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Exosomes as Theranostics for Lung Cancer

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Abstract

Extensive research in genetics and genomics has revealed that lung cancer is a physiologically complex and genetically heterogeneous disease. Although molecular targets that can yield favorable response have been identified, those targets cannot be exploited due to the lack of suitable drug carriers. Furthermore, lung cancer often is diagnosed at an advanced stage when the disease has metastasized. Conventional treatments are not effective for treating metastatic lung cancer. Targeted therapeutics while beneficial has challenges that include poor tumor-targeting, off-target effects, and development of resistance to therapy. Therefore, improved drug delivery systems that can deliver drugs specifically to tumor will produce improved treatment outcomes.

Exosomes have a natural ability to carry functional biomolecules, such as small RNAs, DNAs, and proteins, in their lumen. This property makes exosomes attractive for use in drug delivery and molecular diagnosis. Moreover, exosomes can be attached to nanoparticles and used for high precision imaging. Exosomes are now considered an important component in liquid biopsy assessments, which are useful for detecting cancers, including lung cancer. Several studies are currently underway to develop methods of exploiting exosomes for use as efficient drug delivery vehicles and to develop novel diagnostic modalities. This chapter summarizes the current status of exosome studies with regard to their use as theranostics in lung cancer. Examples from other

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cancers have also been cited to illustrate the extensive applicability of exosomes to therapy and diagnosis.

1. INTRODUCTION

Lung cancer is the leading cause of cancer-related death worldwide (ACS Statistics, 2018). Despite the availability of newer therapeutic methods, such as targeted therapy, immunotherapy, and combined modalities, the poor survival rates of patients with lung cancer are mainly due to late-stage diagnosis and/or the use of inefficient therapeutic regimens. An understanding of the molecular mechanisms, the ability to detect cancer at early stages, and the development of safe and highly efficient targeted therapy methods are crucial for lung cancer treatment. In this context, extracellular vehicles (EVs), especially exosomes, have received recent interest for their role in facilitating early detection and diagnosis and improving treatment outcomes in cancer (Vanni, Alama, Grossi, Dal Bello, & Coco, 2017). Accumulating evidence suggests that the genetic materials, proteins, and lipids carried by exosomes originating from cancer cells may have unique signatures that allow us to identify specific biomarkers for cancer identification and to predict therapy outcome (Kadota, Yoshioka, Fujita, Kuwano, & Ochiya, 2017).

Lung cancer-derived exosomes are known to represent the cell of origin in many aspects. A recent study demonstrated that exosomes derived from lung tumor cells (H292, H1975, and H1650) in culture reflected the mutational status of specific genes (EGFR) of the parental cell lines (Thakur et al., 2014). In a different in vitro study, microvesicles/exosomes derived from epidermoid carcinoma (A431), lung adenocarcinoma (A549), and colorectal carcinoma (DLD-1) cell lines transferred activated EGFR from cancer cells to endothelial cells and induced the mitogen-activated protein kinase (MAPK) and AKT pathways (Al-Nedawi, Meehan, Kerbel, Allison, & Rak, 2009). Further, the study demonstrated that EGFR transfer by microvesicles resulted in the activation of vascular endothelial growth factor receptor 2 (VEGFR-2) expression in the host cell. These studies show that molecular profiling of lung cancer-derived exosomes would be a feasible technique for understanding the tumor's molecular status.

Exosomes are reported to be involved in the underlying mechanism of chemotherapy resistance in lung cancer. Cisplatin resistance is common in lung cancer. Exosomes derived from a cisplatin-resistant lung cancer cell line (A549) had variable contents, suggesting a role for exosomes in mediating cisplatin resistance (Xiao et al., 2014). Exosomes are also involved in inducing treatment resistance to concurrent chemotherapy and tyrosine kinase inhibitor. Recently, Li et al. showed that when exosomes derived from gefitinib-treated lung cancer cells and cisplatin were combined, the cisplatin sensitivity of the cancer cells was significantly reduced, whereas a combination of exosomes from cisplatin-treated cells and gefitinib had no significant effect on gefitinib-induced cell apoptosis (Li et al., 2016). When they blocked the exosome production in lung cancer cells and tested the cisplatin and gefitinib combination, a synergistic effect was observed. These findings underline the importance of exosomes in mediating the antagonistic effects of cisplatin and gefitinib therapy in lung cancer.

The peculiar double-membrane structure of exosomes resembles the structure of a commercial drug delivery vehicle, the liposome. Exosomes are engineered to carry anticancer therapeutics by active (sonication, electro-poration, or freeze–thaw cycles) or passive (simple incubation with exosomes) drug-loading approaches (Luan et al. 2017). Munagala, Aqil, and Gupta (2016) and Munagala, Aqil, Jeyabalan, and Gupta (2016) employed passive loading approaches with milk-derived exosomes to deliver paclitaxel, doxorubicin, and withaferin A. The exosome drug delivery system not only reduced the IC₅₀ values of chemotherapeutics but also induced in vivo antitumor activity in mouse lung cancer models. Kim et al. (2016) tested paclitaxel-loaded exosomes in murine Lewis lung carcinoma cell lines. They observed that paclitaxel-loaded exosomes significantly lowered the IC₅₀ of free drug in cancer cell lines. Results from our own research have shown that exosomes loaded with gold nanoparticles to which anticancer drugs (doxorubicin or cisplatin) were attached by a pH-stimulated linker were able to deliver drugs to H1299 and A549 lung cancer cell lines and produced therapeutic response (Srivastava, Amreddy, et al., 2016).

Thus, new avenues are opening in the field of exosome technology and can be explored for the diagnosis and treatment of lung cancer.

2. EXOSOMES

Exosomes are now the most widely studied extracellular vesicles that are produced in the cell, yet there is no classical definition for exosomes; their characteristics and definition often overlap with that of other cellular vesicles, such as microvesicles or apoptotic bodies (Akers, Gonda, Kim, Carter, & Chen, 2013; Fig. 1). To circumvent this confusion, many studies have used a combined term of extracellular vesicles (EVs) or nanovesicles (NVs); nevertheless, these studies mostly involved exosomes (Jørgensen et al., 2013; Nakano, Minata, & Rak, 2015). It is crucial to understand the basic structure and composition of exosomes in order to use them in nanomedicine.

2.1 Structure and Composition of Exosomes

The extremely small size of exosomes prevents their visualization under conventional optical microscopes. The transmission electron microscope (TEM) is the preferred method of visualizing exosomes. The isolated exosomes are directly mounted on a metallic formvar grid and are observed after negative staining with uranyl acetate. In structure, exosomes are heterogeneous in size, ranging from 30 to 150 nm in diameter. The morphology of exosomes as visualized in TEM images is largely defined as a double-membrane, cup-shaped structure (Jung & Mun, 2018; Srivastava, Amreddy, et al., 2016). However, Sharma et al. used atomic force microscopy and field-emission scanning electron microscopy to visualize exosomes, and described exosomes as having a three-dimensional, round structure. According to the study, the cup- or saucer-shaped structure of exosomes that is usually described is largely based on TEM imaging and is an artifact that is introduced due to the external force exerted on exosomes during the sample preparation (Sharma et al., 2010).

The round structure of exosomes has been confirmed using cryo-EM. Cryo-EM is considered superior to TEM, as cryo-EM does not involve dehydration or staining steps and

preserves the native biological structure by vitrification, which maintains the biological specimens at atomic resolution. Exosome samples were cryo-fixed, which transforms the water present in exosomes into a glass-like state without forming ice crystals, and the samples were then visualized under cryo-EM. The exosomes appeared as rounded electron-dense structures consisting of bilayered lipoproteins (Cizmar & Yuana, 2017; Yuana et al., 2013).

Exosomes can also be characterized based on their biophysical properties. The method used to do this, however, does not illustrate the ultrastructure of exosomes, but provides information regarding the concentration and size of exosomes in the isolated samples. Two companies offer technologies for performing such analysis. The first is qNano® by Izon Science Ltd. which uses a tunable resistive pulse sensor to count the particles passing through a tunable core under the influence of electric current (Maas, Broekman, & de Vrij, 2017; Maas, De Vrij, & Broekman, 2014). The second technique is called nanotracker analysis, which is performed with a device called NanoSight (Malvern, Inc.). This device measures the particles by passing the samples under a laser light. The light scattered by exosomes is recorded by a high-resolution camera attached to an optical system. Later, using a specific computer algorithm, the track formed by each particle under the influence of light is measured, and is represented as the size and number of exosomes present (Dragovic et al., 2011; Oosthuyzen et al., 2013).

Exosomes are reported to have lipoproteins in their membrane structure, which will bear some charge. This charge can be a determining factor in designing a drug delivery vehicle. Researchers have attempted to measure the ζ (zeta)-potential of exosomes so that appropriate fabrications can be done to enable efficient cellular uptake and delivery of therapeutic payload into the recipient cells making exosomes as an amenable drug delivery vehicle. In summary, the research analyzing the characteristics of exosomes describes a size in range of 30–150nm, a lipoprotein bilipid layer, a flat-to-round structure, and a membrane potential of –14 to –24 mV (Wang et al., 2015).

The lumens of exosomes mirror the composition of the cell from which the exosomes originate. This knowledge will be helpful in identifying the biomolecules that can be used for therapeutic purposes, as well as determining the molecules that can be loaded into them. Various researchers have identified many biologically active molecules in the lumen, including RNA, mRNA, miRNA, DNA, proteins, and lipids (Thakur et al., 2014; Théry, Zitvogel, & Amigorena, 2002; Vojtech et al., 2014). Although numerous studies have been performed to understand the composition of exosomal lumen, it is a consistent challenge to identify one class or type of molecule that is universally present in any exosome secreted by any cell type that could be regarded as a marker for exosomes. Although tetraspanins, such as CD9, CD63, and CD81, are the most common and abundant proteins present on exosomes isolated from almost any cell, no study to date has confirmed the universal presence of tetraspanins on exosomes (Srivastava et al., 2015).

Certain adhesion proteins, such as integrins and tetraspanins, are predominantly found on the exosome surface. Such proteins can be useful in using exosomes as drug delivery

vehicles for appropriate receptors on cancer cells to design targeted delivery of therapeutic payloads (Batrakova & Kim, 2015).

Different cytosolic proteins, such as tubulin, actin, ANNEXINS, RAB, SNARE, and GTPase, have also been detected in exosomes originating from different cells. These proteins are involved in biogenesis, intracellular membrane fusion, and transport of exosomes (Hannafon & Ding, 2013). Other proteins of metabolic importance, such as heat shock proteins HSP70, Hsp90, and cytochrome “c,” are also frequently detected in exosomes. The knowledge of exosomal content derived from different types of cells or tumor types can aid in determining and designing exosomes for therapeutic purposes. Vesiclepedia and Exocarta are online databases that meticulously curate updated information about the contents of extracellular vesicles and exosomes and can be used for such experiments (Table 1; Keerthikumar et al., 2015; Mathivanan, Fahner, Reid, & Simpson, 2012; Mathivanan & Simpson, 2009; Simpson, Kalra, & Mathivanan, 2012).

2.2 Biogenesis of Exosomes

Exosomes are produced in cells through a dynamic multistep endocytic pathway that is initiated by maturation of early endosomes into late endosomes. During this process, inward folding of early endosomal membranes sequesters proteins, lipids, and other biomolecules into small vesicles called internal luminal vesicles (ILV). The ILV-containing endosomes are morphologically different and are termed multivesicular bodies (MVBs). MVBs end up in lysosomes, where enzymes such as hydrolase degrade the components of MVBs, ensuring the removal of toxic materials. However, few of the MVBs escape degradation due to the presence of specific surface proteins, such as tetraspanins, CD63, and lysosomal-associated membrane proteins LAMP1 and LAMP2. The presence of these proteins enables the MVB to escape and fuse to the plasma membrane and get released to the extracellular milieu as vesicles termed exosomes (Colombo, Raposo, & Théry, 2014; Hessvik & Llorente, 2018).

In addition to biosynthesis of exosomes, whole cellular machinery consisting of about thirty proteins is involved in selective packaging of cellular cargo into exosomes during biogenesis. The proteins are members of a complex known as the endosomal sorting complex required for transport (ESCRT). The function and contribution of ESCRT in exosome biogenesis has been extensively reviewed by Colombo et al. (2013) and Stoorvogel (2015). Four protein members of ESCRT play a vital role in packaging biomolecules inside the lumens of exosomes and in the release of exosomes from the cell. Other associated or helper proteins, such as Alix and VSP4, are reported to be involved in exosome biosynthesis (Hannafon & Ding, 2013). Some ESCRT-independent mechanisms are also involved in exosome packaging and release from the cells. One such example is the ceramides, which have been shown to influence exosome biosynthesis. Blocking the production of ceramides using an inhibitor of spingomyelinase, an enzyme-involved in ceramide synthesis pathway, resulted in reduced exosome production (Subra, Laulagnier, Perret, & Record, 2007; Trajkovic et al., 2008).

3. THERANOSTICS APPLICATIONS OF EXOSOMES IN LUNG CANCER

Extensive research has resulted in identification of critical molecular targets that can manipulate lung cancer cell growth by perturbing the associated signaling pathways. Researchers have also identified, isolated, and synthesized drugs and molecules to control lung cancer (Zappa & Mousa, 2016). Preclinical studies have shown promising results. In spite of these successes, the majority of such discoveries are far from human applications since there are few modalities available to deliver them efficiently and safely. Biological barriers, poor uptake by cells, and digestion by hydrolyzing enzymes or nucleases are the major reasons that many therapies cannot be used in their naïve form and must be delivered by a vehicle that can provide protection.

The structural and physiological properties of exosomes offer tremendous opportunities to utilize exosomes in lung cancer therapy and diagnosis. In 2004, Bard and colleagues carried out the first study of exosomes in lung cancer when they analyzed exosomes from pleural effusions of patients with lung cancer (Bard et al., 2004). The ability of exosomes to reach almost every part of body and to deliver their content to recipient cells is superior to that of any natural or synthetic drug carrier. The exosomal cargo can also be manipulated to package therapeutic molecules for treatment.

Due to their ubiquitous access to the body and their ability to represent their cell of origin, exosomes have been explored for diagnostic purposes. Various imaging moieties are also being used to develop sensitive and harmless imaging methods in cancers, especially in lung cancer, in which frequent tissue biopsy is not feasible. The presence of exosomes in bodily fluids has made exosomes important candidates for developing noninvasive or minimally invasive diagnostic modalities. In Sections 3.1 and 3.2, the theranostic (therapeutic and diagnostic) capabilities of exosomes are discussed in lung and other cancers.

3.1 Exosomes in Therapeutics

3.1.1 Synthetic Drug Delivery Systems—The advent of nanotechnology brought hope for overcoming the issues of sending therapeutics to cancer sites, especially in lung cancer. Nanocarriers were expected to deliver drugs to tumor cells and produce the intended cytotoxic effects while minimizing the exposure of normal cells to drugs. Liposomes are phospholipid bilayer-containing synthetic vesicles that can be used for drug delivery. With a membrane structure similar to that of cells, liposomes are biocompatible and can deliver hydrophilic (in its aqueous lumen) and hydrophobic (on its phospholipid membrane) drugs.

Liposome-based drug delivery garnered much attention in experimental therapeutics. Doxil[®], a liposomal-based drug delivery vehicle for doxorubicin, has been successful in breast cancer treatment. The side effects of doxorubicin (DOx), such as cardiotoxicity, are considerably reduced in the encapsulated liposomal form (Lao et al., 2013; Perez, Domenech, Frankel, & Vogel, 2002). Due to the large surface area of the lung, a drug delivery system for lung cancer must release drug in controlled manner for a prolonged period. A liposome formulation based on dipalmitoylphosphatidylcholine is under investigation for intratracheal delivery in mice, with a view toward treating lung infections in patients with cystic fibrosis. Studies have shown better drug uptake in pulmonary cells after

administration of the complex. The initial success has encouraged researchers to explore its applications in lung cancer in the future (Rudokas, Najlah, Alhnan, & Elhissi, 2016).

In addition to liposomes, many metallic nanoparticles, such as those involving gold, iron, and galladanium, have been successfully tested for drug delivery. These nanoparticles can be designed in various structures and shapes to incorporate different drugs, different amounts of therapeutics, or multiple types of agents to produce combinatorial effects. Finally, the surfaces of these metal-based nanoparticles can be easily manipulated for functionalization with targeting ligand. The biocompatibility of these nanoparticles is not yet established, but may be explored for drug delivery (Amreddy et al., 2018).

3.1.2 Exosomes: The Natural Delivery Vehicle for Therapeutic Molecules—

Although various nanoparticles have been designed for drug delivery, clinical applications have failed due to poor bioavailability, nontargeted cytotoxicity, and immunogenicity (Blanco, Shen, & Ferrari, 2015; De Jong & Borm, 2008). An ideal drug delivery vehicle should overcome these limitations; natural cellular vesicles, such as exosomes, are now being explored as drug carriers due to their lack of immunogenicity and ability to cross biological barriers (Johnsen et al., 2014). Berry-derived compounds, such as aglycones and anthocyanidins, which have antioxidant, antiproliferative, apoptotic, and antiinflammatory properties, could be useful in cancer treatment. However, due to their poor bioavailability and retention, these compounds cannot be used efficiently. In a recent study, Munagala et al. attempted to encapsulate these compounds in exosomal lumen and showed enhanced therapeutic effect in cancer cells and in lung cancer xenografts in nude mice, suggesting the benefit of using exosomes as drug delivery vehicles (Munagala et al., 2017).

One major disadvantage with currently available drug delivery systems is that they are unable to cross the blood–brain barrier. Brain endothelial cell-derived exosomes could potentially be used to deliver anticancer drug to the brain, as these exosomes can easily cross the blood–brain barrier (Yang et al., 2015). There are two major considerations regarding the use of exosomes as drug carriers: (1) loading of therapeutics and (2) the type of cargo.

3.1.3 Loading of Therapeutic Agents on Exosomes—The key requirements for using exosomes as therapeutic carriers are high encapsulation efficiency of loading molecules and stability of biomolecules and exosomal structure. The ability of exosomes to carry therapeutic molecules/genes is expedited by loading them with molecules of interest extrinsically. This task is achieved by manipulating the donor cells to incorporate the therapeutic molecules, followed by isolating exosomes primed with desired molecules. This method has been useful in loading molecules that are difficult to add to exosomes through physical methods, e.g., hydrophobic molecules that will not cross the lipid bilayer membrane. In addition, molecules such as long noncoding RNAs, miRNAs, and siRNAs can be added through genome engineering of the donor cells, which helps in de novo production and incorporation of these molecules into exosomes (Johnsen et al., 2014; Srivastava, Babu, et al., 2016).

In another method, the exosomes are first purified from the donor cells and are then loaded with desired biomolecules by in vitro methods, such as incubation, electroporation, or using

chemical reagents. Since the vesicle membranes are generally dynamic in nature, small hydrophilic compounds, e.g., synthetic drugs and miRNAs, can be introduced by making reversible, transient changes in exosome membranes, and incorporating the molecules into them (Bryniarski et al., 2013; Melo et al., 2014; Zhang et al., 2010). These methods are straightforward, but there is no control on the incorporation of molecules by the exosomes.

Curcumin is a diarylheptanoid and a tautomeric polyphenolic hydrophobic compound that possesses antiinflammatory and antioxidative properties. Its therapeutic potential has been explored. Curcumin is also the first compound that has been tested for loading on exosomes (Sun et al., 2010). Recently, Kalani et al. loaded curcumin on exosomes by both priming and physically loading on exosomes. They created CUR-EXO by treating mouse brain endothelial cells with 7.5 μ M curcumin for 72h. The exosomes were then isolated by ultracentrifugation and demonstrated enhanced therapeutic effect (Kalani, Kamat, Chaturvedi, Tyagi, & Tyagi, 2014). In another study, the exosomes were isolated from mouse embryonic stem cells and were then loaded with curcumin to create MESC-exo^{cur}, which was checked for ischemia reperfusion (IR) injury in mice. The results showed the combining the potentials of embryonic stem cells exosomes and curcumin showed effective treatment response in IR-injured mouse inking the importance of exosomes as efficient drug delivery carriers (Kalani et al., 2016).

Electroporation involves the use of weak electric pulses to make rehealable transient holes in the exosome membrane. Alvarez-Erviti et al. were among the first investigators to add exogenous siRNA into exosomes. They used electroporation to load exogenous siRNA into exosomes from dendritic cells and noted a 25%–35% siRNA loading efficiency into the lumens of exosomes. The loaded exosomes were injected back into the cells and successfully delivered the therapeutic cargo to targeted brain cells after crossing the blood–brain barrier (Alvarez-Erviti et al., 2011). In addition, siRNA for MAPK was incorporated into exosomes isolated from HeLa cells and HTB-177 lung cancer cells; peripheral blood mononuclear cells were successfully delivered into monocytes and lymphocytes, causing gene silencing and demonstrating the technique’s effectiveness (Wahlgren et al., 2012). Doxorubicin was also electroporated into exosomes derived from mouse immature dendritic cells and then injected intravenously. The doxorubicin-loaded exosomes delivered the drug into targeted tumors and reduced the tumor size and growth, illustrating their potential in clinical applications (Tian et al., 2014).

In order to load therapeutic molecules, we developed a unique strategy in which we combined the synthetic nanotechnology with exosome biology. The new delivery system that we named “nanosomes” consisted of drug-conjugated gold nanoparticles loaded on and inside exosome-derived normal lung fibroblasts. This strategy is unique as it does not involve any cloning or other procedure, and the presence of gold nanoparticles permits functionalizing the GNPs with different targeting molecules or other therapeutics (Fig. 2; Srivastava, Amreddy, et al., 2016).

Electroporation sonication is another method that involves sound waves used to disrupt the membrane, allowing the drug to enter into the lumen. Following this strategy, the hydrophobic drug paclitaxel was loaded into the exosomes derived from macrophage cells.

The exosomes loaded with PTX (exo-PTX) were characterized by any changes in their size, stability, drug release, and were examined for in vitro antitumor efficacy. Interestingly, increased drug loading was observed due to reformation of the membrane as an effect of sonication. Further encapsulation of PTX into exosomes resulted in increased cytotoxicity by more than approximately 50 times in drug-resistant MDCKMDR1 (Pgp +) cells. Anticancer efficacy and colocalization of airway-delivered exosomes with cancer cells were also observed in a model of murine Lewis lung carcinoma pulmonary metastases (Kim et al., 2016). As an extension of this study, the same research group developed a new exosome-based drug delivery system intended for targeted delivery of therapeutic molecules. In their latest formulation, they added targeting moiety aminoethylanisamide-polyethylene glycol (AA-PEG) into the *exo*-PTX formulation. AA-PEG targets the sigma receptors, which are overexpressed in lung cancer cells. The results showed enhanced therapeutic outcome. The combination of targeting ability with the biocompatibility of exosome-based drug formulations offers a powerful and novel delivery platform for anticancer therapy (Kim et al., 2018).

3.1.4 Therapeutic Cargo—Exosomes are natural cellular vesicles, the lumens of which can be manipulated for loading desired drug or other molecules of therapeutic interest. Exosomes isolated from certain cell types, such as immature dendritic cells or mesenchymal stem cells, are unlikely to be immunogenic. Since the function of exosomes in cellular physiology is to carry and deliver cargo from the cell of origin to recipient cells, therapeutically loaded exosomes do not encounter resistance such as engagement with the immune system and interactions with opsonin proteins, complements, antibodies, or coagulation factors. The small size of exosomes enables them to pass through stringent biological barriers like the blood–brain barrier (Yang et al., 2015). The phospholipid bilayered membrane has many fusion and adhesion proteins that aid exosomes in directly attaching to and fusing with the recipient cells to deliver their contents. Thus, exosomes are now being explored as vehicles for efficient and specific delivery of anticancer therapeutics.

Hydrophobic drugs are otherwise difficult to deliver and are generally administered intravenously. Agrawal et al. recently attempted to administer paclitaxel orally by loading milk-derived exosomes with paclitaxel (ExoPAC). Their results showed a better reduction in tumor growth than that observed with PTX delivered intravenously in a nude mouse model of human lung cancer (Agrawal et al., 2017). Similarly, milk-derived exosomes were used to deliver curcumin. The complex was made by mixing curcumin with exosomes in the presence of 10% ethanol:acetonitrile (1:1) to form a complex called ExoCUR. Upon oral administration, the curcumin complex produced significantly higher antiinflammatory response, as measured through NF- κ B activation in human lung and breast cancer cells (Aqil, Munagala, Jeyabalan, Agrawal, & Gupta, 2017).

Celastrol (CEL) is another plant-derived compound that is known to inhibit the Hsp90 and NF- κ B activation pathways and has shown excellent therapeutic response in H1299 and A549 lung cancer cell lines. To explore its utility in in vivo systems, CEL was loaded into exosomes. Compared with free CEL, the CEL-loaded exosomes showed enhanced antitumor efficacy against lung cancer xenografts, without exhibiting any gross or systemic toxicity (Aqil et al., 2016). In addition, Sansone et al. recently showed packaging of the whole

mitochondrial genome into the lumen of exosomes and mediated hormonal therapy-resistant breast cancer (Sansone et al., 2017). Several examples were also compiled by Johnsen et al. (2014) and Aryani and Denecke (2016), who reviewed various miRNA and siRNA loaded into exosomes for their delivery to cancer cells (Aryani & Denecke, 2016; Johnsen et al., 2014).

3.1.5 Translational Application of Exosome-Based Therapeutics: Clinical Trials

—The endpoint of any research performed in experimental therapeutics is translation into clinical applications. A November 20th 2017 search of www.clinicaltrial.gov showed about 15 studies related to exosomes and various cancers, including lung cancer. Although most of the studies are related to development of exosome-based diagnostics, a few investigate the therapeutic aspects (Table 2).

In one trial, the concentration of curcumin is being evaluated for its delivery through plant-derived exosomes. Specifically, the effect of exosomally delivered curcumin on immune modulation, cellular metabolism, and the phospholipid profile of normal and malignant colon cells was evaluated in subjects who are undergoing surgery for newly diagnosed colon cancer. The effect of exosomally delivered curcumin on the production of cytokines, the changes of immune cells, and glucose metabolism by administration of ¹³C-glucose prior to surgical resection will also be characterized (NCT01294072). In another clinical trial, exosomes derived from grape juice are being evaluated for prevention of oral mucositis associated with chemoradiation treatment of head and neck cancer. In addition, the researchers will look for interaction between tumor exosomes and grape extract exosomes by measuring the levels of cytokines in response to tumor antigens present on tumor-derived exosomes (NCT01668849).

A dendritic cell-derived exosome (Dex)-based clinical trial is in phase II. Here, the investigators are examining the use of Dex as vaccine to impart immunotherapy in combination with metronomic cyclophosphamide (mCTX) therapy in patients with lung cancer (NCT01159288). There are also two phase I trials based on immunostimulatory exosomes for antitumor therapy. In these trials, dendritic cells of patients with stage III/IV melanoma were isolated and pulsed with MAGE3 tumor antigens. Exosomes presenting MAGE3 were isolated and readministered to melanoma patients. Therapy appeared to be well tolerated by all patients and induced the desired immune effects in some patients, showing clinical feasibility for exosome-based therapeutics (Escudier et al., 2005).

3.2 Exosomes in Diagnosis

High mortality and failure of various therapeutic interventions occurs in lung cancer because the current diagnostic approaches are not efficient enough to detect the disease at early stages. In general, lung cancer is detected when the disease has already progressed into an advanced stage. Hence, timely diagnosis of lung cancer is a crucial factor for favorable therapeutic response. In clinics, tissue collection is considered the standard practice for lung cancer diagnosis. Unfortunately, this method is insufficient to present a complete picture of disease state, especially in the era of precision and personal medicine. The limitations with tissue biopsies, such as collection of a limited amount of tissue, inability to collect tissues

multiple times, and frequent tumor heterogeneity, mean that the collected localized tissue sample may not be sufficient to provide the global status of the disease. Hence, alternative routes of diagnosis are being explored.

Liquid biopsies, or analysis of bodily fluids, have drawn attention. Since exosomes can be isolated from almost all bodily fluids and their content mirrors the cell of origin, exosomes can be used to determine the state of the body. In several studies, the exosomes are manipulated in such a way that they can also be used for imaging purposes. Section 3.2 describes the various ways in which exosomes are studied for their possible role in diagnostics (Cui, Cheng, Qin, & Jiang, 2018).

3.2.1 Imaging of Exosomes—Many studies in lung and other cancers have shown that exosomes can successfully deliver the therapeutic cargo to recipient cells. Partial or preliminary success in targeted delivery of cargo to the site of cancer cells has been realized. Successful targeted delivery will lead to imaging the exosomes reaching the cancer site, which will pave the way for development of novel high-precision diagnostic and prognostic modalities. Reports of several approaches to image exosomes are available in the literature.

3.2.1.1 Labeling of Exosomes With Nanoparticles for Imaging: As an alternative to conventional fluorescent dyes, a new group of nanosized optical reporter called semiconductor quantum dots are under investigation. These quantum dots are nanosized crystals that are superior to fluorescent dyes, as they are more stable and have tunable optical properties that can be used for a wide range of optical applications, including in vivo imaging and diagnostics (Walling, Novak, & Shepard, 2009). Zong et al. used silicon quantum dots (Si QDs) to image exosomes using a microscopic method called single-molecule localization microscopy (SMLM). The CD63 protein present on the outer membrane of exosomes was tethered with Si QDs to form nanoprobe, which recognized exosomes. Using the SMLM, a high-resolution image of the exosome was obtained. Biocompatible in nature, Si QDs will have minimal cytotoxic effects and could be ideal for live cell imaging and diagnosis (Zong et al., 2018).

In addition, researchers have explored Gold–Carbon quantum dots (GCDs). Tumor-specific antibodies were attached to GCDs, which were attached to exosomes as described for Si QDs. These exosome-labeled nanoprobe successfully demonstrated the fluorescent imaging of exosomes and showed the intrinsic intracellular behavior of exosomes that can be used for studying the role of exosomes in cancer metastasis (Jiang et al., 2018).

In addition to quantum dots, we have demonstrated an application of exosomes as theranostics in lung cancer. We used normal lung fibroblast cell (MRC9)-derived exosomes and attached doxorubicin (Dox), an anticancer drug, and 5–10 nm super paramagnetic iron oxide Nanoparticle (SPION), creating a system we termed “fexosomes.” With fexosomes, we demonstrated that we can deliver anticancer therapeutics (Dox) to lung cancer cells (H1299 and A549) and simultaneously image them using magnetic resonance imaging. This dual capacity of exosomes makes them unique for developing theranostics (Fig. 3). We further intend to exploit the gold nanoparticle part of drug carrier nanosomes described earlier in the chapter for their utility in PET imaging. Thus, the compatibility of exosomes

with different nanoparticles provides an opportunity to develop novel approaches in lung and other cancer diagnosis.

3.2.1.2 Microscopic Methods for Visualizing Exosomes: Imaging exosomes can be a robust way to develop potent diagnostic and prognostic modalities. The tumor-targeted exosomes can be monitored in real time to check the distribution of exosomes and identify the precise location of tumors. Several small lipophilic fluorescent dyes, e.g., DiR, DiD, and PKH67, have been used to label the lipoprotein membranes of exosomes. Although these dyes are suitable for biodistribution studies, clinical applications for diagnosis are yet to be achieved. In addition, exosome membranes have been fluorescently labeled by tagging exosomal membrane proteins with fluorescent molecules, such as GFP or td-Tomato. This kind of labeling is considered more stable and suitable for evaluation of clinical applications.

Surface display technology is a novel approach for targeted theranostics, but we currently lack a suitable system for mammalian surface display. Stickney et al. explored exosomes and tetraspanins by adding fluorescent probes to the inner and outer surfaces of exosomes and observed the fate of exosomes in an in vivo experiment. They were able to track the exosomes in the body and cells after transfection, paving the way to visualize the fate of exosomes used for drug delivery (Stickney, Losacco, McDevitt, Zhang, & Lu, 2016). In another application of microscopic imaging of exosomes, tumor cell-derived exosomes were labeled with photo-switchable probes. This system was used to obtain super resolution imaging by using photo-activated localized microscopy or stochastic optical reconstruction microscopy. This imaging technique was successfully used to study the interaction between breast cancer cell-derived exosomes and normal cells. In the future, methods like this will be valuable for tracking the exosomes carrying therapeutic loads and will help clinicians in developing high precision and personalized treatment modalities, in addition to giving researchers a tremendous opportunity to understand the pathophysiology of cancer (Chen et al., 2018).

3.2.1.3 Spectrometric Methods of Exosome Detection: The method described thus far for detection of exosomes involves manipulation of exosomes that may have their own limitations. One such limitation is that synthesis protocols must be optimized. To avoid these synthesis-related complexities, Park et al. developed an interesting and novel approach for exosome detection. They used surface-enhanced Raman scattering spectrometry, followed by analysis with statistical pattern analysis using principal component analysis. In their study with lung cancer cell-derived exosomes, they clearly distinguished lung cancer exosomes from normal exosomes with 95.3% sensitivity and 97.3% specificity (Park et al., 2017).

3.2.2 Exosomal miRNA and Proteins as Biomarkers for Liquid Biopsy—In the current era of genomic medicine where much of the emphasis is on development of personalized and precision medicine-based standard of care, liquid biopsy is fast gaining attention. In liquid biopsy, bodily fluids (largely blood and urine) are used for diagnosis, instead of collecting tissue samples, which are generally obtained through surgical procedures. Due to its immense potential liquid biopsy-based tests are predicted to become a standard tool for diagnostic, prognostic, and predictive information in nonsmall cell lung

cancer (Molina-Vila et al., 2016). Mainly circulating tumor cells (CTCs), circulating-free DNA (cfDNA), and exosomes are analyzed in liquid biopsies. Exosomes are suitable candidates for developing liquid biopsies, especially compared with CTCs and cfDNA, because the exosome remains more stable and is easily collected from urine or blood. Much of the diagnostic study related to exosomes has focused on miRNA and proteins as biomarkers. In Sections 3.2.2.1 and 3.2.2.2, these molecules are discussed with respect to their diagnostic significance in lung cancer.

3.2.2.1 Exosomal miRNA as Biomarkers: Exosomal miRNAs are considered a source of biomarkers, since they play regulatory roles and changes in physiology should be reflected in terms of changes in miRNA enrichment. Table 3 shows some of the miRNAs that could be used as biomarkers. Liu et al. reported that three miRNAs, miR-23b-3p, miR-10b-5p, and miR-21-5p, appeared to be promising prognostic biomarkers in a patient with NSCLC. They isolated exosomes from blood from 196 patients and used qPCR assay to determine the enrichment of miRNAs (Liu, Vermesh, et al., 2017; Liu, Yu, et al., 2017).

Tumor-specific circulating miRNAs have been developed as early diagnostic biomarkers for lung cancer. Remarkably, some researchers have succeeded in discovering circulating miRNAs with prognostic or predictive significance; Table 3 describes several such studies and miRNAs that have been evaluated for their diagnostic value in lung cancer. In order to substantiate the significance of miRNA in lung cancer physiology and increase confidence in exosomal miRNA-based biomarkers in NSCLC, Lai and Friedman developed a mathematical model for early-stage NSCLC for three highest overexpressed miRs: miR-21, miR-2015, and miR-155. The model provided a simultaneous quantitative relationship between miRNA levels and tumor volumes and tumor mass. The result showed a positive correlation and is viewed as an initial step toward establishing these three miRs as a panel of biomarkers for early diagnosis of NSCLC (Lai & Friedman, 2016).

One of the challenge of using miRNA from exosome as diagnostic tool is the potential difficulty in isolating exosomes followed by purification of miRNA from them in clinical setting. In a study to obtain exosomal miRNAs for quick analysis, Wei et al. developed an electric field-induced release and measurement method. The method employs simultaneous disruption of exosomes and release of their contents for simultaneous monitoring for miRNA/protein biomarkers. This technology is currently in exploratory stages; when fully developed, this method will aid easy exosome-based diagnosis (Wei et al., 2014).

3.2.2.2 Exosomal Proteins as Biomarkers: Like miRNA, exosomes should contain proteins from the originating cell and thus should have a potential source of developing biomarkers. Profiling the whole proteome of exosomes by mass spectroscopy has revealed various biomarkers in prostate cancer and bladder cancer. A study involving 276 patients with NSCLC identified NY-ESO-1, EGFR, PLAP, EpCAM, and Alix as protein biomarkers for overall survival (Sandfeld-Paulsen, Aggerholm-Pedersen, et al., 2016). Exosomes derived from blood plasma were successfully used for evaluation of multimarker models using the Extracellular Vesicle Array (EV Array) system consisting of 37 antibodies targeting lung cancer-related proteins. Using a multimarker system the study was able to distinguish between the lung cancer and noncancer patient group. Further this method can be

useful for future clinical practice as it used only 10 μ L of plasma samples of performing the analysis (Jakobsen et al., 2015). In another study, using the multimarker model system Paulsen et al. found that CD151, CD171, and tetraspanin 8 were important protein markers with diagnostic value for lung cancer with different histological types. Patients with cancer of all histological subtypes vs patients without cancer showed a sensitivity and specificity index of (CD151: AUC = 0.68, P = 0.0002; CD171: AUC = 0.60, P = 0.0002; and TSPAN8: AUC = 0.60, p = 0.0002) (Sandfeld-Paulsen, Jakobsen, et al., 2016).

Most of the proteomic evaluation done for identification of biomarkers for lung cancer was done using blood serum. Leucine-rich α -2-glycoprotein (LRG1) was recently identified in urine-derived exosomes from patients with lung cancer and is now under investigation to explore its utility as a biomarker. Li used a nano-HPLS-chip-MS/MS method to analyze urinary exosomes and identified 18 proteins of interest that showed differential expression. The results were further validated by western blot and by immunohistochemistry of lung tissue; LRG1 was found to be expressed at higher levels in urinary exosomes and lung tissue of patients with NSCLC. These results suggest that LRG1 may be a candidate biomarker for noninvasive diagnosis of NSCLC in urine (Li, Zhang, Qiu, & Qiu, 2011).

4. TECHNIQUES FOR ISOLATION OF EXOSOMES

One bottleneck in the application of exosomes as theranostics is that exosome isolation is tedious and inefficient. At present, there is no standard protocol to ensure consistent yield of exosomes. Much initial material is needed to produce exosomes, which does not seem feasible for clinical applications. The conventional method of isolating exosomes is ultracentrifugation. This method yields a good amount of exosomes, but requires specific instrumentation and technical skill.

Recognizing this limitation, researchers are developing novel approaches to isolate exosomes from a small volume of samples. Several proprietary precipitation reagents are available from different vendors. However, these reagents are often polymer-based, which causes some impurities in the isolation. In addition, there is no defined mechanism for selective isolation of exosomes, but not microvesicles. Methods for exosome isolations using microfluidics are being developed. These methods require a small volume of input material, and isolation and sorting of specific exosomes can be performed simultaneously (Contreras-Naranjo, Wu, & Ugaz, 2017; Liang et al., 2017; Zhao, Yang, Zeng, & He, 2016). In an innovative approach, Ueda et al. constructed anti-CD9 antibody-coupled, highly porous monolithic silica microtips and used it to isolate exosomes from clinical cancer samples, including serum samples from patients with lung cancer. The isolated exosomes showed diagnostic ability, as mass spectrometry performed on the isolated exosomes identified CD91 as a lung adenocarcinoma-specific antigen on exosomes (Ueda et al., 2014).

Liu et al. recently developed a chip-based method for isolation of exosomes. Researchers reported a size-based exosome isolation tool called ExoTIC (exosome total isolation chip), which is simple, easy-to-use, modular, and facilitates high-yield and high-purity exosome isolation from biofluids. ExoTIC achieves a yield ~ 4- to 1000-fold higher than that with ultracentrifugation, and exosome-derived protein and microRNA levels are well-correlated

between the two methods. Moreover, the researchers demonstrated that ExoTIC can sort a heterogeneous population of cancer cell exosomes based on size. They also tested ExoTIC to isolate exosomes from clinical samples, including plasma, urine, and lavage, demonstrating the device's broad applicability to cancers and other diseases (Liu, Vermesh, et al., 2017; Liu, Yu, et al., 2017).

The size-exclusion chromatography method developed by Blans et al. does not require any exosome pelleting, yet results in improved isolation of exosomes from human and bovine milk (Blans et al., 2017). Exploiting the membrane proteins expressed on the surface of exosomes, various chip-based microfluidic methods of exosome isolation have been developed. For example, Fang et al. recently developed a microfluidic chip for immunocapture and quantification of EpCAM-positive exosomes. The researchers also used a similar method to study HER2-positive exosomes from breast cancer samples (Fang et al., 2017). Improvements and innovations in isolation methods are essential for translating exosome research into clinical applications and patient care. With the development of novel methods of isolation, exosome-based theranostics will be realized in the near future.

5. CHALLENGES AND PERSPECTIVES

Research into lung cancer has identified several molecular targets that can be used for therapeutic purposes. Several tumor-targeting moieties have also been identified. Thus, lung cancer is a promising disease in which to study precision and personalized medicine. Due to their unique structure, size, composition, and mechanism of biogenesis, exosomes have a unique place in theranostics interventions (Fig. 4). The delivery of therapeutic agents through exosomes is a novel approach that holds a great future in medicine.

In diseases with complex pathophysiology and genetic makeup, like cancer, the conventional treatment regimens can become more effective if the treatment is administered through efficient, body-specific, or personalized modalities. Due to their exceptional capacity to carry and deliver biologically active molecules to different cells and due to their homing effect, exosomes are ideal candidates for developing such modalities. A number of studies show that exosomes can be loaded with anticancer therapeutic agents and can also act as therapeutic molecules by virtue of its luminal contents that contain several biomolecules that can be exploited for therapeutic effect. Similarly, exosomes are promising for use in liquid biopsy, especially compared with CTCs or circulating cell-free DNAs. In summary, exosomes are unique molecules that have excellent properties to develop as theranostics.

Exosome-based theranostics are emerging as a leader in nanomedicine. However, a number of associated challenges remain, largely because of our lack of knowledge about exosomes. These challenges must be resolved before exosome-based delivery systems are applied in clinical settings.

The first and most important limitation of using exosomes in clinical settings is associated with the isolation of exosomes. Exosomes can be isolated from in vitro cell culture medium and from different bodily fluids, but the abundance of exosomes in these biofluids varies. In addition, the availability of biofluids varies. Hence, a standardized method for exosome

isolation is warranted. Many methods are being investigated, but each has its own limitations. Efforts should be made to develop a standardized method that can ensure a consistent, repeatable supply of exosomes. The exosome preparation should also be of high quality, homogenous in nature, and free from other cellular vesicular contamination. Therapy sometimes involves the use of exosomes derived from specific cells, e.g., “dexosomes,” or exosomes isolated from immature dendritic cells. For such studies, methods should be developed to sort dexosomes from other contaminating exosomes (Le Pecq, 2005).

In order to make use of exosomes in clinical applications for disease treatment and early diagnosis, it is essential to develop a deep understanding about the exosomes, especially their biogenesis, packaging of luminal content, and the mechanism involved in sorting, exchange, and transportation of exosomes. A number of studies that are expected to shed new light on the biology of exosomes are underway.

Finally, the foremost challenge associated with any therapeutic system of biological origin is the maintenance of good manufacturing practices (GMPs). Unlike the production of synthetic drug molecules, a slight protocol change in the production of these molecules can result in dramatic differences in the quality of final product. Hence, robust protocols to ensure the consistent production of exosomes per GMP parameters will be needed before exosome-based therapy enters clinical practice.

6. CONCLUSION

In this chapter, we highlighted the exemplary capacity of exosomes to be used as diagnostic and therapeutic agents in lung and other cancers. The properties of exosomes make them uniquely suitable for use as theranostics, and their application has been explored in many cancers. Although exosome-mediated theranostics appear promising, more research needs to be done before these theranostics are incorporated into clinical applications.

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FURTHER READING

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Fig. 1.
Different vesicles produced by the cells.

Fig. 2. Synthesis of nanosomes—an exosome-based drug delivery vehicle constructed by combining gold nanoparticles and exosomes. *Figure modified from* Srivastava, A., Amreddy, N., Babu, A, Panneerselvam, J., Mehta, M., Muralidharan, R., et al. (2016). *Nanosomes carrying doxorubicin exhibit potent anticancer activity against human lung cancer cells.* Scientific Reports, 6, 38541. <https://doi.org/10.1038/srep38541>. Copyright © 2016, Rights Managed by Nature Publishing Group.

Fig. 3. Exo-MNP-Dox (fexosomes) (A) therapeutic effect was confirmed by molecular analysis of Dox-inducing DNA damage analysis through cleaved Caspase9. (B) Magnetic nanoparticles T2 MR imaging of (i) 0 µg, (ii) 12 µg, and (iii) 62 µg of MNPs in fexosomes confirm the increasing T2 relaxation.

Fig. 4. The theranostic application of exosomes. The exosome can be loaded with various therapeutic molecules (*left panel*) through metallic nanoparticle. Simultaneously, the nanoparticle-loaded exosomes can be used for imaging (*top two right panels*), and the exosomes can be labeled with fluorescent dye and imaged to see the biodistribution of drugs encapsulated in fluorescent-labeled exosomes (*bottom right panel*). *Green circle* shows exosomes as imaged by X-ray and fluorescence in *right panel*.

Table 1

Two Manually Curated Databases Describe the Number of Biomolecules Identified in Contents of Extracellular Vesicles (EVs) in Different Studies

	Vesiclepedia Database ^a	Exocarta Database ^a
Proteins	92,897	9769
mRNA	27,642	3408
miRNA	4934	2838
Lipids	584	1116
No. of Studies	538	286

^aDatabase accessed on: March 8th, 2018.

Current Clinical Trials Based on Exosomes in Cancer

Table 2

Title	Recruitment	Cancer	Interventions
Exosomes as potential prognostic and predictive biomarkers	Unknown status	Gastric cancer	Other: ExoIntelliScore Prostate
Urinary exosome for cancer diagnosis	Completed	Prostate cancer	
Exosome-mediated intercellular signaling	Recruiting	Pancreatic cancer Benign pancreatic disease	
Vaccination trial with dendritic cell-derived exosomes	Unknown status	Nonsmall cell lung cancer	Biological: Dex2
Plant exosomes to deliver curcumin to normal and cancer tissue	Active, not recruiting	Colon cancer	Dietary supplement: curcumin Dietary supplement: Curcumin conjugated with plant exosomes Other: No intervention
Edible plant exosome ability to prevent oral Recruiting mucositis associated with chemoradiation treatment	Head and neck cancer Oral mucositis	Dietary supplement: Grape extract Drug: Lortab, fentanyl patch, mouthwash	
Tumor-derived exosomes as diagnostic and Withdrawn prognostic markers	Breast neoplasms		
Metformin hydrochloride affecting cytokines and exosomes in cancer	Recruiting	Larynx Lip Oral cavity Pharynx	Radiation: External beam radiation therapy Drug: Metformin hydrochloride Other: Placebo
Screening for human papillomaviruspositive oropharyngeal squamous cell carcinoma	Recruiting	Oropharyngeal cancer	
Diagnostic accuracy of circulating tumor cells (CTCs) and onco-exosome	Recruiting	Pancreatic ductal adenocarcinoma (PDAC)	Procedure: Blood samples Procedure: Portal vein blood sample
Consistency analysis of PD-L1 in cancer tissue and plasma exosome	Not yet recruiting	NSCLC	Other: Liquid biopsy
Consistency analysis of PD-L1 in lung cancer tissue and plasma exosome before and after radiotherapy	Not yet recruiting	NSCLC	Radiation: Radiotherapy
Circulating exosome RNA in lung metastases of primary high-grade osteosarcoma	Recruiting	Lung metastases Osteosarcoma	Other: Blood samples
Anaplastic thyroid cancer and follicular thyroid cancer-derived exosomal analysis via treatment of liothyronine and vildagliptin and Pilot Prognostic Study via urine exosomal biological markers in thyroid cancer patients	Not yet recruiting	Thyroid cancer	
ncRNAs in exosomes of cholangio carcinoma	Recruiting	Cholangiocarcinoma Benign biliary stricture	

Table 3

List of exosomal miRNA of diagnostic significance identified in lung cancer

miRNA	Method of Study	Functional Role	References
miR-155, let-7a-2	miRNA-microarray	Differentiates lung cancer from noncancerous lung tissue	Yanahara et al. (2006)
miR-17-3p, hsa-miR-21, hsa-miR-106a, hsa-miR-146, hsa-miR-155, hsa-miR-191, hsa-miR-192, hsa-miR-203, hsa-miR-205, hsa-miR-210, hsa-miR-212, and hsa-miR-214	miRNA-microarray	Validation of diagnostic miRNAs	Rabinowitz, Gerçel-Taylor, Day, Taylor, and Kloecker (2009)
miR-29a-3p and miR-150-5p	Quantitative real-time polymerase chain reaction using Exqon miRNA expression profiling panel	Detection of response of radiotherapy in nonsmall cell lung cancer (NSCLC)	Dinh et al. (2016)
miR-21 and miR-155	miRNA-microarray	Differentiates recurrent tumors from primary tumors	Munagala, Aqil, and Gupta (2016) and Munagala, Aqil, Jeyabalan, and Gupta (2016)
miR-30b-5p, 30c-5p, 221-3p, 222-3p	miRNA-microarray	EGFR status	Giallombardo et al. (2016)
miR-19b-3p, miR-21-5p, miR-221-3p, miR-409-3p, miR-425-5p, and miR-584-5p	Quantitative real-time polymerase chain reaction using Exqon miRNA expression profiling panel	Biomarkers for lung adenocarcinoma detection	Cui et al. (2018)
miR-205-5p and miR-200b	Sequencing	Differentiates patients with lung cancer from patients with pneumonia and pulmonary tuberculosis	Lin et al. (2016)
miR-181-5p, miR-30a-3p, miR-30e-3p, and miR-361-5p and let-7, miR-21, miR-24, and miR-486	Seq	Identify NSCLC adenocarcinoma and squamous cell carcinoma	Jin et al. (2017)
miR-21 and miR-4257	miRNA-microarray	Predictive biomarkers for the recurrence of nonsmall cell lung cancer	Dejima, Iinuma, Kanaoka, Matsutani, and Kawamura (2017)