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Applications of MicroRNAs in the Diagnosis and Prognosis of Lung Cancer

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Abstract

Introduction—Lung cancer is the leading cause of cancer death worldwide, due to its late diagnosis and poor outcome. MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the posttranscriptional levels by either degrading or blocking translation of messenger RNA targets. Accumulating evidence indicates that miRNAs play a pivotal role in the development and progression of human malignancies, including lung cancer.

Areas covered—In this review, the authors focus on 1) application of miRNA-based biomarkers to help classify lung cancer, 2) application of the miRNA biomarkers for the early detection of lung cancer, and 3) use of miRNAs as biomarkers to predict outcomes of lung cancer.

Expert Opinion—MiRNAs provide promising biomarkers for the diagnosis and prognosis of lung cancer. The developed miRNA biomarkers should be comprehensively and prospectively validated in clinical trials before being used in laboratory settings.

Keywords

MiRNAs; biomarker; diagnosis; prognosis; lung cancer

1. Introduction

Lung cancer is the second most common cancer and the number one cancer killer in the USA and worldwide (1). Histologically, 85% of lung cancers are non-small-cell lung cancers (NSCLCs), and 15% are small-cell lung cancers (SCLCs). Despite the numerous efforts have been made, the overall 5-year survival rate of lung cancer is less than 16%, compared to 5-year survivals of 88% for breast cancer, 65% for colon cancer, and almost 100% for prostate cancer, respectively (1). The main reason for poor lung cancer prognosis is that the patients are often diagnosed in late stages with advanced disease. Once lung cancer spreads to other parts of the body, or causes metastases, it becomes extremely difficult to treat, thus worsening the survival rates. Furthermore, there is a challenge in monitoring the treatment of lung cancer and predicting prognosis of the patients (2). Some techniques, such as chest radiography and sputum cytology, have been used for lung cancer early detection and screening. However, the sensitivity is very low (3). A NCI-National Lung Screening Trail recently found 20% fewer lung cancer deaths among those who were screened with computed tomography (CT) compared with those who were screened with chest X-ray (4). Given the continued search for proof that early detection of lung cancer can improve outcomes of the deadliest form of malignancy, this is certainly an exciting and

encouraging finding. However, the widespread use of CT scanning has dramatically increased the number of indeterminate solitary pulmonary nodules (SPNs) seen in asymptomatic individuals. Most malignant SPNs represent stage I NSCLC and can be surgically removed (5). However, because only up to 13% SPNs are malignant (6), CT often produces a high false positive rate for diagnosis of lung cancer among indeterminate SPNs (7). Therefore, it is clinically imperative to make a definitive preoperative diagnosis of indeterminate SPNs, so that lung cancer patients can be found earlier and benefit most from effective and prompt therapy, while sparing ones bearing benign growths from emotional and financial costs of invasive biopsies and treatments (8). Furthermore, lung cancer is a disease with high genetic complexity and heterogeneity. The current classification system for lung cancer is not efficient in predicting clinical behavior of lung tumors. Molecular genetic analysis may provide a new classification system that can improve the conventional histology to more precisely identify lung cancer cases that have aggressive behavior and should receive more effective therapies.

2. Features of microRNAs (miRNAs)

miRNAs are small (20–22 nucleotides) and endogenous noncoding RNAs that have pivotal functions in various biological processes (9,10). miRNAs arise from large precursors (pri-miRNAs). These are transcribed by RNA polymerase II and processed into shorter hairpin sequences of approximately 70 nucleotides (pre-miRNAs) by RNase III Drosha. The pre-miRNAs undergo a processing step in the cytoplasm, which is carried out by RNase III Dicer to form a double-stranded RNA. Single-stranded miRNAs bind through partial or complete sequence homology to the 3' untranslated region of target mRNA, leading to either a block of translation or mRNA degradation. Therefore, miRNA can regulate gene expression at a post-transcriptional level, and thereby control fundamental cellular aspects, such as differentiation, cell growth, proliferation, and apoptosis. miRNAs have the potential to regulate at least 20–30% of all human transcripts (11), and are involved in almost all basic signaling pathways. Furthermore, miRNAs can control the expression of important tumor-related genes in tumorigenesis, including oncogenes and tumor-suppressor genes (12). Therefore, dysregulated miRNAs contribute to a variety of pathological events, including carcinogenesis.

3. miRNA as biomarkers of lung cancer

miRNA expressions in tumors have frequently been observed to be up- or down-regulated compared to normal tissues (13). Furthermore, miRNA expression profiling of different types of tumors has given remarkable insight into the developmental lineage and differentiation states of cancer. Even within a single developmental lineage, there are distinct patterns of miRNA expressions that might reflect mechanisms of transformation. Therefore, miRNA expression patterns could encode the developmental history of human cancers. Furthermore, as important modulators of gene expression, miRNAs provide novel candidate diagnostic and prognostic indicators (14, 15, 16, 17, 18, 19). In addition, characteristics of miRNA make the molecules as ideal tumor biomarkers. The characteristics, particularly, include the following. First, each type of tumor tissues may exhibit a unique expression miRNA profile. Second, miRNAs are more stable compared to mRNAs. For example, miRNA expression can be detected in formalin-fixed paraffin-embedded (FPPE) tissue, plasma, sputum, and urine specimens. Third, quantification of miRNAs' expressions can be efficiently and reliably obtained by using different platforms, such as quantitative reverse transcriptase PCR (qRT-PCR), microarray assay, and next-generation arrays (20, 21). In this review, we will mainly describe 1) application of miRNA-based biomarkers that might help classify lung cancer, 2) application of the miRNA

biomarkers for the early detection of lung cancer, and 3) use of miRNAs as biomarkers to predict lung cancer's outcomes.

4. The use of miRNAs as biomarkers to help classify lung cancer

Lung cancer diagnosis and therapy are largely dependent on accurate classification and subtyping of tumor tissues. WHO published guidelines for pathological typing of lung cancer in 1981 (23). Since then several updates and techniques have been made and added (24). For example, advancements in immunohistochemistry increased our knowledge of lung cancer pathology and greatly improved our diagnostic and subphenotyping accuracy of lung cancer. This technique, however, has several limitations: inconsistency in staining resulting from tumor heterogeneity, variability in sensitivity and specificity of different antigens, and presence of inter-reader variability (25). Furthermore, the current classification system is not suitable for predicting different responds to certain treatments. For instance, based on the current WHO system, adenocarcinoma (AD) and squamous cell carcinoma (SCC) are two major histologic types of NSCLC. Although presenting different histopathological characteristics at distinctive preferential anatomical locations, AD and SCC are still clinically staged using the same system and treated with the same strategy. However, some chemotherapeutic agents show different efficacy between AD and SCC. For example, pemetrexed treatment following platinum-based chemotherapy in locally advanced or metastatic AD had favorable prognosis compared with SCC (26). In addition, the lung tumors classified within one histological type at the same stage may display different responds to the same therapeutic treatment. It is, therefore, important to develop new classification system for NSCLC that can be used for more effective treatments of the malignancy.

By using global miRNA expression profiling, Yanaihara et al. (27) found that 6 miRNAs (mir-205, mir-99b, mir-203, mir-202, mir-102, and pre-mir-204) exhibited different expression profiles between AD and SCC. Two later studies confirmed that mir-205 can differentiate AD from SCC on FFPE samples or preoperative biopsies (28, 29). Furthermore, Landi et al. (30) identified a large panel of 34 miRNAs that could distinguish AD and SCC groups in 165 ADs and 125 SCCs. Most of the miRNA were upregulated in AD, and the top five genes were mir-181a, mir-191, mir-107, mir-103, and let-7b. Furthermore, there was a significant association between expressions of the five miRNAs with those of their target protein-coding genes. For instance, mir-181a is correlated with TUSC3, EEF1E1, and RFC3, mir-191 is correlated with SEC24B, PPIG, and GRPEL1, mir-107 is correlated with CYP27B1, KIAA0241, and MRPS2, and mir-103 is correlated with MDH2, CYP27B1, and NUDT3. These genes are involved in chemical/cellular homeostasis, peroxisomal transport, and G protein signaling.

Because lineage specific miRNA regulation involves in maintaining the stemness, embryonic development, and tissue differentiation (31), miRNA expression profiles could be used to differentiate tumors derived from different tissue origins. Indeed, miRNA expression profiles displayed better results as compared to mRNA expression profiles even in poorly-differentiated clinical samples (13). For instance, measuring miRNA expression levels in FPPE specimens from 22 different tumor tissues and metastases, Rosenfeld et al. (32) constructed a transparent classifier based on 48 miRNAs. By using the miRNA-based classifier, they categorized two-thirds of samples with 90% accuracy. The accuracy was successfully confirmed in an independent set of 83 samples with 89% accuracy. Furthermore, using a different technique, qRT-PCR, the classification power of the miRNAs was also validated in 65 additional samples. These results demonstrated the effectiveness of miRNAs as biomarkers for tracing the tissue origin of tumors that have unknown primary sources. This could also be attributed to the significant differential miRNA expression

within the same lung cancer histological group of either AD or SCC (26), or between histologic subtypes within the NSCLC group (19). For instance, Del Vescovo et al (27) recently studied 50 lung tumor tissues (25 ADs, 24 SCCs, 1 adenosquamous) for miRNA expressions. They found that measuring relative levels of mir-205 and mir-21 offer an important diagnostic tool. Particularly, mir-205 could be a biomarker to distinguish between AD and SCC. Altogether, use of miRNAs as biomarkers would help classify subtypes of lung tumor tissues. Examples of the type of miRNAs are summarized in Table 1.

5. miRNAs as biomarkers for the early detection of lung cancer

To develop biomarkers for the early detection of lung cancer, a variety of molecular and genetic changes have been investigated for decades. However, none has been accepted in clinical settings. Intriguingly, measuring aberrantly expressed miRNAs can discriminate tumor from normal lung tissue samples. For instance, Yanaihara et al. (27) analyzed 352 miRNAs in 144 lung tumors and the matched normal lung tissues. 43 miRNAs were identified that differentially expressed between the tumor and normal specimens. From the miRNAs, 12 miRNAs (mir-17-3p, mir-21, mir-106a, mir-146, mir-155, mir-191, mir-192, mir-203, mir-205, mir-210, mir-212, and mir-214) were further defined that can be used for the early detection of NSCLC. Using qRT-PCR, Liu et al (35) analyzed miRNA expressions in the paired tumor and normal lung tissues of 70 NSCLC patients. Expression levels of mir-21, mir-141, and mir-200c in NSCLC tissues were higher than those in normal tissues. In addition, Vosa et al. (36) investigated expressions of 858 miRNAs in 38 NSCLC samples (stage I and II) and 27 adjacent noncancerous tissue samples using Illumina miRNA arrays. mir-9, mir-205, and mir-210 were significantly higher in tumor tissues compared with normal lung tissues. In a study of 60 SCC patients, Tan et al. (41) found that a 5-miRNA classifier (mir-210, mir-182, mir-486-5p, mir-30a, mir-140-3p) could distinguish SCC from normal lung tissues. This classifier had an accuracy of 94.1% in a training set (34 patients) and 96.2% in a test set (26 patients), respectively. Seike et al. (42) measured miRNA expressions in lung cancer tissues and the matched normal tissues in 28 cases of lung cancer patients who were non-smokers. mir-21, mir-141, mir-210, and mir-200b were upregulated, whereas mir-346, mir-126, mir-126, mir-3a, mir-30d, mir-486, mir-129, mir-451, mir-521, mir-128, mir-30b, mir-30c, mir-516a, and mir-520 were downregulated in tumors compared to normal tissues. Raponi et al. (43) showed that expression levels of mir-210, mir-200c, mir-17-5p, mir-20a, mir-203, mir-200a, mir-106b, mir-93, mir-182, mir-83, mir-106a, mir-20b, and mir-224 were higher, whereas expression levels of let-7e and mir-200a were lower in SCC tissues compared to normal tissues. In another study, Dacic et al. (44) found that some miRNAs having unique expression patterns correlated to mutation types of protein-coding genes, suggesting that the miRNA pathways contribute to the tumorigenesis. For instance, mir-155 was upregulated only in EGFR/KRAS-negative group, whereas mir-25 was solely upregulated in EGFR positive group. In addition, mir-495 was upregulated in KRAS-positive ADs. However, let-7g was downregulated in all above three groups with more significant downregulated in EGFR/KRAS-negative ADs. The studies suggested that measuring miRNAs in tissues would provide useful tool for diagnosis of NSCLC.

It is clinically important to develop miRNAs that can be tested in easily accessible body fluids as noninvasive or mini-invasive biomarkers for the early detection of lung cancer. In particular, sputum is the most easily accessible and noninvasive biological material. Furthermore, cancer-associated-genetic changes found in sputum highly indicate that there might be malignancy in the lungs. We demonstrated that measuring expression levels of mir-210, mir-708, and mir-205 in sputum could discriminate SCC patients from normal subjects (37). Subsequently, we (38) found that high levels of mir-21, whereas low levels of mir-486, mir-375, and mir-200b in sputum could distinguish AD patients from normal

subjects. Because blood is an easily accessible and rich biological fluid for the development of cancer biomarkers, we (39) also validated expressions of the miRNAs in paired lung tumor tissues and blood plasma specimens from a set of 28 stage I NSCLC patients. Our results showed that the miRNA aberrations identified in tissues could be confirmed in blood plasma. In particular, testing mir-21, mir-210, mir-126, and mir-486-5p in patients' plasma could discriminate NSCLC from healthy controls. Recently, studying 152 NSCLC patients and 75 healthy controls, Chen et al. (14) found that higher levels of mir-23 and mir-225 in the circulating exosomes of NSCLC patients as compared with healthy donors. Zheng et al. (40) analyzed plasma miRNAs of 74 lung cancer patient and 68 age-matched cancer-free controls. The levels of plasma mir-155, mir-197, and mir-182 of lung cancer patients were significantly higher compared to the controls ($P < 0.001$). Rabinowits et al. (45) found that the miRNAs that had upregulated expressions in lung primary tumors were also upregulated in the lung cancer patients' blood samples. They also suggested that the tumor could release circulating mirRNAs through exosome particles. Analyzing serum from patients with 220 early-stage NSCLC and 200 healthy controls, Heegaard et al. found that the expression of mir-146b, mir-221, let-7a, mir-155, mir-17-5p, mir-27a, and mir-106a were significantly reduced, while that of mir-29c was increased in the serum of NSCLC cases. Roth et al. (47) studied the clinical relevance of circulating miRNAs in the serum of 35 lung cancer patients (19 NSCLCs, 8 SCLCs, 8 indefinite cancer types), 7 patients with benign growths, and 28 healthy controls. The levels of mir-10b, mir-141, and mir-155 were considerably higher in lung cancer patients compared to patients with benign growths. Furthermore, an association between high serum mir-10b levels and lymph node metastasis was observed. Altogether, these miRNAs could provide potential noninvasive or mini-invasive biomarkers for the early detection of lung cancer.

miRNAs could also have the potential as biomarkers used to screen for lung cancer. Foss et al. (48) evaluated miRNA expressions in a set of 31 controls and 22 patients with early-stage NSCLC. The expressions of serum mir-1254 and mir-574-5p were significantly increased in early-stage of NSCLC patients compared to those in the controls. Interestingly, the aberrant expressions of the miRNAs could be found before clinical diagnosis was made. Using risk score analysis in 400 NSCLC cases and 220 controls, Chen et al. (49) found that testing expression levels of ten serum miRNAs (mir-20a, mir-24, mir-25, mir-145, mir-152, mir-199a-5p, mir-221, mir-222, mir-223, and mir-320) could identify NSCLC up to 33 months ahead of the clinical diagnosis (50). Boeri et al. (51) conducted a miRNA expression profiling study to analyze 13 miRNAs in lung tumors and the paired adjacent normal lung tissues. The miRNA profiles of tumors detected in the first 2 years of the screening were different from those were found after two years, implying that the miRNAs might be associated with distinct aggressive features and fast growth rate. Furthermore, the panel of 13 miRNAs could distinguish aggressive from indolent tumors detected by low-dose CT with 80% accuracy. Their findings also showed that the signature actually appears 12–24 months before the time NSCLC can first be diagnosed by CT in patients whose tumors presented with aggressive clinical behavior. Interestingly, this subgroup of patients happened to be those diagnosed by CT in the 3rd to 5th year rather than first 2 years of CT screening program. The top 10 miRNAs that can discriminate CT-detected lung cancer from normal lung tissue are: mir-7-2-prec, mir-21, mir-200b, mir-210, mir-219-1, mir-324-5p, mir-126, mir-451, mir-30a, and mir-486-5p. Recently, Bianchi et al. (52) measured expressions of miRNAs in serum from 93 patients who were diagnosed with NSCLC in the first 2 years of screening, and serum from 69 individuals without any cancer detected by low-dose CT. The selected 34 miRNAs were weighed according to their accuracy in predicting diagnosis and then linearly combined into a risk score. The risk score generated from 34 miRNAs could differentiate high risk from low risk patients in both AD and SCC with 80% accuracy, and separate malignant tumors from benign growths discovered by CT as well. Validation of the miRNA signature was carried out in an independent cohort,

resulting in similar success rate. We (53) investigated whether plasma miRNAs could be useful in identifying lung cancer among individuals with CT-detected SPNs. By using qRT-PCR analysis, we first determined plasma expressions of five miRNAs in a training set of 32 patients with malignant SPNs, 33 subjects with benign SPNs, and 29 healthy smokers to define a panel of miRNAs that has high diagnostic efficiency for lung cancer. We then validated the miRNA panel in a testing set of 76 patients with malignant SPNs and 80 patients with benign SPNs. miR-21 and miR-210 displayed higher plasma expression levels, whereas miR-486-5p had lower expression level in patients with malignant SPNs as compared to subjects with benign SPNs and healthy controls. A logistic regression model with the best prediction was built on the basis of miR-21, miR-210, and miR-486-5p. The three miRNAs used in combination produced the area under receiver operating characteristic curve at 0.86 in distinguishing lung tumors from benign SPNs with 75.00% sensitivity and 84.95% specificity. Validation of the miRNA panel in the testing set confirms their diagnostic value that yields significant improvement over any single one. The plasma miRNAs provide potential circulating biomarkers for noninvasively diagnosing lung cancer among individuals with SPNs. Therefore, the miRNA-based biomarkers can not only diagnose early stage lung cancer using body fluid samples, but also identify lung tumors in the small pulmonary nodules discovered by CT scan. Future application of the biomarkers in laboratory settings will complement CT to screen high risk individuals (e.g., heavy smokers) for the early detection of lung cancer. Examples of the type of miRNAs are listed in Table 2.

6. miRNAs as biomarkers for predicting outcomes of lung cancer and prognosis of the patients

The risk of dissemination is around 30% for stage I, 50% for stage II and 75% for stage III lung cancer patients, respectively (54). Because tumor-node-metastasis (TNM) classification frequently produces a poor prediction of the individual dissemination risk, new biomarkers are required for better selecting the early-stage lung cancer patients for adjuvant systemic treatments (55, 56). Analyzing miRNAs in tissue specimens might provide biomarkers to predict outcome of lung cancer. Indeed, low expression of pri-let-7a and pri-let-7f was correlated to shorter overall survival in NSCLC, and high disease stage as well (57). Furthermore, high expression of mir-155, mir-17-3p, mir-106a, mir-93, and mir-21 and low expression of let-7a-2, let-7b, and mir-145 were associated with unfavorable outcomes of AD patients. Zheng et al. (40) found that expression levels of mir-155 and mir-197 were higher in the plasma from lung cancer patients with metastasis than in those without metastasis, and remarkably, were significantly decreased in responsive patients during chemotherapy. Tan et al. (41) indicated that a high expression level of mir-31 was associated with poor survival in 60 SCC patients. The result was confirmed in 88 SCC patients. Therefore, mir-31 could be a prognostic biomarker for SCC. Vosa et al. (36) recently analyzed a correlation of miRNAs' levels and cancer progression in 38 NSCLC patients. The data showed that low expression of mir-374a in early-stage NSCLC was associated with a poor overall survival of the patients. Therefore, mir-374a can serve as a prognostic biomarker for the risk stratification of the lung cancer patients. Garofalo et al. (58) analyzed mir-221/222 in 32 snap-frozen normal and malignant lung tissues and lung cancer cells. mir-221 and mir-222 were overexpressed in aggressive NSCLC. Boeri et al. (51) found that mir-486-5p, along with mir-21, mir-126, mir-15b, mir-148a, mir-142-3p, mir-17, mir-197, mir-221, mir-28-3p, and mir-106a, were downregulated in plasma of patients with unfavorable prognosis. Furthermore, Inamura et al. (59) found that let-7 expression was lower in cancer tissues, as compared to normal lung tissues. Interestingly, let-7 expression in bronchioloalveolar carcinoma (considered an AD in situ) was equal to invasive or poorly differentiated AD. However, there was lack of correlation between let-7 expression and overall survival.

DNA methylation has a pivotal role in epigenetically silencing miRNAs in carcinogenesis. Watanabe et al (60) found that mir-34b was methylated in 41% of the primary NSCLC specimens. Furthermore, a multivariate analysis demonstrated that mir-34b methylation was significantly associated with lymphatic invasion. Therefore, mir-34b methylation can be used as a biomarker for an invasive phenotype of lung cancer. Brain metastasis (BM) is common in NSCLC patients. Arora et al. (61) showed that the combination of mir-328 and mir-330-3p could classify BM-positive vs. BM-negative NSCLC patients with 80% accuracy. Therefore, the two miRNAs could be incorporated into clinical treatment decision-making to stratify NSCLC patients at higher risk of developing BM. Navarro et al. (62) found that a overall survival was 23.9 months for lung cancer patients who had high mir-16 expression levels, 97.6 months for those with normal mir-16 levels, and 63.5 months for those with low mir-16 levels ($P < 0.001$). Therefore, high mir-16 levels could be used as a prognostic factor for poor disease-free survival. By comprehensively analyzing 157 miRNAs in 56 early stage NSCLC patients (63), Yu et al. found that aberrant expressions of mir-221, let-7a, mir-137, mir-372, and mir-182 were correlated with favorable prognosis. The risk score based on these 5 miRNA signature predicted progression-free survival and overall survival of lung cancer patients. Furthermore, the miRNA signature was significantly associated with relapse-free survival of patients with stage I disease, stage III disease, AD ($P = 0.009$), and SCC, respectively (All $p < 0.05$). The risk score was, therefore, a prognostic factor in stage I and stage III as well as AD and SCC groups. This study also indicated that the five-miRNA signature could have clinical implications in the molecular pathogenesis of cancer, or selection of high-risk cancer patients for adjuvant chemotherapy. Landi et al. (30) investigated expression of 85 miRNAs in 165 AD and 125 SCC samples. The results showed that miRNA expression profiles strongly differed between AD and SCC, particularly in the early stages. Most miRNAs, including all members of the let-7 family, were downregulated in SCC. A five-miRNA signature (mir-25, mir-34c-5p, mir-191, let-7e, and mir-34a) was further identified that could significantly predict survival for the patients with SCC. Therefore, this miRNA-based prognostic assay can potentially be utilized to identify the patients who may need more additional chemotherapy after surgery. Patnaik et al. (64) investigated 77 cases of stage I NSCLC (37 cases with recurrence and 40 cases without recurrence after clinical follow-up at least 32 months) using FFPE samples. 752 human miRNAs were analyzed to obtain expression profiles by using two different methods, leave-one-out and Monto Carlo cross-validations. The results showed that the identified nine miRNAs could predict lung cancer recurrence with an accuracy of 70% and 83% with the hazard ration of 3.6 (95% CI, 1.8–7.1) and 9.0 (95% CI, 4.4–18.2), respectively. The miRNAs include mir-200b*, mir-30c-1*, mir-510, mir-630, mir-657, mir-146b-3p, mir-124*, mir-585, and mir-708. Raponi et al. (65) analyzed 20 miRNAs' expression levels in 61 SCC samples and 10 normal lung tissues. miR-146b alone had the strongest prediction accuracy for stratifying prognostic groups at 78% and a HR of 5.9 (95% CI, 2.2–13.1). Interestingly, this study indicates that miRNAs may have greater clinical utility in predicting the prognosis of patients with SCC as compared with mRNA-based signatures.

To verify the previously reported prognostic significance of the miRNAs in lung cancer, Saito et al. (66) investigated expressions of mir-21, mir-17, and mir-155 in frozen tumor tissues from a large set of cohort consisting of 317 NSCLC patients. The results showed that high expression of mir-21 was associated with a poor prognosis of the cancer patients. Similarly, Wang et al (67) conducted a study using serum specimens from 88 NSCLC patients and 17 healthy controls to measure mir-21 expression levels. High serum mir-21 expression was significantly correlated with tumor-node metastases stage and lymph node metastasis of NSCLC patients ($P = 0.016$ and 0.026 , respectively). The 3-year overall survival rates in NSCLC patients with high serum mir-21 expression was considerably shorter than those with low serum mir-21 expression ($P < 0.001$). Therefore, serum mir-21 might be used as a prognostic biomarker for NSCLC patients. mir-126 is associated with tumor

angiogenesis and vascular endothelial growth factor-A (VEGF-A). Donnem et al (68) analyzed surgically resected tumor tissues from 335 stage I-IIIa NSCLC patients by using high-throughput tissue microarray, in situ hybridization, and immunohistochemistry. The results showed that the coexpression of mir-126 and VEGF-A had a significant prognostic impact on 5-year survival rates ($P=0.002$). Cho et al. (69) found that upregulation of mir-145 appeared to be a key gene for the proliferation of lung adenocarcinoma cells. Low expression of mir-145 had a strong correlation with the downregulation of EGFR. The mir-145 might be a predictive biomarker for the effect of EGFR-TKI and useful in personalized therapy of AD. Furthermore, in a study using 639 FFPE specimens from patients who participated in International Adjuvant Lung Cancer Trial, Voortman et al. (70) reported that high mir-21 level was associated with unfavorable prognostic effects. Lee et al (71) investigated the expression of 7 miRNAs (mir-21, mir-29b, mir-34a/b/c, mir-155, let-7a) in 31 SCLC tumors, 14 SCLC cell lines, and 26 NSCLC cell lines. However, the results indicated that the expressions of 7 miRNAs were not prognostic in SCLC patients, and mir-34a was unrelated to the malignant behavior of SCLC cells. In a study (72) comprising 303 lung cancer patients, 60 patients were selected for the discovery stage for Solexa sequencing of serum miRNAs, remaining 243 patients with NSCLC were randomly classified as either training ($n=120$) or testing ($n=123$) sets. High levels of serum mir-486 and mir-30d and low levels of mir-1 and mir-499 were correlated with unfavorable prognosis of the patients. Taken together, although there is inconsistent evidence regarding prognostic values for some individual miRNAs, the previous studies imply that some sets of miRNAs could be developed as biomarkers for predicting the outcomes of lung cancer. Examples of the class of miRNAs are summarized in Table 3.

7. Conclusion

Increasing discoveries of the miRNA-based markers provide great opportunities for molecular diagnostics of lung cancer (16). The identification of circulating miRNAs as body-fluid based biomarkers for lung cancer is proceeding at a fast pace, which would become valuable for early diagnosis and follow-up investigations. Furthermore, analysis of clinical specimens reveals that miRNAs can be used as molecular biomarkers for lung cancer classification, prognostic stratification, and drug-response prediction.

8. Expert Opinion

The recent research discoveries and data generated from a variety of studies are very promising, as they provide strong evidence that miRNAs involve in the different regulation pathways and tumorigenesis of lung cancer. Importantly, defining unique patterns of miRNA expressions in lung cancer would provide molecular biomarkers for early detection and prognosis, and prediction of treatment responses. However, some issues should be addressed in the development of miRNA-based biomarkers. First, the commonly used internal controls for determination of target miRNAs are mir-16, U6, U6B, mir-142-3p, 18S rRNA, mir-638, let-7a, mir-1249, mmu-mir-295, 5S RNA, RNU38B, and RNU43. However, none has been widely accepted as a standard control. Therefore, previous studies frequently display conflicting data in the crossing-comparison of the results. It is imperative to identify universal control genes that are expressed at a constant level under all conditions and in all tissues, thereby developing gold standard controls. Second, tumorigenesis is a complex multistep process resulting from abnormal gene expression consequent to accumulation of epigenetic and genetic abnormalities. The functions of dysregulated miRNAs in lung tumorigenesis remain largely unknown (16, 74). Therefore, to develop more reliable and accurate biomarkers, it is important to continually uncover the detail mechanisms that drive lung tumor formation and progression through miRNA aberrations. Third, numerous factors, such as hypoxia, infection, and cytotoxic treatments, could affect the miRNA expressions. For

instance, some diseases also cause abnormal miRNA expressions. In the future, it is important to identify and develop a miRNA signature that not only distinguishes lung cancer from healthy individuals, but also from the subjects who are affected with the factors. For example, a specific panel of miRNAs is required for discriminating lung cancer patients from not only healthy smokers, but also the patients with COPD. Eventually, the biomarker will be able to diagnose lung cancer with high accuracy. Fourth, tumor cells release miRNAs into the extracellular space and the miRNAs could be detected in the blood and other body fluids (20, 73). How the miRNAs are protected from degradation and how they enter blood system and other body fluids remains largely unknown. Furthermore, because miRNAs in the circulation systems also can be released by non-cancer tissues, whether dysregulation of miRNAs in peripheral blood and other body fluids are consistent with that in tumor tissues are uncertain (45, 51). For example, Boeri et al. (51) found that miRNAs deregulated in tissue specimens were rarely detected in plasma samples. Therefore, it is also important to have comprehensive understanding the mechanisms of the protection of miRNAs from degradation and the way of miRNAs circulating in body fluids before we are able to successfully develop reliable biomarkers. Fifth, to develop prognostic biomarkers, previously published studies usually used overall survival as main end-point prevails, which are insensitive measures for prognostic assay development (19). In the future, it is imperative to define whether the miRNA expression is correlated with distant metastases, local recurrence or both. Finally, all the developed potential biomarkers are required to be validated in multi-institutional endeavor to conduct independent validation before they can be considered suitable for prospective clinical trials.

Acknowledgments

Declaration of interest

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List of abbreviations

NSCLC	non-small-cell lung cancer
SCLC	small-cell lung cancer
CT	computed tomography
SPN	solitary pulmonary nodule
miRNA	microRNA
AD	adenocarcinoma
SCC	squamous cell carcinoma
FFPE	formalin-fixed paraffin-embedded
EGFR/KARS	epidermal growth factor receptor/KARS
TNM	tumor-node-metastasis
COPD	chronic obstructive pulmonary disease
BM	Brain metastasis

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Table 1

Examples of miRNAs as potential biomarkers for classification of lung cancer

MicroRNA	Functions	Reference
mir-205, mir-99b, mir-203, mir-202, mir-102, and pre-mir-204	Distinguish between adenocarcinoma and squamous cell carcinoma	27
mir-205	Distinguish between adenocarcinoma and squamous cell carcinoma	28, 29, 34
mir-181a, mir-191, mir-107, mir-103, let-7b	Distinguish between adenocarcinoma and squamous cell carcinoma	30
mir-205, mir-31	Distinguish between adenocarcinoma and squamous cell carcinoma	33

Table 2

Examples of miRNAs as potential biomarkers for the early detection of lung cancer

MicroRNA	Functions	Reference
mir-17-3p, mir-21, mir-106a, mir-146, mir-155, mir-191, mir-192, mir-203, mir-205, mir-210, mir-212, and mir-214	Distinguish lung tumors from normal tissues	27
mir-21, mir-141 and mir-200c	Distinguish NSCLC from normal tissues	35
mir-9, mir-205 and mir-210	Distinguish NSCLC from normal tissues	36
mir-210, mir-708, and mir-205	Discriminate SCC patients from normal subjects	37
mir-486, mir-375, mir-200b	Distinguish AD patients from normal subjects	38
mir-21, mir-210, mir-126, and mir-486-5p	Distinguish NSCLC from healthy controls	39
mir-25 and mir-223	Diagnose early stage NSCLCs	14
mir-155, mir-197, mir-182	Discriminate lung cancer from controls	40
mir-210, mir-182, mir-486-5p, mir-30a, mir-140-3p	Distinguish SCC from normal lung tissues	41
mir-21, mir-141, mir-210 and mir-200b	Upregulate in lung tumor tissues compared to normal tissues	42
mir-346, mir-126, mir-126, mir-3a, mir-30d, mir-486, mir-129, mir-451, mir-521, mir-128, mir-30b, mir-30c, mir-516a, mir-520	Downregulate in lung tumor tissues compared to normal tissues	42
mir-210, mir-200c, mir-17-5p, mir-20a, mir-203, mir-200a, mir-106b, mir-93, mir-182, mir-83, mir-106a, mir-20b, and mir-224	Upregulate in lung SCC tissues compared to normal tissues	43
let-7e, mir-200a	Downregulate in lung SCC tissues compared to normal tissues	43
mir-146b, mir-221, let-7a, mir-155, mir-17-5p, mir-27a, mir-106a	Downregulate in serum NSCLC compared to normal controls	46
mir-29c	Upregulate in serum NSCLC compared to normal controls	46
mir-10b, mir-141 and mir-155	Upregulate in lung cancer patients than in benign tumor patients	47
mir-1254 and mir-574-5p	Upregulate in early-stage of NSCLC serum samples compared with control	48
mir-20a, mir-24, mir-25, mir-145, mir-152, mir-199a-5p, mir-221, mir-222, mir-223, mir-320	Early NSCLC diagnosis	49
mir-7-2-prec, mir-21, mir-200b, mir-210, mir-219-1,	Discriminate CT-detected lung cancer from normal lung tissue	51
mir-324-5p, mir-126, mir-451, mir-30a, mir-486-5p		
mir-17, mir-660, mir-92a, mir-106a, mir-19	Lung cancer diagnosis	52

Table 3

Examples of miRNAs as prognosis biomarkers for lung cancer

MicroRNA	Functions	Reference
pri-let-7a and pri-let-7f	Correlated to shorter overall survival in NSCLC	57
pri-let-7	Correlated to high lung cancer disease stage	57
mir-155, mir-17-3p, mir-106a, mir-93, mir-21	High expression were associated with unfavorable outcomes of AD patients	27
let-7a-2, let-7b, mir-145	Low expression were associated with unfavorable outcomes of AD patients	27
mir-31	Associated with poor survival in SCC	41
mir-374a	Associated with poor patient survival in early-stage NSCLC	36
mir-221, mir222	Correlated with aggressive NSCLC	58
mir-486-5p, mir-21, mir-126, mir-15b, mir-148a	Downregulated in plasma of patients with unfavorable prognosis	51
mir-142-3p, mir-17, mir-197, mir-221, mir-28-3p		
mir-106a		
let-7	Lower expression in cancer tissues than in normal lung tissues	59
mir-34b	Methylation can be used as a biomarker for an invasive Phenotype of lung cancer	60
mir-328, mir-330-3p	Classify BM-positive vs. BM-negative NSCLC patients	61
mir-16	prognostic marker in NSCLC	62
mir-221, let-7a, mir-137, mir-372, and mir-182	Correlated with favorable prognosis	63
let-7e, mir-34a, mir-34c-5p, mir-25, mir-191	Low expressions were correlated with poor survival of the NSCLC patients	30
mir-200b*, mir-30c-1*, mir-510, mir-630, mir-657,	Predict lung cancer recurrence	64
mir-146b-3p, mir-124*, mir-585, mir-708	Predict the prognosis of patients with SCC	65
miR-146b		
mir-21	High expression was associated with unfavorable prognosis	66, 70
mir-21	High serum level was significantly correlated with tumor-node metastases stage and lymph node metastasis of NSCLC patients	67
mir-126	A strong and independent negative prognostic factor in NSCLC	68
mir-145	A predictive biomarker for patients of lung adenocarcinoma	69
mir-486, mir-30d	High levels are correlated with unfavorable prognosis	72
mir-1, mir-499	Low levels are correlated with unfavorable prognosis	72