cell culture media; 0.5–1.4 ml plasma or serum; 1 ml cerebrospinal fluid; 2–5 ml urine	~2 h
System Biosciences	ExoQuick	Serum, ascites	0.25 ml	~1.5 h serum; ~13 h ascites
	ExoQuick-TC	Cell culture media, cerebrospinal fluid, urine	5–10 ml	~13 h
BioVision	Exosome Isolation	Cell culture media, plasma, serum, urine	2–4 ml cell culture media; 0.1–0.5 ml plasma or serum; 5–20 ml urine	~2 h
	Exosome Isolation (Bio Fluids)	Amniotic fluid, breast milk, bronchoalveolar lavage, cerebrospinal fluid, gastrointestinal fluid, inflammatory fluid, lymph fluid, saliva	0.5–2 ml	~40 min
Cell Guidance Systems	Exo-spin™	Cell culture media, plasma, serum, saliva, urine	1–50 ml cell culture media, urine, saliva; 0.5 ml plasma or serum	~3 h cell culture media, urine, saliva; ~ 2 h plasma or serum
Norgen Biotek Corporation	Exosome Purification	Cell culture media, plasma, serum, urine	5–35 ml[a] cell culture media; 0.05–10 ml[a] plasma or serum; 0.25–30 ml[a] urine	~45 min cell culture media; ~30 min plasma, serum, urine
101Bio	PureExo® Exosome Isolation	Cell culture media, plasma, serum	2–4 ml cell culture media; 0.1–0.5 ml plasma or serum	~2 h
Izon Science	qEV Size Exclusion Column	Cell culture media, plasma, saliva, serum, urine	0.1–0.5 ml	~20 min

[a] Volume depending on the kit format (mini, midi, maxi).

Circulating free miRNAs and exosome miRNAs in lung cancer

Among the circulating nucleic acids miRNAs are probably the most commonly investigated. miRNAs are a family of small noncoding RNAs of 20–25 nucleotide length able to regulate gene expression at the post-transcriptional level by degrading or repressing target mRNAs [44]. A single miRNA can regulate the expression of hundreds of mRNAs with crucial roles in diverse physiological processes. Moreover, miRNAs can also act as modulators of gene expression in different diseases including malignancies [45]. One of the first descriptions of circulating free miRNAs was reported by Chen et al. in 152 lung cancer patients – two highly expressed miRNAs (miR-25 and miR-223) compared with 75 healthy donors were identified [46]. Successively, numerous circulating miRNAs have been described as biomarkers for diagnosis, prognosis and response to treatment in lung cancer [47]. However, the instability of circulating cell-free miRNAs as a result of physiological conditions, such as the presence of ribonuclease, extreme pH and the difficulty in normalization procedures, can be crucial in the analytic workflow to select reliable biomarkers [48]. Conversely, miRNAs encapsulated into extracellular vesicles were demonstrated to be more resistant to ribonuclease than their free counterparts [49,50]. In addition, emerging evidence also indicated how the tumor-derived exosomes containing miRNAs could potentially modulate the behavior of the recipient cells, facilitating progression and metastasis.

Exosome miRNAs as diagnostic, prognostic and predictive biomarkers in lung cancer

The ability of exosome miRNAs to distinguish lung cancer patients from healthy individuals has been shown in a few studies. One of the first investigations was carried out by Rabinonowits et al. who employed a lung cancer signature previously identified in tumor tissue by Yanaihara et al. [51,52]. The expression of a 12 miRNA-signature in the plasma exosomes from 27 patients with lung adenocarcinoma and 9 healthy individuals was evaluated. Interestingly, the mean exosome miRNA was significantly higher in cancer patients than control group and the 12 tumor-linked miRNAs were overexpressed in the patients with lung cancer only [51]. Another study examined 365 miRNAs in the exosomes isolated from the plasma of 28 lung cancer patients and 20 healthy individuals. Five miRNA candidates were selected (let-7f, miR-20b,

TABLE 3

Exosome miRNAs as biomarkers in lung cancer

Body fluid	*In vitro*/*in vivo* studies	Patients/cell lines number	miRNAs evaluated	Refs
Plasma	*In vivo*	7 patients with adenocarcinoma (ADC) (stages I–IV); 9 controls	467 human miRNAs by microarray	[52]
Plasma	*In vivo*	28 patients with NSCLC (stages I–IV); 20 controls	365 human miRNAs by real-time PCR	[53]
Plasma	*In vivo*	30 patients: 10 lung ADC; 10 lung granulomas; 10 control smokers	742 microRNAs by real-time PCR	[54]
BAL and plasma	*In vivo*	30 patients with NSCLC; 75 controls	84 miRNAs by real-time PCR	[55]
Tumors and serum	*In vivo*	7 primary and 18 recurrent tumors of mouse models inoculated with H1299 cells	84 miRNAs by real-time PCR	[56]
Culture medium	*In vitro*	1 cancer cell line (A549)	6 miRNAs by real-time PCR	[38]
Serum	*In vivo*	60 patients with lung cancer	179 human miRNAs by real-time PCR	[58]
Plasma	*In vivo*	5 patients with NSCLC (stage IIIA)	~1900 human miRNAs by real-time PCR	[59]

miR-30e-3p, miR-223 and miR-301) and then validated in an independent set of patients (78 lung cancer and 48 healthy). The emerging results showed that let-7f, miR-20b and miR-30e-3p were statistically different between the two populations and that the levels of miR-30e-3p and let-7f were associated with shorter disease-free survival and overall survival, respectively [53]. Similarly, Cazzoli et al. identified four miRNAs (miR-378a, miR-379, miR-139-5p and miR-200b-5p) in the plasma exosomes from 30 subjects to screen and distinguish patients with lung lesions (adenocarcinoma or lung granuloma) from healthy controls. Moreover, a diagnostic signature of six miRNAs (miR-151a-5p, miR-30a-3p, miR- 200b-5p, miR-629, miR-100 and miR-154-3p) was selected to discriminate between lung adenocarcinoma and granuloma [54]. Exosome miRNAs were also investigated in other body fluids such as BAL. In a study by Rodriguez et al. the levels of exosome miRNAs isolated from BAL were compared with the exosome levels from the plasma of lung cancer patients ($n = 30$) and controls ($n = 75$). Although plasma samples contained more exosomes than BAL, the exosome miRNAs from plasma and BAL were higher in tumor patients than controls [55]. At the same time, 84 miRNAs were profiled and specific signatures, according to the source of exosomes (plasma or BAL) and pathology (tumor or control), were identified as follows: miR-126 and miR-144 in plasma samples (tumor and control); miR-302a and miR-302c in BAL samples (tumor and control); miR-128 in plasma of control individuals only; and miR-143 in tumor BAL only. In addition, miR-122 was the only tumor-specific miRNA irrespective of the source (plasma or BAL).

The role of exosome miRNAs in the regulation of tumor progression and metastasis was also investigated. A recent study in a nude mouse model xenografted with subcutaneous primary and recurrent lung cancers reported that two miRNAs (miR-21 and miR-155) were significantly upregulated in the exosomes from mice with recurrent tumors compared with primary tumors [56]. These findings were in agreement with those from a previous clinical study reporting that miR-21 and miR-155 can predict recurrence and poor survival in lung cancer patients [57].

Exosome miRNAs have also been described as predictors of treatment response in lung cancer. An *in vitro* study by Xiao et al. showed that the exposure of a lung adenocarcinoma cell line (A549) to cisplatin led to an increase of exosomes shuttling miRNAs. In particular, the levels of exosome miR-21 and miR-133b were upregulated after treatment and were also able to induce cisplatin resistance in the parental cells [38]. More recently, two clinical studies investigated the exosome miRNAs in patients undergoing radiation therapy. The first one by Tang et al. showed that serum exosomes from lung cancer patients exhibited a dose-related overexpression of miR-208a following radiotherapy [58]. The second one by Dinh et al. screened 752 miRNAs in the plasma exosomes of locally advanced patients at baseline and at two-week intervals upon radiotherapy, reporting that the levels of miR-29a-3p and miR-150-5p decreased with increasing radiotherapy dosage [59]. These studies suggest that tumor-derived exosome miRNAs, isolated from different biological fluids, could be potential tools in the diagnosis and prognosis of lung cancer as well as in treatment monitoring (Table 3).

Exosomes as drug deliverers in lung cancer treatment

Nowadays, chemotherapy is still the mainstay management of advanced lung cancer patients who do not harbor targetable driver mutations; therefore, the identification of novel therapeutic approaches is strongly needed. Because exosomes are secreted by almost all cell types in all body fluids and naturally deliver proteins, lipids, mRNAs, miRNAs and DNA to recipient cells, they might represent potential carriers of drugs and biological molecules. Various drug delivery systems, such as liposomes and nanoparticles, have been developed but they present several drawbacks [60]. Exosomes conversely disclose a number of advantages over other drug delivery systems: (i) less immunogenic and poorly toxic; (ii) widely distributed in human body fluids; (iii) non-mutagenic compared with all other existing nanoparticle-based delivery systems; (iv) able to cross the cell membrane (including blood–brain barrier) and deliver carried materials to target cells thanks to natural lipid bilayers; (v) suitable to be genetically engineered to display peptides and ligands on their surface improving their targeting ability and uptake by specific recipient cells. Similarly to the other nanoparticle-based delivery systems, exosomes show higher accumulation in the kidney, liver and spleen and lower concentrations in the destined organs and tissues when administered unmodified, as reported in mouse models [61]. Exosomes can be vehicles of several biological molecules, including proteins, membrane receptors and nucleic acids (miRNA mimic or antagonist) [62]. The load of genetic materials inside exosomes can be performed either during exosome biogenesis

TABLE 4

Clinical trials on exosome delivery systems as therapeutics in lung cancer

Vesicles type	Disease	Drug	Exosome source	Isolation/ purification	Effect	Refs
Autologous dexosomes (Intradermal/ subcutaneous administration)	13 NSCLC III/IV (Phase I)	MAGE3 peptides	Dendritic cells	Filtration/ UC sucrose cushion	Toxicity < Grade I–II; 9/13 patients completed therapy; DTH reactivity against MAGE peptides in 3/9 patients; MAGE-specific T cell responses in 1/3, NK lytic activity increased in 2/4;	[67][a]
Autologous dexosomes (Intradermal administration)	22 advanced unresectable NSCLC (Phase II)	Autologous IFN-γ matured monocyte-derived dendritic cell loaded with MHC class I- and class II-restricted cancer antigens	Dendritic cells	Filtration/UC sucrose cushion	1 patient had grade III hepatotoxicity; 7 patients (32%) experienced stabilization of >4 months; Primary endpoint (50% of patients with progression-free survival at 4 months) not reached; No induction of T cell responses. Increase in NKp30-dependent NK cell functions in patients with defective NKp30 expression	[68][b] NCT01159288

[a] Completed trial.
[b] Currently recruiting participants.

or after exosome isolation by different techniques as electroporation, transfection, cell activation and incubation [63]. *In vivo* unmodified or modified exosomes can be administered by intradermal, intramuscular, intravenous and intraperitoneal injections. Although the use of exosomes as drug delivery vehicles appears more advantageous in respect to the synthetic vehicles, data on their therapeutic application in lung cancer are not available yet.

At present, the use of drug delivery systems in lung cancer treatment has been restricted to one study investigating the efficacy of a liposomal nanoparticle loaded with miR-34a mimics (MRX34) in a syngeneic mouse model (tumor induced by 344SQ murine lung adenocarcinoma cells) [64]. The administration of miR-34a, a negative modulator of PDL1, by MRX34 increased tumor-infiltrating CD8+ cells and reduced tumor-infiltrating PD1+ T cells, macrophages and T regulatory cells (Tregs). These effects improved upon combination of MRX34 with radiotherapy and the authors concluded that miR-34a delivery might represent a novel immunotherapeutic approach for lung cancer patients. A multicenter Phase I clinical trial of MRX34 evaluating the safety profile in patients with hematologic malignancies and primary solid tumors including lung cancer is ongoing (https://clinicaltrials.gov/ct2/show/NCT01829971).

Exosomes as a lung cancer vaccine

Recent evidence suggests that the use of dendritic-cell-derived exosomes (DEX), tumor-cell-derived exosomes (TEX) and ascitic-cell-derived exosomes (AEX) is emerging as a promising avenue in the development of cancer vaccines. DEX can be a powerful tool to trigger the immune system in lung cancer patients by enhancing antitumor T cell responses, suppressing cancer cell proliferation and eradicating established tumors [65,66].

The administration of DEX as a cancer vaccine in lung cancer patients was evaluated in two clinical trials (Table 4). The first Phase I trial dated back to 2005; the safety, feasibility and efficacy of autologous DEX loaded with tumor antigens were assessed in advanced lung cancer patients. Despite DEX therapy being well tolerated and some patients experiencing stability of disease, only a minimal increase in antigen-specific T cell activity was observed in three out of nine patients [67]. Successively, to enhance the limited DEX-induced T cell response observed in the previous trial, the clinical benefit of a second generation of DEX (IFN-γ-DEX: exosome derived from interferon-γ-matured DEX loaded with MHC class I- class II-restricted cancer antigens) was assessed in a Phase II study. Patients with advanced lung cancer, not progressed after chemotherapy, were treated with IFN-γ-DEX. Because only 32% of the patients experienced disease stabilization longer than four months after treatment cessation compared with the expected 50%, the primary endpoint was not reached; in addition, no antigen-specific T cell activity was reported. The only antitumor immunity, associated with longer progression-free survival, was observed in a fraction of patients with defective NKp30 expression who showed an increase in natural killer (NK) function [68].

Concluding remarks and future perspectives

The identification of diagnostic, prognostic and predictive markers in lung cancer is becoming particularly relevant and liquid biopsy is one of the most promising approaches in screening and/or diagnostic programs and to monitor treatment efficacy. Ideally, the exosomes can be isolated from all body fluids and in recent years notable efforts have been made to develop protocols able to isolate high yield of pure and intact exosomes at best. Because exosomes are stable sources of genetic materials, such as DNA, RNA and proteins, their potential application in lung diagnosis and the clinical setting is widely pursued. The possibility to detect targetable mutations (such as EGFR) from plasma exosomes of patients with lung cancer could open new perspectives in diagnosis when tumor biopsy is not feasible. In addition, different protein and lipid exosome signatures have been proposed as predictive tools. More recently, a growing interest has also been focused on the exosome miRNAs for their ability to regulate gene expression post-transcriptionally. Indeed, the evidence that exosome miRNAs can mirror the profile of origin cells has led to the identification of

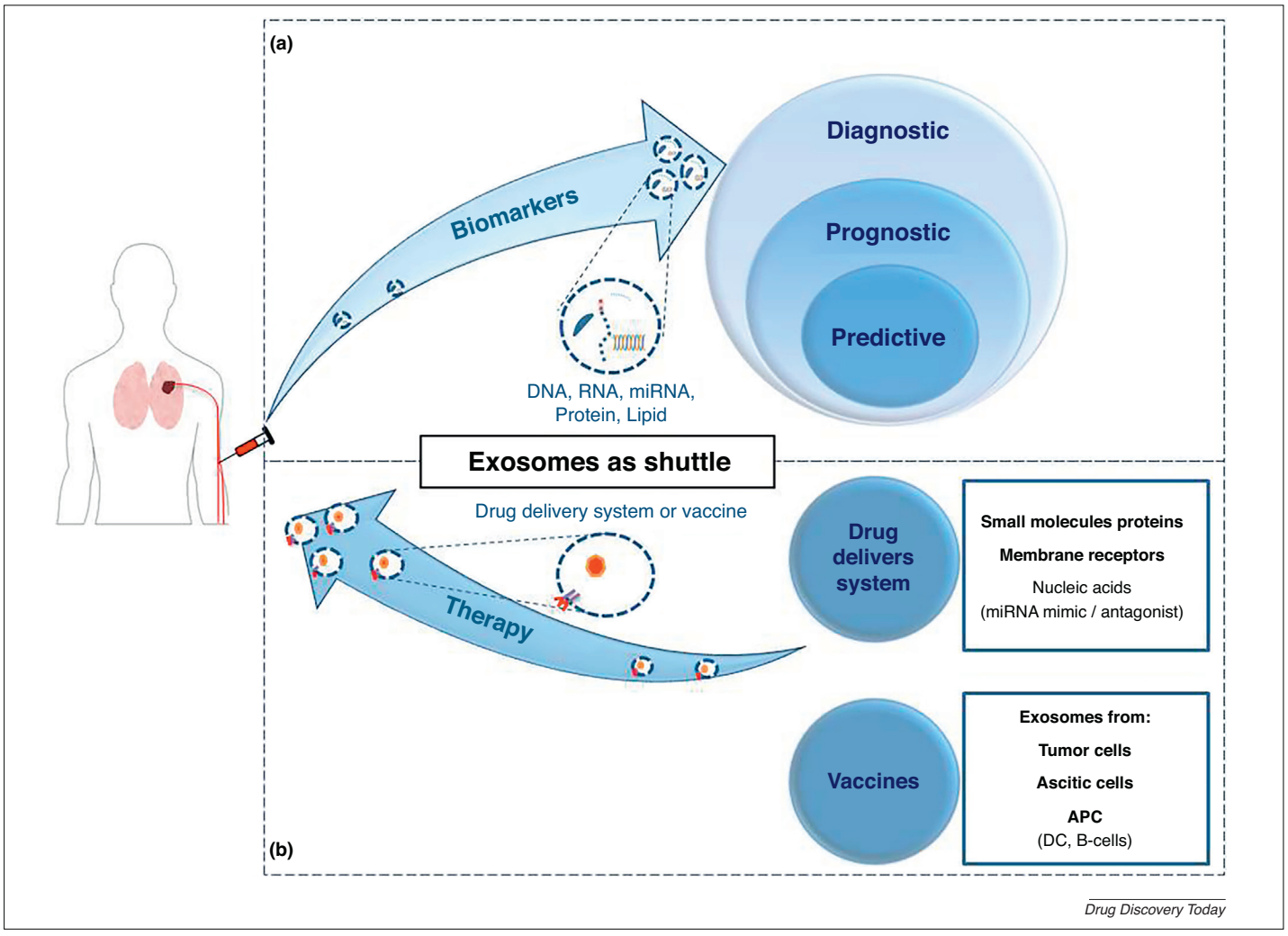

FIGURE 2

Potential applications of exosomes in lung cancer. Schematic representation of the potential use of exosomes in lung cancer patients. **(a)** Exosomes as candidate biomarkers in cancer diagnosis, prognosis and monitoring therapeutic efficacy. **(b)** Exosomes as a drug delivery system or vaccine in cancer therapy. Exosomes as a drug delivery system can be loaded with small molecules, proteins, membrane receptors, nucleic acids (miRNA mimic or antagonist); whereas exosomes as a vaccine can be derived from tumor cells, ascitic cells and antigen-presenting cells (APCs) [e.g., dendritic cells (DCs), B cells].

different signatures able to distinguish healthy from lung cancer patients, as well as predict response to treatment and clinical outcome. However, the majority of the present signatures were generated from studies with a limited number of patients thus being poorly reproducible and requiring further large multicenter studies to be translated into screening and diagnostic protocols.

The use of exosomes in antigen-presenting cell systems involving DEX is emerging as a powerful technique in lung cancer. Although the administration of DEX was well tolerated and a positive effect on NK cell activity was also reported in a subgroup of patients vaccinated with a second generation of DEX [68], clinical data are still limited and not completely satisfactory. To establish the efficacy of exosomes for a lung cancer vaccine, more extensive clinical trials need to be conducted.

In conclusion, although the discovery of exosomes is recent, a number of exciting results in lung cancer are emerging: (i) as vehicles of genetic material, exosomes are the most promising liquid biopsy-derived markers in diagnosis and/or prognosis and treatment response compared with the circulating cell-free counterpart; (ii) as drug delivery vectors exosomes can be an emerging therapeutic strategy in advanced disease as an alternative to less effective treatments (Fig. 2). In the perspective of the translation into the clinical setting, additional studies on exosomes in lung cancer should better elucidate their role and mechanism of action to reduce the risk of off-target effects or therapeutic failures.

Conflicts of interest

The authors declare that they have no conflicts of interest to disclose.

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