Lung Cancer Diagnosis

Subjects: Oncology

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Lung cancer is the leading cause of cancer-related deaths in North America and other developed countries. One of the reasons lung cancer is at the top of the list is that it is often not diagnosed until the cancer is at an advanced stage. Thus, the earliest diagnosis of lung cancer is crucial, especially in screening high-risk populations, such as smokers, exposure to fumes, oil fields, toxic occupational places, etc. Based on the current knowledge, it looks that there is an urgent need to identify novel biomarkers. The current diagnosis of lung cancer includes different types of imaging complemented with pathological assessment of biopsies, but these techniques can still not detect early lung cancer developments.

lung cancer diagnosis biomarkers

1. Introduction

Lung cancer is the most common cause of cancer-related deaths in North America and other developed countries. According to the 2020 special report on lung cancer, this disease is the most commonly diagnosed cancer and the leading cause of cancer death in Canada [1]. The impact imposed is highlighted by statistics reporting a higher number of Canadians dying of lung cancer than colorectal, pancreatic, and breast cancers combined. For instance, approximately 30,000 Canadians will be diagnosed with lung cancer, with a projection of 21,000 death in 2020. Globally, the cancer burden is projected to double by 2050, with lung cancer at the top of the list [1].

People die from lung cancer because it is often not diagnosed until the cancer is at an advanced stage. Detailed pathogenesis, effective early detection, and suitable drugs help in the effective therapy of lung cancer. Thus, the earliest diagnosis of lung cancer is crucial, especially in screening high-risk populations (e.g., smokers, exposure to fumes, oil fields, toxic occupational places, etc.) with an urgent need to identify novel biomarkers. Furthermore, accurate diagnosis is vital for the most suitable treatment of individual patients with lung cancer. Thus, there is an urgent need to identify sensitive and specific biomarkers for early diagnosis.

Currently, low-dose CT (LDCT) is routinely used for lung cancer screening. In addition, a trial (NELSON) has shown that this particular screening has a selectivity of 85% and a specificity of 99% compared to no screening [2]. A recent study showed that the overall false-positive rate reached 81% [3]. This very high number required additional imaging or testing to confirm the results.

2. Classification

As mentioned above, lung cancers are classified into two main histological types, SCLC and NSCLC [4]. SCLCs are aggressive lung tumors often caused by smoking and encompass 15–20% of all primary lung cancers. Interestingly, the gene amplification of the transcription regulators *MYC* is common in SCLC [5][6]. NSCLC can be divided into four subtypes: Lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), large-cell carcinoma, and bronchial carcinoid tumor. Among these, LUAD is the most prevalent subtype of NSCLC and the most common primary lung tumor. LUAD frequently arises among female non-smokers, a category often missed on screening. It adopts a histologically glandular pattern with activating mutations affecting driver genes such as the oncogenes *KRAS* and *BRAF* and the epidermal growth factor receptor *EGFR* [7][4].

3. Transition to Biomarker Applications

In practice, the cornerstones of lung cancer assessment are radiology and tissue biopsies, as discussed earlier. Between missing early diagnosis, cost, and their risks, especially thoracic oncology biopsies, introducing the use of techniques as simple as a blood test provides a much safer and faster option. A review at the MD Anderson Cancer Center assessing cancer biopsies showed more than 17% adverse effects for thoracic biopsies [8].

3.1. cfDNA

This is a liquid biopsy that analyzes circulating free DNA (cfDNA) and CTCs via a non-invasive method, such as a routine blood draw or urine sample. cfDNA is released by normal cells and cells exhibiting pathologic processes (e.g., inflammation and neoplasia). Circulating tumor DNA (ctDNA) is a subset of cfDNA released by tumor cells that occurs through a combination of apoptosis, necrosis, and secretion. Analysis of the genetic alterations include point mutations, methylation patterns, chromosomal rearrangements, structural rearrangements, and copy number variations. Examples of cells contributing to cfDNA include the cells turning over due to: (i) Normal processes (e.g., lining of the gut), (ii) inflammatory events or other immune-mediated processes, and (iii) neoplastic phenomena. Thus, ctDNA is a tumor shed product. Normally, phagocytes clear cellular debris; however, this does not happen competently in solid tumors since cellular debris accumulates and is released into the blood [9]. Epigenetic screening is concerned with structural changes in chromosomal regions unrelated to DNA changes that mark altered activity states and show potential lung cancer diagnostic markers. DNA methylation and histone modification modulate gene expression that could influence early lung cancer detection [10].

Although the use of cfDNA showed promising results, further analyses of the published studies showed a different picture. For instance, a metanalysis comprised of 10 studies using cfDNA showed that a pool sensitivity of 0.8 was calculated with a range of 0.48 to 0.91 across the studies [11]. In the case of the specificity, a pool specificity of 0.77 was calculated, ranging between 0.47 to 1. Following this line of variation between the studies, an inconsistency of 86.6% and 93.4% was calculated by I-square [11]. These results signify that the pooled sensitivity and specificity of the studies are the result of heterogeneity rather than chance.

3.2. Blood Circulating Antigens

A number of antigens found in blood have been assessed over the years as potential biomarkers of lung cancer. The most studied biomarkers include CYFRA 21-1, carcinoembriogenic antigen (CEA), neuron specific enolase (NSE), and squamous cell carcinoma antigen (SCC-Ag). The following table is provided as an illustration of the sensitivities and specificities reported by clinical trials (**Table 1**).

Table 1. Sensitivity and specificity analysis of common antigens found in lung cancer.

Antigen Name	Type	Sensitivity (%)	Specificity (%)	Reference
CYFRA				
	SCLC	34	95	[12]
	NSCLC	49	95	[12]
	ND	43	89	[13]
	ND	85.1	88.3	[14]
	NSCLC	59	94	[15]
	SQC	68	94	[15]
	SCLC	19	94	[15]
	NSCLC	40	95	[16]
CEA				
	NSCLC	29	95	[12]
	ND	69	68	[13]
	ND	55	79.6	[14]
	NSCLC	42	95	[16]
SCC				
	NSCLC	17	95	[12]
	ND	35.6	71.2	[14]
	SQC	95	32	[17]
	NSCLC	19	95	[16]
NSE				
	SCLC	54	95	[12]

Antigen Name	Type	Sensitivity (%)	Specificity (%)	Reference
	ND	23.4	91.2	[14]

ons could
ed for the

analysis, such as differences in the ELISA kits from different suppliers, including the threshold of the antigen values set by the company. Taken together, it appears that a unique antigen biomarker is not valuable for diagnostic and likely, a multi-antigen approach should be considered in combination or not with other biomarkers.

3.3. Cell-Free DNA (cfDNA) and Circulating Tumor Cells (CTCs)

The first discovery of circulating DNA and RNA in the plasma of healthy and sick individuals commenced in 1948 [18]. This discovery was later acknowledged more than 30 years later when increased amounts were found in cancer patients. Throughout the decade of 2000–2010, studies implying a direct relation between cfDNA and cancer found an increase in the tumor size and the quantity of cellular debris [18]. It was also found that cfDNA exists at stable levels with a concomitant increase due to cell injury. Hence, cfDNA was proposed as a marker of cancer cell death. Efforts to use cfDNA as a diagnostic and screening biomarker have been shown to identify early-stage lung cancer. The detection of ctDNA in plasma depends upon cfDNA shed, which is calculated via the difference in rates between the release of DNA by tumor cells vs. the renal clearance. Among the key variables are the mitotic rate and tumor. For example, it would favor ctDNA detection when metastasis, bone, or the liver is involved. On average, the amount of cfDNA found in a normal person ranges 5–10 ng/mL [18]. In cancer patients, depending on the type of cancer and stage, the cfDNA concentration might range up to 50 times more than the normal concentration.

Another way to investigate the cfDNA is by the use of the polymerase chain reaction (PCR). A study examined the levels of plasma DNA in 84 patients with NSCLC, which was compared to 43 healthy blood donor controls [19]. This study stated that healthy controls could be distinguished from patients with lung tumors. Even in patients with stage 1A, the amount of cfDNA in plasma was significantly higher than in the control patients. However, another study that also measured cfDNA as a screening tool found that cfDNA could not distinguish differences in a cohort of approximately 1000 high-risk smokers, suggesting that progression to lung cancer could not be predicted. Although cfDNA might not be an effective marker for screening high-risk smokers, it could still play a role in diagnosing whether nodules identified by LDCT are either benign or malignant. Studies have revealed limitations towards the advanced use of biomarkers in the clinic to facilitate physician adoption as part of their standard of care, mainly validity and maintaining general mass use of biomarkers in liquid biopsies [9][20][21][22].

Another predictor for cancer development is the quantification of CTC. CellSearch can perform this quantification. This platform uses whole blood to evaluate CTCs of epithelial origin in extensive clinical studies for breast and prostate cancer as markers of response to therapy and indicators of prognosis. Studies have revealed that CTCs in the blood are associated with a decrease in overall survival in patients treated for metastatic breast, colorectal, or prostate cancer. Thus, CTCs offer the opportunity to capture and profile individual aspects of a patient's malignancy and have proven to be a vital cornerstone of precision medicine. Technical advances have now made it possible to detect and characterize single CTCs in the blood of patients. The identification of CTCs in the blood torrent platform measures the epithelial cell adhesion molecule (EpCAM) [23]. Non-EpCAM approaches for CTC

capture and quantification are also under investigation. For instance, a further classification of CTCs using markers of transition from epithelial to mesenchymal markers could be used to monitor the progress of the disease.

4. Liquid Biopsies Use in Lung Cancer

The use of liquid biopsies could be in the form of introducing lung cancer genetic, transcriptomic, and epigenetic screening biomarkers to determine potential high-risk subjects as a preliminary screening before the use of CT. Thus, early diagnostic using biomarkers could diagnose intermediate nodules identified by CT, leading to selecting subjects that need a surgical biopsy and saving others who do not need it.

Liquid biopsies have clinical applications in early detection, tracking primary and metastatic foci, assessing and monitoring treatment, and treatment resistance. However, they come with a setback regarding mass implementation in that they require complicated analytical methods to analyze. Nevertheless, projects like the FDA Sequencing Quality Control Phase II (SEQC2) project and the Blood Profiling Atlas in Cancer (BloodPAC) consortium have focused on these aspects [18].

Liquid biopsies allow for the non-invasive analysis of body fluids for DNA-shed products and aberrant circulating cells. They have also been assessed as a pillar in the precision medicine field, as genetic analysis provides quantitative feedback and monitors patient responses, enabling a more precise, personalized, and practical approach towards individualized treatment.

The ability to use these non-invasive methods of analyzing liquid biopsies, such as plasma, saliva, pleural effusions, CSF, or urine at the clinic, is considered technological progress in immune-oncology.

Extensively studying and subtyping NSCLC through genetic analyses enables molecular understanding, resulting in more effective therapeutic options, significantly reducing toxicity profiles through target treatment of NSCLC subtypes (e.g., *EGFR*, *ALK*, and *ROS1*). Unfortunately, not many patients use target therapies. Nearly 80% of cancer patients do not have genetic mutation results available during the initial consult with an oncologist, and approximately25% begin cancer treatment before receiving results. Molecular diagnostic companies offer rapid services to overcome this issue, where whole blood is shipped overnight to identify ctDNA mutations (in *EGFR* and *KRAS*) using commercially available droplet digital PCR (ddPCR), and the results are reported within 72 h [24].

One of the challenges in using liquid biopsies to detect mutations arises from the difficulty of very low-frequency mutation detection. This might happen after lung cancer surgery for a curative intent (i.e., a small, localized tumor) as the source of ctDNA shed was removed, due to the continued clearance of ctDNA by the kidneys. In these cases, postoperative blood potentially requires the detection of mutations of ≤0.1%. Therefore, the improvement of specificity and sensitivity of lung biopsies is a field of technological research that could be used as adjuvant therapy and cancer screening.

5. Applications of Biomarkers in Clinical Samples

The clinical samples available for biomarker measurements are:

5.1. Sputum

Although cytological examination of sputum is a helpful screening tool for early diagnosis of lung cancer, peripheral tumors, such as adenocarcinomas arising from the smaller airways, can be missed.

PCR techniques have been used for the possible detection of molecular biomarkers for early lung cancer. This was highlighted in a study performed on 15 patients from a project called The Johns Hopkins Lung Project (JHLP) [25] [26]. In this study, approximately 50% of the recruited patients (n = 15) with adenocarcinoma or large-cell carcinoma were detected by mutations in sputum cells before the clinical diagnosis (1–13 months) when traditional methods would have probably missed them.

Another gene of interest is the p16 gene, commonly inactivated or mutated in lung cancer [27]. The measurement of hypermethylation of the CpG islands in the sputum of lung cancer patients demonstrated a high correlation with early stages of NSCL cancer, suggesting that p16 CpG hypermethylation could be helpful in the early diagnosis of lung cancer.

The potential use of plasma microRNAs (miRNAs) as novel biomarkers for early detection of lung cancer has been studied. miRNA biomarkers also have the potential for lung cancer screening and early detection. These are non-coding RNAs with a length of 22 nucleotides targeting specific regions or mRNA sequences, usually found in the 3′ untranslated regions of mRNA, which either prevent translation or promote mRNA degradation and lead to down-regulation of particular genes. Being more stable than mRNA, miRNA used as a marker for lung cancer risk or diagnosis is more practical for clinical application.

Studies have shown that these miRNAs differentially circulate in plasma samples of lung cancer patients. These miRNAs include miR-155, miR-197, and miR-182, which have demonstrated high specificity and sensitivity to discriminate all cancer stages, including stage I of lung cancer, from cancer-free controls. Once validated in a large-scale clinical trial, these markers may be used as a non-invasive confirmatory screening test complementary to the LDCT screening procedure and used as a clinical test for monitoring and clinical follow-up of patients with lung cancer [24].

Several studies have explored the utility of miRNA-based biomarkers in sputum samples. miRNA profiles in the sputum could be used to identify NSCLC. More recently, studies were also able to identify and distinguish miRNA profiles that could be used in the early detection of SCC or adenocarcinoma. For example, an SCC signature of three miRNAs diagnosed the presence of a stage I SCC in patients' sputum. The adenocarcinoma signature composed of four miRNAs detected no overlap between the two signatures in sputum in patients with stage I adenocarcinoma. Seven different miRNAs were identified in these two signatures, and these miRNAs could be used as risk factors for lung cancer. In bronchial tissue studies profiling miRNA in pre-malignant airway lesions, 69 miRNA were found to evolve in high-risk patients from a pre-invasive stage to a higher stage in the multistep process of lung carcinogenesis [28].

Although airway miRNA expression may serve as an early detection biomarker, it is limited to bronchial biopsies of pre-malignant airway lesions [29].

microRNA and methylome in plasma or urine.

5.2. Bronchoalveolar Lavage (BAL)

Routine cytopathological analysis of bronchoalveolar lavage (BAL) specimens has been used as a common diagnosis over the years. Currently, BAL is another specimen sample where the use of molecular biomarkers for early diagnosis is exploited. BAL involves the infusion and respiration of a sterile saline solution in distal segments of the lung via a fiberoptic bronchoscope. Molecular markers including *p53* mutations, *KRAS* mutation, the methylation status of the CpG island of the *p16* gene, and microsatellite alteration were studied in BAL samples. In addition, a study examined a series of 50 resected NSCLC tumor patients and compared the tumors and BALs concerning those molecular biomarkers. With the possible exception of the test for microsatellite alteration, all trials had relatively high sensitivity, detecting mutant cells in the presence of a significant excess of normal cells. The results showed that *p53* mutations were predominant in squamous cell tumors, whereas the *KRAS* mutations were predominant in adenocarcinoma tumors. Except for microsatellite alterations, the exact genetic change in the BAL sample as in tumors was always found. Unfortunately, small, peripherally located tumor results were the least specific, representing tumors where early intervention would be of great value. Further studies using these markers are necessary to apply to populations with increased risk, such as smokers without lung cancer and survivors of previous cancer [30].

5.3. Peripheral Blood

As discussed before, the limited accessibility of lung carcinomas has led to efforts to identify tumor-associated soluble markers in more accessible and non-invasive samples like serum or plasma. With the development of DNA technologies and the use of PCR techniques able to detect nanogram quantities of DNA circulating in the blood, it was found that the plasma and serum of cancer patients are enriched four times in DNA compared to free DNA from normal controls [31].

A comparison of microsatellite alterations in tumor and plasma DNAs was made in SCLC patients. Results showed that 93% of the patients with microsatellite alterations in tumor DNA also showed modifications in the plasma DNA [32]. These results suggest that modifications of circulating DNA can be used as an early detection biomarker. Another type of modification in circulating DNA is related to aberrant DNA methylation. The hypermethylated DNA was found in all cancer stages, opening up the possibility of an early lung cancer detection marker. Other gene mutations like *p53* and *RAS* gene mutations settled as markers in the plasma and serum of patients of other cancers like colorectal and pancreatic malignancies have not yet been established in lung cancer [10]. In addition, gene-expression alterations in circulating white blood cells have been identified in lung tumors.

Although the identification of a gene-expression biomarker in the blood is desirable by measuring mRNAs in the blood, studies have been relatively limited because of RNA degradation, restricting the use of blood-based

transcriptomic biomarkers for early detection of lung cancer. For instance, a study analyzed gene expression in peripheral blood mononuclear cell samples of smokers with histologically diagnosed NSCLC tumors [33], and identified a signature of 29 genes that separate patients with and without lung cancer. Another study analyzed gene expression of lung tissue using serum RNA in whole peripheral blood collected using PAXgene blood RNA tubes. The study included patients with adenocarcinoma and controls to identify differential expression patterns of lung cancer genes that could be tested in blood to improve the identification of risky patients in the future. They showed that RNA-stabilized whole-blood samples could potentially be developed into a gene-expression-based classifier to discriminate between NSCLC patients and controls [31][34].

The stability of miRNA is attractive to explore because of its potential use as a blood biomarker for the early detection of lung cancer. Previous studies showed the applicability of miRNA in cancer diagnosis. For instance, a panel of seven miRNAs was differentially expressed in patients with cancer, as demonstrated by ultra-deep sequencing of blood samples from 10 patients with NSCLC and 10 healthy controls [35]. These results showed that miRNA signatures that predict lung cancer development and prognosis were identified.

When miRNA was analyzed in parallel to oncogene mutations, a better predictive occurrence of cancer was observed after finding a signature of six miRNA specific for lung cancer [36]. This finding is important because it connects a potential link between genetic damage and postgenomic control generated by the miRNA mechanism. This analysis is non-invasive, and both the miRNA and the oncogene mutations can be detected in the same sample. Moreover, deciphering oncogenic mutations, which might represent an individual signature, could be applied in the development of a personalized medicine in cancer prevention.

In summary, the use of miRNA is still questionable as more studies should demonstrate its applicability as a diagnosis of lung cancer.

The ELISA-based method for detecting the open reading frame 1 protein (ORF1p) in serum biomarkers can be used to identify patients at high risk of developing lung cancer based on LDCT findings. As such, ORF1p quantification in serum may provide a minimally invasive technique that can complement current lung cancer screening with LDCT [37].

5.4. Urine

Urine is seldom examined in the search for biomarkers. However, urine shows potential for use as a biomarker of lung cancer. Different analytes, such as a signature of volatile organic compounds (VOCs) and proteomic analyses, have been proposed as potential biomarkers for lung cancer diagnosis.

In the case of the VOC signature, it is expected that each individual would develop a unique signature. A study that aimed to assess the feasibility of VOC measurement to find biomarkers recruited patients with various lung cancer types. Urine samples were collected and analyzed using a urine cartridge sensor with an array of 73 spots [38]. Results showed that accuracies with sensitivities and specificities varied, with values of 36–95.5% and 60–97.6, respectively. These variations were obtained when different cancers were compared to the controls [38]. Although

VOCs are very promising, more studies are necessary to validate the test for clinical. Moreover, it is clear that the VOC signature will fluctuate according to the phenotype of the individual as well as the diet and ethnic group. Thus, all these factors would have a direct effect on the baseline of the VOCs. Another limitation of the study is the sample size, but preliminary results are encouraging.

Another recently evaluated test is the conversion of the antiviral FDA-approved drug amantadine. It has been reported that the drug amantadine is acetylated by the enzyme spermine spermidine N^1-acetyltransferase or SSAT-1 [39], and the levels of acetylated amantadine can be detected in urine samples as the acetylated product is not catalyzed anymore [40]. SSAT-1 is an enzyme upregulated in lung cancer, probably because of its function in the cell cycle [41]. The amantadine test is simple, and an individual is required to consume the amantadine pill and provide a urine sample (time = 0). After 2 h, a second urine sample is collected (time = 2 h). The measurement of acetyl amantadine levels in urine can be used to indicate the progress of cancer, and it is non-invasive (urine sample), simple, with clinical applicability for lung cancer diagnosis. For example, a receiver operating characteristic analysis showed an area under the curve = 0.689 when comparing lung cancer patients vs. healthy controls [40] in a clinical trial. Thus, the levels of acetylated amantadine could be used as a helpful and straightforward screening test for early diagnosis of lung cancer [39][42][43][44]. Other applications could be monitoring individuals working with carcinogenetic materials (e.g., asbestos), smokers, high-risk populations, and monitoring cancer recurrence after therapy.

Recently, the potential use of extracellular vesicles was proposed [45]. These vesicles were isolated from different body fluids, including urine, BAL, and serum of mice. The EpCAM levels were measured in these vesicles, as this protein has a role in tumorigenesis as a cell-cell adhesion mediator [46]. When mice were subjected to smoking, an increase in the vesicle concentration in BAL was observed, suggesting that the detection of vesicles in different body fluids might be potentially applicable for an early diagnosis of lung cancer. However, it is noteworthy that vesicles have not been tested in humans, and more studies should be performed for the validation of the test.

5.5. Metabolomics

Metabolomics data have the advantage of providing information on the levels of metabolites that can characterize the stage of the disease. Recently, the application of metabolomics for predicting cancer development has been reported in different fluids, such as serum, sputum, urine, and sweat, with promising results [47][48][49][50][51].

Metabolomic studies using serum showed that the discriminating metabolites aspartic acid and pyruvic acid differentiated individuals with lung cancer from healthy controls [48]. However, another study showed a different set of discriminating metabolites, such as glycerophospho-β-arachidonoyl ethanolamine and sphingosine with sensitivities and specificities of 77% and 93%, and 97% and 90%, respectively [50].

When sputum was analyzed to discriminate lung cancer patients from healthy controls, the metabolites cardiolipin (derivatives), hexanal, cysteic acid, and hydroxypyruvic acid were significant, with AUC ranging between 0.81–1.0 [47]. In sweat analysis, the trisaccharide MG (22:2), nonanedioic acid, and unidentified tetrahexose and trihexose

showed sensitivities and specificities of 80% and 79%, which were calculated with a false-positive and -negative factor of approximately 20% [49].

Lastly, hypothesizing that the level of SSAT-1 increased in lung cancer and taking into consideration that this enzyme is involved in the polyamine metabolism [52], it is reasonable to assess the polyamine pathways fluctuations. Thus, a panel of six metabolites corresponding to the polyamine pathway discriminated lung cancer patients from healthy controls with an area under the curve (AUC) = 0.97 [51]. Interestingly, an AUC >0.9 was measured when liquid biopsies corresponding to the early stage of NSCLC were assessed using five metabolites. These data allowed for the differentiation of stages I and II from healthy controls [53].

Although metabolomics is an emergent field in cancer diagnosis, more studies should be implemented to validate its use consistently. Many factors might contribute to heterogenic results when comparing metabolomics in the same fluid. Some of the fluctuations might originate from the time of sampling, daily changes of the metabolites, methods used for the analysis, including variations in the commercial kits or in-house analysis, stage of the lung cancer, and the individual size of the study. Taken together, the validation of metabolomics requires confirmatory studies for its use in clinical diagnosis.

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