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# Circulating Nucleic Acids as Promising Biomarkers: A New Frontier of Personalized Medicine

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*Circulating DNA and microRNA are useful biomarkers in non-small cell lung cancer prognosis and treatment.  
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## Abstract

*Biomarkers are a range of biological signals that measure the presence and severity of the disease. This literature review assesses circulating DNA (ctDNA) and microRNA (miRNA) biomarkers detected in liquid and tissue biopsies and their importance in the prognosis, outcomes, and treatments for non-small cell lung cancer (NSCLC). These biomarkers have the potential for clinical use; however, further studies with requisite data and sufficiently large trials are required to refine our understanding of their applicability. The prognostic significance of ctDNA and miRNA biomarkers in NSCLC care has demonstrated that liquid biopsy and molecular diagnostic testing may provide a feasible and noninvasive method for tailoring treatment plans to the specific mutational landscape of diverse NSCLC patients. However, further testing must be conducted to analyze the significance and benefit of ctDNA and miRNA biomarkers in larger cohorts and substantiate the standardization of liquid biopsy in clinical practice.*

## Introduction

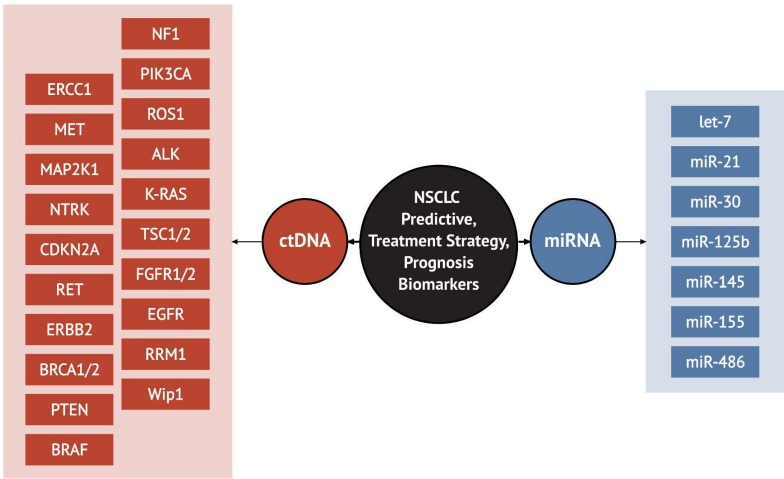
Despite representing 13% of all cancer diagnoses, lung cancer accounts for 24% of all cancer deaths in the United States each year. Among the two subsets of lung cancer (non-small cell and small cell), NSCLC accounts for 80% of all lung cancers. With only 19% of the diagnosed patients surpassing the five-year survival rate, there is an urgent need for early detection, diagnosis, and prognosis.

Recently, studies have shown that these predictions for NSCLC can be assessed via biomarkers (Howlader et al., 2020). A biomarker is broadly defined as an indicator of the presence and severity of a disease state. It can refer to a range of biological signals, including pulse, blood pressure, blood tests, and tissue tests. Hundreds of novel biomarker-related articles are published each year, but only a few biomarkers are currently used in practice (Rinaldi et al., 2011). Notably, the current approach relies primarily on the use of tissue biopsy-based biomarker testing. Tissue biopsy refers to an invasive procedure in which solid matter from the body, usually coming directly from the tumor or bone marrow, is sampled and assessed for the presence of clinically actionable biomarkers. Although this method is deemed the gold standard for NSCLC testing, it is time-intensive, invasive, and often fails to capture tumor heterogeneity (Rijavec et al., 2019).

Liquid biopsy is an emerging, minimally invasive process that can address this demand for early disease monitoring. Via this method, clinicians can quantify biological components circulating in bodily liquids, such as tumor cells and nucleic acids, including DNA and miRNA. Circulating DNA (ctDNA) is derived from DNA and released by cancerous cells and tumors into the bloodstream. As a tumor grows, newer cells replace older cells. The dead cells are broken down into their main components, releasing DNA into the bloodstream (Mader et al., 2017). MicroRNA (miRNA) are single-stranded, non-coding RNAs in the blood and function as antisense RNA to regulate the target genes. Multiple miRNAs can target the same gene (Wang et al., 2018). The pathways of ctDNA and miRNA are depicted in **Figure 2** and **Figure 3**, respectively.

There are three main classes of biomarkers. Predictive biomarkers interact with specific treatments to affect the outcome, prognostic biomarkers are

associated with particular outcomes regardless of the course of treatment, and treatment-focused biomarkers are any biomarkers that influence the selection of a specific course of treatment (Stein, n.d.). This paper outlines several ctDNA and miRNA biomarkers detected from liquid biopsies and uses previous data to assess their value in delineating NSCLC prognosis, outcome prediction, or treatment strategies. Studies regarding these biomarkers were reviewed according to sample size, procedure, and clinical relevance. The specific ctDNAs and miRNAs addressed in this paper are summarized in **Figure 1**.



**Figure 1:** List of NSCLC ctDNA and miRNA biomarkers. This list is not comprehensive; It serves as a brief summary of the ctDNA and miRNA biomarkers discussed in this paper.

### Common Methods utilized for circulating nucleic acid assessment:

Several primary technologies have been used to assess circulating nucleic acid biomarkers.

#### 1. Fluorescence in situ hybridization (FISH):

FISH utilizes a DNA sequence probe with a fluorescent dye attachment to locate and label a specific complementary DNA strand on a chromosome. This fluorescent marker can be visualized through microscopy. This method is applicable to blood, cytology smears, and bone marrow to detect duplications, deletions, and total chromosomal loss or gain (Green).

#### 2. Immunohistochemistry (IHC):

During IHC, fixed tissue is exposed to antibodies, which enter and adhere to antigenic determinants. Once this antibody-antigen substrate is created, it catalyzes an oxidation reaction to form a visible colorized marker. This can

be used as a tool for measuring protein and nucleic acid expression (Schildhaus et al., 2020).

### 3. Polymerase chain reaction (PCR):

PCR allows for the rapid amplification of small DNA fragments. First, the sample DNA is denatured at a high temperature to form two single-stranded DNA fragments. Second, Taq polymerase, a thermostable enzyme, attaches complementary DNA strands (known as primers) to each exposed strand. This process results in the duplication of the original DNA strand once elongation has been completed. Millions of copies of a specific DNA strand can be produced after several cycles of this process (Waters & Shapter, 2014).

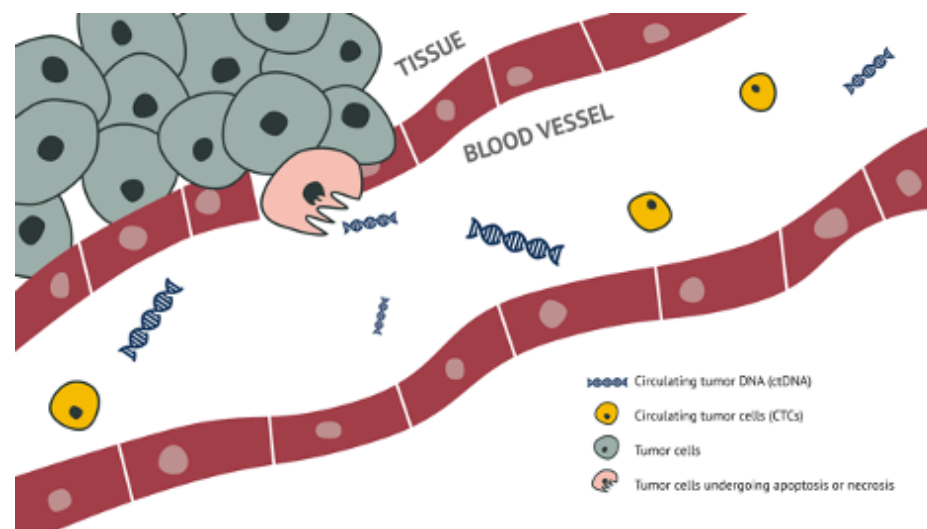
**4. Next generation sequencing (NGS):** NGS refers to the parallel sequencing of genomic segments. There are three main kinds of NGS:

**a. Illumina Sequencing:** In illumina sequencing, the DNA bases are identified by their unique fluorescent signals.

**b. Roche 454:** Roche 454 sequencing relies on the release of visible fluorescent pyrophosphate that occurs upon nucleotide addition to a new strand of DNA.

**c. Ion Torrent:** Ion torrent sequencing measures the release of protons due to the incorporation of nucleotide bases in the DNA.

## Circulating DNA Biomarkers



*Figure 2: An illustration of the ctDNA pathway. At the top, the tissue cells are shedding into the bloodstream. An immune response occurs in order to eliminate the tumor cells entering the bloodstream and to eliminate any CTCs. As part of this elimination, many tumor cells are*

*phagocytized, thus increasing the concentration of ctDNA in the bloodstream, which can be analyzed through liquid biopsy (Delmonico et al., 2020, Bettgowda et al., 2014).*

## ERCC1

Excision repair cross-complementation group 1 (ERCC1) is a part of the nucleotide excision repair (NER) pathway that acts as a mechanism for DNA repair, and also removes the cytotoxic elements from genomic DNA. For example, platinum is a cell-damaging agent that causes apoptosis, therefore regulating cell growth and death in the body. Overexpression of ERCC1 can cause lower platinum absorption, resulting in decreased cell death and subsequent increased cell growth and carcinogenesis. Underexpression of ERCC1 can cause carcinogenic factors to enter genomic DNA and hinder DNA repair mechanisms because ERCC1 typically repairs any DNA damage done by platinum (Li et al., 2016). Hence, ERCC1 expression level directly correlates to platinum sensitivity (Hamilton et al., 2018).

Programmed death receptor-1 is an immune-regulatory protein with two ligands, PD-L1 and PD-L2, that is expressed by immune cells and tumor cells. In a study done by Buderath et al., PD-L1, PD-L2, and ERCC1 levels were measured in 83 patients with epithelial ovarian cancer through liquid biopsy. Results showed that higher ERCC1 expression was associated with lower PD-L2 levels. Lower PD-L2 levels were found to be associated with platinum resistance ( $p < 0.0001$ ), which supports the results from previous studies (Li et al., 2016; Hamilton et al., 2018). Higher PD-L2 levels were associated with lower ERCC1 expression and reduced progression free survival (PFS) and overall survival (OS), while lower PD-L2 levels were associated with platinum resistance and higher ERCC1 expression (Buderath et al., 2019). This demonstrates that ERCC1 expression levels detected through liquid biopsies can be used to predict the outcomes of multiple cancers, including NSCLC and epithelial ovarian cancer.

## MET

MET is a proto-oncogene involved in cell growth, wound healing, and post-physical injury response. It encodes the receptor tyrosine kinase c-MET, for hepatocyte growth factor, which causes c-MET dimerization and autophosphorylation. This results in the activation of mitogen-activated protein kinases (MAPK), phosphatidylinositol-3' phosphate kinases (PI3K), viral oncogene homologs, and signal transducers

and activators of transcriptional signaling pathways. Mutated MET initiates the growth of cancer cells. Unusual MET expression has been shown to correspond with NSCLC, as well as gastrointestinal cancer and hepatocellular carcinoma. If the MET receptor is overexpressed via genomic amplification, mutation, or alternative splicing, cellular degradation of MET can occur (Mo et al., 2017).

c-MET receptor activation is associated with EGFR-TKI resistance and has poor prognosis. A trial consisting of 167 patients examined the prognosis, determined by PFS, after treatment with MET inhibitors, erlotinib and tivantinib. 84 patients were randomly assigned to receive oral erlotinib and tivantinib (ET group), and 83 patients were randomly assigned to receive erlotinib plus placebo (EP group). The average PFS was 3.8 months for ET and 2.3 months for EP, which is statistically significant ( $P = .024$ ) (Sequist et al., 2011). Deng et al. also studied NSCLC patients with MET amplification and EGFR mutation. For one patient, a combination of crizotinib and osimertinib was administered, which greatly improved his overall health. Results showed a 20-40% partial response rate to dual therapy with and without prior treatment with TKIs. Unfortunately, MET mutations have occasionally been shown to cause resistance to crizotinib treatment (Deng et al., 2018).

In a study done by Bardelli et al., 7 patients with metastatic colorectal tumors who had responded to panitumumab or cetuximab-based treatment and later relapsed were analyzed. MET expression was higher in post-relapse patients compared to first-occurrence patients. In relapse patients with KRAS mutations, MET expression was low or undetectable. MET amplification was seen in the blood before relapse, indicating that MET monitoring could be used to predict relapse. MET amplification is detected in 5-20% of EGFR-mutated lung cancers (Bardelli et al., 2013). In metastatic colorectal tumors and NSCLC, MET can be used for predictive outcomes and may help direct the course of treatment.

## **NTRK**

Neurotrophic tyrosine kinase receptor (NTRK) mutations are found in many solid malignancies, including NSCLC. Trk receptors include three transmembrane proteins: TrkA, TrkB, and TrkC, which are encoded by NTRK1, NTRK2, and NTRK3, respectively. In carcinogenesis, TrkA, TrkB, and TrkC undergo changes that lead to activation of signaling



pathways involved in cell growth and proliferation. Mutations to NTRK1, NTRK2, and NTRK3 have also been observed in 2-3% of NSCLC patients. For example, overexpression, in-frame deletions, and alternative splicing of NTRK1 have been demonstrated to be potential oncogenic mechanisms. Several NTRK inhibitors have been developed, but only LOXO-101 and entrectinib are under clinical evaluation (Ricciuti et al., 2017). Of 1,378 patients with locally advanced or metastatic solid tumor malignancies with NTRK1, NTRK2, NTRK3, ROS1, or ALK mutations, two showed NTRK1 gene rearrangements on anchored multiplex PCR. One patient, a 45-year-old male with a history of smoking, was treated with 400 mg/m<sup>2</sup> oral entrectinib daily. CT scans of his lungs 26 days after beginning the treatment showed no tumor growth. After 155 days, CT scans showed a 77% overall tumor reduction (Farago et al., 2015).

## CDKN2A

The CDKN2A mutation is uniquely present in NSCLC. Studies have suggested that the CDKN2A mutation is associated with the increased expression of the extracellular matrix and metabolic gene sets in NSCLC cell lines (Kim et al., 2015). This heightened expression has been suggested as an indicator of increased mortality in lung, breast, and gastric cancers (Gilkes, Semenza, and Wirtz, 2014). As a result, early detection of CDKN2A mutations is critical. The efficacy of liquid biopsy for mutation detection has been suggested in breast cancer studies, but further testing is needed to determine whether the detection of CDKN2A alterations via liquid biopsy would yield significant clinical benefit for NSCLC patients possessing alterations in this gene (Veldore et al., 2018; Delmonico et al., 2019).

CDKN2A alterations in NSCLC can be categorized into several discrete classes, including copy number changes, hyperphosphorylation, and deletion (Liu et al., 2020). A study by Wen et al. suggests that the relative frequency of CDKN2A alterations exhibits significant variability among different racial groups, with prevalence ranging from 5.1 to 21.5 percent in lung adenocarcinomas and 23.2 to 43.6 percent in squamous cell lung carcinomas. Percentages in this study were drawn from NGS assessment of 1200 Chinese NSCLC patients and The Cancer Genome Atlas (TCGA) data for American and European cohorts (Wen et al., 2019). Liu et al., also assessed data from TCGA to pinpoint important genes implicated in lung cancer tumor biology related to CDKN2A. A549 and H322 cell lines were

cultured and evaluated through real-time PCR, Western blot analysis, 2,5-diphenyl tetrazolium bromide (MTT) assay, and cell counting, invasion, wound healing, and migration assays. The researchers observed a marked decrease in survival among patients with CDKN2A depletions and found a direct correlation between CDKN2A knockdown and increased cell invasion, migration, and proliferation in experiments in A549 and H322 cell lines. A TCGA Provisional and Pan-Cancer Atlas survival analysis and further studies on other diseases have suggested decreased survival rates and other adverse effects in patients with CDKN2A depletion. (Reis et al., 2015; Zeng et al., 2018; Dacic et al., 2008). While CDKN2A loss is suggested to have a negative influence on prognosis and survival outcomes, more research is necessary to understand CDKN2A's specific implications in lung cancer (Liu et al., 2020).

With CDKN2A's potential to be a clinically actionable biomarker, testing and genomic panels with this gene have been beneficial and often included in biomarker testing. Recent advances in assay techniques have established ctDNA as a viable biomarker for detection. One study suggests that CDKN2A ctDNA, along with the ctDNA of several other cell-cycle related genes, exhibit only an 81.6% concordance rate to their corresponding solid tumor biopsies (Mao et al., 2017). Studies analyzing the concordance of CDKN2A mutation in both liquid and tissue biopsies from breast cancer patients also revealed differences in mutations detected through cell-free DNA analysis and tissue biopsy, although overall mutational prevalence was quite similar to frequencies observed in tissue samples (Delmonico et al., 2020). Hence, the impetus for further exploration of ctDNA's clinical value is essential to develop increasingly accurate, noninvasive, and timely methods of detection to ensure that CDKN2A mutations are subject to earlier interventions, which may ultimately lead to a better prognosis for NSCLC patients.

## **RET**

Rearranged during Transfection (RET) activating fusion mutations are found to occur in approximately 1% to 2% of NSCLC patients and have been suggested to show heightened prevalence in patients who never/lightly smoked and who are younger (Kohnno et al., 2013; Kato et al., 2017). RET codes for a receptor tyrosine kinase that recognizes growth factors for the neurotropic factor family, which is derived from glial cell lines. Fusion of the RET proto oncogene can activate the receptor tyrosine kinase in the



absence of its ligand. Such an activation can have downstream effects on other pathways such as those including PI3K and MAPK (Kato et al., 2017). The existence of FDA-approved therapies for RET-altered cancers such as vandetanib and cabozantinib underscores the value of early RET detection to improve prognosis for patients with NSCLC presenting with RET-altered cancers. Clinical trials with vandetanib and everolimus have demonstrated the potential of these drugs to serve viable treatments for RET-altered cancers that have metastasized to the brain (Subbiah et al., 2015). In addition, Kodama et al. assessed the efficacy of alectinib in RET fusion-positive CCDC6-RET and KIF5B-RET genes. The researchers determined that alectinib can preclude phosphorylation of RET, thus providing substantial inhibitory control. Furthermore, alectinib demonstrated substantial efficacy in treating V804L and V804M mutated cancers compared to cabozantinib and vandetanib. The suggested basis of this difference is supported by alectinib's structure, which is not influenced by the steric effects of the V804L and V804M gatekeeper mutations (Kodama et al., 2014).

Mao et al. found that plasma samples of RET ctDNA from patients with histologically confirmed lung cancer demonstrated 96.2% concurrence with solid tumor tissue DNA samples. Concordance rates in this study were determined by an analysis of 40 tissue and plasma samples collected from participants aged 18-80 who did not qualify for first-line surgical treatment (Mao et al., 2017). Similar studies have also demonstrated high concurrence rates between tissue and liquid biopsies, although the concurrence percentage was shown to drop significantly in one study when analysis was limited to specific subtypes of genomic alterations, such as copy number variations, which demonstrated concordance rates as low as 3.5%; however, sample size in this cohort was limited to 45 patients (Chae et al., 2017). The variability in concordance rates between ctDNA and tissue biopsy shows that further analysis is required to determine whether ctDNA is a viable standalone method of detection for RET-altered cancers, given that RET mutations are included in the National Comprehensive Cancer Network's recommendation for genomic targets in NSCLC (Yang et al., 2018; Thompson et al., 2016).

## **ERBB2 (HER2)**

Erb-b2 receptor tyrosine kinase 2 (ERBB2), human epidermal growth factor receptor 2 (HER2), has become widely acknowledged as a potential

target for therapy (Kirs et al., 2015; Chuang et al., 2017). While alterations in ERBB2/HER2 have primarily been studied in breast cancer, recent research has assessed the potential for targeted therapies in treating ERBB2/HER2- positive lung cancers (de Melo Gagliato et al., 2016; Chuang et al., 2017; Veatch et al., 2019). Some studies have shown that HER2 commonly presents in female patients with adenocarcinoma histology with no history of smoking (Garrido-Castro and Enriqueta Felip, 2004). ERBB2/HER2 receptors can also be phosphorylated to form heterodimers with other epidermal growth factor receptors (EGFR), such as HER1, and play a critical role in signaling pathways that regulate transcription, cell proliferation, and prevention of apoptosis (de Melo Gagliato et al., 2016). Approximately

1-5% of lung cancers were found to harbor ERBB2/ HER2 aberrations, but some studies have demonstrated that the percentage for the NSCLC subtype likely lies between 1-2% (Kris et al., 2015; Chuang et al., 2017). Regarding detection methods, Mao et al. demonstrated that ERBB2, along with other driver genes, exhibits a 96.2% concordance between tissue and liquid biopsy methods (2017).

Chuang et al. performed a retrospective assessment with nine patients, seven of whom presented with ERBB2 mutations and advanced NSCLC between 2013-2016 and two of whom met similar criteria from a 2014-2015 cohort. All patients were treated with a regimen of paclitaxel, vinorelbine, and trastuzumab. To pinpoint ERBB2 mutations, NGS-based methods Solid Tumor Actionable Mutation Panel, PCR-based ERBB2 sizing assay, Geneseq assay, or NGS-based FoundationOne assay were executed on tissue samples, while NGS-based profiling using deep sequencing was performed on ctDNA samples. Therapeutic response was assessed according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. The results of this study demonstrated that 44% of the patients exhibited response to targeted therapies. Although the sample size was small, clinical indications and availability of targeted therapies for ERBB2/ HER2 may warrant the use of these treatments. The authors caution that genotypic differences in ERBB2 mutations may result in differential efficacy of targeted therapies. Trastuzumab in conjunction with chemotherapy has demonstrated therapeutic promise in clinical settings, although it has not been shown to penetrate the blood-brain barrier, rendering it less useful for metastases to the brain and spinal cord. Other potential therapies include dacomitinib and neratinib (NCT01953926) (Chuang et al., 2017).

In conjunction with targeted therapies, detection remains a critical factor in prognosis. Early detection is optimal, but tissue sampling can be difficult at certain periods of cancer development. As such, ctDNA analysis may provide a reliable baseline for determining tumor mutational landscape. In other cancer types, such as cholangiosarcoma, ctDNA analysis resulted in the successful detection of ERBB2 (HER2) mutations. Liquid biopsy was selected in one case study in lieu of tissue biopsy due to challenges in tissue acquisition. As a result of ERBB2 (HER2) mutation detection via liquid biopsy, the patient was able to begin dual anti-HER2 pertuzumab/trastuzumab therapy as an off-label treatment regimen (Yarlagadda et al., 2019).

Another study in 29 colon cancer patients demonstrated that proper clinically verified circulating free tumor DNA analysis may provide a method of detection for HER2 copy number changes (concordance with tissue equaled 96.6%), allowing clinicians to identify patients who may benefit from anti-HER2 therapies. In addition to high concurrence rates, ctDNA has also been suggested to surmount the issue of tumor heterogeneity. Nevertheless, more studies are required to determine whether the observed concordance rates are affected by differential release of tumor DNA into circulation (Siravegna et al., 2019).

## BRCA1/2

Accurate DNA replication is integral to genomic integrity and protection against deleterious mutations. The BRCA1/2 genes work sequentially to ensure that this integrity is maintained, specifically by assisting in DNA lesion repair. Despite deriving its name from its frequent association with breast cancer, BRCA1/2 genes have also been implicated in NSCLC (Hu et al., 2019; Remon et al., 2020; Ji et al., 2020). With regards to targetability, germline BRCA-mutated (*gBRCAm*) cancers have demonstrated promising responses in poly-(ADP ribose) polymerase (PARP) clinical trials and other studies (Hu et al., 2019; Ji et al., 2020). In a study by Remon et al., analysis was performed on advanced NSCLC patient specimens negative for ALK rearrangements and positive for activating EGFR mutations. NGS and single nucleotide polymorphism (SNP) arrays were performed on the samples in order to assess genomic features in patients new to targeted therapy. Using molecular data, the authors determined the prevalence of pathogenic mutations in BRCA genes, RECIST-assessed response rate to platinum-based chemotherapy, and characteristic

clinicopathological features of BRCA mutated patient subgroups. Reported BRCA prevalence without respect to mutation type was 5.3% (20 out of 379 patients), although pathogenic mutations in BRCA1/2 constituted only 2.1% of advanced NSCLC patients. The results of Ramon et al.'s study demonstrated that there may not be a significant link between BRCA mutation in NSCLC and improved response to platinum based chemotherapy; a similar inconclusiveness on BRCA's role in predicting treatment outcomes was noted in another study on tumor samples that exhibited positive IHC staining for BRCA1 (Ramon et al., 2020; Watchers et al., 2005). Based on study data, it appeared that BRCA variants of unknown significance (VUS) exhibit longer OS ( $p=0.07$ ), whereas tumor protein p53 (TP53) mutation status in BRCA-positive subgroups did not yield a statistically significant difference in prognosis ( $p=0.3$ ). Although the authors found that biallelic mutations in BRCA may warrant further study to determine their prognostic significance, they noted that data gathered in their patient cohort did not yield sufficiently robust evidence to support implementation of specific targeted therapies in monoallelic BRCA1/2 patients (Ramon et al., 2020). In contrast to the data presented by Ramon et al., Ji et al. also analyzed BRCA1/2 in determining efficacy of targeted olaparib (PARP inhibitor) therapy. Assessment of data demonstrated that BRCA1 and BRCA2 depleted cells are more susceptible to olaparib. Similar to its role in breast cancer cells, olaparib was found to exhibit a similar apoptosis-inducing function in BRCA-mutated NSCLC cells (Ji et al., 2020). Moreover, other studies have demonstrated that heightened BRCA1 expression correlates with lower overall survival rates (Joerger et al., 2011).

While a common concern with the use of ctDNA assays is their ability to provide an accurate indication of a patient's mutational landscape, recent studies have assessed the utility of a BRCA1 selective polyethylene glycol, gold nanoparticle-infused biosensor. This biosensor showed substantial selectivity and a BRCA1 lower limit of detection of 1.72 femtomolar (Wang et al., 2015). These results suggest that biosensor-based detection of specific biomarker sequences may serve as a viable method of detection to address the issue of sensitivity in liquid biopsies (Wang et al., 2015; Yang et al., 2016).

## PTEN

Phosphatase and tensin homolog (PTEN) serves as a phosphatase for phosphatidylinositol-(3,4,5)-triphosphate (PIP3). PTEN decreases PIP3 concentration, which in turn serves to downregulate phosphorylated AKT (P-AKT). In healthy cells, PTEN serves as an indirect downregulator of P-MAPK and P-AKT, resulting in lower rates of cell division and increases in apoptosis (Teresi et al., 2006). Patients carrying EGFR mutations have reaped significant benefit from EGFR-TKIs; however, resistance to these TKIs poses a significant obstacle in treating patients. PTEN loss represents one mechanism of resistance to TKIs such as gefitinib and erlotinib, and continuing studies have set out to discover methods to circumvent the effects of this resistance mechanism (To et al., 2018).

Peroxisome proliferator-activated receptor agonists (PPARs) are implicated in the development and differentiation of cells, tumorigenesis, and metabolism. Studies have demonstrated that PTEN loss is correlated with resistance to gefitinib (To, Wu, and Loong, 2018; Lee et al., 2006). Increases in apoptosis were observed in cell lines treated concurrently with PPAR $\gamma$  agonists and gefitinib ( $P < 0.017$ ) (To et al., 2018). Hence, PPAR $\gamma$  agonists in conjunction with EGFR-TKIs may serve as a viable treatment strategy to circumvent EGFR-TKI resistance in patient subsets that exhibit PTEN loss (To, Wu, and Loong, 2018; Ni et al., 2017; Lee et al., 2006). Although future studies require further analysis of PTEN's ctDNA and solid tumor concordance rates in NSCLC, comparative analysis of liquid and tissue biopsies in prostate cancer patients revealed an 88.9% agreement between individual copy number calls of PTEN (Wyatt et al., 2017).

Despite the higher concordance rate observed in some studies, others caution that PTEN copy number alterations, when present in low circulating concentrations, can go undetected (Vandekerkhove et al., 2019). Thus, a predominant factor in the standardization of ctDNA assessment is the development of sufficiently sensitive assays that have lower limits of detection, which may preclude the aforementioned issue.

## BRAF (V600E and non-V600E)

BRAF (v-RAF murine sarcoma viral oncogene homolog B) is a serine/threonine kinase involved in the RAS/RAF/MAPK pathway. BRAF mutations can be categorized in three classes. BRAF V600/E/R/K

mutations are class I mutations that result in strong activation of BRAF's kinase activity and MAPK pathway. The mechanism of V600E mutations is that it can form salt bridges with K507 and the c-terminal of the alpha-C helix. They signal as monomers but mimic the dimerization process and respond to BRAF inhibitors (Dankner et al., 2018).

Class II and Class III mutations are non-V600E mutations. Class II mutations have high or intermediate kinase activity and serve as RAS-independent mutations, which signal as dimers and can respond to MEK and Pan-RAF inhibitors. Class III mutations show low kinase activity and are also called RAS-dependent mutant BRAF-wild-type. Class II and class III non-V600 BRAF mutants dimerize with BRAF or CRAF (Dankner et al., 2018).

A study by Lin et al. was performed to determine the frequency of BRAF mutations in Chinese individuals. The study determined that 1.2% to 4.2% of NSCLC patients in the Chinese population had the BRAF mutations. Authors also determined that only 30% of patients with BRAF V600E mutation benefited from BRAF inhibitors. Furthermore, Lin et al. also reported that the Class I mutation is predominantly found in females, while class II and III mutations co-occur with the KRAS mutation. This study's impact is limited because of its analysis of the BRAF mutation in specific races and ethnicities, and thus the findings cannot be readily extrapolated to other groups (Lin et al., 2019). In the clinical setting, detection of BRAF mutation via liquid or tissue biopsy appears equally reliable, as one study demonstrated that assessment of BRAF alteration yields a 96.2% concordance rate between liquid and tissue biopsies (Mao et al., 2017).

## NF1

NF1 is a tumor suppressor gene that shuts down activity of RAS protein via a protein called neurofibromin, which under normal physiological conditions stimulates GTPase activity of RAS and converts active RAS-GTP complex to its inactive RAS-GDP complex. This inactivation can lead to the shutdown of RAS associated GTPases (i.e. KRAS, NRAS, MRAS, HRAS, RRAS, and RRAS2). In patients with the NF1 mutation, there is a loss of functional neurofibromin, which can lead to accumulation of active RAS GTPases. This results in excessive cellular proliferation and tumor formation which can lead to the development of additional mutations, apart from those affecting RAS activity. Beau-champ et al.



found that loss of NF1 has shown response to dasatinib in dasatinib-resistant lung cancer (2014). Furthermore, low levels of NF1 with MAP-ERK expression can be sensitive to erlotinib and reverse the erlotinib resistance (Tao et al., 2020). TKI resistant NF1 mutations develop by the following mechanisms:

1. Activation of heat shock factor 1
2. Inhibition of tumor cell apoptosis
3. Promotion of epithelial-mesenchymal transformation
4. Promotion of sustained angiogenesis

Chian et al. performed a case-control study in the blood samples of 187 patients with NSCLC and 310 non-cancer patients. Authors used quantitative PCR to identify age and gender-based biomarkers (CPEB4, DUSP6, EIF2S3, GRB2, MCM4, MDM2, NF1, POLDIP2, RNF4, STAT2, and WEE1). The majority of the population was comprised of males 66 years and older, as well as smokers. Participants who never smoked expressed NF1 at lower levels. NF1 also had protective effects compared to MDM2 gene. NF1 was shown to be an age-dependent marker (OR: 0.16, CI: 0.14 to 0.88, P-value: 0.0255).

## PIK3CA

Hyperactivation of the PI3K/AKT pathway is common in NSCLC patients. This pathway is involved in interfering with various cellular mechanisms such as proliferation, migration, invasion, and resistance to therapy. Mutations in AKT, the catalytic subunit of PIK3CA, or PTEN downregulation can activate the AKT pathway. Sawa et al. determined a relationship between PIK3CA mutation and lung cancer in patients with COPD. The researchers extracted DNA from the surgical specimens of 197 patients and analyzed using deep sequencing techniques. The frequency of *PIK3CA* mutation was significantly higher in the COPD group than in the non-COPD group (10.4% vs. 1.7%,  $p = 0.015$ ).

The PIK3CA gene mutation can affect the prognosis of NSCLC through several mechanisms. A systematic review and meta-analysis performed by Wang et al. showed that PIK3CA expression status was not an independent risk factor for OS of NSCLC patients (HR = 0.80; 95% CI: 0.58-1.12;  $P = 0.193$ ), which is consistent with the results of 3 included studies.

## ROS1

ROS1 is a receptor tyrosine kinase similar to ALK. It is a single-pass transmembrane protein with an intracellular C-terminal tyrosine kinase domain and an extracellular N-terminal domain. ROS1's extracellular domain has similar amino acid sequences to that of cell adhesion molecules and the extracellular matrix. Typically, ROS1 is expressed more in the kidneys than in the lungs. The mechanism of ROS1 mutations is currently unknown. In general, ROS1 rearrangement occurs in the absence of other known oncogenic drivers (EGFR mutations, KRAS mutations, ALK rearrangements). There are some exceptions as well, which exclude it to be useful in screening. ROS1 can be identified via the FISH and RT-PCR techniques, but RT-PCR sometimes yields false negatives. IHC is another technique used to detect mutations of ROS1 (Luk et al., 2018). Concordance rates for detecting ROS1 alteration is 96.2% between liquid and tissue biopsies, according to analysis by Mao et al. (2017).

A multicenter prospective cohort study by Mezquita et al. was conducted on 128 patients to determine liquid biopsy's clinical usefulness in detection of ALK/ROS1 fusion and mutation to extrapolate with efficacy of tyrosine kinase inhibitors. Patients aged 18 years and older with ALK and ROS1-fusion-positive were enrolled during October 2015 and August 2018. Samples were collected at diagnosis and during radiation therapy. Blood samples were collected, and ct-DNA analysis as well as fusion load were evaluated. Absent mutation showed better OS. The median OS was 58.5 months in patients with ALK mutations (95% CI, 26.9 to not reached [NR]) while 44.1 months in patients with non-ALK mutations (95% CI, 21.7 to NR) and it was found to be 105 months in patients who were detected negative for ctDNA ( $P = .001$ ). Also, patients who had complex ALK mutations showed poor OS (median: 26.9 months; 95% CI, 13.9 months to NR) compared to patients with single ALK mutations (median: NR; 95% CI, 57.0 months to NR;  $P = .003$ ). The median PFS was 20.7 months (95% CI, 6.3 to NR) in the negative ctDNA group while it was found to be 2.8 months (95% CI, 1.2 to NR) in the patients with one or more ALK mutations ( $P = .03$ ).

## ALK

ALK is most common in NSCLC patients, presenting itself in about 3-5% of cases per year. ALK alteration is not hereditary and is most commonly present in younger patients (55 years old or below) or patients who have never smoked. IHC or FISH remain the most common strategies to identify this biomarker. ALK normally encodes for a protein that plays a role in neural development. Alterations in this gene are caused by chromosomal translocations and are almost always mutually exclusive to EGFR and KRAS. ALK mutations are most common among patients with a never/light smoking history, an adenocarcinoma histology, of a younger age, of the female gender, and in tumours with the wild type for EGFR and KRAS. The most common rearrangement of ALK in NSCLC is EML4-ALK (echinoderm microtubule-associated protein-like 4 (EML4)-ALK). This mutation leads to uncontrolled proliferation, migration, and tumorigenesis. Increasing research in fusion genes such as ELM4-ALK show that alongside coding for proteins, they generate non-coding RNAs that contribute to tumor progression (Camidge et al., 2010).

In 2011, the FDA approved the first TKI for ALK mutations, crizotinib. Since then, researchers began studying ALK as a predictive biomarker. For instance, Vincent et al. outlined biomarkers involved in clinical practice in 2012, indicating the importance and extensive research that has been done on ALK over the past couple years. Currently, IHC combined with crizotinib is considered a gold standard for the detection and treatment of ALK-positive NSCLC (Shaw et al., 2011). EML4-ALK fusion variant 3 (V3) serves as a new potentially targetable biomarker for higher-risk cases. Evidence shows that the presence of the V3 protein in ELM4-ALK leads to acceleration of disease, failure at early stages of treatment, and a poorer OS. ELM4-ALK V3 is also associated with shorter PFS, even after non-TKI treatments and chemotherapy (Christopoulos et al., 2018). This suggests the differences between the V3 variant and other more common variants, such as EML4-ALK V2, are clinically distinguishable due to V3's stark differences ( $P < 0.001$ ) in PFS regardless of previous treatment to other ALK tumor types. V3 variants progress faster in response to TKI treatments, which is likely due to the developments of TKI resistant mutations. A study conducted by Madsen et al., analyzed ctDNA of NSCLC patients with the ALK gene. The results showed the presence of ctDNA before treatment initiation is associated with inferior PFS as well as the presence of ctDNA shortly after the start of ALK TKI treatment. This study

emphasizes the value of genomic profiling through ctDNA during all stages of the treatment. Although there are multiple targeted therapies for ALK, fusion variants of ALK that show poorer PFS and OS still need to be studied (Madsen et al., 2020).

## KRAS

Kirsten rat sarcoma viral oncogene homolog (KRAS) is the most frequent oncogenic mutation in western countries for NSCLC (Liu et al., 2020). It is involved in the RAS/MAPK pathway and codes for the K-ras protein (GTPase), the most commonly mutated gene in its pathway.

Mutations in the KRAS gene impair the GTPase activity, resulting in a constantly active GTPase, which leads to cell survival, proliferation, and differentiation. The most common nucleotide mutation in KRAS is the G12C mutation, which has been directly linked to smoking. Further research is necessary to establish a relationship between variant KRAS types and prognosis. Cox regression and multinomial logistic regression were used to differentiate the effect of KRAS mutation subtype on OS. G12C subtype was associated with poor OS ( $P = 0.021$ ), compared to G12D (Aredo et al., 2019). Data was extracted from patients in the Thoracic Malignancies Cohort (TMC) to compare clinical features and OS of the wild type KRAS to KRAS G12C, amongst other mutated types. 100% of the patients with the KRAS G12C subtype were active smokers. Results showed that the treatment and survival of KRAS mutant subtypes were similar (Cui et al., 2020). However, KRAS G12C in particular proved to be an important predictive biomarker compared to the other KRAS mutant types, such as wildtype KRAS and mutated KRAS ( $P = 0.74$ ) (Cui et al., 2020). Although there are no current KRAS inhibitors on the market, it has been marked as a clinically applicable biomarker in a paper by Vincent et al. in 2012. Subsequently, research conducted since then corroborates the potential for KRAS to serve as a potential predictive biomarker in NSCLC. Data collected from plasma based liquid biopsies by NGS showed that 36 (18.6%) out of the 194 liquid biopsies were a KRAS G12C variant. Since tissue biopsies are often rejected (17%) due to insufficient cellularity, KRAS detection in ctDNA may benefit from clinical implementation.

## TSC1/2

The TSC1-TSC2 complex is a key regulator of the mTORC1 (mammalian target of rapamycin complex 1), which controls cell growth. Hamartin (TSC1) and tuberlin (TSC2) are the mutations of genes found in the

tuberous sclerosis complex (TSC). Inhibition of TSC1/2 results in inactive mTORC1, leading to uncontrolled cell growth. Fuchs et al., determined the inverse correlation between hamartin and p-mTOR (the catalytic subunit of two distinct protein complexes which controls cell proliferation) expression in NSCLC and SCLC samples. FISH and western blot analysis were performed on 166 NSCLC and SCLC cell lines. Furthermore, cytoplasmic hamartin expression was expressed in greater than 50% of NSCLC cell lines (Fuchs et al., 2014). Fuchs et al., concluded that activation of lung cancer cell lines is caused by the inhibition of the mTORC by the TSC. Unlike most other NSCLC mutations, TSC1/2 may be caused by EGFR expression, but is not dependent on it. Wang et al. utilized 144 NSCLC patients to study the characteristics of TSC1 and TSC2 in NSCLC. The researchers found 27 of the 144 NSCLC patients exhibited TSC1 mutation and 40 exhibited TSC2 mutation. It must also be noted that most patients with TSC mutations expressed other oncogenic gene alterations as well. The expression of TSC1/2 was slightly more common in NSCLC compared to SCLC (53.7% vs. 43.6%) (Wang et al., 2020). Furthermore, the TSC1 mutation had a median OS of 14.1 months, whereas patients with TSC2 mutation had a median OS 110.6 months; however, the difference was not statistically significant ( $P = 0.201$ ). Wang et al. concluded that TSC1/2 defines a unique NSCLC population and often coincides with other mutations such as TP53. In an experiment conducted by Rulli et al., liquid biopsies were used to measure the number of circulating tumor cells (CTCs), the quantity of cell free tumor DNA (cftDNA), and the mutational profile of DNA from CTCs (ctcDNA) and cftDNA in early stage breast cancer patients. NGS of ctcDNA and cftDNA showed that 52% of the patients expressed mutations in multiple genes including TSC1 (Rulli et al., 2020). In addition to the significance of TSC in NSCLC prognosis and treatment, initial research shows strong promise for it to be implemented via liquid biopsies. Currently, there are no targeted therapies on the market for TSC1/2, although PD-1 and/or CTLA-4 inhibitors show success in clinical trials for TSC1/2 associated tumors (Liu et al., 2018).

## **FGFR1/2**

Fibroblast growth factor receptor 1 and fibroblast growth factor receptor 2 are members of the fibroblast growth factor receptor family. FGFR1 and FGFR2 span the cell membrane and interact with fibroblast growth factors outside the cell. This results in a cascade of downstream signaling within

the PI3K and MAPK pathways, which control embryonic development, cell proliferation, differentiation, and migration (Theelen et al., 2016). In a study conducted by Theelen et al., 653 early stage NSCLC samples were assessed. The levels of expression in FGFR1 and FGFR2 directly correlated to clinicopathological features. FGFR1 expression was associated with light smoking ( $p = 0.02$ ), FGFR2 correlated negatively with age for the whole cohort ( $p = 0.27$ ), and there was a negative correlation between tumour stage and FGFR2 expression ( $p = 0.002$ ). FGFR1 was expressed in 10.6% and FGFR2 in 12.9% of all NSCLC tumor samples (Theelen et al., 2016). Protein expression of FGFR is related to worse OS. Moreover, while FGFR1 is associated with light smoking, FGFR2 is more common in females and younger age patients (age 31 to 53 years). The study by Theelen et al. demonstrated an abundance of FGFR mutations present in NSCLC, suggesting that they play a role in growth and malignant progression of NSCLC. A study by Santiago-Walker et al. demonstrated a 63% concordance rate for detecting FGFR mutations in temporally unmatched blood and tissue, which supports the potential for patient selection with blood-based testing. Clinical differences were observed between ctDNA-FGFR positive and negative patients, but they were not statistically significant (Santiago-Walker et al., 2019). Furthermore, FGFR1's indication of a worse OS suggests that it may serve as a prognostic biomarker (Theelen et al., 2016).

## EGFR

Epidermal growth factor receptor (EGFR) mutations are observed in 10-35% of all patients with adenocarcinomas. EGFR-targeting TKIs (EGFR-TKIs) have shown higher objective response rates and longer PFS. EGFR-TKIs are considered more successful than chemotherapy for EGFR mutated patients. Unfortunately, about 30% of patients develop resistance to these EGFR TKIs. A study done with 502 EGFR-mutated NSCLC patients was conducted between 2003 and 2014 to examine the relationship between EGFR mutations and TP53 mutations. TP53 is a tumor suppressor that is commonly mutated in many cancers including NSCLC. TP53 mutations are found in 35-55% of NSCLC patients. Of the 502, 43 had both TP53 and EGFR mutations. Those with mutant TP53 had relapse-free survival (RFS) for a median of 42.2 months whereas those with wildtype had RFS for a median of 37.7 months ( $P=0.59$ ). 60 patients received EGFR-TKIs as treatment. Of these 60 patients, 24 had mutant TP53 and 36 had wildtype TP53. The results of the treatment were not



statistically different (Labbe et al., 2017). EGFR can be used to direct the course of treatment.

## RRM1

Ribonucleotide reductase catalytic subunit M1 (RRM1) is a biomarker involved in tumor proliferation, invasiveness, and metastasis. In a study done by Mlak et al., 60 NSCLC patients' RRM1 expression was measured through use of liquid biopsy. All of the patients had not been treated for NSCLC yet. High RRM1 expression was associated with higher risk of oral mucositis, but it was not significantly associated with disease-free survival (DFS) or OS shortening (Mlak et al., 2018). In another study done by Zhang et al., RRM1 and ERCC1 levels were measured through liquid and tissue biopsy in NSCLC patients using PCR. Patients with lower level RRM1 expression, as seen in the liquid biopsy, had longer median OS. Their OS was 18.5 months, as opposed to patients who had higher level expression whose OS was 13.0 months ( $P = 0.043$ ).

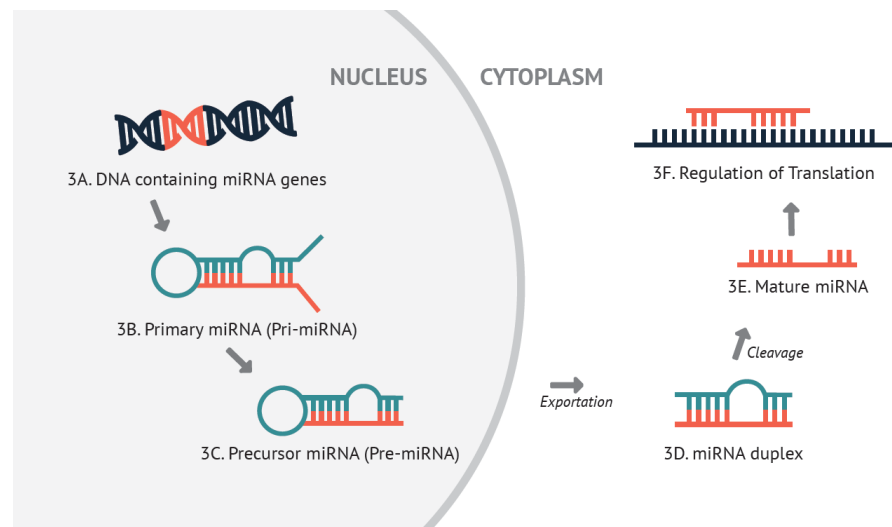
Higher level RRM1 was also associated with prolonged PFS. Patients with low expression level had a median PFS of 6.0 months whereas patients who had lower expression level had a median PFS of 4.0 months ( $P = 0.044$ ) (Zhang et al., 2012). RRM1 levels, measured through use of liquid and tissue biopsy, can be used to predict outcomes such as OS and PFS.

## Wip1

Overexpression of wildtype p53-induced phosphatase 1 (Wip1) is commonly found in many types of tumors and is associated with poor prognosis. In a study by Zhao et al., 117 NSCLC patients were examined and Wip1 expression was determined through IHC. The patient cohort had a mean age of 56.9 years, of which 87 were male and 30 were female. In normal lungs, Wip1 is not expressed; the results of this study showed that Wip1 was expressed in 69.3% of the NSCLC patients. Wip1 overexpression was observed more in lung adenocarcinomas than other subtypes of NSCLC. Wip1 negative patients survived for a longer time than Wip1 positive patients. After 80 months, the survival of Wip1 positive patients was less than 20% but the survival of Wip1 negative patients was over 40% ( $P=0.014$ ) (Zhao et al., 2016). In another study, 84 patients, comprising 46 males and 38 females, were examined and treated. All of them had lung adenocarcinomas. Wip1 levels were examined through IHC. Patients were considered Wip1 positive if 10% or more cancer cells within the tumor were strongly stained for Wip1 after IHC. Wip1 expression was positive in

64.3% of the patients. The survival of Wip1 negative patients was significantly greater than Wip1 positive patients. The OS rate was around 50% for Wip1 positive patients 100 months after surgery whereas the OS rate was around 90% for Wip1 negative patients ( $P = 0.0099$ ). These results demonstrate that Wip1 expression correlates with negative prognosis (Sato et al., 2011). Wip1 can be used as a predictive outcome biomarker in order to foresee the prognosis of the patients.

## miRNA Based Biomarkers



**Figure 3.** This figure depicts the miRNA pathway. 3A: miRNA genes are transcribed to primary miRNA (Pri-miRNA). 3B, 3C: Pri-miRNA is processed to precursor miRNA (Pre-miRNA). 3D: Pre-miRNA is exported from the nucleus to the cytoplasm, maturing into miRNA duplex. 3E: miRNA duplex is cleaved to produce mature miRNA. 3F: Mature miRNA participates in the regulation of cellular pathways (Kwak et al., 2010).

## Let-7

The miR let-7 family regulates components of cellular development and differentiation. Although typically a tumor suppressor, some cases have been recorded in which let-7 acts as an oncogene (Chirshev et al., 2019). Xie et al. examined the role of let-7 in detecting time of acceleration re-proliferation, a key contributor to radiotherapy failure. The study enrolled 19 eligible patients and via qRT-PCR and statistical analyses found a high level of serum let-7 was associated with a better OS rate ( $P=0.024$ ), suggesting that serum let-7 could reflect the proliferation of tumor tissue (Xie et al., 2016). Another study tested let-7 prognostic capability as part of a miR panel consisting of let-7e, 125a-5p, miR-30a, miR-30e, and

miR-30-3p. The study found NSCLC dedifferentiation was associated with reduced expression of miR-125a-5p, let-7e and miR-30e ( $P=0.038$ ); the loss of expression of let-7e and miR-125a-5p was associated with shorter postoperative survival in NSCLC patients ( $P=0.007$ ) (Zhu et al., 2014). Dedifferentiation is a process required for tumorigenesis, occurring when specialized cells become less specialized and acquire tumor cell plasticity or self-renewal ability (Friedmann-Morvinski & Verma, 2014, p. 245). A more recent study performed qRT-PCR analysis on 120 NSCLC patients and 360 healthy controls and found plasma let-7c and miR-152 expressions were lower in NSCLC patients. Furthermore, receiver operating characteristic curves displayed an association between low let-7c level and poor differentiation status ( $P<0.001$ ), cancer metastasis ( $P=0.021$ ), and advanced stage classification ( $P=0.013$ ). Lastly, comparing postoperative and preoperative plasma from 96 NSCLC cases showed an increase in let-7c expression level post-operation: suggesting the potential of let-7c as a prognosis biomarker for NSCLC (Dou et al., 2015).

## **MiR-21**

miR-21 is an extensively studied oncogenic miRNA due to its association with tumor suppressor genes that regulate proliferation, apoptosis, and invasion (Feng et al., 2016). More recently, researchers have sought to utilize the presence of miR-21 as a predictive marker of NSCLC. Wang et al. utilized serum samples of 88 NSCLC patients and 17 healthy controls obtained from Jiangsu Province People Hospital. The researchers found that the 3-year OS in NSCLC patients with high serum miR-21 expression was lower (39.8%) compared to 58.2% in patients with low serum miR-21 ( $P<0.001$ ) (Wang et al., 2011). Liu et al. reported consistent results with the prior study regarding OS, but noted that the miR-21 levels in serum and tumor miR-21 levels had no significant correlation (2012).

miR-21 levels have also been used to predict NSCLC tumor recurrence in patients post surgical resection. Munagala et al. studied cancer recurrence in mouse models and found that miR-21 was upregulated in recurrent tumors compared to primary tumor tissue, and more notably, serum miR-21 also mirrored the tumor profile tumor miRNA results (Munagala et al., 2011). Dejima et al. confirmed this observation by examining miR-21 levels in 195 NSCLC patients and 30 healthy patients; finding that exosomal miR-21 levels showed significant increase in NSCLC patients with recurrent tumors ( $P<0.01$ ) (Dejima et al., 2017). In the clinical

setting, Han-Bo Le et al. found that miR-21 levels in post-operative patients' serum samples was lower than pre-operative levels, which further suggests miR-21's role as predictive biomarker for tumor recurrence.

The use of miR-21 to evaluate treatment options has also been investigated. Wei J et al. followed up with 53 patients who received platinum-based chemotherapy to treat NSCLC. They found that patients with partial response to the therapy had miR-21 plasma levels several fold lower than patients with stable or progressive disease (Wei et al., 2011). Gao et al. noted similar results via tumor miRNA analysis of 58 patients, with high levels of miR-21 expression correlating with increased platinum resistance (Gao et al., 2012).

## **MiR-30**

The miR-30 family serves in a regulatory capacity during tissue and organ development. The miR-30 family possesses tumor suppressor abilities, which include its roles in the pathogenesis of cancers, including breast cancer, thyroid cancer, colon cancer, and lung cancer (Mao et al., 2018).

A study investigated the prognostic value of plasma miR-30b and miR-30c from EGFR-mutated lung cancer patients undergoing erlotinib treatment. Blood qPCR analyses from 29 erlotinib-treated, EGFR-mutated lung cancer patients demonstrated associations between low plasma levels of miR-30b and miR-30c and increased efficacy of erlotinib in EGFR-mutated NSCLC patients as indicated by PFS ( $P < 0.05$  for both miRNAs). The study concluded that miR-30b and miR-30c may serve as potential biomarkers to predict erlotinib efficacy in EGFR-mutated NSCLC patients (Hojbjerg et al., 2019).

Another study on 104 lung cancer cases with benign lesions and 20 healthy controls from West China Hospital investigated the prognosis value of miR-30a-5p. Through qRT-PCR, high plasma miR-30a-5p levels correlated with increased tumor size ( $P = 0.02$ ), advanced tumor differentiation ( $P = 0.03$ ), and advanced tumor node metastasis (TNM) stage ( $P = 0.0001$ ). The plasma level of miR-30a-5p was also found to significantly decrease post-surgery ( $P < 0.0001$ ) (Liang et al., 2019). Through Kaplan–Meier survival analysis, low miR-30a-5p expression was associated with longer survival compared to high miR-30a-5p expression ( $P = 0.0001$ ). Kaplan–Meier survival analysis is an approach to measure the portion of

samples surviving post-treatment after a certain time period (Kishore et al., 2010). Finally, Cox multivariate regression analysis confirmed the correlation between miR-30a-5p, advanced TNM stage, and OS, signifying the prognostic value of miR-30a-5p in NSCLC (Liang et al., 2019).

### **MiR-125b**

The dysregulation of miR-125b is a common feature across many cancers that affects tumor cell proliferation, differentiation, invasion, migration, drug resistance, and tumor immunity. Due to its tendency to act as both tumor-suppressor and oncogene, depending on the cancer type and molecular contexts, miR-125b has not been utilized for clinical purposes. Recently, however, several studies have noted miR-125b's potential as a prognostic biomarker for advanced NSCLC patients. Cui et al. compared serum miRNA-125b across 260 inoperable advanced NSCLC patients and 260 healthy patients. Utilizing qRT-PCR to measure circulating miR-125b, and evaluating efficacy of chemotherapy in accordance with the Radiologic RECIST, the study found that 99 patients (38%) responded to chemotherapy with partial or complete response; 161 (62%) patients were not responsive, but instead exhibited stabilization or disease progression. miR-125b was significantly associated with chemotherapeutic response, with nonresponsive patients exhibiting significantly higher expression levels than responsive patients ( $P=0.003$ ). The authors postulate that miR-125b may act as a viable biomarker to predict chemotherapy resistance. Confirming this observation, Shi et al. measured the relative expression of miR-125b in 74 patients with advanced NSCLC pre- and post chemotherapy using RT-qPCR and noted that sensitivity to chemotherapy in NSCLC patients with high expression of miR-125b was lower than those with low expression of miR-125b ( $p<.05$ ) (2020).

The utility of miR-125 to predict survival outcomes has also been studied. Yuxia et al. compared miR-125b serum levels in 193 patients with varying stages of NSCLC, following surgery and therapy. Patients with miR-125 expression levels lower than 2.79 were put into the low-expression group, and those above, in the high expression group. Kaplan-Meier survival curves revealed that high expression significantly correlated with poor survival ( $p<.00001$ ).

Multivariate Cox hazard analysis also showed miR-125b to be an independent prognostic marker on NSCLC (Yuxia et al., 2012).

## MiR-145

miR-145 has been reported to have decreased levels in many different cancers, such as pancreatic cancer, prostate cancer, breast cancer, and colorectal cancer (Liu et al., 2018). miR-145's tumor suppressive abilities and its prevalence in many types of cancer makes it a potential biomarker for prognosis. A study on immune responses and cancer progression assessed 345 NSCLC patients and focused on a 5 miRNA panel, which consisted of miR-191, miR-28-3p, miR-145, miR-328 and miR-18a. Through the use of liquid biopsy and PCR, these miRNA levels were measured; a high expression level was defined as greater than 80%. The median survival time for those with high expression of all 5 miRNAs was 3.6 to 5.7 months shorter than those with low expression. Out of the miRNAs investigated in the study, miR-145 demonstrated higher expression in normal patients than in NSCLC patients. Nevertheless, higher levels of miR-145 in NSCLC patients is associated with poorer survival. High miR-145 expression had an 88.4% 3 year death rate as opposed to low miR-145 expression which had an 79.2% 3 year death rate ( $P = 5.21E-03$ ). miR-145 is associated with Ras, mitogen-activated protein kinase, ATM and estrogen receptor signaling pathways, which are closely associated with resistance to chemoradiotherapy and/or targeted therapy in advanced NSCLC (Zhang et al., 2019).

## MiR-155

MiR-155 is overexpressed in many diseases and is significant in carcinogenesis. It is most commonly overexpressed in solid tumors and hematopoietic malignancies. Its overexpression in NSCLC has foreshadowed its potential as a biomarker for prognosis and predictive purposes. MiR-155 has exhibited high expression in NSCLC patients and has been seen to induce proliferation of NSCLC cancer cells. In a study with 180 total NSCLC patients and 80 control patients, miR-155 and miR-21 expression levels were compared between one group of 68 newly diagnosed patients and 112 patients with recurrent or metastasized NSCLC. The expression of miR-155 and miR-21 was higher in those with NSCLC when compared to the control group ( $P < 0.01$ ), while expression of both miR-155 ( $P < 0.05$ ) and miR-21 ( $P < 0.01$ ) was higher in those in the recurrence group than those in the newly diagnosed group. Higher levels of these miRNAs were found to affect the prognosis negatively, with a mortality rate of 91.96% and a 19 month median survival time in the



recurrence group compared to a 57.35% mortality rate and a 28 month median survival time in the newly diagnosed group ( $P < 0.05$ ) (Xu et al., 2019). In combination of a three-miRNA signature panel of high level miR-155-5p, high level miR-223-3p, and low level miR-199a-5p, there was a mean DFS of 46 months in 52 resectable NSCLC patients (Sanfiorenzo et al., 2013). In three independent cohorts of NSCLC patients in Maryland, Norway, and Japan, miR-155 was concluded in addition with miR-17 and miR-21 in association with mortality rates and DFS (Saito et al., 2011).

The prognosis impact of miR-155 expression is influenced by other factors as well. In another study, 335 NSCLC patients were examined, and it was discovered that squamous cell carcinoma expressed higher levels of miR-155 than adenocarcinomas. Higher miR-155 expression has a negative role in disease specific survival (DSS) prognosis in patients with adenocarcinomas, but a positive role in DSS in patients with squamous cell carcinomas. The median survival for those with high miR-155 expression was 84 months whereas the median survival rate for those with low miR-155 expression was 190 months ( $P=0.43$ ). Thus the prognostic impact of miR-155 differs by histological subtype (Donnem et al., 2011). Hanafi et al. also found that in 52 patient serum samples high miR-155 expression level correlated with poor prognosis, and is correlated with a median survival (MS) of 69 days ( $P=0.034$ ) in adenocarcinomas and a MS of 58 days ( $P=0.023$ ) in patients with positive EGFR gene mutations (Hanafi et al., 2020).

## **MiR-486**

While miR-486 is widely controversial, with many studies reporting conflicting results in miR-486's role as an oncogene or tumor suppressor, it has been proven to be a significant, non-invasive biomarker for prognosis. Sromek et al. measured miR-486 levels in patients that underwent tumor resection and found increased miR-486 levels expressed in their plasma samples ( $n=14$ ) one year after surgery (Sromek et al., 2017).

A meta-analysis conducted by Jiang et al. among 7 studies demonstrated that there was no increased risk of poor outcome with lower expression of miR-486 (Jiang et al., 2018). Poor outcome was defined using 3 factors: OS, PFS, and RFS. Conversely, in a study by Gao et al. high levels of miR-486 in 140 NSCLC patients was directly correlated with shorter OS, specifically in Stage I NSCLC cancer (Gao et al., 2020). When using

miR-486 in combination with miR-30d, miR-1, and miR-499 on a 4 signature miRNA signature panel, high serum levels of miR-486 also showed similar results of unfavorable survival. More specifically, it was shown that within the 60 patient cohort, those that carried two or more of the high-risk miRNA would have significantly shorter survival ( $P$  all  $< 0.001$ ) compared to those who had none or one high-risk miRNA (Hu et al., 2010). In patients that showed down-regulated miR-486 levels in plasma after surgical removal of tumors, the recurrence free survival (RFS) was higher than in those that had stagnant levels of miR-486, the un-reduced group, that did not have reduced miR-486 levels or miR-486 levels did not change. The group with down-regulated miR-486 had a median unreached survival, while the un-reduced group had a median survival of 19 months ( $P=0.056$ ) (Li et al., 2015).

In studies that focused on clinical phenotype of metastasis, it was shown that miR-486-5p suppressed migration and invasion abilities of NSCLC cells, preventing cancer progression and metastasis. In one study, miR-486-5p downregulated NSCLC through *ARHGAP5*, a protumorigenic gene. miR-486-5p had a statistically significant inverse relationship with *ARHGAP5* ( $P=0.0156$ ), which correlated with further cancer progression ( $n=76$ , frozen NSCLC samples) (Wang et al., 2014). Moreover, in correlation with the P13K-Akt signaling pathway, miR-486-5p and the P13R1 gene had regulatory effects. In A549 and H1299 miR-486-5p transfected NSCLC cell lines, inhibited A549 and H1299's abilities of migration and invasion after 48 hours ( $p < 0.05$ ), showed higher apoptosis rates after 72 hours ( $p < 0.05$ ), and inhibited cell proliferation through miR-486-5p suppression of P13R1 ( $p < 0.001$ ) (Tian et al., 2019). Furthermore, it was found that miR-486-5p was an effective therapeutic agent for cisplatin resistant NSCLC treatment. miR-486-5p improved the susceptibility to cisplatin through miR-486-5p's suppression of TWFI1, twinfilin actin binding protein 1, a gene that plays a role in tumor invasion and chemotherapy resistance. An *in vivo* investigation of cisplatin resistance showed that miR-486-5p expression correlated with reduced tumor size in nude mice ( $P < 0.001$ ) (Jin et al., 2019).

## Conclusion

In the literature about miR, we found several articles that indicate significant promise for their utility as a biomarker for prognosis, outcome prediction, or treatment strategies. First, recent studies regarding miR

biomarkers have begun to diversify from general metrics such as OS. More specific metrics now include predicting tumor recurrence, resistance to treatments, medication performance, and much more. Second, the articles we reviewed have indicated miR testing to be feasible in most clinical settings. We found the measurement of miR typically involves some variation of PCR procedure performed on plasma, serum, and saliva samples.

Lastly, while we failed to observe standardization in the studies we reviewed, each independent study demonstrated significant promise for miR utility as a biomarker for prognosis, outcome prediction, or treatment strategies. These studies consisted of large sample sizes with little error indicated in the observed results. If these studies are verified, they present great potential for miR biomarkers that would assist clinicians in determining treatment strategies.

Similar trends were found in the ctDNA literature. Our review paper noted that many ctDNA biomarkers indicate predictive outcomes including PFS and OS. Many ctDNAs are used for directing the course of treatment, including MET, BRCA, PTEN, and EGFR. This is often because abnormal expression of these biomarkers can lead to resistance, which requires the use of alternate therapies. Furthermore, while comparing ctDNA from liquid biopsies with tissue biopsies, we found high concordance rates between liquid biopsies and tissue biopsies. There is a 96.2% concordance rate between liquid and tissue biopsies for RET and ROS1 and an 81.6% concordance rate for CDK2NA. High concordance rates between tissue and liquid biopsies furthers the case for clinical implementation of liquid biopsy as a means to detect genetic alterations in ctDNA. Unlike standard tissue biopsy options, ctDNA and miRNA are obtained through less invasive methods (usually via a simple blood test). However, the question remains whether ctDNA or miRNA will serve as viable standalone methods in the detection of actionable or prognostic biomarkers. Based on current literature, circulating nucleic acid biomarkers appear to hold promise in indicating targeted treatment plans for NSCLC patients presenting with specific genomic profiles. With the increasing number of emerging biomarkers, it is critical to analyze the clinical utility of these biomarkers in light of their prognostic value and clinical indications. Clinical actionability remains a significant factor in determining biomarker value.

Ease of implementation and invasiveness of testing procedures are important considerations in developing assay methods that ease patient discomfort without diminishing specificity and accuracy of detection. The impetus to ensure reliable and streamlined testing measures is paramount, as the current literature suggests that early detection of specific biomarkers can provide a remarkable clinical benefit. Moreover, in an effort to ensure equal access to advanced cancer care, the financial burden of testing should be assessed. Ultimately, the prognostic significance of ctDNA and miRNA biomarkers in NSCLC care has demonstrated that liquid biopsy and molecular diagnostic testing hold promise for the future of personalized cancer care. Further testing must be conducted to analyze the significance of ctDNA and miRNA biomarkers in larger cohorts and to determine the precise clinical benefit of their standardization in clinical practice.

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