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MicroRNAs in non-small cell lung cancer: Gene regulation, impact on cancer cellular processes, and therapeutic potential

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Abstract

Lung cancer remains the most lethal cancer among men and women in the United States and worldwide. The majority of lung cancer cases are classified as non-small cell lung cancer (NSCLC). Developing new therapeutics on the basis of better understanding of NSCLC biology is critical to improve the treatment of NSCLC. MicroRNAs (miRNAs or miRs) are a superfamily of genome-derived, small noncoding RNAs that govern posttranscriptional gene expression in cells. Functional miRNAs are commonly dysregulated in NSCLC, caused by genomic deletion, methylation, or altered processing, which may lead to the changes of many cancer-related pathways and processes, such as growth and death signaling, metabolism, angiogenesis, cell cycle, and epithelial to mesenchymal transition, as well as sensitivity to current therapies. With the understanding of miRNA biology in NSCLC, there are growing interests in developing new therapeutic strategies, namely restoration of tumor suppressive and inhibition of tumor promotive miRNAs, to combat against NSCLC. In this article, we provide an overview on the molecular features of NSCLC and current treatment options with a focus on pharmacotherapy and personalized medicine. By illustrating the roles of miRNAs in the control of NSCLC tumorigenesis and progression, we highlight the latest efforts in assessing miRNA-based therapies in animal models and discuss some critical challenges in developing RNA therapeutics.

KEYWORDS
Cancer, miRNA, NSCLC, regulation, therapy, tumorigenesis

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1 | INTRODUCTION

Lung and bronchus cancer is the second most commonly diagnosed cancer in the United States, with over 220,000 estimated new diagnoses in 2019, accounting for almost 13% of all cancer diagnoses. One in 15 men and 1 in 17 women will be diagnosed with lung cancer during their lifetime and the average age at diagnosis is 70 years. Lung cancer accounts for the highest cancer-related deaths in the United States, causing 23% of all cancer-related deaths which is more than colon, breast, and prostate cancers combined. The five-year survival rate of all lung cancer diagnoses is 19%, which is lower than colon, breast, and prostate cancers. When the disease is detected while still localized to the lung, the five-year survival rate is 56%; however, only 16% of cases at diagnosed at that stage. By contrast, the five-year survival rate for metastatic lung cancer patients is only 5%. More than half of patients diagnosed with lung cancer will die within one year. It is estimated that lung cancer care may be increased to 173 billion dollars in 2020 in the United States.2

The lung epithelium undergoes a series of morphological changes before becoming invasive, such as hyperplasia, metaplasia, and finally dysplasia and carcinoma in situ. Lung cancer is classified by the site of origin, and method of diagnosis, prognosis, and treatment. The two main types of lung cancer are small cell lung cancer, accounting for 15% of all cases, and non-small cell lung cancer (NSCLC) which is any type of epithelial lung cancer, and accounts for 80% to 85% of all cases (Figure 1). The three most common histological forms of NSCLC are epidermoid or squamous cell carcinoma, large cell carcinoma, and adenocarcinoma; among them adenocarcinoma accounts for 40% of all lung cancer cases. Squamous cell carcinoma occurs inside the airways, adenocarcinoma occurs in the cells lining the alveoli located in the outer part of the lungs, and large cell carcinoma is in any other part of the lung. A major risk factor for NCSLC is smoking; other risks include secondhand smoke, radiation exposure, air pollution, family history, and human immunodeficiency virus infection. Lung cancer may present as a persistent cough, chest pain, weight loss, malaise, difficulty breathing, pleural effusion, pneumo-

2 | MOLECULAR FEATURES OF NSCLC

Molecular features of NSCLC tumors may not only predict the prognosis and outcome the cancer but also serve as targets for therapies. The most frequent mutations in NSCLC occur in the TP53 gene, occurring in about 50% of NSCLC cases (Table 1).9 Mutations in EGFR, a tyrosine kinase receptor, account for 10%-35% of cases and can cause dysfunction of the AKT and MAPK signaling which enhances cell survival and stimulates proliferation.10 The most common mutations of EGFR are in-frame deletions of exon 19, and the second most common EGFR mutation is single nucleotide substitutions L858R in exon 21.11 The most common mutation detected after treatment with EGFR inhibitors is T790M in exon 20 which can confer drug resistance.12 The third most frequent mutations occur in KRAS, accounting for 15%-25% of cases.13 Usually mutations in KRAS and EGFR are mutually exclusive and non-overlapping. Another common molecular feature of NSCLC is the presence of ALK fusion gene, which encodes a receptor tyrosine kinase not normally expressed in the lung.13 At least nine different variants of fusion of ALK with an upstream partner EML4 have been identified causing constitutive activation of the kinase.13 The HER2 protein, a HER family receptor tyrosine kinase, is overexpressed in 20% of all NSCLC and gene amplification occurs in 2%.14,15 These mutations commonly lead to constitutive activation of the HER2 signaling pathway.16 Mutations in the main catalytic subunit, PIK3CA, of phosphatidylinositol 3-kinase occur in about

FIGURE 1 Lung cancer classifications and frequency of diagnosis. There are two main types of lung cancer: small-cell lung cancer and non-small cell lung cancer. Small-cell carcinoma occurs in the outer edges of the lungs and accounts for about 15% of all cases. Non-small cell lung cancer (NSCLC) makes up 85% of all lung cancer cases, and can be further classified into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Adenocarcinoma, the most common NSCLC subtype, occurs in the cells lining the alveoli, squamous cell carcinoma is generally found in the airways or bronchi, and large cell carcinoma is in the edges of the lungs.
2% of NSCLC cases. These tumors can activate the protein kinase B signaling pathway without growth factors. Protein kinase B is encoded by AKT1, which is mutated from a glutamate to a lysine at position 17 in 1% of NSCLC cases and causes PI3K-independent activation of protein kinase B. BRAF is a member of the RAF kinase family which confers signaling of the MAPK family from the RAS GTPases to control cell proliferation. BRAF mutations are mostly found in adenocarcinomas and lead to higher kinase activity and constitutively active MAPK2 and MAPK3. MAPK2 and MAPK3 are downstream of BRAF and three mutations in the non-kinase portion of these proteins have been found in cancer. Amplification of the gene MET, which codes hepatocyte growth factor receptor (HGFR) causes resistance to EGFR tyrosine kinase inhibitors. The molecular heterogeneity of NSCLC tumors increases the complexity of treatment for NSCLC patients.

### 3 | CURRENT TREATMENTS FOR NSCLC PATIENTS

Lung cancer that is diagnosed at the early stages is commonly treated with resection surgery or lobectomy, chemotherapy, and radiation. Surgery may range from removing an entire lung to removing part of a lobe, depending on the size and location of the tumors. Radiation, alone or concurrent with surgery or chemotherapy is also commonly utilized. External beam radiation therapy, the most widely used form of radiation for NSCLC, consists of administering 1.5 to 2.5 Gray to the lungs usually 5 days a week for 5-7 weeks, while stereotactic radiotherapy consists of a larger dose, around 22 Gray, in usually fewer than 5 doses. Brachytherapy, or internal radiation therapy, involves the inserting a radioactive pellet in or near the tumor for a short amount of time or permanently. The radiation stays localized and gets weaker over time. Radiofrequency ablation uses radio waves emitted from a probe guided by a computed tomography scan.

Molecular medicine or pharmacotherapy spans from chemotherapy to targeted therapy and the most recent immunotherapy, which utilize small-molecule and protein or antibody drugs (Table 2). Commonly used chemotherapies for the treatment of NSCLC include cisplatin, carboplatin, docetaxel, paclitaxel, pemetrexed, and vinorelbine that usually interfere with DNA synthesis or replication to achieve the inhibition of cancer cell proliferation and growth (Table 2). Nevertheless, chemotherapy may not be effective for all patients and many cancers will eventually become resistant to the drugs. Furthermore, chemotherapy kills cancer cells less specifically and could cause some side effects like pain, nausea, vomiting, blood disorders, and hair-loss.

Pharmacotherapy for NSCLC has been benefited greatly by the development of targeted and personalized medications, either small molecules or antibodies, which act more selectively on particular molecular targets including transmembrane and cell surface proteins or receptors (eg, EGFR, PD-1, PD-L1, etc) as well as signal proteins (eg, cytokines, VEGF) and cytoplasmic kinases (eg, MEK) (Table 2). While all the therapies listed in Table 2 are approved in the United States, many are also approved in Europe and elsewhere. The response rate for most of the therapies is generally consistent across subtypes with higher rates for tumors with high mutational burden. The effectiveness of two antibody drugs, anti-VEGF bevacizumab and anti-VEGFR ramucirumab, is attributable to the inhibition of angiogenesis. NSCLC patients with an overexpression of EGFR mRNA or increased copy number have a 70% or higher response rate to small-molecule EGFR inhibitors like gefitinib or EGFR antibodies like necitumumab. Furthermore, some targeted therapies are approved for specific subsets of NSCLC patients. Osimertinib is used to treat NSCLC patients with T790M mutations of EGFR. The EML4-ALK tumors are mostly responsive to small-molecule tyrosine inhibitors of ALK like crizotinib. Dabrafenib and trametinib, which target BRAF and MEK1/2, respectively, are prescribed for patients with BRAF V600E mutations. Immunotherapies such as PD-1 and PD-L1 antibodies (eg, nivolumab and atezolizumab) are also effective for the treatment of some NSCLC patients regardless of the subtype. While targeted and immunotherapies are generally less toxic and personalized for particular patients, some patients do exhibit primary or acquire resistance or show severe adverse effects such as diarrhea and pneumonitis. In addition, targeted therapies have the greatest response rate for patients with the indicated mutation, therefore, due to the high heterogeneity of mutations within NSCLC, targeted therapies may not work in every patient. Large efforts are underway to advance the understanding of NSCLC biology and assess novel therapies.

### 4 | GENOME-DERIVED MICRORNAS ARE DYSREGULATED IN NSCLC

As less than 5% of the human genome is processed to functional proteins in cells, the majority is transcribed into enormous numbers of functional noncoding RNAs. Among them, microRNAs (miRNAs or miRs) are a superfamily of short RNAs that act on corresponding transcripts via complementary binding to achieve mRNA degradation or translation inhibition (Figure 2). The biogenesis of miRNAs starts with the transcription of miRNA-coding genes into primary
| Treatment         | Classification | Target or Action | Approval | Overall Response Rate |
|-------------------|----------------|------------------|----------|-----------------------|
| Bevacizumab       | antibody/protein | VEGF             | Non-squamous NSCLC | 35% with carboplatin and paclitaxel 150 |
| Ramucirumab       | antibody/protein | VEGFR            | Metastatic non-squamous NSCLC | 23% with docetaxel 151 |
| Erlotinib         | small molecule  | EGFR             | EGFR L858R mutation, metastatic NSCLC | 74.4% 152 |
| Necitumumab       | antibody/protein | EGFR             | Metastatic squamous NSCLC | 48.1% with cisplatin and gemcitabine 153 |
| Gefitinib         | small molecule  | EGFR             | Advanced or metastatic NSCLC with L858R EGFR mutations | 76.9% 152 |
| Afatinib          | small molecule  | EGFR             | Metastatic squamous NSCLC with non-resistant EGFR mutations | 56% 154 |
| Osimertinibe      | small molecule  | EGFR T790M mutations | Advanced or metastatic NSCLC with T790M EGFR mutations | 77% 155 |
| Crizotinib        | small molecule  | ALK/CD246, ROS   | Advanced or metastatic ALK-positive NSCLC | 74% 156 |
| Ceritinib         | small molecule  | ALK/CD246        | Metastatic ALK-positive NSCLC | 58% 157 |
| Brigatinib        | small molecule  | ALK/CD246        | Metastatic ALK-positive NSCLC | 71% 158 |
| Alectinib         | small molecule  | ALK/CD246, RET   | Metastatic ALK-positive NSCLC | 82.9% 159 |
| Dabrafenib        | small molecule  | B-Raf            | Metastatic NSCLC with B-Raf V600E mutation | 67% in combination with trametinib 160 |
| Trametinib        | small molecule  | MEK              | Metastatic NSCLC with B-Raf V600E mutation | See dabrafenib |
| Entrectinib       | small molecule  | ROS1/NTRK fusion | Metastatic, ROS1/NTRK-positive NSCLC | 78% 161 |
| Nivolumab         | antibody/protein | PD-1/CD279       | Metastatic squamous NSCLC | 47% in patients with a high tumor-mutation burden 162 |
| Pembrolizumab     | antibody/protein | PD-1/CD279       | Advance or metastatic squamous NSCLC | 44.8% 163 |
| Atezolizumab      | antibody/protein | PD-L1/CD274/B7-H1 | Metastatic non-squamous NSCLC | 63.5% with bevacizumab, carboplatin, and paclitaxel in patients with no EGFR or ALK alterations 164 |
| Ipilimumab        | antibody/protein | CTLA4/CD152      | Metastatic NSCLC | 45.3% with nivolumab 165 |
| Carboplatin & cisplatin | small molecule | Inhibition of DNA replication | Advanced or metastatic NSCLC | 62% carboplatin with paclitaxel 166 |
| Irinotecan        | small molecule  | Topoisomerase I   | Advanced NSCLC | 43.7% with cisplatin 167 |
| Etoposide         | small molecule  | Topoisomerase II  | Metastatic NSCLC | 21.9% with cisplatin 168 |
| Docetaxel         | small molecule  | Microtubules; inhibition of mitosis | Advanced NSCLC | 9% in patients previously treated with chemotherapy 169 |
| Paclitaxel        | small molecule  | Tubulin; inhibition of mitosis | Advanced or metastatic NSCLC | See carboplatin & cisplatin |
| Vinorelbine       | small molecule  | Tubulin; inhibition of mitosis | Advanced NSCLC | 43% with cisplatin 170 |

(Continues)
miRNA (pri-miRNA) transcripts. The pri-miRNA is thus processed by the Drosha-DGCR8 complex within the nucleus to produce a precursor miRNA (pre-miRNA) that can be exported into the cytoplasm by Ran-GTP-dependent Exportin-5 (XPO5). The pre-miRNA is cleaved into a miRNA duplex by the RNase Dicer in the cytoplasm. The miRNA duplex is then unwound to offer two strands, among which the guide strand is preferably incorporated into the RNA-induced silencing complex (RISC) consisting of the Argonaute family of proteins while the passenger strand is readily degraded. The RISC proteins stabilize and aid the mature miRNA in binding to the 3’-untranslated region (3’UTR) of a target transcript to accomplish the regulation of target gene expression.

Many miRNAs are involved in the control of target gene expression behind various cancer cellular processes (see the following section), exhibiting tumor suppressive or promotive activities. Specifically, a miRNA that reduces the expression of tumor suppressors acts as a tumor promoter, and a miRNA that degrades oncogene transcripts functions as a tumor suppressor. Interestingly, some miRNAs are dysregulated in NSCLC (Table 3) that may be indicative of disease status or therapeutic outcome. With some exceptions, generally there is a decrease of tumor suppressive miRNAs (e.g., miR-34a-5p and miR-124-3p) and increase of tumor promotive miRNAs (e.g., miR-21-5p and miR-183-5p) in many human cancers including NSCLC (Table 3), as compared to normal tissues; however, the magnitude of dysregulation varies by case. Dysregulation of miRNA expression may be caused by different mechanisms such as chromosomal deletion or methylation, or dysregulation of their transcription factors, enzymes, or binding proteins involved in miRNA biogenesis. Dicer, the RNase responsible for the processing of pre-miRNAs, is essential for mouse development and stem cell maintenance. Dicer was reported to be downregulated in some lung cancer patients, leading to a global decrease in miRNAs and associated with poor prognosis, and conditional deletion of Dicer led to increased lung tumorigenesis in mice. Actually, the role of Dicer in cancer remains contradictory. It has been suggested that a partial loss or downregulation of Dicer is oncogenic, while a complete loss is tumor protective. In addition to Dicer, the expression of some miRNAs is also modulated by the tumor suppressive transcription factor p53. During DNA damage, p53 associates with the Drosha/DGCR8 complex and facilitates processing of pri-miRNA to pre-miRNA. p53 is mutated and inactivated in many cancers, including lung, reducing the total levels of pre-miRNAs. Taken together, a global decrease in miRNA levels, by either decreased Dicer expression or loss of p53, might be involved in tumor initiation and progression. Ultimately,
restoration of tumor suppressive miRNAs and inhibition of tumor promotive miRNAs represent new anti-cancer strategies.

5 | MICRODRNAs ARE INVOLVED IN THE CONTROL OF MULTIPLE NSCLC CELLULAR PROCESSES

5.1 | Epithelial to Mesenchymal Transition

The epithelial to mesenchymal transition (EMT) is a process in which an epithelial-like cell loses its attachment to the basal membrane and assumes mesenchymal characteristics like greater motility and invasiveness. EMT allows for cancer cells to metastasize by migrating from the primary tumor through the blood stream and invading other organs. Comprehensive reviews of the process of EMT have recently been published.44 Some miRNAs can affect cells’ ability or likelihood to undergo EMT by regulating the expression of EMT-related genes. One important EMT signaling cascade involves TGFβ, a signaling cytokine, that binds to its receptor, TGFβR1/2 to transduce a signal through RAS, PI3K, RhoA/ROCK, or Smad2/3 and activate transcription factors, like ZEB1/2, Twist and Snail. This ultimately results in the loss of epithelial attachment proteins, such as E-cadherin, and gain of intermediate filament or cell-cell adhesion proteins, such as vimentin and N-cadherin.45 The EMT phenotype is reduced in NSCLC by the action of miR-17-5p, 20a-5p, and 20b-5p that directly target TGFβR2.46 miR-148a-3p and miR-101-3p that target ROCK1 and ROCK2 respectively,47,48 and miR-132-3p, miR-638-5p, and miR-338-5p which target transcription factors ZEB2, SOX2, and SOX4, respectively49-51 (Figure 3). Both miR-149-5p and miR-509-3p target the transcription factor FOXM1 to reduce invasion in H1299 cells as determined by Matrigel invasion assay.52 In addition, miR-186-5p targets CDC42 leading to the inhibition of migration and related EMT processes.53 Likewise, dysregulation of these miRNAs, as evident in NSCLC, leads to greater EMT and a more invasive, migratory, and potentially metastatic phenotype.

5.2 | Signal transduction in lung cancer survival and proliferation

Oncogenesis is driven by an over-expression or activation of growth signaling, such as growth factors, receptors, or downstream signaling molecules. Growth factor ligands bind to their corresponding receptors to relay a signal and induce proliferation. Cancer cells can hijack signaling by over-expressing or mutating growth factor receptors to increase proliferative signals and miRNAs target certain receptor to modulate signaling.
miR-7-5p, miR-133b-3p, miR-134-5p, miR-200a-3p, miR-206-3p, 31-5p, 139-5p, 329-3p, 27b-3p, miR-570, miR-320a-3p, miR-195-5p, miR-152-3p, 130a-3p, 449a-5p, 34a-5p, 200a-3p, 206-3p, 31-5p, 139-5p, 329-3p, 27b-3p, miR-186-5p, 135a-5p, 133b-3p, 134-5p, 200a-3p, 139-5p, 30a-5p, 140-3p, 320a-3p, 195-5p, 486-5p, 99b-5p, 100-5p

EGF, IGF, FGF, and HGF signaling results in activation of RAS or PI3K pathways and downstream growth signaling. A decrease in the miRNAs that target growth factors and their receptors, as well as downstream targets, as evident in NSCLC, results in an increase in growth signal transduction and an increase in cancer cell proliferation and growth.

5.3 | Angiogenesis

Angiogenesis is the process of building new blood vessels for nutrients and gas exchange which is essential for cancer cells to survive and proliferate. Dysregulation of miRNAs in cancer cells can lead to increased angiogenesis through multiple pathways, including vascular endothelial growth factor (VEGF) or placenta growth factor (PIGF) binding to VEGF receptor (VEGFR) and activating hypoxia-inducible factor-1α (HIF1α). These miRNAs that target VEGF or HIF1α may reduce angiogenesis essential for tumor progression.
angiogenesis and blood vessel formation, as measured by tube formation assay. 77,78 miR-210-3p is overexpressed in late-stage NSCLC and protects against hypoxia induced apoptosis by indirectly stabilizing HIF1α to promote angiogenesis and increase glycolysis. 79 miR-21-5p directly targets PTEN and activates AKT and ERK1/2 which leads to higher levels of HIF1α and VEGF expression. 80 miR-378-5p is over-expressed in NSCLC tumors in patients with brain metastasis and leads to increased VEGF expression and angiogenesis. 81 miR-206-3p directly suppresses the expression of protein 14-3-3ζ which consequently decreases VEGF, HIF1α, and phosphorylated STAT3 and results in a lower degree of angiogenesis as assayed by HUVECs recruitment as well as inhibition of intratumoral capillary tube formation in vivo. 82 In one study, coculture of NSCLC cell lines with vascular endothelial cells leads to higher levels of miR-494-3p in the vascular endothelial cells, in addition, a miR-494 antagonist decreases tumor vascularization, suggesting that miRNAs may be transferred to vascular endothelial cells to control angiogenesis. 83 Such miRNAs are important in the control and development of vascularization which is critical for tumorigenesis and metastasis.

5.4 | Cell Cycle

The cell cycle is altered among almost all cancer cells to allow for uncontrolled growth. Cyclins and cyclin-dependent kinases (CDKs) are partly responsible for entry into the different cell cycle stages. G1 begins with cyclins D1, D2, and D3 associating with CDK4 and 6 84 to phosphorylate Rb and repress the E2F transcription factor. 85 G1/S transition is characterized by cyclin E complexing with CDK2 86 and cyclin A/CDK2 complex during S phase. 87 Cyclin A complexes with CDK1 to transition to M phase, then cyclin B and CDK1 are complexed during M phase. 88 Such proteins regulating G1 and S phases of many types of cancers, including NSCLC, are dysregulated, and some are direct targets of particular miRNAs (Figure 4 and Table 3). Tumor suppressive miR-34a-5p directly targets CCND1 and CDK6, leading to the arrest of the cell cycle in G1 phase. 89 Furthermore, miR-15a-5p and miR-16-5p, down-regulated in NSCLC, directly targets CCND1, CCND2, and CCNE1, and arrests the cell cycle in G1 to G0 in an Rb-dependent manner. 90 Combination of miR-34a-5p and miR-15a/16 produces synergism in G1 cell cycle arrest in an Rb-dependent manner due to an increase in miRNAs targeting more cell cycle related miRNAs. 91 In addition, miR-30d-5p targets CCNE2, 92 miR-186-5p targets CCND1, CDK2, and CDK6, 93 and miR-129-5p targets CDK6 94 to arrest the cells in G1. Generally, cell cycle-regulating miRNAs exhibit tumor suppressive actions by targeting cell cycle promotive genes to induce a cancer cell cycle arrest.

5.5 | Evading Apoptosis

Cell death mechanisms, including apoptosis or necrosis, are important for cells to maintain homeostasis, and dysregulation of these pathways leads to an alteration of cell proliferation, including NSCLC cells. Some miRNAs regulate certain proteins involved in cell death (Figure 5), and dysregulation of such miRNAs may make the cells evade death signals and continue to proliferate. In brief, apoptosis occurs when a death ligand, such as tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), binds to a death receptor, including TNF receptors 1 and 2 (TNFR1/2), causing receptor multimerization and activation of the death inducing signaling complex (DISC). 95 This can result in direct activation of caspase-8 mediated cleavage of effector caspases, like caspase-3, 96 or caspase-8 cleavage of Bid which releases mitochondrial cytochrome c to associate with Bax and Bak and forms the apoptosis and cleaves effector caspases. 97 BCL2, an anti-apoptotic protein that mainly functions to inhibit release of cytochrome c from the mitochondria, 98 is targeted by miR-181-5p, 99 miR-7-5p, 100 miR-503-5p, 101 miR-200bc/429 cluster, 102 and miR-497-5p 103 (Figure 5). In addition, BCL2L2 and BCL6, also anti-apoptotic proteins, are directly targeted by miR-15a-5p and miR-187-3p, respectively, to enhance apoptosis. 104,105 TRAIL expression induces apoptosis; however, NSCLC can confer resistance to TRAIL-mediated apoptosis through many mechanisms including loss of PTEN and constitutive activation of AKT 106 or increased matrix metalloproteases. 107 Therefore, over-expression
miRNAs that inhibit the metabolic potential of NSCLC cells may alter tumor progression.

6 | MICRORNAS AFFECT THE SENSITIVITY OF NSCLC CELLS TO CURRENT THERAPIES

Dysregulation of miRNAs can confer the resistance to current therapies including chemotherapy and radiation therapy. For instance, upregulation of miR-21-5p leads to a reduction of apoptosis and decrease of sensitivity to two chemotherapeutics, docetaxel and cisplatin.\(^{110}\) Induced by radiation, miR-155-5p does confer resistance to radiation therapy by indirectly increasing HK2 to promote aerobic glycolysis.\(^{111}\) Furthermore, chronic treatment with EGFR inhibitor gefitinib reduces the expression of miR-155-5p and miR-200c-3p and may decrease the sensitivity to gefitinib.\(^{118}\) Therefore, miRNA profiles from tissue or body fluids may also be used as predictive biomarkers for the sensitivity of NSCLC tumors to certain therapies and to determine optimal therapies for the treatment of NSCLC. As an example, miR-143-3p is downregulated in NSCLC and suppresses NSCLC cell proliferation, migration, and invasion by regulating EGFR expression.\(^{119}\) NSCLC patients showing lower miR-143-3p levels and higher EGFR levels may benefit from anti-EGFR therapy like gefitinib. PI3K or VEGF inhibitors may be beneficial for patients with decreased expression of miR-126-3p because dysregulation of miR-126-3p may lead to an increase in PI3K and VEGF-A.\(^{7,112}\) In addition, miRNAs can play an important role in drug uptake and efflux via direct targeting of drug transporters. For example, miR-31-5p is upregulated in cisplatin resistant cell lines and directly regulates the expression of ABCB9, a drug transporter, to confer cisplatin resistance.\(^{121}\) Let-7c-5p modulates the expression of ABC22 transporter to sensitize cisplatin resistant cell lines.\(^{122}\) ABC24 involved in the transport of many anti-cancer drugs such as methotrexate and topotecan and is directly targeted by miR-124-3p and miR-506-3p.\(^{123}\) Excellent reviews on the potential of miRNAs as biomarkers in lung cancer have been recently published.\(^{124}\) Understanding miRNA-controlled regulation may not only improve the understanding of multidrug resistance mechanisms but also offer clues to the development of new therapeutic strategies.\(^{125,126}\)

7 | MICRORNAS IMPACT THE TUMORIGENESIS OF NSCLC CELLS

To determine the effect of a miRNA on tumorigenesis, investigators transiently or stably express the target miRNA in a cell line for implantation in vivo. While this does not necessarily model therapeutic potential of miRNAs, it does provide important information beyond cell-based findings regarding the importance of miRNAs in the control of tumor initiation and development or tumorigenesis (Table 4). For example, subcutaneously or tail vein injected A549 cells expressing miR-124-3p, miR-126-3p, miR-143-3p, miR-34a-5p, Let-7b-5p, or miR-182-5p showed reduced tumor growth and in some cases reduce lung metastasis

**FIGURE 5** miRNAs affect the ability to evade of apoptosis. BCL6, BCL2L2, and BCL2 are anti-apoptotic as they inhibit cytochrome c release from the mitochondria. Dysregulation or malfunction of miRNAs in NSCLC that inhibit the anti-apoptotic cascade may reduce apoptotic capacity and enhance cancer progression. Therefore, restoration of such miRNA expression or function represents a novel therapeutic strategy of miR-148a-3p can sensitize NSCLC to TRAIL by targeting MMP15.\(^{108}\) miR-221-3p and miR-222-3p can confer TRAIL resistance by targeting tumor suppressors PTEN and tissue inhibitor of metalloproteases 3 (TIMP3),\(^{110}\) while miR-130a-3p can reverse this effect by targeting miR-221-3p and miR-222-3p.\(^{111}\) miR-21-5p also targets PTEN and results in the inhibition of apoptosis, which can be reversed by the transfection of anti-miR-21-5p.\(^{110}\)

5.6 | Metabolism

Some miRNAs can alter the metabolic potential of cancer cells. A higher metabolic rate may enhance the tumorigenesis and growth of NSCLC cells. miR-155-5p promotes aerobic glycolysis by indirectly upregulating HK2, as determined by a hexokinase colorimetric assay as well as glucose and L-lactate test kits.\(^{111}\) The increase in glycolysis leads to greater degree of cell viability. miR-143-3p directly targets HK2, the first rate-limiting enzyme in glycolysis, to decrease glycolysis and proliferation as well as tumorigenesis in vivo.\(^{112}\) miR-124-3p overexpression decreases glucose consumption, lactate production, and ATP content by decreasing HK2 and glucose transporter 1 (GLUT1), leading to a lower extent of cell proliferation.\(^{113}\) miR-449-5p directly targets lactate dehydrogenase A (LDHA) and suppresses glycolysis.\(^{114}\) miR-182-5p and miR-31-5p target HIF1AN and FIH, respectively, both of which are HIF-1α inhibitors and lead to enhanced glycolysis.\(^{115,116}\) miR-210-3p directly targets two genes important in the electron transport chain, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4 (NDUFA4), and succinate dehydrogenase complex subunit D (SDHD), which leads to alteration of the physical structure of the mitochondria, visualized by electron microscopy as well as alterations of the mitochondrial membrane potential that are phenotypic of mitochondria dysfunction.\(^{79}\) miR-145-5p and miR-138-5p directly target phosphoinositide-dependent protein kinase-1 (PDK1), an important enzyme in glucose and fatty acid metabolism.\(^{115,117}\) Dysregulation or loss of those miRNAs that inhibit the metabolic potential of NSCLC cells may alter tumor progression.
as compared to control cells. Lewis lung carcinoma cells transiently transfected with miR-101-3p displayed a smaller increase of tumor volume over time when subcutaneously injected into the flank of mice, as well as a reduction of metastasis to the lung when intraperitoneally injected. Two miRNA-based therapeutic strategies have been established, aiming to restore tumor suppressive miRNAs and inhibit tumor promoting miRNAs, respectively. Many studies were thus conducted to define the effectiveness of specific miRNA therapeutics for the treatment of NSCLC in animal models in vivo (Table 5). AntagomiRs were employed for the inhibition of tumor promoting miR-21-5p, miR-183-5p, and miR-206-3p and shown to inhibit subcutaneous A549 tumors. Let-7c-5p and let-7a-5p were both shown to decrease the progression of NSCLC in vivo. Synthetie miR-34a-5p injected either intratumorally or through the tail vein was effective in inhibiting the growth of subcutaneous xenograft NSCLC, and it did not show major impact on the cytokine profiles or liver or kidney functions. A specific type of chemical modification of miR-145-5p, namely locked nucleic acid, delivered with a polyethylene-short branched-polyethyleneamine, led to significant inhibition of tumor growth and the effects were enhanced by radiation and cisplatin therapy.

Orthotopic NSCLC tumor growth, metastasis, and vascularization were decreased in mice treated with miR-200a/b. miR-29b-3p decreased cell proliferation and increased apoptosis in subcutaneous NSCLC tumors. Combination treatment with miR-34a-5p and

### Table 4: Some miRNAs shown to affect tumorigenesis of NSCLC cells in animal models

| miRNA     | Cell line | Mouse strain | Finding                                                                 | Reference |
|-----------|-----------|--------------|--------------------------------------------------------------------------|-----------|
| miR-124-3p| A549      | nude BALB/c  | Reduced lung metastasis from tail vein injected cells                    | [176]     |
| miR-126-3p| A549      | nude BALB/c  | Reduced tumor weight                                                     | [77]      |
| miR-143-3p| A549      | nude BALB/c  | Reduced tumor weight                                                     | [225]     |
| miR-34a-5p| A549      | nude BALB/c  | Reduced tumor weight and lung tumor metastasis                           | [226]     |
| let-7b-5p | A549, H460| nod/scid     | Reduce tumor growth                                                      | [227]     |
| miR-101-3p| LLC       | C57BL/6      | Reduced tumor weight, metastasis from IP injected cells                  | [127]     |
| miR-100-5p| SPC-A1/DTX| nude         | Reduced tumor volume in response to docetaxel                           | [201]     |
| miR-145-5p| A549      | nude         | Reduced tumor volume                                                     | [128]     |
| miR-486-5p| H460-luc2 | athymic Swiss| Reduced lung metastasis from tail vein injected cells                   | [62]      |
| miR-451-5p| A549      | nude BALB/c  | Reduced tumor volume in response to cisplatin                           | [228]     |
| miR-21-5p | CAG-miR-21;K-rasL2 | | Reduced tumor burden and increased survival                             | [130]     |
| miR-205-5p| H460      | BALB/c       | Reduced tumor volume                                                     | [131]     |
| miR-31-5p | H1993/H1437/H460| nude   | Reduced tumor volume                                                     | [132]     |
| miR-200a/b| 344SQ     | nude         | Reduced tumor volume                                                     | [129]     |
| miR-182-5p| A549      | nude         | Reduced tumor volume and weight and increased survival                   | [229]     |

Two miRNA-based therapeutic strategies have been established, aiming to restore tumor suppressive miRNAs and inhibit tumor promoting miRNAs, respectively. Many studies were thus conducted to define the effectiveness of specific miRNA therapeutics for the treatment of NSCLC in animal models in vivo (Table 5). AntagomiRs were employed for the inhibition of tumor promoting miR-21-5p, miR-183-5p, and miR-206-3p and shown to inhibit subcutaneous A549 tumors. Whereas, tumor suppressive miRNAs let-7b-5p or miR-34a-5p reduced KRAS-activated tumor burden in vivo. Let-7c-5p and let-7a-5p were both shown to decrease the progression of NSCLC in vivo. Synthetie miR-34a-5p injected either intratumorally or through the tail vein was effective in inhibiting the growth of subcutaneous xenograft NSCLC, and it did not show major impact on the cytokine profiles or liver or kidney functions. A specific type of chemical modification of miR-145-5p, namely locked nucleic acid, delivered with a polyethylene-short branched-polyethyleneamine, led to significant inhibition of tumor growth and the effects were enhanced by radiation and cisplatin therapy. Orthotopic NSCLC tumor growth, metastasis, and vascularization were decreased in mice treated with miR-200a/b. miR-29b-3p decreased cell proliferation and increased apoptosis in subcutaneous NSCLC tumors. Combination treatment with miR-34a-5p and
miRNAs were formulated with lipid-based technologies, such as those established for the production of new miRNA reagents for the assessment of miRNA therapies. Very recently, a novel RNA bioengineering technology has been established for the production of new miRNA reagents for the assessment of miRNA therapies. Biologic or recombinant miR-34a-5p or miR-124-3p molecules produced in bacteria and delivered with viral vectors, such as lentivirus or adenovirus, as well as positively charged polyethylenimine, which associates with the negatively charged RNA, were also used. In most cases, miRNA therapeutics were administered through the tail vein or intra-tumoral injection. These in vivo findings demonstrate the promise of miRNA-based therapies for the treatment of NSCLC.

One tumor suppressive miRNA, namely MRX34 or liposomal miR-34a mimic, was also moved into first-in-human Phase I clinical trials. It was evaluated as dose-escalating intravenous infusions under a regimen of twice a week in three-week cycles. This study consisted of 47 patients with advanced solid tumors, including one patient with NSCLC who had stable disease for 8 cycles of treatment. While efficacy of MRX34 was obvious among some patients, 96% of all patients

### Table 5: Some miRNA-based therapies for the treatment of NSCLC assessed in animal models in vivo

| miR | Mouse model | Delivery | Findings | Reference |
|-----|-------------|----------|----------|-----------|
| let-7b-5p and miR-34a-5p (synthetic) | Cre-Kras mutant | Neutral lipid emulsion (NLE) | Lower tumor burden, increased apoptosis, decreased proliferation | [135] |
| let-7b-5p (synthetic) | Subcutaneous H460 cell line | siPORTamine (lipid based) | Decreased proliferation | [136] |
| let-7a-5p (lenti-let-7) | Cre-Kras mutant | Lentiviral | Decreased proliferation | [136] |
| miR-34a-5p (synthetic) | Subcutaneous H460 | MaxSuppressor in vivo (lipid based) | Decreased proliferation, increased apoptosis, minimal change in blood chemistry or cytokine profile | [137] |
| miR-145-5p (synthetic; LNA) | Subcutaneous, intrabronchial or intravenous patient derived primary lung adenocarcinoma CD133+ | Cationic polyurethane-short branched polyethyleneimine (PU-PEI) | miR-145-5p alone showed moderate tumor inhibition, increased tumor inhibition and survival in combination with radiation and cisplatin | [138] |
| miR-200a/b (synthetic) | intrapulmonary 344SQ (murine) cell line | 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) nanoliposomes | Reduced proliferation, metastasis, and tumor vasculature permeabilization | [129] |
| miR-29b-3p (synthetic) | Subcutaneous A549 cell line | Lipoplex | Suppressed target expression, reduced proliferation, increased apoptosis | [139] |
| miR-34a-5p & miR-124-3p (biologic RNA) | Intravenous A549 cell line, (metastatic) | In vivo-jetPEI | Decreased lung lesions, minimal change on blood chemistry or cytokine release | [144] |
| miR-34a-5p (biologic RNA) | Subcutaneous A459 cell line | In vivo-jetPEI | Decreased tumor size, minimal change on blood chemistry or cytokine release | [145] |
| miR-34a-5p (synthetic) | Intramuscular H460 or H1299 cell line | NOV340 (Liposomal nanoparticle) | Sensitized tumor to irradiation | [146] |
| miR-34a-5p and let-7b-5p (synthetic) | Kras/p53 mutant, Cre-adenoviral activated | NOV340 (liposomal nanoparticle) | Combination of miR-34a-5p and let-7c-5p reduced tumor burden, decreased proliferation, and increased survival with minimal cytokine induction | [140] |
| anti-miR-21-5p (synthetic) | Subcutaneous A549 cell line | QTsome (cationic lipids) | Stable tumor growth or tumor regression after treatment, increased survival | [133] |
| anti-miR-183-5p (synthetic) | Subcutaneous A549-LUC-GFP | Adenovirus (intra-tumoral injection) | Decreased tumor growth as measured by luminescence | [134] |
| miR-206-3p-ago-mir (synthetic) | Subcutaneous A549 cell line | No vehicle mentioned (intra-tumoral injection) | Decreased tumor volume and formation of intra-tumoral capillary tubes, and increased apoptosis | [82] |

let-7c-5p was effective in improving overall survival and reducing tumor burden in KRAS mutant mice. Nevertheless, the majority of miRNAs used for in vivo therapies are made by chemical synthesis or in vitro transcription, or achieved through viral vectors or plasmids, where RNAs or mimics are often delivered with lipids or polymers. Very recently, a novel RNA bioengineering technology has been established for the production of new miRNA reagents for the assessment of miRNA therapies.
experienced immune-related adverse effects where multiple deaths also occurred with complex and uncertain causes. The most common adverse effects were fever, fatigue, nausea, diarrhea, and vomiting, and laboratory abnormalities included lymphopenia, neutropenia, and increased AST among others. The study does not distinguish whether the toxicity resulted from the RNA or the liposomal carrier, however, both components have shown immune toxicities in previous studies.148,149 The immune-related toxicity suggests that more studies are warranted to understand the effect of miRNA therapies and the carriers on the immune system. The termination of this trial reiterates the importance of safety study in addition to efficacy during drug development.

9 | CONCLUSIONS AND PERSPECTIVES

Functional miRNAs derived from the human genome are critical factors in posttranscriptional regulation of target gene expression underlying many cellular processes, including metabolism, proliferation, apoptosis, and disease initiation and progression. Uncontrolled NSCLC cell growth and tumor development is associated with dysregulated miRNA expression in addition to the alterations of proteins and signaling pathways, among which tumor suppressive miRNAs are generally downregulated and tumor promotive miRNAs are commonly upregulated. With the improved understanding of miRNA biology in NSCLC, new miRNA-based therapies are under active investigations, in particular, the restoration of tumor suppressive miRNAs and inhibition of tumor promotive miRNAs. Nevertheless, many challenges remain for the development of new therapeutics. Although a number of RNA drugs have been approved for clinical practice,142 the pharmacokinetic and pharmacodynamic properties of RNA molecules are still of concern since RNA molecules are generally susceptible to serum RNases and cannot pass freely through cell membrane barriers. Chemical modifications and formulation with biocompatible lipids or polymers have proven useful for improving the metabolic stability and delivery of RNA therapeutics. As chemical modifications undoubtedly lead to different RNA folding, stability, biologic activity, and safety profiles, there are growing interests in producing biologic or recombinant RNA molecules in living cells for RNA research and drug development.141,142 similar as the success of protein-based therapeutic modalities. Improved formulations with lipid or polymer-based drug delivery systems may aid in protecting the RNA from degradation or recognition by the immune system. In any case, evidence is required to address two fundamental questions: whether the drug is effective against the disease and whether the drug is safe for the patients, which warrants more extensive studies.

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DISCLOSURES

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Research design/conducting experiments: n/a. Literature research & analysis: Petrek & Yu. Writing and revising the manuscript: Petrek & Yu.

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