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Review

Molecular Markers in Guiding Lung Cancer Diagnosis and Treatment

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Abstract

Background: Lung cancer has the highest mortality rates and one of the lowest 5-year survival rates amongst cancer types in the world. Although there are constant advancements in treatment, the overall prognosis for lung cancer continues to be poor. In order to achieve early detection and personalized targeted treatment, an effective method is needed to make prognostic and treatment decisions. **Methods**: A thorough literature search was conducted to identify tumor tissue, blood, and expired breath markers that have been discovered in lung cancer. Articles were chosen by determining main markers holding promise for future clinical use. **Results**: Data suggests significance in using tumor tissue markers as promising diagnostic, prognostic and predictive of treatment response and outcome. Epidermal Growth Factor Receptor (EGFR) and Anaplastic Lymphoma Kinase (ALK) and ROS-1 biomarkers can be used to decide to treat with EGFR-TKI and ALK-TKI, respectively. KRAS and p53 mutations suggest a likelihood of developing EGFR-TKI resistance. And c-MET is showing pertinence in predicting disease recurrence. Blood and expired breath markers are two more novel sources for biomarkers that is gaining more ground in lung cancer research. Circulating tumor cells (CTC) and DNA (ctDNA) were shown to be important markers in lung cancer prognosis and treatment response prediction. Circulating tumor cells suggest negative prognosis and increased likelihood of recurrence, while ctDNA data indicates use in treatment monitoring to help make decisions without keeping patients on disagreeable therapies. Volatile organic compounds (VOC) are the least studied, but investigators have noticed changes in VOC profiles between healthy and lung cancer patients. Blood and expired breath markers continue to be studied as these would be a welcome alternative to invasive biopsies. Recently there had been interest in using specific tumor biomarkers for imaging to localize tumors and determine disease progression. **Conclusions**: Years of research have elucidated multiple candidates as biomarkers found in tumor tissues, circulation, and even in exhaled air. Although more studies need to be performed on some markers mentioned in this review, such as EGFR, KRAS, ALK, and ROS-1, there is enough evidence for some use of these biomarkers to guide decisions in clinic, as well as evidence for promising future developments.

Keywords: lung cancer; biomarkers; predictive biomarker; prognostic biomarker

1. Introduction

Lung cancer is currently the leading cause of cancer-related death in men and women worldwide, with 1.8 million deaths being attributed to lung cancer in 2020 [1]. In the United States alone, there were an estimated 235,760 new cases and 131,880 deaths in 2021 [2]. 5-year survival rates are also low in comparison to other cancers at 21% overall. This is likely because late diagnosis is common, with most patients being diagnosed after the cancer has significantly advanced to later stages. Notably, 5-year survival for lung cancers diagnosed at early stage is vastly higher at 59%, which unfortunately only accounts for 17% of lung cancers [3]. In addition, more men than women historically have succumbed to lung cancer, due to a higher percentage of males with smoking history. Early detection and subsequent treatment are extremely important and can greatly improve lung cancer mortality. As a result, an effective method to detect lung cancer in early stages as well as to determine personalized treatment therapies, and predict treatment resistance or disease recurrence is needed.

Research in this field has elucidated the presence of tumor markers in the form of driver mutations, with treatments already developed for the commonly seen mutations. These include EGFR, ALK, KRAS, ROS-1, and many others. Circulating biomarkers such as circulating tumor cells or circulating tumor DNA can also predict metastasis or recurrence. A portion of the research is also now focusing on volatile organic compounds. Since lung cancer begins in the lungs, which are connected to the outside environment through our airway, VOCs are also being investigated as potential biomarkers.

Biomarker research has mainly focused on cellular markers, but the existence of imaging biomarkers must also be mentioned. Standard imaging performed during lung cancer treatment includes computed tomography (CT) scans, positron emission tomography (PET) scans, and magnetic resonance imaging (MRI) [4]. Through these scans, biological particularities within the primary tumor and metastases can be identified, however these are not specific for lung cancer. Fluorodeoxyglucose-F18 is an example of a suggested imaging biomarker in PET scans, by indicating tumor presence through glucose metabolism [4].

Imaging can also be used to detect lung cancer specific features within the tumor and metastatic patterns which are correlated to the common driver mutations [5]. While there is not enough evidence for imaging biomarkers to be used on their own for prognostic or therapeutic purposes, they may be useful in localizing tumor tissue during intervention. By modifying the chemo/immuno therapeutic agent to target specific tumors are currently under investigation and very possible future development that can be considered in precision or personalized therapy.

Current application of this field has been focused on the mechanism and potential benefits of lung cancer markers as prognostic and to predictive treatment response. It is also now known that there are differences in biomarker expression based on gender. We provide a comprehensive review discussing the molecular alterations seen in lung cancer detected through tissue, liquid, or expired breath methods which can be used as biomarkers guiding diagnosis and treatment in lung cancer.

2. Tumor Markers

A tumor marker is anything present in or produced by tumor cells that can provide more information about the cancer to help with diagnosis or staging, such as aggressiveness or responsiveness to targeted therapy. The use of tumor tissue markers, especially genomic markers, is already well-established in the field of lung cancer.

2.1 EGFR

Epidermal growth factor receptor (EGFR) is a member of the HER family of transmembrane tyrosine kinases. EGFR is activated by epidermal growth factor (EGF), which is a protein involved in signaling pathways that control cell division and survival. When EGF binds to EGFR, the receptor homo-dimerizes or hetero-dimerizes with other HER family receptors and activates its tyrosine kinase function. Activation of tyrosine kinase can then activate RAS and subsequently activate the RAS-RAF-MAPK pathway (Fig. 1). EGFR can also activate PI3K and the PI3K-AKT pathway [6]. Both pathways promote signaling pathways that increase cell proliferation and protein synthesis [7]. However, mutations within the EGFR gene can result in EGFR dysregulation. This leads to an oncogenic phenotype by mediating cell proliferation, increasing cell motility resulting in increased metastasis, and enhancing angiogenesis. EGFR is the most commonly mutated gene in non-squamous cell lung cancer (NSCLC). Mutations are mostly found within exons 18 to 21 of the EGFR gene, with the most important being deletions in exon 19 and point mutations in exon 21 [8]. Women tend to have higher frequency of EGFR mutations compared to men [9].

The knowledge of EGFR mutations has also led to the development of tyrosine kinase inhibitors (TKI), a small molecule inhibitor that blocks the binding of ATP which is crucial for signaling activity. There are currently two FDA approved EGFR-TKIs to treat NSCLC: gefitinib and erlotinib [7]. Lynch *et al*. [10] showed that the patients with the most dramatic response to gefitinib were patients whose cancers had EGFR mutations in exons 18–21.

It has been suggested that EGFR could be useful as a prognostic marker in post-operative NSCLC patients. In a study performed by Kosaka *et al*. [11], 397 patients with lung adenocarcinoma who underwent curative surgical resection were included. The study found that patients with EGFR mutations had a longer overall survival time compared to patients with EGFR wild-type. Jeon *et al*. [12] found that EGFR was an independent prognostic factor for post-recurrence survival using multivariate analysis (HR 0.552; $p = 0.013$). Similarly, D'Angelo *et al*. [13] enrolled 1118 patients over 8 years. He discovered patients with EGFR mutation had longer overall survival and a survival period of longer than 10 years.

However, there were also studies that did not support the use of EGFR as a prognostic marker [14].

Studies suggest that gender differences influence the status of EGFR as a predictive marker for EGFR-TKIs. A meta-analysis by Pinto *et al*. [15] observed gender differences in outcome measures of overall survival and progression-free survival in patients treated with EGFR-TKI compared with chemotherapy. The study found that women were less likely to die from NSCLC compared to men. The women treated with NSCLC also saw a 10% increase in progression-free survival compared to men. A separate meta-analysis confirmed women had a significantly higher overall survival compared to men after treatment with EGFR-TKIs [16]. These gender differences could be attributed to higher frequency of EGFR mutations or increased levels of estrogen receptors in women, but studies have been contradicting in their conclusions [15,17].

It is prudent to determine if a patient has EGFR mutation, as this can result in a treatment decision using EGFR-TKIs. The presence of an EGFR mutation in relation with the patient's gender can also elucidate the benefit of using EGFR-TKIs as a treatment.

2.2 KRAS

The Ras oncogene family, including KRAS, encodes for GTPases, which are responsible for hydrolyzing GTP to GDP. These G proteins are located on the intracellular side of the plasma membrane and bind guanine nucleotides. In a resting cell, Ras is bound to GDP and inactivated. Extracellular stimuli such as growth factors bind to the tyrosine kinase receptor and activate guanine nucleotide exchange factor to exchange GDP for GTP bound to Ras. Activated Ras-GTP then activates the RAS-RAF-MEK, JAK3, and PI3K-AKT pathways. Intrinsic GTPase activity of Ras hydrolyzes GTP to GDP and inactivates Ras-GDP. Activated Ras mutation prevents GTPase activity, leaving Ras stuck in its active configuration. This leads to constitutive activation of the downstream signaling pathways, driving tumori-

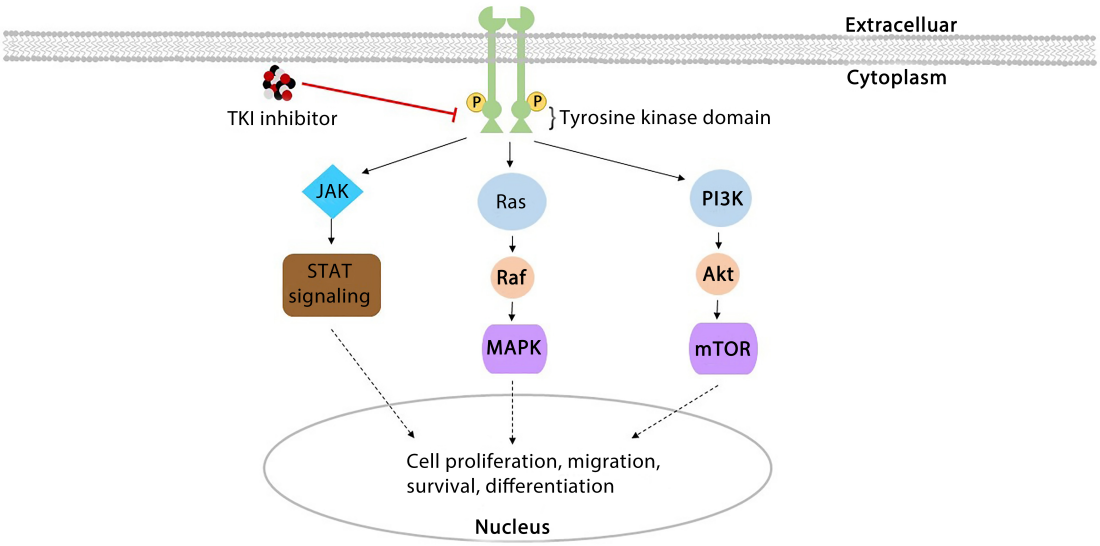

Fig. 1. Simplified cartoon showing signaling pathways of cell proliferation and site of Tyrosine Kinase inhibition (TKI).

genesis. In NSCLC, KRAS mutations are mainly found in exon 2, codon 12 or 13 and account for 30% of NSCLC cases in patients with smoking history [7]. KRAS mutations are mutually exclusive with ALK and EGFR. Most treatments for KRAS-mutant NSCLC targets the downstream signaling pathways as it was previously thought to be too difficult to directly target KRAS. However, in the recent year, several novel inhibitors directly targeting KRAS have emerged. Although these inhibitors are not yet FDA-approved, several have already entered Phase 1 clinical trials [17].

For many years, the presence of KRAS mutation in NSCLC patients was associated with poorer outcomes. Even though multiple studies have been done, meta-analyses have shown significant heterogeneity between studies, with differences in end point, patient population, and NSCLC stage [18]. Renaud *et al.* noted patients who had KRAS mutation had worse overall survival and a higher time to recurrence compared to the rest of the cohort [19,20]. Similarly, Nadal *et al.* found that patients with KRAS mutation had shorter disease-free survival and overall survival times compared to patients with KRAS wild-type [20,21].

KRAS mutations have been identified as a predictor of resistance to EGFR-TKI therapy. Massarelli *et al.* studied 73 patients with advanced NSCLC previously treated with EGFR-TKI and found the presence of KRAS mutation correlated with progression of the disease [21,22].

Women are more likely than men to possess the KRAS mutation [9]. However, there have been no studies performed on the effect of gender on KRAS' biomarker ability, likely because there is no consensus on KRAS as a prognostic or predictive biomarker. As of now, the use of KRAS as a biomarker is limited to its ability to exclude EGFR and ALK mutations, due their mutual exclusivity, and as a negative predictive marker of EGFR-TKI response.

2.3 ALK

Anaplastic lymphoma kinase gene (ALK) is a tyrosine kinase receptor similar to the human insulin receptor and is a driver oncogene. It was first discovered in anaplastic large-cell lymphoma [22]. In a subset of NSCLC patients, there is the presence of the EML4-ALK fusion gene. Through an inversion process, ALK translocates to join EML4 on the short arm of chromosome 2 to form the EML4-ALK fusion gene. This results in a chimeric protein with constitutive kinase activity. Like EGFR, this results in the activation of the RAS-RAF-MEK, JAK3, and PI3K-AKT pathways. 2–7% of NSCLC cases contain the EML4-ALK biomarker [23]. This fusion gene was detected in NSCLC patients of young onset who were non-smokers or light-smokers. ALK rearrangements are also mutually exclusive with EGFR or KRAS mutations [24]. Due to the unique clinic-pathological features, ALK-positive NSCLC represents a specific subtype targetable by ALK-TKIs.

Crizotinib is a small molecule TKI that targets several tyrosine kinases, one of which is ALK. Camidge *et al.* in a Phase 1 study showed that 250 mg of crizotinib given two times daily in cycles of 28 days leads to an objective response in 60.8% of patients with progression-free survival, at 9–10 months [25,26]. Another clinical trial investigating the efficacy of crizotinib against chemotherapy showed that progression-free survival was higher in the crizotinib-treated group compared to the chemotherapy-treated group, 7.7 months and 3 months respectively. Patients treated with crizotinib also reported a greater improvement in symptoms

and quality of life compared to the chemotherapy group [26]. Due to the eventual acquired mutations that lead to crizotinib resistance, Certinib, a second-generation ALK-TKIs was developed. Certinib has shown benefit in patients with ALK-positive patients who developed resistance to crizotinib and superior clinical efficacy over chemotherapy [27,28].

ALK mutations are found in never smokers, and more frequently in women than men. However, there has been no evidence to prove a difference in ALK mutations due to gender differences [9]. In addition, the predictive value of ALK biomarker is similar between genders. A meta-analysis study has shown that the benefit for ALK-inhibitors in patients with NSCLC is similar across genders [15]. There have been no studies performed elucidating the value of ALK as a prognostic factor for NSCLC.

2.4 ROS-1

The ROS1 gene encodes for a tyrosine kinase receptor that is related to the ALK and insulin receptor family. Although it has these relations, ROS-1 is an orphan receptor tyrosine kinase as there is currently no known ligand or role for ROS-1 in the human body. Certain solid tumors have ROS-1 rearrangements, which represents a new subtype of NSCLC, seen in 1–2.5% of all cases. NSCLC patients with ROS-1 rearrangements have similar patient characteristics to EML4-ALK positive NSCLC patients, with both markers affecting East Asian never-smokers who have earlier-than-average onset of disease [29,30]. ROS-1 rearrangement occurs with the fusion of the intact tyrosine kinase region of ROS-1 with another gene that is usually on another chromosome. Fourteen ROS-1 fusion partner genes have been discovered in relation to lung cancer, including CD74, SLC34A2, FIG, TPM3, LRIG3, CLTC, LIMA1, TMEM106B, MSN, CCDC6, SDC4, TPD52L1, EZR, and KDELR2. Of these, the most common fusion partner in NSCLC is CD74. ROS-1 rearrangement results in the constitutive activation of the ROS-1 kinase and promotes cellular transformation, proliferation, and survival through the PI3K-AKT, RAS-RAF-MEK, and SHP-1/SHP-2 pathways [30].

Given that ROS-1 NSCLC cases are rare and were discovered recently compared to other NSCLC tissue biomarkers, there are few studies on the prognostic value of ROS-1 biomarker. However, due to the homology between ALK and ROS-1, studies have been done pinpointing the possibility of ROS-1 as a predictive biomarker for response to crizotinib. In a phase I study determining efficacy of crizotinib in advanced ROS-1 positive patients, 53 patients saw an objective response rate of 72%. They also had a median overall survival of 51.4 months, which was the longest overall survival observed to date using ROS-1 targeted therapy [31]. A separate study included 35 Chinese patients with advanced or metastatic ROS-1 positive NSCLC. Efficacy was demonstrated with an overall response rate of 71.4% and a disease control rate of 94.3% [32]. There are currently other ongoing studies not only further investigating efficacy of crizotinib, but also investigating other ALK-TKIs and a novel dual ALK/ROS1 TKI. Although studies have shown a significantly higher rate of ROS-1 fusion detection in women compared to men with NSCLC this gender difference has no implications on the effect of ROS-1 as a predictive treatment biomarker [33].

ROS-1 fusion detection is confirmed using a break-apart FISH assay which is expensive and labor intensive. As the number of ROS-1 NSCLC cases are still low, further studies are needed to determine diagnostic algorithms and cost-efficient screening methods for patients with ROS-1 rearrangements. For now, it seems beneficial to screen patients without markers for EGFR, KRAS and ALK mutations for ROS-1 fusion rearrangements as they can be given targeted treatment of crizotinib, regardless of gender.

2.5 c-MET/HGF

c-MET is a tyrosine kinase receptor expressed in epithelium that responds when bound to its ligand, hepatocyte growth factor (HGF). HGF is secreted by mesodermal cells in development and is responsible for regulating proliferation and motility. Activating c-MET with its ligand HGF results in the activation of several downstream pathways including the RAS-RAF-MEK, PI3K-AKT, and PLC-γ pathways, triggering both mitogenesis and morphogenesis. c-MET is overexpressed in many cancers, including NSCLC and SCLC. However, c-MET is more likely to show tumorigenic activity when mutated. Mutations of c-MET in its juxtamembrane domain have been found in cases of both NSCLC and SCLC; this causes constitutive activity of the tyrosine kinase activity, resulting in tumorigenesis [34–37].

Overexpression of c-MET has been thought to correlate with a poorer prognosis in lung cancer. Park *et al.* [38] evaluated the prevalence and prognostic role of c-MET overexpression in 380 patients with surgically resected NSCLC. Patients with overexpressed levels of c-MET had significantly shorter overall survival and disease-free survival rates. These patients also had a significant increased risk of death with a hazard ratio (HR) of 1.618 (p = 0.024) Cappuzzo *et al.* [39] also showed the association of c-MET overexpression with disease stage. They performed a retrospective study using the stored tissue biopsy samples of 447 patients with NSCLC and discovered that overexpression of c-MET was not only associated with shorter overall survival but also with a more advanced stage of disease. Their results also showed an increased risk of death in patients with overexpressed c-MET [38]. Furthermore, studies have implicated c-MET in the diagnosis and detection of residual disease. A study examined the levels of c-MET expression in paired tumor and normal lung tissues and peripheral blood of patients with NSCLC. 68% of the patients who showed overexpressed c-MET also had an increase in circulating c-MET, between 1.4 to 8 times higher

than that of control patients. Overexpression of c-MET showed a correlation with nodal involvement and early recurrence. After Cox regression multivariate analysis, the study found that circulating c-MET was an independent predictor of early recurrence with a HR of 3.94 ($p = 0.027$) [39].

c-MET has also been implicated as a biomarker for EGFR-TKI resistance. A retrospective study was conducted on 51 tumor samples from patients with NSCLC treated with EGFR-TKI. They found that increased c-MET expression was significantly associated with disease progression and shorter time to progression [40]. Another study retrospectively analyzed 1199 NSCLC patients within two years. Although there was no difference in response rate to EGFR-TKI treatment between patients with overexpressed c-MET and patients without, the study did show that progression-free survival was significantly shorter in patients who had overexpressed c-MET [41]. There are no gender differences in c-MET expression, however studies have shown a higher risk of death in men with overexpressed c-MET NSCLC compared to women [42]. As mentioned earlier, women have better survival on EGFR-TKIs. With c-MET acting as a predictive marker for EGFR-TKI resistance and as a negative prognostic marker for men, there is a suggested role in gender on the effect of EGFR-TKI for c-MET overexpressed lung cancers.

These studies show a role for c-MET expression as a prognostic biomarker for adverse survival and recurrence. c-MET overexpression could also be a biomarker indicating possibility of developing resistance to EGFR-TKIs.

2.6 p53

p53 codes for a tumor-suppressor protein, given its name due to its function in regulating the cell cycle by blocking cell cycle progression from G1 phase. At high levels, this protein stimulates transcription for the cyclin dependent kinase inhibitor (CDKI) p21. p21 binds to and inactivates the G1/S-Cdk complex which arrests the cell in G1. This CDKI can also inhibit the G1/K cyclin complex and prevent Rb phosphorylation. Unphosphorylated Rb will then bind to and inhibit E2F from activating gene transcription of essential S-phase genes, which also blocks the G1/S transition. p53 is not only helpful in routine cell cycle regulation, but also is extremely important in response to DNA damage. Mutations in p53 result in a loss of inhibition of the cell cycle, resulting in uncontrollable cell division and proliferation. Mutations in p53 responsible for NSCLC are missense and reported in 30–60% of NSCLC as well as SCLC [43,44]. In a meta-analysis examining p53 mutations in patients with surgically resected lung cancer, p53 mutations were found in 47% of NSCLC. They also found more p53 mutations in squamous cell and large cell histology that are often associated with smoking [45]. Higher frequency of p53 mutations have been found in the lung cancer tumors of women compared to men [9].

As p53 mutation is widespread in cancers, the use of p53 as a prognostic biomarker in lung cancer is still debated. A study by Tan *et al*. obtained clinicopathological data on 179 patients with NSCLC and correlated the p53 expression on the respective surgically resected tumors, detected using a monoclonal antibody. They found a relationship between strong p53 expression and patient survival, suggesting the use of p53 protein expression as an independent prognostic marker in NSCLC [46,47]. Another study examined the effect of p53 expression on 482 patients with resected NSCLC who were randomly assigned to a control group or a chemotherapy treatment group. Half of the patients in the chemotherapy treatment group were positive for overexpression of p53. Patients with overexpressed p53 tumors in the control group had shorter overall survival. However, the patients with overexpressed p53 tumors in the chemotherapy treatment group had significantly increased survival benefit from adjuvant chemotherapy [47].

There is increasing evidence that p53 may have clinical use as a predictive biomarker for treatment options. Shih *et al*. [48] examined differences in p53 expression of 87 lung cancer patients before treating them with cisplatin chemotherapy. Half of the enrolled patients were chemo-effective, meaning they showed previous response to chemotherapy, whereas the other half was chemo-ineffective, meaning they received no previous benefit with chemotherapy. The data showed that p53 expression was 27% higher in chemo-ineffective patients compared to chemo-effective patients, suggesting that higher levels of p53 expression could be a predictive marker for resistance to cisplatin chemotherapy [49]. Two more recent studies indicate a use for p53 in determining treatment with small-molecule inhibitors or immunotherapy. Effects of p53 in sensitivity and primary resistance to NSCLC cells *in vitro* showed p53 mutations resulted in primary and acquired resistance to EGFR-TKIs [48]. The second study evaluated the association between p53 mutation and lung cancer patients treated with anti-PD-L1 immunotherapy. Of the 186 patients studied, the data showed increased progression-free survival in p53 mutation patients treated with immunotherapy compared to p53 wild-type patients who had been treated with immunotherapy [50]. The differences seen in these studies suggest the possible benefit of p53 as a predictive marker for the appropriate treatment for patients. Studies have yet to elucidate a gender difference between use of p53 as a prognostic or predictive biomarker. However, given the gender association with EGFR-TKI treatment and the suggestion of p53 as a predictive biomarker of EGFR-TKIs, studies regarding gender differences of p53's predictive ability should be performed.

3. Blood Markers

The use of liquid biopsy is expanding in the field of cancer diagnosis, with several reasons detailing the usefulness of liquid biopsy. First, a significant subgroup of patients is unable to undergo conventional tumor biopsy pro-

cedure and be under anesthesia for surgery either due to poor clinical condition or the location of the tumor. Second, liquid biopsies could spare the patient from undergoing an invasive procedure and any related complications. Third, an invasive procedure such as surgery would be more costly than a blood draw, making liquid biopsies more cost-effective. Finally, due to the heterogeneous nature of the tumors, a tissue biopsy performed at a certain location may not represent the overall molecular landscape of the tumor. Because of the above reasons, potential blood biomarkers have been proposed for clinical application. Specifically in NSCLC, the use of liquid biomarkers is mainly to monitor treatment response and resistance mechanisms. The most used blood biomarkers from liquid biopsy are circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA).

3.1 Circulating Tumor Cells

One of the most important biomarkers seen in liquid biopsy are CTCs, which are cells shed from the primary tumor or metastases and found in circulation. These cells can contribute to tumor progression through metastasis to a secondary site. CTCs fall in two categories: single circulating cells or CTC clusters, a group of at least two or three tumor cells traveling in the bloodstream. CTC clusters can occur from aggregation or proliferation of single cell CTCs and are considered rare but highly metastatic [51]. Metastasis is the primary culprit responsible for disease progression and death in not only lung cancer, but all cancers. Traditional metastatic models suggest a single cell seeding from the primary tumor results in metastasis. Larger CTC clusters result in more metastases than smaller clusters. CTC clusters undergo epidermal-mesenchymal-transition (EMT) through the secretion of TGF-β by platelets who have also aggregated within the cluster [52]. CTC detection in early-stage NSCLC is negatively correlated to overall and disease-free survival, whereas CTC detection in advanced NSCLC is related to worsening prognosis [53].

The primary method of detection for CTCs is by liquid biopsy. There are multiple techniques to isolate, characterize, and count CTCs. CTCs are quite difficult to isolate from peripheral blood, due to the low number of cells. There is only one cell per milliliter of blood with a confounding background of millions of leukocytes [54]. CTCs can be isolated using two methods based on either physical or biological characteristics. Physical characteristics include size, density, and electric charge of the cells. Biological properties include using positive selection to target CTC tumor-specific biomarkers to select for CTC or using negative selection to remove other cells in the sample by targeting common lymphocyte biomarkers. Epithelial cell adhesion molecule (EpCAM) is a protein that is not normally expressed by blood cells but found on epithelial cells, so EpCAM could be used as a positive selector to isolate CTCs. There is a test kit that uses anti-EpCAM antibodies to detect and isolate CTCs named CellSearch, which is the only FDA-approved test for CTCs. However, EpCAM-based tests for CTCs have their limitations. During EMT, EpCAM can be downregulated, resulting in an underestimated level of CTCs. EpCAM is also not expressed in all tumor types, which results in a lower detection rate of CTCs [55]. Overall, CTCs exist at a low level in peripheral blood, resulting in the rareness of CTC detection. However, they are a viable alternative for invasive tissue biopsies. Compared to a single-site tissue biopsy, the analysis of CTCs can give an improved comprehensive picture of overall tumor content and tumor heterogeneity [56,57]. While CTCs have been extensively studied for many years, only recently has their role as a prognostic and predictive treatment marker emerged.

In general, CTC has been posited as an independent negative prognostic marker in both early and advanced NSCLC. It has also been suggested as a predictive biomarker in either response to treatment or determining proper treatment. A retrospective study enrolled 347 patients with Stage I-IIIA NSCLC who underwent surgical resection for their disease. Blood samples were taken from patients prior to the operation, which were used to later isolate CTCs using a positive selection marker. Patients who later experienced disease recurrence and subsequent metastasis had higher pre-operative CTC concentrations. Higher CTC concentrations were also associated with worse recurrence-free survival. Given that the researchers found no correlation between CTC concentrations and the previously noted tumor stage, yet CTC had such a high prognostic value, this study concluded that CTC could be used as an independent prognostic biomarker for NSCLC [58]. A separate study on advanced NSCLC looked at 46 patients treated with chemotherapy. CTC levels were measured at baseline and before each chemotherapy cycle. Higher baseline CTC count corresponded to worse progression-free and overall survival, also indicating CTC as a negative prognostic biomarker in advanced NSCLC [59].

It is also possible for CTC to be a predictive biomarker for treatment. 92 patients with Stage I NSCLC were treated with stereotactic body therapy (SBRT) in this next study. CTCs were detected from peripheral blood using a telomerase probe. Measurements were obtained before, during, and up to 24 months after treatment. Higher CTC detection before treatment resulted in a higher chance for persistent detection in the 3 months following SBRT treatment. Overall, the higher CTC detection before treatment as well as persistent detection both were correlated with an increased risk of recurrence [60]. Another study enrolled 50 patients who were undergoing surgical resection for a lung mass. These patients' CTCs were measured before surgery, on the day of surgery in post-operative recovery, at post-operative day 1, and at post-operative day 3. All patients saw a decrease of CTC levels in recovery after surgery. However, patients with an earlier CTC rebound

level on post-operative days 1 and 3 resulted in a recurrence of disease months later [61]. This data suggests CTC could also be a determinant biomarker for lung cancer recurrence. The study by Taminga *et al*. enrolled 104 patients with advanced NSCLC treated with checkpoint inhibitors. The study included both patients who had experienced a response and did not experience a response to treatment. Blood was drawn at baseline and 4 weeks after treatment, using CellSearch to detect CTCs levels. The presence of CTC at the baseline was associated with worse overall survival. Increased CTC at baseline also was a predictive marker for treatment response [62,63].

Studies discussing gender differences of CTC have focused on investigating differences in detection rates. A study comparing detection rates of CTC in patients with early NSCLC and healthy volunteers also looked at correlation between CTC detection and gender. A separate study investigated the detection of CTC in correlation with tumor tissue markers and clinical variables, including gender. In both studies, there was no significant correlation found between positive CTC detection and gender [63,64].

Although there are many benefits in using CTC as a prognostic or predictive treatment biomarker, the techniques to isolate CTC must be further developed and more reliable to confirm the results of these studies.

3.2 Circulating Tumor DNA

Circulating tumor DNA (ctDNA) are single- or double-stranded DNA fragments shed into circulation. This concept was discovered in 1977, but only gained traction in research and clinical practice in recent years due to the advances made in gene sequencing technologies [65]. Dead or dying tumor cells discard these fragments into the circulation when undergoing apoptosis or necrosis due to increased tumor burden and growth or treatment with anti-tumor therapy. ctDNA is also increased in patients without cancer due to a variety of other pathological processes, however levels are more significantly increased in cancer patients than these other patients [66].

The difficulty in using ctDNA is that it is more challenging to detect ctDNA in the circulation as its levels are lower when compared to circulating germline DNA. As such, sensitive assays are required for an accurate result. ctDNA detection technology has evolved from conventional karyotyping and PCR-based assays to molecular cytogenetics to modern technologies. Molecular cytogenetics combines the ability to identify a specific gene with the ability to directly visualize the cells of interest under a fluorescent microscope. These techniques include spectral karyotyping, comparative genomic hybridization (CGH), and fluorescence in-situ hybridization (FISH). Modern technologies include microarray-based CGH, single nucleotide polymorphisms (SNP), and next-generation sequencing (NGS) [67]. NGS and PCR-based assays can be used in clinical practice to personalize treatment therapies.

As a biomarker, ctDNA has been implicated in use as a diagnostic or prognostic marker for NSCLC. It has also been suggested to have the ability to guide decisions for targeted therapy of NSCLC. Several studies have been performed showing the benefit of ctDNA as a diagnostic marker for NSCLC. Levels of ctDNA is significantly increased, almost 4–5 times higher, in patients with NSCLC compared to control patients [68–70]. A separate study also showed that ctDNA levels increased as the cancer progressed, but these levels also decreased with successful treatment, accurately indicating cancer progression and regression [71]. As a diagnostic biomarker, it seems ctDNA can accurately discern between NSCLC patients with healthy patients.

In addition to its proven usefulness as a diagnostic marker, ctDNA could also be used in indicating prognosis and treatment responses in NSCLC. A study enrolling 446 patients with advanced NSCLC treated by chemotherapy concluded ctDNA was a negative independent prognostic factor for longer time to progression and overall survival. Blood samples were collected from patients using a PCR-based assay before chemotherapy treatment to determine ctDNA levels. Higher ctDNA levels before chemotherapy treatment were correlated with longer time to progression and a lower overall survival compared to patients with lower levels of ctDNA [72]. Use of ctDNA as a treatment predictor could prevent patients who might not respond as well to chemotherapy from undergoing the harsh and difficult side effects of this treatment. ctDNA has also been implicated as a marker for treatment response in EGFR-TKIs. 45 NSCLC patients who had received EGFR-TKI therapy were enrolled in a study that concluded use of ctDNA as a monitoring biomarker for response to EGFR-TKI treatment. The study investigated changes in the EGFR mutation in ctDNA using a PCR-based assay. 26.7% of the patients went from EGFR positive to EGFR negative after the therapy and 31.1% of patients went from T790M mutation negative to T790M mutation positive after completion of therapy. T790M mutation is a known mutation in EGFR positive NSCLC that indicates acquired resistance to EGFR-TKI [73]. Monitoring of ctDNA during treatment could promptly inform a clinician of positive response or acquired resistance to therapy, allowing patients to end their course of treatment early, as immunotherapies such as small molecule inhibitors are costly.

Few studies have been performed on the effect of gender on ctDNA utility as a biomarker due to its novelty. However, there have been results implicating a gender difference in ctDNA of lung cancer patients. A recent study examined ctDNA correlation to overall survival in NSCLC patients and included baseline clinical parameters such as gender. The ctDNA clearance levels, which were defined as lack of detectable mutation in blood was experienced in men more than in women with treatment [74]. Given this study that showed positive correlation between ctDNA

clearance and increased overall survival, it is reasonable to suggest that ctDNA is a useful prognostic predictor for NSCLC in this cohort for men.

ctDNA is developing to take on a main role as a diagnostic, prognostic, and treatment biomarker in the assessment of NSCLC. Specifically, ctDNA could help monitor treatment therapy in EGFR-positive NSCLC, improving prognosis.

4. Expired Breath Markers

In a similar vein to liquid biopsies mentioned above, expired breath markers are beginning to receive more attention as an alternative to the traditional invasive tumor tissue biopsies. For patients who are unable to undergo or have no clinical indication for surgical resection due to advanced disease, a less-invasive, easily applicable method to function as a diagnostic or treatment response predictor is beneficial. Expired breath tests rely on the existence of volatile organic compounds (VOCs) within exhaled breath and composition of these compounds can change with the appearance of cancer.

Common VOCs found in expired breath analysis include isoprene, acetone, ethanol, methanol, and alkane and benzene derivatives [75]. VOCs can be either endogenous or exogenous. Exogenous VOCs are compounds inhaled through the nose or mouth or absorbed through the skin due to environmental exposures. Examples include chemicals inhaled from paint, pollutants, and microbes. Endogenous VOCs are compounds that are end-products of biochemical metabolic processes in the body such as oxidative stress, energy metabolism, or cell membrane function [76,77]. Isoprene is formed during cholesterol synthesis in connection with mevalonate derivation [78]. Acetone is produced during free fatty acid oxidation which can be part of the glucose metabolism process. In times of decreased food intake, prolonged fasting, or increased energy demands due to pathological process, fat will be used as energy and is released through fatty acid oxidation [79]. Alkanes and hydrocarbons are produced during lipid peroxidation in response to free radicals such as reactive oxygen species, which usually happens in times of oxidative stress [80]. Certain VOC compounds such as isoprene are gender-specific, but the significance of this regarding expired breath biomarker value is not yet known [81].

As VOCs are found in trace amounts in expired breath, it is quite challenging to detect them accurately [82]. As a result, equipment and tests need to be not only accurate, but very sensitive. Methods of VOC collection include gas chromatography – mass spectrometry (GC-MS), portable devices, electric noses (eNose), or even canines. Unfortunately, GC-MS is difficult to use, expensive, and extra labor is required in the form of a specialized analyst to take samples and interpret results, so GC-MS is not found in clinical practices. Even canines have been trained to smell the difference between the exhaled breath of a patient with and without lung cancer [83]. The devices that would be indicated for clinic use are the portable devices and eNose.

Different studies have been performed on exhaled breath to determine differences in VOC of patients with lung cancer compared to healthy patients. They compared VOC profiles and discovered extensive lists of possible VOC breath biomarkers. One of these studies was performed by Poli *et al.* and compared VOC levels from NSCLC patients against healthy smokers, healthy nonsmokers, and chronic obstructive pulmonary disease patients. Using GC-MS, 13 VOCs were analyzed as part of the profile. There was no one VOC that was more significant than the rest in the results, suggesting that VOC as a diagnostic or screening marker would be in the difference in profiles as opposed to the existence of a singular marker. The VOC profiles accurately classified 80% of the NSCLC patients as lung cancer [84,85]. A similar study compared VOC profiles of 97 patients with lung cancer and 182 control individuals who were considered at-risk. The patients with lung cancer were biopsy-confirmed, but no treatment was initiated yet. The VOC profiles were accurate at distinguishing patients with lung cancer from at-risk individuals [85]. Besides characterization, studies have also focused on VOCs as a prediction model. In a study with 193 lung cancer patients and 211 control individuals with a negative chest CT, the developed prediction model predicted lung cancer accuracy with 84.6% sensitivity and 80% specificity. The prediction model used 16 VOCs and was found to not be affected by smoking status or TNM stage [85]. Another study enrolled 37 lung cancer patients with 23 healthy controls who were age-matched. They investigated a 24 VOC profile using GC-MS and discovered that lung cancer patients had increased levels of oxygenated VOCs [86]. This finding supports the idea that oxidative stress is active in cancer processes.

Analysis of expired breath VOCs are composed of a panel of organic compounds that could be potential markers, instead of just one marker like we see in tumor tissue or liquid biopsy. Given that the goal of expired breath analysis is to determine a difference between healthy and diseased patients, particularly in lung cancer, studies have applied various statistical algorithms to determine effect. These statistical algorithm have not been compared to each other, so the significance in using one method over the other is unknown. Additionally, the number of VOCs used in each expired breath analysis differs, which could also give cause to why these studies have not reached a consensus on the efficacy of expired breath analysis.

Another concerning issue is the selection of a control group for these studies. The main goal is to eventually use expired breath VOCs as a marker in diagnosing or screening for lung cancer. There is an undeniable link between tobacco smoking and lung cancer due to years of research showing a cause and effect between these two. Most lung cancers are caused by tobacco smoking. However, tobacco

smoke can also be considered an exogenous VOC as it is a compound that is inhaled through the external environment and then can be exhaled during an expired breath analysis. In this manner, the effect of tobacco smoke on expired breath analysis results are extremely important. And so, the studies above recruited smokers without lung cancer as a control group. Two concerns arise from this. It is known that tumorigenesis occurs in the body before symptoms become noticeable to the patient and usually before cancer can be diagnosed [87]. The individuals who smoke in the control group could already have changes in their body because of tobacco smoking that are tumorigenic which would affect their expired breath VOCs. Another concern is that smoking is also a main risk factor for chronic obstructive pulmonary disease (COPD). COPD is also a pathological diseased state like cancer and VOC changes have been found in COPD patients compared to control. A solution could be to conduct studies comparing COPD and lung cancer patients.

Although there is convincing evidence that expired breath analysis can provide valuable information, it is not yet possible to use current expired breath techniques to accurately screen or diagnose individuals with risk factors or lung cancer. However, the analysis of expired breath and its contained VOCs has the promise to become a diagnostic and early lung cancer screening tool in patients [88].

5. Molecular Imaging

Combining cancer specific molecular markers with imaging can improve the diagnosis of indeterminate findings during lung cancer screening and intervention [89,90]. This is further assisted by applying artificial intelligence using the data available using lung cancer phenotypes and tumor microenvironment. Currently investigational and will need further validation for routine clinical application [91]. Another interesting development in lung cancer is intraoperative imaging of lung nodules that are difficult to localize during minimally invasive surgery or not found in conventional imaging to facilitate localization [92]. Specifically targeting using biomarkers to determine treatment response or disease progression [93,94].

6. Gender Differences

Gender plays an important role as a prognostic marker in lung cancer. Although the predominant factor for lung cancer is tobacco smoke, there has been a demographic shift of lung cancer patients towards never-smoker females [17]. Gender also results in a distinct set of risk factors, which is currently not represented in screening guidelines [17]. Exogenous exposures seen more in women include indoor cooking fumes or HPV infection, while men have higher smoking rates [17]. Given the gender differences in lung cancer risk and mortality, it seems advisable to determine the existence of an effect of gender differences on diagnostic, prognostic, or predictive biomarkers. This determination could develop a more personalized treatment plan not just based on the unique characteristics of the patients' lung cancer, but on the patients themselves, bringing an even more concise interpretation of personalized medicine.

In terms of tissue markers, men are less likely to have EGFR, ROS-1, and p53 changes within their tumors. KRAS, ALK, and c-MET have no gender differences in mutational changes. Gender differences have been most pronounced regarding treatment response prediction of EGFR-TKIs. Men with NSCLC have a poorer treatment response to EGFR-TKIs compared to women. c-MET has also been correlated with poorer prognosis in men with NSCLC. This data reveals the use in considering a patient's gender when determining treatment with EGFR-TKI or in c-MET overexpressed lung cancer.

Due to the recent proposal of using CTCs, ctDNA, and especially VOCs as diagnostic, prognostic, or predictive biomarkers, there are limited studies examining gender differences. There is implication in gender differences in detection of ctDNA and gender-specific VOC profiles. This information could be useful after the value of these factors as biomarkers has been elucidated. Currently, there seems to be minimal effect of gender on these biomarkers.

Although precision medicine is a new and exciting field to explore, we must not forget the use of basic prognostic markers in parallel with precision medicine. Gender is one of the oldest prognostic markers in use which unfortunately has been reduced to a variable in recent oncology research. There is a need for further gender difference studies and inclusion of gender differences as a factor in clinical trials to fully clarify sex-based disparities in lung cancer.

7. Conclusions

Lung cancer is responsible for the highest mortality from cancer globally and it is a complex and heterogeneous disease. The high mortality is from a prevalence of late diagnoses and resistance to treatments. The discovery of biomarkers as a method of personalizing treatment for patients depending on their subtype of lung cancer changed the field of thoracic medicine. Tissue biomarkers such as EGFR, ALK, KRAS, ROS-1, c-MET, and p53 are able to inform clinicians of specific mutations within the cancer. This can assist with treatment decisions as well as predict resistance to targeted immunotherapy and chemotherapy. These markers also have prognostic value which can contribute to a clinician's plan for their patient. The discovery of liquid biopsy biomarkers was a boon for patients as this removed the need for invasive biopsies and provided a cost-effective alternative to tissue biopsy. ctDNA and CTCs also provide prognostic and treatment response value that would benefit lung cancer patients. Expired breath VOCs are new to the biomarker scene. Although more studies are needed, there is early evidence that gives hope for the efficacy of VOC profiles as a diagnostic or screening tool, especially for at-risk individuals. In the coming years, it will

be possible to perform diagnostic, prognostic, and treatment tests for lung cancer using tissue, blood, or expired breath biomarkers which will provide information to clinicians to personalize treatment and diagnose early.

Author Contributions

VL—performed the initial literature search, involved in the analysis and interpretation, critical review, drafting the manuscript and final approval. WTV—involved in the analysis and interpretation, critical review, drafting the manuscript and final approval.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

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