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Review Article

A Comparative Review of the Detection of Early-stage Lung Cancer by Exosomal and Free Nucleic Acids and Standard Screening Methods

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Abstract

Lung cancer (LC), with its high incidence and less effective treatment strategies, is often not detected until it is in an advanced stage, which contributes to its high mortality rate. Hence, screening the DNA and RNA content in exosomes offers a promising method for its early diagnosis. New technologies for the early detection of LC are absolutely necessary to improve patient outcomes. Such an approach could be the exploitation of exosomes and their content. Exosomes contain DNA, different RNA species, proteins, ceramides, and cholesterol. They can transport their oncogenetic cargo derived from tumor cells to healthy cells over a range of distances to help propagate genetic information that could contribute to the initiation of cancer. This review provides an overview of the involvement of exosomes and their DNA/RNA together with cell-free DNA, summarizes potential biomarkers and describes the application of technologies for the characterization of exosomes with their possible tumor markers. Current radiological and scanning methods for the early detection of LC are also described and compared with nucleic acid analysis. The strengths and weaknesses of both approaches are considered.

Introduction

Lung cancer

Worldwide, lung cancer (LC) is the leading cause of death because of its late detection. Specifically, LC represents 12% of all incident cancer diagnoses and 18% of cancer mortality. About 40% of LC patients are diagnosed with stage IV disease, for whom treatment aims to reduce disease-related adverse events and prolong life.[1] The World Health Organization divides LC into two main categories: small-cell LC (SCLC) and non-small cell LC (NSCLC), accounting for 10–15% and 80–85% of LC cases, respectively. NSCLC is the most studied cancer type with a 5-year overall survival rate of 23%, whereas SCLC has a median survival time of less than 1 year. Further histological types comprise squamous cell LC (SQCLC), adenocarcinoma (ADC) and large cell LC.[2] LC is mainly caused by cigarette smoke. The identification of major driver mutations genes including epidermal growth factor receptor (EGFR), Kirsten rat sarcoma (KRAS), anaplastic lymphoma kinase (ALK), v-raf murine sarcoma viral oncogene homolog B1 (BRAF), human epidermal growth factor receptor 2 (HER2), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), protein kinase B (AKT1), mitogen-activated protein kinase kinase 1 (MAP2K1) and mesenchymal epithelial transition (MET) has improved therapeutic strategies and introduced multiple targeted therapies. Current treatment options for LC include combinations of cytotoxic chemotherapy and targeted biological therapies such as bevacizumab and erlotinib. While NSCLC patients are treated with multiple targeted therapies, SCLC patients are mainly treated with platinum-based chemotherapy.[3] For detection of LC, the National Comprehensive Cancer Network recommends computed tomography (CT; chest and upper abdomen including adrenals), biopsy (image-guided transthoracic biopsy), bone x-rays, bone scintigraphy, and brain CT with surgical exploration, radiotherapy, and chemoradiation. CT is the first-line modality for accurate T staging, whereas chest radiography has limited utility in T staging but shows chest wall invasion with advanced disease or large tu-

Keywords: Lung cancer; Exosomes; Cell-free DNA; microRNAs; screening methods.

Abbreviations: ADC, adenocarcinoma; CEA, carcinoembryonic antigen; CT, computed tomography; dscfDNA, double-stranded cell-free DNA; EGFR, epidermal growth factor receptor; EVs, extracellular vesicles; Exs, exosomes; LC, lung cancer; miRNAs, microRNAs; MRI, magnetic resonance imaging; ncRNA, non-coding RNA; NGS, next-generation sequencing; NSCLC, non-small cell lung cancer; PEG, polyethylene glycol; PET, positron emission tomography; sscfDNA, single-stranded cell-free DNA; SCLC, small-cell lung cancer; SQCLC, squamous cell lung cancer.

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mors.[4,5] Although CT-based population screenings are performed to identify high-risk individuals and can identify smaller nodules more often than chest radiography in lung screening, minimally invasive biomarkers, such as exosomes (Exs), to detect LC early are urgently needed.

Exosomes

Major populations of extracellular vesicles (EVs) include Exs, microvesicles and apoptotic bodies.[6] Exs form part of a range of vesicles that are released from both healthy and cancer cells and are 30–100 nm in size.[7] They carry both transmembrane and interluminal proteins, i.e. tetraspanins cluster of differentiation 9 (CD9), CD63, CD81 and CD82; heat shock protein 70 (HSP70) and HSP90; as well as integrins. In addition, some proteins play roles in both membrane transport and fusion such as Rab GTPases, annexins and flotillins plus proteins involved in the formation of multivesicular bodies (e.g., ALG-2-interacting protein X and tumor susceptibility gene 101).[8] These surface molecules have been identified as biomarkers for Exs derived from various normal wild-type cells. Those cells undergoing pathological processes secret Exs with distinct compositions reflecting benign and malignant disease patterns and stages. Proteins, such as EGFR, ephrin type-A receptor 2 and epithelial cellular adhesion molecule (EpCAM) on the membrane surface of Exs are commonly used to distinguish tumor-derived Exs from nontumor-derived Exs.[9] The expression levels of Ex lipopolysaccharide-binding proteins and E-cadherin have been used to identify metastatic LC.[10] The changes in these compositions are often accompanied by rising Ex levels.[11]

Exs act as a transport system carrying material and informative molecules between cells over a range of distances. This can be for transport from healthy cells to healthy cells, tumor cells to tumor cells and tumor cells to healthy cells. Exs are able to transport both genomic (100 bp to 17 kb) and mitochondrial DNA, both cellular and mitochondrial-specific RNA (including mRNA, miRNA, ribosomal RNA [rRNA], transfer RNA [tRNA], long non-coding RNA [lncRNA], Piwi-interacting RNA [piRNA], small nuclear RNA [snRNA] and small nucleolar RNA),[12] protein, ceramides, and cholesterol. This means that they help cancer cells propagate genetic information, which may allow the inception of cancer progression and metastases. They are also involved in moving chemotherapeutic drugs from cell to cell. Such activities involving Exs can aid in chemotherapy resistance initiation. While other EV subgroups may harbor these features, Exs are the best characterized subgroup.[13] Exs carry DNA as DNA strands, DNA-protein complexes and mitochondrial DNA either as free DNA or in damaged mitochondria or whole active mitochondria. These mitochondria are likely to be specifically inserted into Exs by either the healthy or cancer cells from which they are derived.[14–16] They are unlikely to contain mitochondria normally found in the blood.[17–19] Since 90% of plasma DNA is found in Exs[20] with some of the DNA occurring on the Ex surface as opposed to being internalized.[21] This will leave 7% of the DNA free in the plasma[20] including virtosomal DNA.[22]

Deep-sequencing analysis has been used to characterize human plasma-derived exosomal RNAs. The major fraction was mRNA (42.32%) together with rRNA (9.16% of all mappable counts), lncRNA (3.36%), piRNA (1.31%), tRNA (1.24%) and snRNA (0.18%). A total of 593 miRNAs were found with miR-99a-5p, miR-128, miR-124-3p, miR-22-3p, and miR-99b-5p being the five most common, accounting for 48.99% of all mappable miRNA sequences.[12] In particular, miRNAs, lncRNAs, and circular RNAs (circRNAs) have been exploited in a variety of liquid biopsy methods for the detection of primary LC.[23] The movement of Exs containing a range of these DNA and RNA molecules from tumor cells to healthy cells can result in modification of the biology of the recipient cells. Hence, the Exs contain either DNA or RNA molecules that can be identified with the tumor induction process—either primary or secondary—and so can act as possible indicators of tumors at a very early stage via the liquid biopsy approach.[24] Alternatively, employment of the more commonly used methods to screen for tumors frequently occurs when patients present with specific symptoms (e.g, for LC). This is likely to be initially by radiology followed by the more sensitive methods of either CT/positron emission tomography (PET) scan or magnetic resonance imaging (MRI), although the sputum test for tumor cells and bronchoscopy with biopsy for nodule samples are likely follow-up processes. The present review compares the efficacy of the two different approaches for the early detection of LC and their possible limitations.

Non-exosomal circulating tumor DNA

About 7% of cell-free DNA (cfDNA) is not present in Exs.[20] These DNAs include the naturally released DNA present in virtosomes in which it has been shown that DNA in tumor-derived virtosomes can be readily passed to healthy cells and convert them to tumor cells. Virtosomes remain in solution after the preparation of Exs[22] and so are not measured. Additionally, bacterial and viral DNAs represent 3% of total cfDNA,[25,26] including tumor virus DNA released as cfDNA capable of use as a biomarker.[27] Hence, 4% of non-Ex DNA still needs to be accounted for. Healthy mitochondria are present in the bloodstream, possibly together with those released from platelets.[17–19,28] Nucleosomes may also be present[29] along with double-stranded cell-free DNA (dscfDNA) and single-stranded cell-free DNA (sscfDNA) in small amounts due to their release into the bloodstream from apoptotic cells. The dscfDNA will most likely be complexed with lipoprotein.[28] Hence, the remaining 4% of non-Ex DNA is readily accountable.

Non-exosomal RNA

It appears to be no similar study for total versus Ex RNAs. Grunt *et al*.[30] did develop an analytical approach of miRNA extraction measuring either cell-free or Ex miRNAs in plasma, serum and other bodily fluids. However, they did not give a percentage distribution.

Exosome removal from the bloodstream

About 1.5 L of blood flows through the liver, spleen, kidney and lung each minute. Since the average human blood volume is ~5 L, the total volume of blood can be calculated to pass through these structures in an average of 3–4 min.[31] This results in blood flow through the body about 350 times daily. It follows that, during this time, Exs can be removed by the liver, spleen, kidney and lungs as was demonstrated by Sun *et al*.[32] For the liver, lungs and spleen just 1 h after intraperitoneal injection of fluorescent Exs. Similar results with fluorescence-labeled Exs were subsequently identified by Takahashi *et al*.[33] on *in vivo* injection when they were demonstrated to have a half-life of about 2 min due to being taken up by various tissues - primarily the spleen and lungs. A further study exploiting hexamethylpropyleneamine oxime (HMPAO)-labeled Exs in mice was developed by Hwang *et al*.[34] (99m)-Tc HMPAO is a scintillation compound that is normally used to determine brain blood flow. However, in this case, the study permitted the uptake of Exs primarily by the liver and spleen. An alternative approach was exploited by Morishita *et al*.[35] who employed B16-BL6 cell ^{125}I-labeled Exs *in vivo* injected into mice. Four hours after injec-

tion, the radioactivity was found in the liver (28%), spleen (1.6%) and lungs (7%).

It appears that the macrophage populations are major players in the removal of Exs and are present in all three organs. In the liver, they are referred to as Kupffer cells and as discussed by Khier & Gahan,[28] are capable of removing mitochondria,[36–38] nucleosomes,[39,40] and chromatin[41] from the blood in addition to Exs. In demonstrating Ex removal, Imai *et al*.[42] exploited B16BL6 Exs labeled with the lipophilic, red fluorescent dye PKH26. Such Exs were taken up by the macrophages in both the liver and spleen but not in the lungs, where they were found to be present in the epithelial cells. Depletion of macrophages from the liver Kupffer cells was made by injecting clodronate-containing liposomes. When there was a subsequent injection of 16BL6 exosomes labeled with PKH26, much slower clearance occurred from the blood i.e., in the absence of macrophages. Thus, 4 h after injection, measurement of luminescence showed that the signal intensity of liver, spleen, and lung in macrophage-depleted mice was increased by ~29-, ~26- and ~15-fold, respectively, compared to untreated mice. An injection of either 1.25, 2.5 µg, or 5 µg Exs into healthy mice ended in their total clearance from the blood. However, upon macrophage depletion, the serum contained 285 times the number of Exs that were found in the undepleted serum. Luo *et al*.[43] were also able to demonstrate the uptake of AML12 cell-derived Exs by hepatic stellate cells, which they exploited for CRISPR/dCas9-VP64 delivery. It was also noted that the Exs were also taken up by the lungs and to a lesser extent by the spleen. Thus, the system for uptake of Exs by these organs is through endocytosis, though clearly, not all of them will be destroyed dependent upon the membrane signal carried by them.

cfDNA removal from the bloodstream

CfDNA can be readily removed from the blood. ^{35}S-DNA *in vivo* injected uptake by the liver lysosomal system was first demonstrated by Watteaux *et al*.[44] Analysis of an isolated liver showed 60% of injected ^{35}S-DNA to be present at animal death, with a reduction of ~45% at 30 min and a further ~20% at 2 h. On centrifugation, the DNA was found to precipitate together with caspase C activity, indicating its presence in lysosomes. Further treatment with Triton WR1339 led to the separation of two fractions corresponding to the lysosomes and an endosomal fraction. Given that the hepatocyte nuclei were labeled, it was concluded that they were responsible for uptake of the DNA. Earlier studies on the uptake of sscfDNA during liver perfusion indicated its attachment to specific DNA binding sites on the surfaces of both Kupffer cells and endothelial parenchymal cells, with no association with the hepatocytes.[45] However, DNAse treatment resulted in a rapid elimination of the single-stranded DNA, indicating that a part of the DNA was cell surface-associated. This might involve DNA binding to surface receptors for DNA on both cell types. In a comparative study of the uptake and removal of sscfDNA and dscfDNA by the liver, sscfDNA was demonstrated to be more readily taken up than dscfDNA with its breakdown products being excreted at a faster rate.[45–47] The varying fragment sizes were eliminated at similar rates.[45] Thus, it is possible that a portion of the non-Ex DNA will be removed continuously from the blood. However, Exs will also be removed, a situation that could delay the identification of metastases by Ex nucleic acids.

Techniques

In the following, the single steps of Exs and nucleic acid preparation are described and summarized briefly in Figure 1.

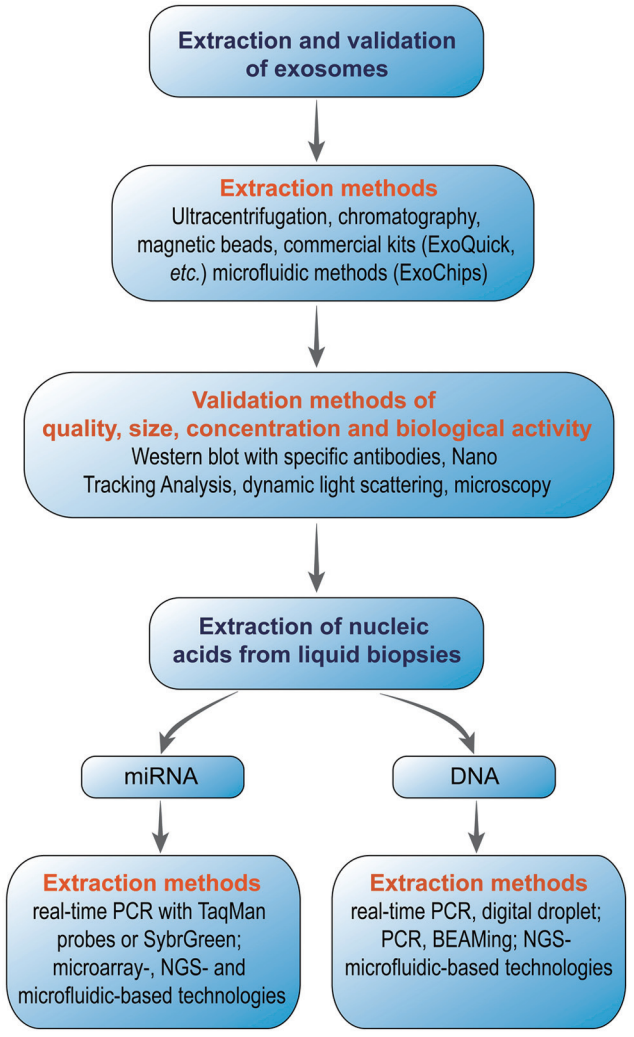

Fig. 1. Summary of the exosome- and nucleic acid-based technologies.

Exosome preparation

To date, a variety of methods have been described to isolate Exs: differential ultracentrifugation, polyethylene glycol (PEG)-based precipitating agents, size exclusion chromatography (SEC) and capture methods that employ many Ex surface proteins.[48] Table 1 shows a short summary of the most used Ex extraction methods together with their advantages and disadvantages. The most widely used laboratory technique is differential ultracentrifugation, which first removes dead cells, cell debris and large-size EVs in a series of low-speed centrifugation steps, and then separates pure EVs including Exs at a higher speed of a centrifugal force of 100,000 g. The performance of this procedure is time consuming taking more than 10 h and requiring an expensive ultracentrifuge. Also, the final ultrahigh speed is suspected to adversely affecting the quality and activity of EVs.[49]

For many years, polymer precipitation approaches have been used to precipitate EVs. Historically, PEG, a hydrophilic polymer, has been mostly used as a precipitating agent for EV extraction. In a few steps, the polymer is mixed with the sample, incubated for

Table 1. Common exosome extraction methods[48]

Technique	Equipment/Chemicals	Benefits	Drawbacks
Differential Ultracentrifugation/Density gradient Ultracentrifugation	Ultracentrifuge	High sample throughout/ High separation efficacy	Expensive instrument/Time-consuming/ Long running time/Impact on quality and activity by the high speed
Immunomagnetic beads	Magnetic beads antibodies	No need for an expensive instrument/Quick and simple method steps	High chemical costs/Low capacity/Low yields
Exoquick/Exoprep/Total Exosome/Isolation Reagent	Kits	Quick and simple method steps/ For small plasma/serum proteins	Expensive kits/High content but contaminated with protein
PEG-based precipitating agents	PEG	Corresponding to Exoquick but much cheaper	
Chromatography/SEC	Gel filtration columns	High purity/Similar size/Separation of large and small molecules	Extensive method/Small sample volumes

a short time period and centrifuged at low speed. However, along with Exs, PEG-based polymeric reagents also co-purify protein complexes that can disrupt downstream analyses.[30,50] Among other commercial kits, ExoQuick (System Biosciences), Total Exosome Isolation Reagent (Invitrogen) and Exoprep (HansaBioMed) are based on PEG. To overcome the inconvenient co-precipitation of protein, we developed a new technique that is based on a mannuronate-guluronate polymer and entraps Exs in a polymeric net to avoid co-precipitating proteins.[30,51]

SEC is based on the separation of large and small molecules on a gel-packed column (e.g., qEV separation columns, EVSecond purification columns, or Exo-spin exosome purification columns). Macromolecules cannot enter the gel pores and therefore, are eluted through the gaps between the porous gels with the mobile phase, whereas small molecules enter the gel pores and are eluted by the mobile phase. Although the isolated Exs have a uniform size and their biological characteristics are not adversely affected, they can be impure by contamination of other particles of a similar size.[52]

Immunoaffinity chromatography separates and purifies Exs from serum, plasma and urine or other fluids by specific binding with antibodies. The binding affinity is dependent on the specificity of the antibodies, elution conditions and matrix carriers. Target proteins are commonly present on the surface of Exs, such as tetraspanins (CD9, CD63, CD81, CD82), heat-shock proteins (Hsp60, Hsp70, and Hsp90), or specific cancer-associated markers. By labeling the columns with specific antibodies, this method has high specificity and sensitivity, as well as high purity and yield. Enzyme-linked immunosorbent analysis uses microplates to enrich Exs from body fluids.[53]

There are numerous commercially available kits based on the traditional extraction platforms described above including exoEasy Maxi kit (Qiagen) or MagCapture™ Exosome Isolation Kit PS (Wako). The advantages of these kits are short preparation time and high yields. However, these kits are expensive and the purity of Exs is not very high. A recently developed method with a high efficiency exceeding 70% uses micro-vortex chips packed with Morpho Menelaus butterfly wings and modified with lipid nanoprobes. By passing the Ex isolation fluid through the chip, the generated micro vortices increase the interaction force between the EVs and butterfly wings.[54]

However, these techniques have different disadvantages, such as high acquisition costs, long preparation times, low yields and impurity by contaminations. The recent application of microfluidic technology is a promising method because it addresses these shortcomings and involves the entire analytical process on a single chip. The chip integrates separation, purification and detection of Exs with a high efficiency and also allows a high-throughput of the liquid samples. Microfluidic chips apply the filtration method using nanomembranes or nanowires. For example, the Exosome Total Isolation Chip can separate Exs from small (10–100 μL) volumes of various samples including urine, plasma, serum and cell culture medium in less than 3 h.[55] In addition, the acoustic microfluidic technology is a label-free Ex separation method and applies acoustic waves with a high repeatability. In this respect, Wu *et al*.[56] developed an Ex separation module that integrates acoustics and microfluidics, and could obtain Exs from an EV mixture with a recovery rate and purity of 82.4% and 98.4%, respectively. To date, several microfluidic devices have been developed, which are based on either electrical fields or viscoelastic flow or immunoaffinity and described in detail by Wu *et al*.[55]

To improve the efficacy of the Ex preparation, a combination of different isolation methods can also be applied to enhance both efficiency and enrichment to obtain the ideal Ex fraction. Ryu *et al*.[57] performed ultracentrifugation prior to the use of polymer-based precipitation kits to isolate small EVs from human serum in large sample-based translational researches. Quality, size, concentration and biological activity of the isolated Exs can be examined by Western blotting with specific antibodies against Ex surface protein markers, Nanoparticle Tracking Analysis, dynamic light scattering and confocal microscopy. Based on their origin, Exs have specific markers that can be used to separate cancer-associated Exs from normal Exs using an affinity selection approach.[48]

Fluorescence-activated cell sorting (FACS) can be applied for the enrichment of cancer-associated Exs. The procedure is based on the high binding affinity of streptavidin to biotin. Streptavidin dynabeads or magnetic beads coupled to a primary biotinylated antibody specific for tumor-associated Ex markers (e.g. EpCAM) are incubated with the Ex solution and stained with a fluorescent dye such as fluorescein isothiocyanate or Alexa. The fluorescently labeled cancer-associated Exs are FACS-sorted and separated from normal non-fluorescent Exs.[48] Recently, the tri-channel electrochemical immunobiosensor was used for enzyme- and label-free detection of carcino-embryonic antigen, neuron-specific enolase and cytokeratin 19 fragments (Cyfra21-1) from Exs for the specific early diagnosis of LC.[58]

microRNA extraction and quantification

The release of the Ex cargo containing among others different

forms of RNAs and DNA into the solution occurs by the lyses of Exs. Quantitative PCR (qPCR) using either TaqMan probes or SybrGreen are the gold standard methods for extracting miRNAs. These methods produce fluorescence signals proportional to the amount of the generated PCR product. qPCR using SybrGreen has lower specificity than PCR using TaqMan probes due to the non-specific binding of SybrGreen to DNA. The specific DNA binding activity of the TaqMan probes relies on the fluorescence energy resonance transfer and 5′-nuclease activity of the Taq-polymerase.[59] qPCR-based TaqMan arrays are commercially available and mounted with 48, 96, 384, etc. miRNAs. Other microarray-based technologies rely on RNA hybridization to specific probes. This may cover thousands of mature human miRNA sequences listed in numerous miRNA databases (e.g., the Sanger miRNABase). The earlier frequent use of Northern blotting to detect both mature and precursor forms of miRNAs is seldom applied nowadays since this technique has a low sensitivity and requires high amounts of starting material. It is comprised of small RNA molecule separation by gel electrophoresis, their transfer from the gel to a membrane, followed by their hybridization with labeled probes.[48]

To profile a large number of different miRNAs, next-generation sequencing (NGS) has been extensively employed. This involves amplification of adapter-ligated sample RNA and cDNA libraries along with a following sequencing step of the PCR products. The output data deliver sequencing reads of varying lengths identifying a broad range of miRNAs to allow the identification of known or unknown miRNAs. The shortcomings of this technique are potential miRNA sequence biases introduced by constructing the library, the high costs of the technical platform and the demanding computational analyses of the extensive data output. However, numerous companies offer NGS analyses.[60]

Microfluidic devices can also be used for miRNA extraction and are based on either electrochemical or colorimetric or optical biosensors or molecular techniques. They have several advantages over common miRNA extraction methods including low sample volume, short preparation time and no need for an expensive laboratory equipment. There are various compositions of microfluids. They can be combined with either optical biosensors or fluorescent antibodies or probes detecting particular target miRNAs and identified by fluorescence imaging. In this respect, a fluorescent biochip combined with microfluidic channels was developed for the detection of miR-21 in LC. This approach includes extraction, amplification and detection of miR-21 found in EVs in plasma.[61]

DNA extraction and quantification

After lysing the Exs, DNA can be extracted by a variety of commercial kits (e.g., Qiagen, Machery Nagel). qPCR is a commonly used method for amplifying DNA. It uses specific probes that hybridize to mutated DNA and emit fluorescent light. The intensity of the fluorescence is measured in every cycle. Based on the number of cycles and a threshold fluorescent signal, the ctDNA samples can then be quantified. In this regard, the TaqMan qPCR is widely used method.[59,62] The cobas *EGFR* mutation test from Roche Molecular Diagnostics is a PCR-based assay for detecting early exon 19 deletions plus L858R and T790M mutations of EGFR.[63] Digital droplet PCR (ddPCR) is based on the microfluidic technology. It partitions 1 or 0 DNA molecules into droplets for amplification. The initial ratio of mutant-to-*wt* DNA is calculated by measuring the signal from each droplet combined with the use of the Poisson distribution.[62] Beads, emulsions, amplification and magnetics (BEAMing) uses DNA templates bound to magnetic microbeads before suspension into droplets. Optical scanning or flow cytometry are applied to quantify the DNA on the beads.[64] A large number of known and unknown DNA alterations can be detected by NGS. The most used NGS-based platforms are Illumina and Ion-Torrent (Thermo Fisher Scientific). Illumina simultaneously identifies DNA bases while incorporating them into a DNA strand, via four-color optical imaging of fluorescently labeled nucleotides. Rather than employing optical signals, Ion-Torrent applies a semiconductor to measure a change in pH referring to the release of an H+ ion following the addition of a nucleotide.[65] Furthermore, microfluidic and lab-on-a-chip technologies can be used for DNA extraction. Compared to the common isolation kits, microfluidic devices have several advantages such as high throughput, purity and sensitivity. This technology involves various extraction methods that are based on solid phase microfluidic chips with microchannels, miniaturized fluidic chips with silica membranes, or silica beads.[66]

Standard screening methods

Currently, LC screening methods mainly include chest X-ray examination, bronchoscopy, low-dose CT (LDCT), sputum exfoliative cytology and lung biopsy. Due to the high false-negative rates, the examination of early pulmonary lesions by chest X-ray is questionable.[67] A study by Bradley *et al*.[68] to determine the chest X-ray sensitivity in the detection of LC in symptomatic people involved a review of 21 studies present in databases including MEDLINE, EMBASE and the Cochrane Library. Many of these were considered to be of poor quality. Nevertheless, these workers suggested that the chest X-ray sensitivity for symptomatic LC is only 77–80%. Hence whatever the outcome, it is still necessary to use alternative (scanning and bronchoscopy) methods.

Bronchoscopy

Bronchoscopy is the initial diagnostic method for diagnosing potential LC patients. A bronchoscope is employed to both visualise and remove samples from suspected lung tissue and is essential for the diagnosis of airway lesions and parenchymal lung disorders. In a detailed review by Fullmann *et al*.,[69] its utility for the diagnosis of peripheral pulmonary nodules is shown to be limited. Since lung tissue is heterogeneous and affects the early detection of tumors, tissue biopsies may not be unambiguous.[70]

Sputum exfoliative cytology

As an alternative, sputum exfoliative cytology has a high specificity though only a reduced sensitivity.[71]

CT scan

A CT scan employs a rotating X-ray tube to scan body tissues from different angles. The measurements are then processed by computer to yield cross sectional images.[72] A major advantage over MRI is that CT scans can be performed on patients with metal implants whereas MRI scans are perturbed by them. Since CT scans can employ 100–1,000 times the X-ray dose as conventional X-ray imaging, care needs to be taken concerning the frequency of use with each patient as DNA damage can occur leading to radiation-induced cancer initiation with over-use (Radiological Society of North America, 2021, www.rsna.org).

The National Lung Screening Trial considered a LDCT (abdomen and chest), which has become the current standard for LC screening because of its high sensitivity for detecting lung lesions

but with the risk of radiation exposure.[73] However, LDCT chest scans have a high false-positive rate and current guidelines do not recommend this test for either patients without a smoking history or those who have not smoked for many years.[74] Considering these detection methods and that the majority of LC patients only present with symptoms at an advanced stage that are accompanied by high mortality, improved sensitivity and specificity of LC screening are required. A step in the right direction is provided by computer-assisted detection and diagnosis (CAD) systems.[75] These are important in image interpretation, effectively improving both detection accuracy and consistency of pulmonary nodules in chest X-ray and CT.[76] A very important characteristic of CAD systems concerns the false positives rate. Their application is an important part of lung nodule detection and hence, an important apsect of LC detection leading to early treatment.[77,78] As well as the use of CT for general screening, either alone or linked to CT-guided biopsy (CT-gb), is also a useful CT application. Chang *et al*.[79] CT-gb used with 94 nodules ≤ 8 mm had a sensitivity of 87.1%, a specificity of 100% and a diagnostic accuracy of 90.4%.

PET scan

PET scans allow the three-dimensional (3D) measurement of either molecular or metabolic activity in a tissue through positron-derived emssions from an initially injected radioactive molecule of known biological properties. This tracer emits positrons that, on breakdown, yield two photons that are detected by the scanner and hence, the organ localization of the activity. Thus, cell metabolism can be visualized by functional imagery rather than just structural imagery with tumor cells offering greater activity than healthy cells.[80]

PET-CT scan

PET and CT have been combined to form a combination of the two approaches of X-ray and radioactive material emission whereby the two images are combined into one image. Nevertheless, technological limitations of PET-CT include metastatic lesions < 1 cm may look 'cold' on the PET-CT image as the result of the scanner resolution whilst some lesions of only micro-metastases, low metabolic neoplasms and well differentiated tumours may be determined as benign.[81] ^{18}F-fluorodeoxyglucose (FDG), a radiolabeled glucose analogue, has significantly advanced the involvement of radiology in managing LC. Malignant tumors may have increased glucose metabolism that permits the ready uptake of FDG, detectable as hot spots. Nevertheless, either small tumors or bronchoalveolar type tumors frequently have lower standardised uptake value.[81–83] Gould *et al*.[84] made a meta-analysis in which FDG-PET was shown to permit diagnosis of malignant pulmonary lesions with an estimated sensitivity of 94.2% and specificity of 83.3%. This use of FDG-PET-CT currrently has an important and central role in both diagnosis and the management of LC.

MRI scan

MRI is a non-invasive imaging technology producing 3D, detailed anatomical images. It is based on excitation and detection of a change in the direction of the rotational axis of protons present in the water of living tissues. MRIs exploit a strong magnetic field forcing protons in the body to align within the field. A radiofrequency current pulsed through the patient results in the protons present spinning out of equilibrium against the pull of the magnetic field. On removing the radiofrequency field, the protons realign with the magnetic field and MRI sensors detect the energy released. The time taken for protons realigning with the magnetic field plus the energy released alters as a function of both the environment and the molecules present to reveal the various tissue types.[85] A major advantage of MRI over CT/CT-PET is that the patient is not irradiated and so follow-up scans are not likely to be detrimental to the patients health. In a study of 10,000 healthy individuals for LC detection, Wu *et al*[28] found that contrasted MRI scans could detect peripheral one-third parenchymal vascular markings of as small as ~2.0 mm in diameter. This was better than the predicted ~3.0 mm diameter and better than non-contrasted MRI scans (~5.0 mm diameter).[86]

Exosomal nucleic acids

There has been growing attention in combining clinical and radiological data with molecular data. Thus, advances in the development of new biomarkers, such as the investigation of Ex nucleic acids are indispensable. As described above, the upregulated levels of circulating Exs and their disease-depending contents have elucidated their potential role in guiding diagnosis of LC patients.[87] The similarity of their Ex ncRNA pattern with the pattern of the cell of source may be useful as a screening test for the early detection of LC. Therefore, it is interesting to investigate whether Exs and their nucleic acids analysis might contribute to the improvement of early LC screening that includes all high-risk group.

Exosomes for detection of early-stage LC

Cancer develops from non-controlled proliferation of cells that harbor an altered expression and activation of cell cycle genes. High amounts of Exs are released by this high cell proliferation rate. This allows increased Ex transfer from cell to cell to propagate cell transformation and tumor growth.[7] The upregulation of Ex levels has been detected in different body fluids of LC patients, suggesting that the increasing Ex levels may play a role in the development of LC and their measurement to be an additional tool for screening LC.[87] In addition, a lipid profile of Exs from peripheral blood was shown to differ between early and late-stage NSCLC patients.[88]

Exosomal miRNA biomarkers for detection of early-stage LC

To date, numerous studies on extensive investigation of Ex ncRNAs have been performed (Table 2). Recent studies have indicated that Ex miRNAs participate in the occurrence and development of LC and are, therefore, potential tumor biomarkers for early diagnosis of NSCLC.[60,89,90,91,92–103]

Since 2009, miRNA presence has been considered a potential biomarkers in NSCLC because miRNA isolated from blood and Exs of NSCLC patients reflect the development and progression of the primary tumor. As summarized in Table 2, several Ex miRNA panels have been suggested for LC diagnosis isolated from different body fluids. In this respect, Wu *et al*.[104] showed that the H1299 human lung adenocarcinoma cell line secreted Exs containing miR-96 that inhibited the expression of the tumor suppressor gene LIM-domain. LC adenocarcinoma cell line secreted Exs containing miR-21 and miR-29a that bound to the Toll-like receptor TLR8 in immune cells and triggered the activation of NF-κB and the secretion of inflammatory cytokines, so supporting tumor growth.

In LC patients, a combination of Ex miR-151a-5p, miR-30a-3p, miR-200b-5p, miR-629, miR-100 and miR-154-3p could discriminate these patients from granuloma patients with a sensitivity of

Table 2. miRNA Biomarkers

miRNAs	Cancer subtype	Ref.
miR-9-3p, miR-205-5p, miR-260, miR-1169	NSCLC	92
miR-20b-5p, miR-3187-5p	NSCLC	91
miR-21, miR-25, miR-155, miR-210, miR-486	NSCLC	93
miR-21, miR-155, miR-205	NSCLC	94
miRNA-96	NSCLC	95
miR-125b-5p, miR-5684	NSCLC	96
miR-146a-5p, miR-486-5p	NSCLC	97
miR-210-5p, miR-1269a	NSCLC	90
miR-1246	NSCLC	98
miR-3182	NSCLC	99
miR-15b-5p, miR-10b-5p, miR-320b	SQCLC	60
miR-7, miR-17, miR-19, miR-21, miR-126, Let-7a	ADC	100
miR-21-5p, miR-126-3p, miR-140-5p	ADC	101
miR-29c-5p, miR-103b, miR-877-5p	ADC	102
miR-30e-3p, miR-30a-3p, miR-181-5p, miR-361-5p	ADC	60
miR-342-5p, miR-574-5p	ADC	103

96% and specificity of 60%.[105] The panel of Ex miR-9-3p, miR-205-5p, miR-210-5p, miR-1269a could differ NSCLC patients from healthy controls with an area under the curve (AUC) of 0.91, a sensitivity of 77% and a specificity of 89%.[90] The panel of Ex let-7b-5p, let-7e-5p, miR-23a-3p and miR-486-5p identified early-stage NSCLC patients with a sensitivity of 80% and a specificity of 92%. miR-181b-5p and miR-361b-5p were mainly expressed in ADC patients whereas miR-10b-5p and miR-320b expressed in SQCLC patients.[60] Other diagnostic biomarker candidates in early-stage NSCLC are Ex miR-20b-5p and miR-3187-5p.[91] Interestingly, Ex miR-23b-3p had a diagnostic efficiency with receiver operating characteristic of 0.915, which was much higher than those observed for the classical serological markers CEA and CYFRA 21-1.[106] In addition, the ratio of Ex miR-21/Let-7a discriminated LC patients from healthy individuals with a sensitivity of 56% and specificity of 100%, and from patients with pulmonary benign nodules with a sensitivity of 56% and a specificity of 82.6%.[107]

In pleural effusions, Ex patterns can also be used to detect LC. So, miR-205-5p and miR-200b of 254 miRNAs could differentiate malignant effusions from those of pneumonia and tuberculosis patients.[108] In addition, the Ex miR-182 and miR-210 pair could differ malignant pleural effusions from benign ones with an AUC of 0.87 and 0.81, respectively.[109]

The novel developed point-of-care device detected even miR-205 in saliva and urine suggesting the diagnostic utility of non-invasive assays.[110]

Exosomal lncRNA biomarkers for detection of early-stage LC

As summarized in Table 3,[111–115,116] Ex lncRNAs have been analyzed more rarely than Ex miRNAs (Table 2). For example, the utility of Ex MALAT-1 as a diagnostic biomarker in NSCLC has been reported. Its upregulation promotes cell proliferation.[111] Interestingly, Ex linc01125 can distinguish NSCLC patients from disease-free and tuberculosis individuals with an AUC of 0.662 and 0.624, respectively.[112] Both in a testing and a multicentric validation cohort, higher levels of Ex RP5-977B1 were detected in NSCLC patients than in healthy controls and the diagnostic capability was confirmed in early-stage NSCLC patients. The AUC of 0.8899 was superior to that of the tumor markers CEA and CYFRA21-1.[113] TBILA and AGAP2-AS1 may also be promising biomarkers for the diagnosis of NSCLC. Notably, the combination of both Ex lncRNAs and Cyfra21-1 improved the diagnostic accuracy for NSCLC patients.[114] In NSCLC patients, GAS5 was downregulated compared with healthy controls. Ex GAS5 could distinguish stage I NSCLC patients from the normal controls with an AUC of 0.822, while combined with CEA with an AUC of 0.929.[115]

Exosomal circRNA biomarkers for detection of early-stage LC

As listed in Table 4, there are only a few studies assessing the role of Ex circRNAs in the early detection of LC.[117,118,119] For example, a panel of Ex circ_0047921, circ_0056285Xian and circ_0007761 could differentiate NSCLC patients from healthy controls as well as from patients with pulmonary disease.[117] In addition, a panel of four Ex circ_0001492, circ_0001346, circ_0000690, and circ_0001439 was upregulated in plasma Exs from early-stage lung ADC patients and could discriminate these patients from healthy individuals.[118] Finally, circRNA-002178 was upregulated

Table 3. lncRNA Biomarkers

lncRNAs	cancer subtype	Ref.
MALAT-1	NSCLC	111
linc01125	NSCLC	112
RP5-977B1	NSCLC	113
SNHG15	NSCLC	116
TBILA, AGAP2-AS1	NSCLC	114
GAS5	NSCLC	115

Table 4. circ_RNA Biomarkers

Circ_miRNAs	Cancer subtype	Ref.
circRNAs, circ_0047921, circ_0056285, circ_0007761	NSCLC	117
circ_0001492, circ_0001346, circ_0000690, circ_0001439	ADC	118
circRNA-002178	ADC	119

in Exs from plasma of ADC patients and these Exs entered $CD8^+$ T cells to induce expression of programmed cell death protein 1.

Exosomal ctDNA for detection of early-stage LC

To date, in contrast to Ex RNAs, the literature search has not found any study that analyzed specific mutations in Ex DNA of LC patients. During cancer initiation, the Ex cargo is significantly modified and can act as a diagnostic biomarker. Essential for the early detection of LC are mutations or translocations in *EGFR*, *KRAS* and *ALK*. Given that Exs contain >90% of cfDNA,[20] their DNA might reasonably include these cancer-specific mutations. Whole genome sequencing revealed that Exs contained the entire genomic double-stranded DNA comprising all chromosomes with mutated KRAS and p53 DNA in the serum of patients with another tumor type, namely pancreatic cancer.[120]

Exosomal proteins for detection of early-stage LC

In their study, Zannetti-Domingues *et al*.[121] showed that EGFR protein is loaded in Exs. The receptor is involved in the biogenesis of specific EV subpopulations and influences the uptake of Exs by recipient cells. The *wt* and mutant EGFR as well as its downstream signal molecules are expressed in LC Exs released from varying sources.[122,123] Huang *et al*.[122] found that about 80% of Exs from LC patients were EGFR-positive, whereas only 2% of Exs from chronically inflamed lung tissue were EGFR-positive. These findings show that EGFR protein might be useful for the early detection of LC. In addition, it may be interesting to investigate whether Exs contain mutated EGFR DNA.

Discussion

The diagnosis of LC at a sufficiently early stage remains a main hindrance to improving the overall survival rate of these patients. The various standard screening methods are the current methods of choice, especially given that using either CT/CT-PET or MRI can identify nodules as small as ~2mm diameter.[86] The current review considered the possibility of the exploitation of circulating nucleic acids as offering a new and more sensitive approach to achieve an earlier identification of the presence of LC. Such nucleic acids are found circulating in bodily fluids primarily via Exs of which there are two forms, those released from healthy cells and those from tumor cells. In particular, the tumor-derived Exs differ in the protein composition on their membrane surface from normal Exs. Various methods have been described permitting the isolation of the tumor cell Exs containing the relevant marker nucleic acids. It is clear that there needs to be technical improvements to increase the sample purity and to enable a better isolation of the relevant markers nucleic acids. Nevertheless, the currently available methods have permitted the isolation and purification of a number of both ctRNA and ctDNA early markers of the presence of LC. If such markers are to be exploited, then they should be able to be detectable in molecular numbers lower than those found with nodules as small as ~2 mm diameter that are identifiable by standard screening methods. In a theoretical study of apoptotically released ctDNA from an epithelial cancer cell, Fiala and Diamantis[124] used a model system in which they considered a tumor measuring 12.5 mm diameter, weighing 1.0 g and yielding a volume of 1.0 mL. This size was chosen as it was smaller than palpable and scan-identified tumors at that time and because it represents dimensions of tumors of epithelial origin i.e., some 85% of all human tumors[125] and may have already developed an independent blood supply. If DNA is released from such a tumor, then the ctDNA content will be 0.1% of the total DNA and contain ~6 cancer genomes per 4.0 mL plasma. In addition, given its size, the total cell number would be ~10^8. Given that some tumor cells become apoptotic, it will result in the release of ~36 × 10 ctDNA fragments into the bloodstream, each with a mean of 167 bp of which, perhaps, only two fragments will be mutated.[126] This led to the conclusion that there will be only a very low number of mutant fragments present in the cfDNA so representing a lower limit of ctDNA to identify.[124] To compare this calculation with screening-sized nodules of ~2 mm means dividing the number of released ctDNA molecules by five resulting in just 0.4 of a 167 bp fragment. Hence, although methods exist for the detection of single mutant NA fragments,[127] the amount of ctDNA available at a very early LC stage may not be enough to better the standard screening approach. Indeed, given that some DNA is removed by the liver, in particular, this amount may be even lower.

Similar data do not appear to have been calculated for Ex RNAs but this would appear to be an alternative way forward in the quest for suitable NA markers to allow an early determination of the presence of LC. This is especially so using a panel of markers as suggested by a number of researchers. Thus, for miRNA a variety of panel compositions have been exploited[60,90,91,105] of which one panel yielded a specificity of 92% and a sensitivity of 80% [60]. In the case of lncRNAs, the use of Ex RP5-977B1 for the early detection of NSCLC patients yielded an AUC of 0.8899.[115] In addition, the current studies have a high heterogeneity concerning the experimental platforms through using too small and heterogenous patient cohorts and technologies, e.g., PCR or NGS. Therefore, a standardization of methodologies for an optimal biomarker search remains a necessity. International consortia are needed to validate sets of several biomarkers derived from different sources (e.g., plasma, bronchial lavage, sputum) of large homogeneous cohorts of patients or risk-groups with long-term follow-up.

The foundation of the European Liquid Biopsy Academy (ELBA) ITN Consortium is a step in the right direction. It includes seven academic and two non-academic beneficiaries and 12 partner organizations from 10 European countries. ELBA Consortium will combine new techniques, including ultra-deep and massive parallel Illumina sequencing, ddPCR and Nanostring technology, and test nucleic acid-based biomarkers in functional assays such as targeted NGS. Computational algorithms shall calculate which combination of biomarkers have the highest sensitivity and specificity for the detection of NSCLC.[128]

It is clear that while there are a number of possible early circulating marker mutated DNA fragments and RNA fragments for LC present in Exs, it is too early for their clinical use. Hence, no de-

liberate comparison of the specificity and sensitivity between the standard screening methods and the nucleic acid analysis has been offered. Although some RNA and perhaps, DNA markers can have a high specificity and sensitivity, no one marker has been validated under clinical conditions (e.g., fetal nucleic acid markers for fetal abnormalities).[129,130] The latter approach has been verified under strict clinical conditions in a number of hospitals in a number of countries and has now replaced amniocentesis in routine prenatal clinics.[129] To date, there are no similar data for LC markers. The automation and application of artificial intelligence for such nucleic acid analyses will be necessary for hospital use in order to reduce the human time initially involved as well as speeding-up the obtaining of the results.[129] It will be of interest to eventually compare hospital costs with respect to the two approaches.

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Conflict of interest

The authors have no conflicts of interest related to this publication.

Author contributions

Contributed to study concept and design (PBG and HS), acquisition of the data (PBG and HS), drafting of the manuscript (PBG and HS), critical revision of the manuscript (PBG and HS).

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