Review

Prognostic, therapeutic and diagnostic potential of microRNAs in non-small cell lung cancer

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Abstract

Non-small cell lung carcinomas (NSCLC) account for about 80% of lung cancers and their remarkable heterogeneity manifests in histology, pathogenesis, prognosis, and response to treatments. Recent advances in molecular characterization help stratifying NSCLC patients for their potential benefit from targeting therapies. However, the fundamental mechanisms underlying the tumoral heterogeneity remain poorly understood. Expression profiling of many microRNAs (miRNAs) in various normal and disease tissues demonstrated unique spatial and temporal expression patterns and some miRNAs have been functionally characterized as oncogenes or tumor suppressor genes. Genome-wide screening identified specific miRNA expression signatures associated with clinical outcome of NSCLC patients. A group of miRNAs that has enriched expression in normal lung was found down regulated in NSCLC and may function as tumor suppressor genes. In this review we: a) summarize the current understanding of the critical role that miRNAs play in normal cell functions and in disease biology especially in lung cancer tumorigenesis; b) highlight their potential as biomarkers for lung cancer risk stratification, outcome prediction and classification of histologic subtypes; c) critically assess current knowledge on lung-enriched miRNAs and expression of their predicted target genes in NSCLC and d) evaluate their potential as circulating biomarkers and therapeutic targets in lung cancer.

Keywords: diagnosis; microRNAs; non-small cell lung cancer; prognosis; tumor biomarkers.

Introduction

Lung cancer is the leading cause of cancer-related death in both males and females worldwide. There are an estimated 222,520 new cases and 157,300 deaths from lung cancer in the United States in 2010 (1). Despite years of research, the prognosis for patients with lung cancer remains dismal. Lung cancers are classified according to the histological types and this classification has important implications for the clinical management and prognosis of the disease (2). There are two main histological groups of lung cancer including non-small-cell lung cancer (NSCLC, 85%) and small cell lung cancer (SCLC, 15%). Non-small cell lung carcinomas (NSCLC) comprises three major histological subtypes: adenocarcinoma (AD), squamous cell carcinoma (SCC), and large cell carcinoma (LCC) (3).

Standard treatment strategies include surgical resection followed by radiation and/or chemotherapy. Chemotherapy is usually palliative rather than curative due to resistance (4) so more effective systemic therapy is in urgent need. The disease is usually diagnosed at advanced stages when the prognosis is poor. When the disease is at earlier stages, the clinical behavior of each histological subtype appears to be different. In a retrospective study on 1119 completely resected stages I and II NSCLC patients, five-year survival is between 30% and 54% in general, but the AD patients had a significantly better survival than the non-AD patients in stage I, whereas the SCC patients had a better survival than the non-SCC patients in stage II (5).

Over the past decade, it has become evident that subsets of lung cancer, particularly those with AD histology, can be defined at the molecular level by mutations. Because the presence or absence of such mutations can heavily influence treatment outcomes in cases of targeted therapy, genetically informed lung cancer medicine that involves the prospective genotyping of lung cancers is becoming a new standard of care. The use of tyrosine kinase inhibitors to target the epidermal growth factor receptor (EGFR) in patients with NSCLC is effective but limited by the emergence of drug-resistance mutations. The identification of mutations in the EGFR has changed how clinicians approach certain groups of individuals with lung cancer. Mutations in the EGFR tyrosine kinase in NSCLCs can cause oncogenic transformation and change the level of sensitivity to tyrosine kinase inhibitors, such as gefitinib and erlotinib (6, 7) while KRAS mutations are negative predictors of radiographic response to these EGFR inhibitors. In a recent study it was shown that first-line gefitinib treatment for patients with advanced NSCLC who were selected on the basis of EGFR mutations improved progression-free survival, with acceptable toxicity as compared with standard chemotherapy (8).

Various molecular factors have been evaluated as prognosis biomarkers including markers of nucleotide excision
Critical roles of microRNAs in normal cell functions and in disease biology

MicroRNAs (miRNAs) are small non-coding, 18 to 25 nucleotide-long, naturally occurring RNA molecules that post-transcriptionally modulate gene expression (13) that were first identified in C. elegans (14). miRNAs by binding to the 3′-untranslated region (3′-UTR) of target mRNA can cause translational repression (15) or degradation (16). There are currently at least 1000 loci encoding miRNAs in humans (17). Some miRNAs may have as many as a few thousand targets and bioinformatics data indicate that miRNAs have the potential to regulate at least 20%–30% of human genes. The biogenesis of miRNA is a multistep process beginning in the nucleus and culminating in the cytoplasm and involves numerous enzymes and accessory proteins. Within the nucleus, a long primary (pri)-miRNA transcript ranging from hundreds to thousands of nucleotides in length is transcribed by RNA polymerase II (18) and the processing of this pri-miRNA to a smaller stem loop, of approximately 70-nucleotide precursor (pre)-miRNA molecules is facilitated by RNAse III endonuclease (19). This pre-miRNA is subsequently processed in the cytoplasm to form the final active form of mature miRNA (Figure 1).

Recent emerging evidence suggests that microRNAs (miRNAs) have the potential to regulate translation in a cell cycle-dependent manner, which opens new horizons in advancing our understanding of cancer at the molecular level (12). Changes in the miRNA expression level have been detected in many human tumor types, and recent studies have demonstrated the critical roles of miRNAs in cancer pathogenesis. In this review we: a) summarize the current understanding of the critical role that miRNAs play in normal cell functions and in disease biology especially in lung cancer tumorigenesis, b) highlight their potential as biomarkers for lung cancer risk stratification, outcome prediction and classification of histologic subtypes, c) critically assess current knowledge on lung-enriched miRNAs and expression of their predicted target genes in NSCLC and d) evaluate their potential as circulating biomarkers and therapeutic targets in lung cancer.

miRNAs are involved in a myriad of biological processes, including proliferation, apoptosis, metabolism, differentiation, and epithelial-mesenchymal-transition (EMT). Examples include miR-273 and the miRNA encoded by lys-6 which are involved in patterning the C. elegans nervous system (20, 21), miR-181 in the differentiation of mammalian pancreatic cell development and the regulation of insulin secretion (22), miR-1 which is involved in mammalian heart development (23), miR-375 which regulates pancreatic insulin secretion (22), miR-181 which influences the differentiation of hematopoietic cells toward the B-cell lineage (24), and miR-430 which is regulated for zebra fish brain development (25). MicroRNAs are also involved in stem cell division and development, and as Hatfield et al. have shown the miRNA pathway might be part of a mechanism that makes stem cells insensitive to environmental signals that normally stop the cell cycle at the G1/S transition (26). Further characterization of miRNAs might reveal other gene regulators that coordinate proper organ formation, embryonic patterning and body growth, and might also provide insight into the mechanisms of human diseases, such as cancer. Just as miRNAs are important in the normal functioning of cells, a dysfunction of the miRNA regulation system would result in disruption of normal cell functions and cause diseases as well.

Changes in the miRNA expression level have been detected in many human tumor types, and recent studies have dem-
Figure 2  Publications on miRNAs in cancer and NSCLC (PubMed, keywords: microRNAs, cancer, non-small cell lung cancer).

onstrated the critical roles of miRNAs in cancer pathogenesis (27–30). Calin et al. first showed that miR-15a and miR-16-1, located in the fragile chromosomal region 13q14, were frequently under-expressed in patients with chronic lymphocytic leukemia (CLL) (31), and they further determined that both miRNAs are likely to function as tumor suppressors in CLL. Hayashita et al. found that miR-17-92 was markedly overexpressed in lung cancer, especially in SCLC (32). Chang et al. showed that miR-34a is frequently absent in pancreatic cancer cells (33). MiR-21 is overexpressed in six types of cancer including breast, lung, gastric and prostate (34) and let-7 miRNA family negatively regulates the RAS oncogenes and down regulation of let-7 expression is a characteristic of NSCLC (35). At the present time the main mechanism that underlies changes in the function of miRNAs in cancer cells seems to be aberrant gene expression, characterized by abnormal levels of expression for mature and/or precursor miRNAs compared to the corresponding normal tissues.

miRNA profiling in most types of tumors has shown significant different miRNA profiles when compared to normal cells from the same tissue. A systematic analysis of 334 leukemias and solid cancers has shown that miRNA-expression profiles can classify human cancers according to the lineage and differentiation state of the tumors; in particular, miRNA expression in tumors was found globally lower than in corresponding normal tissues (36). Another signature of up-regulated 21 miRNAs common to at least three tumor types was described by Volinia et al. (34). Because of the limited sample size and experimental expense, the statistical power of individual research projects is not sufficient to yield a robust conclusion. However, collected microarray datasets of expression profiles provide opportunities to compile the information of individual studies. A recent meta-analysis of miRNA expression microarray datasets from 28 published
tumor studies has comprised 33 comparisons and nearly 4000 tumor and corresponding non-tumoral samples and reported 52 miRNAs as common signatures that are deregulated in tumors. According to this study, in addition to the commonly altered miRNAs, five solid cancers displayed specific tissue patterns of altered miRNAs as well. This meta-analysis also revealed some novel tumor-related miRNAs, such as miR-144, miR-130b, miR-132, miR-154, miR-192, and miR-345 (37).

Tellez et al. have studied the role of EMT and epigenetic silencing through DNA methylation of tumor suppressive microRNAs, such as miR-200b, miR-200c, and miR-205, which were implicated in the dedifferentiation program in primary lung tumors after exposure to tobacco carcinogens (38). Melo et al. suggest that a cancer-specific mechanism guides the subcellular distribution of miRNA precursors and prevent them from being processed to the active mature miRNA. Controlling the miRNA biosynthesis pathway is emerging as an important mechanism in defining the spatiotemporal pattern of miRNA expression in cancer cells (39).

miRNAs as biomarkers in NSCLC

Recent data from multiple studies strongly support the potential of microRNAs as biomarkers in NSCLC. There is increasing evidence in the recent literature (Figure 2) that altered microRNA expression is associated with tumor progression and survival in lung cancer patients.

The let-7 family

The let-7 family is a cluster of miRNAs whose genes map to different chromosomal regions that are frequently deleted in lung cancer (40). Reduced let-7 gene expression in NSCLC patients has been correlated with poor prognosis (41, 42),
suggesting a role of this miRNAs cluster as tumor suppressors. In addition, let-7 can negatively regulate multiple oncoproteins, including RAS (35), MYC (43), HMGA2 (44) and cyclins (45). A single nucleotide polymorphism in let-7 complementary site 6 of the KRAS mRNA 3’-UTR has been found significantly associated with an increased risk of NSCLC (46). High expression of miR-155 and low expression of let-7a-2 were strongly associated with poor survival of patients with lung AD (42). Using quantitative RT-PCR-based analysis, poor prognosis was shown associated with reduced let-7 and miR-221 expression and increased levels of miR-137, miR-372 and miR-182 (47). Epigenetic factors regulate miRNAs expression as well. For example, let-7a-3 is found heavily methylated in normal lung cells, but hypomethylated and expressed in a subset of lung AD (48).

**miR-17-92, miR-126, miR-125a and miR-206**

miR-17-92 acts as an oncogene and may be a potential therapeutic target in lung cancer. miR-17-92 overexpression is associated with retinoblastoma (RB) inactivation (49). miR-17-92 is also involved in regulation of angiogenesis. Vascular Endothelial Growth Factor (VEGF) induces miR-17-92 expression in endothelial cells (50). Disruption of miR-17-92 clusters was shown to cause lethal abnormalities, including lung hypoplasia, ventricular septal defects and inhibition of B cell development (51). The 3’-UTR of VEGF mRNA has a binding site for miR-126; expression of miR-126 was down regulated in eight lung cancer cell lines, and may alter lung cancer cell invasive capacity and growth by targeting Crk (52, 53). miR-126 overexpression was also detected in metastatic vs. primary tumor in a study using microarray in formalin-fixed paraffin-embedded (FFPE) tissues (54). Recently, miR-125a and miR-206 have been shown to associate with invasive and metastatic capabilities of various lung cancer cell lines (55, 56).

**The miR-29 family**

Among the reported down-regulated miRNAs in lung cancer, the miR-29 family (29a, 29b, and 29c) has intriguing complementarities to the 3’-UTRs of DNA methyltransferase (DNMT)-3A and -3B, two key enzymes involved in DNA methylation that are frequently up regulated in lung cancer and associated with poor prognosis. It was found that the expression of miR-29s is inversely correlated to DNMT3A and -3B in lung cancer tissues, and that miR-29s directly target both DNMT-3A and -3B. Enforced expression of miR-29s in lung cancer cell lines restores normal patterns of DNA methylation, induces re-expression of methylation-silenced tumor suppressor genes and inhibits tumorigenicity in vitro and in vivo. These findings support a role of miR-29s in the epigenetic normalization of NSCLC, providing a rationale for the development of miRNA-based strategies for the treatment of lung cancer (57).

**miR-21**

miR-21 is overexpressed in several solid malignancies including breast and lung cancer (58). Inhibition of miR-21 in a breast cancer cell line has a subtle effect on cell growth both in vitro and in vivo but significantly reduces invasion and lung metastasis in animals (59). Overexpression of mature miR-21 was shown to be an independent negative prognostic factor for overall survival in NSCLC patients (60). Changes in the expression of mature miR-21 are more remarkable in the presence of EGFR mutations and miR-21 post-transcriptionally down-regulates the expression of the tumor suppressor PTEN and subsequently stimulates growth and invasion in NSCLC (61). Abruptly increased expression of miR-21, which is enhanced further by the activated EGFR signaling pathway, plays a significant role in lung carcinogenesis in never-smokers, as well as in smokers, and is a potential therapeutic target in both EGFR-mutant and wild-type cases (62). There seems to be an intricate balance between EGFR and miR-7 under tissue-specific context, in that EGFR promotes lung tumorigenesis by activating miR-7 expression (63), whereas miR-7 suppressed EGFR expression and functions in glioblastoma as a tumor suppressor gene (64). Finally, miR-21 drives tumorigenesis through inhibition of negative regulators of the RAS/MEK/ERK pathway and inhibition of apoptosis (65). Very recently Saito et al. have shown that increased miR-21 expression is associated with disease progression and survival in stage I lung cancer. This suggests that expression of miR-21 may contribute to lung carcinogenesis and serve as a therapeutic target or early stage prognostic biomarker for lung adenocarcinoma (66).

**The miR-34 family**

This family is composed of miR-34a, miR-34b, and miR-34c that are part of the p53 network and their expression is directly induced by p53 in response to DNA damage or oncogenic stress (67). MiR-34a is lost or down-regulated in many tumors (68, 69), and in vitro miR-34a overexpression leads to decreased proliferation and activation of apoptosis in multiple tumor cell types (70–72), indicating a role for miR-34a as a tumor suppressor gene. The miR-34 family is down-regulated in NSCLC when compared to normal tissues and NSCLC patients with low miR-34a expression have a higher risk of relapse (73).

**miR-221, miR-222 and miR-210**

miR-221 and miR-222 are overexpressed in aggressive NSCLC by targeting PTEN and TIMP3 tumor suppressors, induce TRAIL assistance and enhance cellular migration through the activation of the AKT pathway and matrix metalloproteinases (74), making these two miRNAs promising therapeutic targets or diagnostic tools for TRAIL resistance in NSCLC (75). miR-210 is overexpressed in late stages of lung cancer and mediated mitochondrial alterations associated with modulating activity of hypoxia-inducible factor-1 (76).

**miR-451**

Very recently, Wang R et al. analyzed the miRNA expression profiles in NSCLC by use of a miRNA microarray platform
and identified 40 differentially expressed miRNAs. They showed that miRNA (miR)-451 was the most downregulated in NSCLC tissues. The expression level of miR-451 was found to be significantly correlated with tumor differentiation, pathological stage and lymph-node metastasis. Moreover, low miR-451 expression level was also correlated with shorter overall survival of NSCLC patients (P < 0.001). Their findings suggest that miR-451 regulates survival of NSCLC cells partially through the downregulation of RAB14. They propose that targeting with the miR-451/RAB14 interaction might serve as a novel therapeutic application to treat NSCLC patients (77).

A recent study by Voortman et al., determined whether expression levels of a panel of biologically relevant miRNAs can be used as prognostic or predictive biomarkers in patients who participated in the International Adjuvant Lung Cancer Trial (IALT), the largest randomized study conducted to date of adjuvant chemotherapy in patients with radically resected non-small cell lung carcinoma (NSCLC). Expression of miR-21, miR-29b, miR-34a/b/c, miR-155, and let-7a was determined by quantitative real-time PCR in FFPE tumor specimens from 639 IALT patients. No significant association was found between any of the tested miRNAs and survival, with the exception of miR-21 for which a detrimental prognostic effect of lowered expression was suggested. Otherwise, no single or combinatorial miRNA expression profile predicted response to adjuvant cisplatin-based chemotherapy. Results indicated that the miRNA expression patterns examined were neither predictive nor prognostic in a large patient cohort with radically resected NSCLC, randomized to receive adjuvant cisplatin-based chemotherapy vs. follow-up only (78).

Despite undergoing curative resection, nearly a third of patients with stage I NSCLC die of recurrent disease. There are no reliable clinical or molecular predictors of relapse in patients with resected stage I NSCLC. Identifying patients at

### Table 1 Prognostic significance of miRNAs in NSCLC tissues.

<table>
<thead>
<tr>
<th>Tested miRNAs</th>
<th>Patients</th>
<th>Cancer type</th>
<th>Differently expressed miRNAs</th>
<th>Prognostic significance</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Technology used: microarray platform</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All human miRNAs</td>
<td>363</td>
<td>Breast, lung, stomach, colon cancer</td>
<td>Up-regulated: miR-21, miR-17-5p, miR-191 Down-regulated: miR-128, miR-155</td>
<td>Not evaluated</td>
<td>(34)</td>
</tr>
<tr>
<td>177</td>
<td>Normal tissues</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All human miRNAs</td>
<td>104</td>
<td>Pairs of NSCLC</td>
<td>Up-regulated: miR-155 Down-Regulated: let-7a-2</td>
<td>OS (p=0.006) OS (p=0.033)</td>
<td>(42)</td>
</tr>
<tr>
<td>700 mature miRNAs</td>
<td>33</td>
<td>Primary lung</td>
<td>Up-regulated: miR-182 Up-regulated: miR-152</td>
<td>Not evaluated</td>
<td>(54)</td>
</tr>
<tr>
<td>43</td>
<td>Metastatic lung</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All human miRNAs</td>
<td>62</td>
<td>Squamous carcinoma Adenoma carcinoma</td>
<td>miR-205 as biomarker of squamous carcinoma</td>
<td>Not evaluated</td>
<td>(84)</td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>713 mature miRNAs</td>
<td>125</td>
<td>Squamous carcinoma Adenoma carcinoma</td>
<td>Down-regulated: miR-29a, let-7b Up-regulated: miR-21, miR-26a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>165</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All human miRNAs</td>
<td>8</td>
<td>Pairs of NSCLC</td>
<td>Down-regulated: miR-181a, miR-143 Up-regulated: miR-21</td>
<td>OS (miR-181a: p=0.050 and miR-143: p=0.386) OS (p=0.002)</td>
<td>(80)</td>
</tr>
<tr>
<td>All human miRNAs</td>
<td>23</td>
<td>Pairs of NSCLC</td>
<td>Up-regulated: miR-451</td>
<td>OS (p&lt;0.001)</td>
<td>(77)</td>
</tr>
<tr>
<td>Technology used: real-time RT-qPCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>let-a</td>
<td>143</td>
<td>NSCLC tissues</td>
<td>Down-regulation</td>
<td>OS (p=0.0003)</td>
<td>(42)</td>
</tr>
<tr>
<td>157 mature human microRNAs</td>
<td>112</td>
<td>NSCLC tissues</td>
<td>miR-221, let-7a, miR-137, miR-372, miR-182</td>
<td>OS (p=0.026) DFI (p=0.024)</td>
<td>(47)</td>
</tr>
<tr>
<td>miR-21 and miR-205</td>
<td>48</td>
<td>Pairs of NSCLC</td>
<td>Up-regulated: miR-21</td>
<td>OS (p=0.027)</td>
<td>(60)</td>
</tr>
<tr>
<td>miR-21</td>
<td>20</td>
<td>Pairs of NSCLC</td>
<td>Overexpression</td>
<td></td>
<td>(61)</td>
</tr>
<tr>
<td>miR-34 family</td>
<td>70</td>
<td>Pairs of NSCLC</td>
<td>Down-regulation</td>
<td>DFI (p=0.039)</td>
<td>(73)</td>
</tr>
<tr>
<td>miR-17, miR-21,</td>
<td>317</td>
<td>NSCLC tissues</td>
<td>Overexpression: miR-21</td>
<td>Yes</td>
<td>(66)</td>
</tr>
<tr>
<td>miR-21 and miR-205</td>
<td>25</td>
<td>Adenoma carcinoma Squamous carcinoma</td>
<td>Down-regulated: miR-205</td>
<td>Not evaluated</td>
<td>(88)</td>
</tr>
<tr>
<td>24</td>
<td>Adenocarcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>let-7a, miR-7, miR-21, miR-155, miR-221</td>
<td>46</td>
<td>Pairs of NSCLC</td>
<td>Down-regulation: miR-221</td>
<td>OS (p=0.0036)</td>
<td>(79)</td>
</tr>
</tbody>
</table>
risk for relapse after surgical resection is one of the important challenges today. In an exploratory study, Duncavage et al. determined whether the expression of six miRNAs (let-7a, miR-7, miR-21, miR-155, miR-210, and miR-221) were associated with tumor recurrence in patients with resected stage I NSCLC and according to their findings, and if confirmed in prospective studies, miRNA expression in resected NSCLC could potentially identify patients at high risk of relapse after surgery (79).

Gao et al. explored the global expression profile of miRNAs in NSCLC and its potential relevance to clinic-pathological characteristics and prognosis. By using LNA miRNA microarrays in eight surgically removed lung carcinoma tissues and their corresponding normal lung tissues they selected miR-21, miR-143 and miR-181a for further study in another 47 paired samples by qRT-PCR using Taqman miRNA assays. Their data indicate the potential of miR-21, miR-143 and miR-181a as novel diagnostic or prognostic biomarkers for NSCLC (80).

In Table 1 we summarize the prognostic significance of miRNAs as evaluated so far in NSCLC tissues, while in Figure 3 we outline the network of the most studied so far lung-enriched miRNAs and their predicted target genes in NSCLC.

**miRNAs in classification of histologic subtypes of NSCLC**

Accurate classification of NSCLC is of paramount clinical relevance, as novel chemotherapeutic agents show different efficacy in AD compared with SCC. Cyto- and histomorphology may sometimes be insufficient for this distinction and immunohistochemistry may improve diagnostic accuracy. AD and SCC are two major histologic subtypes in NSCLC that present unique histopathological characteristics at distinct preferential anatomical locations, and yet are classified together in traditional diagnosis, sharing similar staging system and treatment in clinical management. However, precise molecular mechanisms that differentiate the histopathology and characterize the tumor initiation and progression in these two subtypes are not completely understood. Recent clinical trials have revealed that histologic subtypes of NSCLC respond differently to certain treatments. For example, bevacizumab plus platinum-based chemotherapy have been approved in unresectable, locally advanced, recurrent or metastatic non-SCC lung cancer (81). Advanced-stage SCC patients tend to have more severe side effects from treatment with bevacizumab. Pemetrexed treatment following platinum-based chemotherapy in locally advanced or metastatic AD and LCC had superior outcome when compared to patients with squamous histology (82).

Global miRNA expression profiling demonstrated differential expression of 6 miRNAs (miR-205, miR-99b, miR-203, miR-202, miR-102, and pre-mir-204) between AD and SCC (42). Among these 6 miRNAs, miR-205 was confirmed using FFPE samples or preoperative biopsies in later studies (83, 84). A separate investigation did not reproduce this finding but rather identified a larger panel of 34 miRNAs that were differentially expressed between AD and SCC, with most of them up-regulated in AD, and among the top 5 miRNAs (miR-181a, miR-191, miR-107, miR-103, and let-
transforming growth factor (TGF) protein, which plays a role in the regulation of cancer-associated genes in tissues. Control of gene expression by miRNAs influences many cellular processes and the presence of miRNAs is mainly for the regulation of cancer-associated genes in tissues.

Very recently, Boeri et al. explored miRNA expression profiles of lung tumors, normal lung tissues and plasma samples from cases with variable prognosis identified in a completed spiral-CT screening trial with extensive follow-up. They observed that serum miRNAs are promising prognostic biomarkers. Hu et al. demonstrated that 11 serum miRNAs were found to be altered more than five-fold between longer-survival and shorter-survival groups, and the levels of four miRNAs (miR-486, miR-30d, miR-1 and miR-499) were significantly associated with overall survival (108).

Futhermore, serum miRNA profiles among different cancer types including NSCLC have been analyzed. When Chen et al. investigated the expression profile of miRNAs in various patients and compared it with that of normal subjects, several miRNAs were found to be significantly differentially expressed among these two groups. Concerning NSCLC, 28 miRNAs were missing and 63 new miRNAs were detected after the comparison between healthy subjects and lung cancer patients (109). Another group has studied the expression levels of the 22 miRNAs selected in the study of Chen et al., in 18 malignant and 12 benign effusions and after discarding nine lowly expressed miRNAs, a panel of 13 miRNAs were measured in 30 samples and found that miR-24, miR-26a and miR-30d were expressed differently between the two groups (110). Finally, very recently, Foss et al. showed that miR-1254 and miR-574-5p were significantly increased in plasma of early-stage NSCLC patients with respect to the control volunteers suggesting that these two miRNAs can be used as serum-based minimally invasive biomarkers (111). Shen et al. validated expressions of the miRNAs in paired lung tumor tissues and plasma specimens from 28 stage I NSCLC patients by real-time quantitative reverse transcription PCR, and then evaluated the diagnostic value of plasma miRNAs in a cohort of 58 NSCLC patients and 29 healthy individuals. According to their findings, altered expressions of miRNAs in plasma would provide potential blood-based biomarkers for the clinical laboratory (112).
Table 2  Detection of circulating miRNAs in NSCLC.

<table>
<thead>
<tr>
<th>Tested miRNAs</th>
<th>No of samples</th>
<th>Plasma/serum</th>
<th>Techniques</th>
<th>Significantly expressed</th>
<th>Normalization</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>All human miRNAs</td>
<td>11</td>
<td>Healthy donors</td>
<td>Microarrays</td>
<td>miR-1254, miR-574-5p</td>
<td>External control: cel-miR-39 Internal control: U6</td>
<td>(111)</td>
</tr>
<tr>
<td>miR-21, 126, 145, 139, 182</td>
<td>29</td>
<td>Healthy donors</td>
<td>RT-qPCR</td>
<td>Up-regulated: miR-21, miR-210</td>
<td>External control: cel-miR-238</td>
<td>(112)</td>
</tr>
<tr>
<td>200b, 205, 210, 375, 429, 486-5p, and 708</td>
<td>58</td>
<td>NSCLC</td>
<td>RT-qPCR (Taquin low-density arrays)</td>
<td>Down-regulated: miR-126, miR-486-5p</td>
<td>Internal control: miR-16 and U6</td>
<td></td>
</tr>
<tr>
<td>365 human mature miRNAs</td>
<td>20</td>
<td>Healthy donors</td>
<td>RT-qPCR</td>
<td>Down-regulated: miR-30e-3p, let-7f</td>
<td>Directly normalized to total RNA</td>
<td>(115)</td>
</tr>
<tr>
<td>All human miRNAs</td>
<td>11</td>
<td>NSCLC (pool)</td>
<td>Solexa sequencing and RT-qPCR for Validation</td>
<td>Up-regulated: miR-25, miR-223</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Healthy male donors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Healthy female donors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All human miRNAs</td>
<td>30</td>
<td>Patients with longer survival</td>
<td>Solexa sequencing</td>
<td>Up-regulated: miR-486, miR-30d, Down-regulated: miR-1, miR-499</td>
<td>External control: miR-168 for plant</td>
<td>(108)</td>
</tr>
<tr>
<td>miR-20a, miR-21, miR-22, miR-24,</td>
<td>18</td>
<td>Malignant effusions</td>
<td>RT-qPCR</td>
<td>Down-regulated: miR-24, miR-26a and miR-30d</td>
<td>External control: ath-miR-156a</td>
<td>(110)</td>
</tr>
<tr>
<td>miR-25, miR-26a, miR-26b, miR-27a,</td>
<td></td>
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<tr>
<td>miR-27b, miR-29a, miR-30d, miR-145,</td>
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<td>miR-146a, miR-152, miR-199a,</td>
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<td>miR-200c, miR-221, miR-222, miR-223,</td>
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<td>miR-320, miR-375, miR-382</td>
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According to their data, miRNA expression patterns significantly distinguished: (i) tumors from normal lung tissues, (ii) tumor histology and growth rate, (iii) clinical outcome, and (iv) year of lung cancer CT detection. According to this study, miRNA profiles in normal lung tissues also displayed remarkable associations with clinical features, suggesting the influence of a permissive microenvironment for tumor development. It is impressive that miRNA expression analyses in plasma samples collected 1–2 years before the onset of disease, at the time of CT detection and in disease-free smokers enrolled in the screening trial, resulted in the generation of miRNA signatures with strong predictive, diagnostic, and prognostic potential (area under the ROC curve ≥0.85). These signatures were validated in an independent cohort from a second randomized spiral-CT trial. These results indicate a role for miRNAs in lung tissues and plasma as molecular predictors of lung cancer development and aggressiveness and have a strong clinical implication both for lung cancer management and in the clinical laboratory (113).

By using a microarray platform that enables the simultaneous analysis of all human microRNAs by either fluorescent or electrochemical signals, Lodes et al. have shown that sufficient miRNAs are present in one milliliter of serum to detect miRNA expression patterns, without the need for amplification techniques. According to their findings these expression patterns could correctly discriminate between normal and cancer patient samples (114). Silva et al. analyzed 365 human miRNAs in the plasma from 28 NSCLC patients and 20 controls. They selected five miRNAs (let-7f, miR-20b, miR-30e-3p, miR-223 and miR-301) and validated them independently by real-time PCR in plasma from 78 NSCLC and 48 controls and correlated with pathologic parameters and survival. They found that let-7f, miR-20b and miR-30e-3p were decreased in plasma vesicles of NSCLC patients, and that let-7f and miR-30e-3p levels could distinguish between two groups of patients for stage of disease and therefore possibility of surgery. Plasma levels of miR-30e-3p and let-7f were associated with short disease-free survival and overall survival, respectively. NSCLC patients and healthy controls differ in vesicle-related miRNAs in plasma. Levels of let-7f and miR-30e-3p in NSCLC patients are associated with poor outcome (115). Moreover, very recently it
was shown by Yu et al. that miRNAs in sputum can be used as highly sensitive and specific non-invasive markers for early detection of lung adenocarcinoma (116).

In conclusion plasma miRNAs obtained by non-invasive methods could serve as circulating tumor biomarkers of discriminating and prognostic value in NSCLC. However, there is still a lot of work to be done before the establishment of miRNAs as biomarkers in the clinical laboratory, especially towards the standardization of analytical methodologies used, the inclusion of internal and external controls in each assay, and the consensus towards normalization of these results. In Table 2 we summarize findings presented so far on the detection of circulating miRNAs in plasma and serum of NSCLC patients.

miRNAs as therapeutic targets in lung cancer

Many recent findings implicate that miRNAs could play an important role for the design of innovative therapies for NSCLC. Numerous studies have documented the implications of miRNAs in nearly every carcinogenesis process of lung cancer, including tumor development, apoptosis, invasion and metastasis, as well as anti-cancer drug resistance. Forced expression or suppression of specific miRNAs can regulate the biological alteration during carcinogenesis, underscoring the therapeutic potential of miRNAs in lung cancer. Recent reviews have shown that some key microRNAs can modulate the lung cancer carcinogenesis process, and discuss the perspectives of microRNAs as therapeutic targets for lung cancer (117–119). By exploiting the unique characteristics of miRNAs, clinicians can come ever closer to achieving the goal of individualized cancer treatment.

Esquela-Kerscher et al. have shown that the let-7 microRNA directly represses cancer growth in the lung (120). They found that let-7 inhibits the growth of multiple human lung cancer cell lines in culture, as well as the growth of lung cancer xenografts in immunodeficient mice. These findings provide direct evidence that let-7 acts as a tumor suppressor gene in the lung and indicate that this miRNA may be useful as a novel therapeutic agent in lung cancer. Weiss et al. investigated if the loss of microRNA-128b, that is a putative regulator of EGFR, correlated with response to targeted EGFR inhibition (121). Loss of microRNA-128b would be equivalent to losing a tumor suppressor gene because it would allow increased expression of EGFR. They found that microRNA-128b loss of heterozygosity (LOH) was frequent in tumor samples and correlated significantly with clinical response and survival following administration of gefitinib.

Tumor suppressor miRNAs provide a new opportunity to treat cancer. This approach, miRNA replacement therapy is based on the concept that the reintroduction of miRNAs depleted in cancer cells reactsivates cellular pathways that drive a therapeutic response. Wiggins et al. described the development of a therapeutic formulation using chemically synthesized miR-34a and a lipid-based delivery vehicle that blocks tumor growth in mouse models of NSCLC. Their data provide proof of concept for the systemic delivery of a synthetic tumor suppressor mimic, obviating obstacles associated with viral-based miRNA delivery and facilitating a rapid route for miRNA replacement therapy into the clinic (122). Paxillin (PXN) gene mutations are associated with lung adenocarcinoma progression and PXN is known to be a target gene of microRNA-218 (miR-218). Wu et al. have shown that miR-218 expression in lung tumors was negatively associated with PXN expression and that PXN and miR-218 might independently predict overall survival and regression free survival, respectively, in NSCLC (123). Their findings suggest that PXN overexpression induced by miR-218 suppression is an independent predictor of survival and relapse in NSCLC, highlighting PXN as a potential therapeutic target to improve clinical outcomes in this disease.

Recent results by Chen et al. demonstrate that miR-145 inhibits proliferation of NSCLC cells through c-Myc and suggest that increasing miR-145 expression may provide a novel approach for the treatment of NSCLC (124). Frezzetti et al. have very recently shown that a LNA directed against miR-21 slows down tumor growth in mice (125). Consistently, a search for miRNAs downregulated by miR-21 shows an enrichment for miRNAs encoding cell cycle checkpoints regulators, suggesting an important role for miR-21 in oncogenic RAS-induced cell proliferation.

Identification of miRNA targets is a critical step to design novel therapies and interrogate molecular mechanisms underlying miRNA signatures, but two major hurdles still exist. First, correct prediction of miRNA target genes through computation algorithms is still a major challenge. It has been shown that the union of miRNA target genes predicted by three computational algorithms (miRanda, PicTar, and TargetScan) is one of the strategies that give the highest sensitivity (126), but such sensitivity will be undoubtedly compromised by a large list of false-positive prediction and hence appropriate data filtering would be required. Secondly, as the number of published miRNA expression profiles in lung cancer starts to grow, it appears that the miRNA signatures from different groups are non-overlapping. This interestingly coincides with the lack of consensus in mRNA signatures from at least two dozens of gene expression profiling datasets in lung cancer that have been published so far. In a report using three most prominent lung adenocarcinoma gene expression profiles (127), no common gene signature was found, although the gene expression profile in each of the three datasets can reproducibly stratify the adenocarcinoma into three subtypes. This could be caused by the heterogeneity in histologic subtypes and ethnic origins of lung cancer, platform-to-platform discrepancies, batch effect and sample preparation within the same platform, and even the statistic methods being used (128). All these highlight the importance of meta-analysis of multiple datasets crossing different platforms for either in silico marker identification, or experimental validation of any gene signature using samples from independent sources.

We believe that in the near future all these difficulties, concerning identification and verification of critical miRNA targets and lack of safe and specific delivery system will be
Conclusions

In conclusion, miRNAs have the potential to serve both as biomarkers and therapeutic agents in cancer, by personalizing diagnosis and therapy (129). There is increasing evidence that altered microRNA expression is associated with tumor progression and survival in lung cancer patients. Recent data from multiple studies strongly support the potential of microRNAs as biomarkers in NSCLC. Expression profiles of miRNAs were able to distinguish tumors derived from different tissue origins enabling classification of histologic subtypes of NSCLC. Especially cell-free miRNAs, circulating in body fluids like plasma and serum comprise today very promising novel tumor biomarkers that will play a critical role in the clinical laboratory in the near future. miRNAs could play an important role for the design of innovative therapies for NSCLC and by exploiting the unique characteristics of miRNAs, clinicians can come ever closer to achieving the goal of individualized cancer treatment.

However, there is still a lot of work to be done before the establishment of miRNAs as biomarkers in the clinical laboratory, especially towards the standardization of analytical methodologies used, the inclusion of internal and external controls in each assay, and the consensus towards normalization of these results.

Conflict of interest statement

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