miRNAs as Non-Invasive Biomarkers for Lung Cancer Diagnosis

Paola Ulivi * and Wainer Zoli

Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori (IRST) IRCCS, Via Maroncelli 40, 47014 Meldola (FC), Italy

* Author to whom correspondence should be addressed; E-Mail: paola.ulivi@irst.emr.it; Tel.: +39-0543-739-277; Fax: +39-0543-739-921.

Received: 15 March 2014; in revised form: 10 June 2014 / Accepted: 11 June 2014 / Published: 17 June 2014

Abstract: Lung cancer is a leading cause of cancer death and late diagnosis is one of the most important reasons for the high mortality rate. Circulating microRNAs (miRNAs) represent stable and reproducible markers for numerous solid tumors, including lung cancer, and have been hypothesized as non-invasive diagnostic markers. Serum, plasma or whole peripheral blood can be used as starting material, and several methodological approaches have been proposed to evaluate miRNA expression. The present review provides an in depth summary of current knowledge on circulating miRNAs in different types of biological samples used as diagnostic markers of lung cancer. We also evaluate the diagnostic accuracy of each miRNA or group of miRNAs in relation to the different housekeeping miRNAs used. Finally, the limitations and potential of miRNA analysis are discussed.

Keywords: miRNAs; NSCLC; diagnosis; non-invasive biomarker

1. Introduction

Lung cancer represents the most common cancer worldwide, with a high mortality rate due mainly to the fact that the disease generally only becomes clinically apparent at advanced stages [1,2]. More than 75% of lung cancers are diagnosed when the disease is locally advanced or metastatic, which results in a current 5-year survival of less than 15% [1]. Several screening strategies have been proposed to reduce cancer mortality, e.g., results from the National Lung Cancer Screening Trial showed a 20% decrease in the mortality of heavy smokers who underwent annual low-dose
A non-invasive blood biomarker assay that could complement or even replace LDCT in a lung cancer screening program would have to have two potentially important advantages over other known methods, *i.e.*, it would have to be able to identify individuals at high risk of NSCLC who require further investigation with spiral CT, and it would also have to be capable of discriminating between neoplastic and non-neoplastic diseases in individuals with suspicious CT-detected nodules, thus reducing the need for serial CTs or invasive biopsies. Several circulating molecular markers have been proposed, e.g., free DNA [3,4], gene methylation [5,6] and multiple marker approaches [7–9]. More recently, microRNAs (miRNAs) present in body fluids have been proposed as stable and reproducible biomarkers [10,11]. The aim of this review is to provide an overview of the diagnostic potential of circulating miRNAs in lung cancer, and to evaluate the feasibility of their large-scale determination in a screening program or clinical setting.

2. miRNAs and Mechanisms of Release into the Bloodstream

miRNAs are endogenous 19–22 nucleotide-long non-coding RNA molecules that mediate post-transcriptional gene silencing by RNA degradation or the inhibition of translation initiation [12–14]. miRNAs concurrently target multiple genes and a single miRNA has the potential to regulate the activity of a molecular pathway, making these molecules attractive therapeutic targets to reverse cancer phenotypes or sensitize tumors to chemotherapy [15,16]. miRNAs are also capable of providing important information on the aggressiveness of the disease and of predicting response to specific treatment [17]. Alterations in miRNA expression have been observed in various solid tumors, including hepatocellular carcinoma [18] and lung [17], breast [19], pancreatic [20], gastric [21] and prostate cancer [22], and could potentially be used for cancer diagnosis, prognosis and response to treatment [23,24].

Previous studies have shown that miRNAs are detectable in the blood [11,25,26]. Three possible mechanisms have been hypothesized for miRNA release: energy-free passive leakage of cellular miRNAs from broken cells; active secretion of miRNAs in the form of microvesicle-free miRNAs in response to different stimuli; and active release of miRNAs through microvesicles following specific stimuli [10]. The passive release of miRNAs from broken cells occurs in conditions such as tissue damage, cell apoptosis, inflammation and metastasis, all common processes in cancer [27].

With regard to the active secretion of miRNAs through cell-derived membrane-bound microvesicles, miRNAs are carried mainly by specific exosomes, *i.e.*, 50–90-nm vesicles exocytically released by numerous cell types, and by membrane-bound particles up to 1 μm in size that are produced by the shedding of the plasma membrane [28]. Membrane-bound microvesicles have been detected in various body fluids such as serum, plasma, urine, bronchoalveolar fluid and saliva, and their release may occur under both physiological and pathological conditions [29,30]. Conversely, some studies have shown that miRNAs exist in vesicle-free form associated with protein or high-density lipoprotein complexes [31,32]. In particular, Arroyo et al. [32] reported that the vast majority (over 90%) of circulating miRNAs are present in ribonucleoprotein complexes in a non-membrane-bound form,
and that only a minority are associated with vesicles. Furthermore, Vickers et al. [31] observed that miRNAs in plasma are transported and delivered to the recipient cells by high density lipoproteins.

3. miRNA Stability

Unlike mRNA, miRNAs show high stability in different types of biological samples, i.e., formalin-fixed, paraffin-embedded clinical tissue, fresh snap-frozen material, plasma or serum, and saliva [11,26,33,34]. This high stability is due to their resistance to endogenous and exogenous RNase activity, extreme temperatures, extremes of pH (pH 1 or 13), extended storage in frozen conditions, and repeated freeze-thaw cycles [26,35]. Resistance to such arduous conditions has been attributed to the above-described encapsulation and association with protein complexes [31,32,36–38]. In fact, it has been demonstrated that synthetic miRNAs or purified plasma miRNAs spiked into human plasma are promptly degraded [26].

4. miRNAs as Circulating Biomarkers

Circulating miRNAs are found in healthy individuals, and some authors have reported that miRNA deregulation can occur under different physiological or pathological conditions. Bye et al. [39] demonstrated differences in circulating miRNAs in proportion to aerobic fitness level and hypothesized miR-210, miR-21 and miR-222 as potential biomarkers of cardiovascular health. Moreover, increased circulating levels of miR-1, miR-423 and miR-208 have been correlated with myocardial infarction [40], heart failure [41], and myocardial damage [42], respectively. A recent review analyzed the role of miRNA expression in different non-cancerous diseases, the authors reporting that only a subset of known/identified blood-based microRNA biomarkers have specificity for a particular disease. Levels of miR-1, miR-133a, miR-133b, miR-499, miR-21, miR-208a and miR-208b were found to be higher in the blood of individuals experiencing myocardial injury such as myocardial infarction, viral myocarditis or acute coronary syndrome with respect to healthy subjects. Moreover, 24 different microRNAs were also reported as biomarkers for hepatic injury, in particular miR-122. Numerous other miRNAs have also been found in different types of diseases with a low specificity [43].

Some studies have also revealed a correlation between serum miRNAs and different virus infections, e.g., five miRNAs (miR-197, miR-629, miR-363, miR-132 and miR-122) were shown to be capable of distinguishing varicella patients from healthy controls [44]. Furthermore, a combination of four miRNAs (miR-17, miR-20a, miR-106a and miR-376c) was able to identify patients with avian-origin influenza A (H7N9) virus [45].

Several studies have observed that circulating miRNA levels are higher in cancer patients than in healthy individuals. Lawrie et al. were the first to report that specific miRNAs were elevated in the serum of patients with large B-cell lymphoma with respect to healthy donors [25]. In particular, they found that three miRNAs, miR-155, miR-210 and miR-21, were significantly upregulated (5.24-, 4.15- and 2.56-fold expression change, respectively) in the serum of patients with respect to healthy donors. Chen et al. performed genome-wide expression profiling by Solexa sequencing, observing significant differences between miRNAs in serum and in the blood cells of cancer patients, but not in healthy individuals. This would seem to indicate that specific serum miRNAs probably derive from tumor cells
or tumor tissue [11]. Moreover, Mitchell et al.’s 2008 study on mouse xenograft models of prostate cancer revealed a clear correlation between the amount of miRNAs found in the blood and tumor growth [26]. These researchers used a mouse model to show that human miRNAs can be detected in the blood of mice after prostate cancer xenograft transplantation, and that the amount of miRNAs was correlated with the xenografted tumor mass.

Numerous miRNAs show the same pattern of alteration, i.e., increased or decreased expression, in the plasma/serum and tumor tissue of patients with various tumor types. For example, the expression of miR-17-5p has been found to be increased in both the serum [46] and tumor tissue [47,48] of lung cancer patients. An elevated level of miR-155 has also been reported in tumor tissue/cells [48,49] and plasma [25] of lymphoma patients. In some cases, however, an inverse correlation has been observed between specific circulating and tissue miRNAs. For example, although Coulouarn et al. found a lower level of liver-specific miR-122 in liver cancer tissue [50], an increase in the serum of patients with hepatocellular carcinoma (HCC) carrying the hepatitis B virus has also been reported [51]. These findings indicate that in some instances it might be more opportune to consider secreted miRNA rather than tissue/cellular miRNA in studies on miRNA expression. Overall, the correlation between tissue and circulating miRNAs supports the hypothesis that circulating miRNAs may reflect various aspects of human physiological status and serve as fingerprints for disease diagnosis [27].

5. Circulating miRNAs in NSCLC

The study by Chen et al. [11] represents the first comprehensive analysis of miRNAs in the serum of NSCLC patients. After miRNA profiling by Solexa sequencing, the authors identified two NSCLC-specific miRNAs, miR-25 and miR-223, which were more highly expressed in the serum of NSCLC patients than in healthy donors. In the wake of these findings, several other studies analyzed the potential diagnostic role of miRNAs in different types of biological samples, e.g., serum, plasma and sputum [4,11,52–77] (Table 1). Results were highly heterogeneous, even in studies using similar biological material. However, all of the works reported an alteration of circulating miRNA expression in cancer patients with respect to healthy donors or individuals with other non malignant lung diseases. Normalization of quantitative real-time polymerase chain reaction (qRT-PCR) data was performed in different ways, some authors using total RNA [11], others opting for serum volume [55], specific miRNAs e.g., RNU6B [56,58,65] or miR-16 [59,62,63,66,68], or miRNA ratio [4,67]. Unexpectedly, miRNAs used as normalizers in some studies proved to be promising biomarkers in others. Bianchi et al. identified miR-197 and miR-24 as among the most stable and least variable miRNAs in serum and used them as normalizers [53]. Conversely, other authors reported that miR-24 was upregulated in the serum of NSCLC patients with respect to healthy donors [55,59] and that miR-197 was upregulated in the plasma of NSCLC cases [61]. It can thus be concluded that there are several biases in the different methodological approaches that can only be resolved by method standardization and confirmatory studies. The majority of published works considered panels of miRNAs and a number of single miRNAs were consistently found to be deregulated. miR-21, one of the most frequently deregulated, was at least twofold upregulated in numerous studies [59,62,65,66,68,71]. Heterogeneous results were also obtained using whole peripheral blood as starting material (Table 1). Although various miRNA signatures were identified as markers capable of distinguishing between NSCLC patients and healthy
donors, different studies identified different promising miRNAs [72–78]. The most consistent results for miRNAs were obtained when sputum was used as starting material; in these studies, RNA U6 was frequently used as normalizer. In particular, miR-21 and miR-210 were found to be the most deregulated miRNAs in sputum of NSCLC patients [79–84] (Table 1).

6. Diagnostic Potential of Circulating miRNAs

The strong stability of miRNAs in biological samples suggests that they could potentially be used as diagnostic, prognostic and predictive biomarkers for different tumor types [24,85,86]. The availability of non-invasive biomarkers would be a valuable tool for screening programs and to monitor suspicious CT-detected nodules. Numerous studies have focused on identifying miRNAs that are capable of discriminating between NSCLC patients and healthy donors or individuals with non-neoplastic diseases, with sensitivity and specificity ranging from 60% to 100% (Table 1) [4,51,53,56,60–63,65–68,71,73–77,79–84,87]. In the majority of these studies, initial miRNA profiling was performed using “large spectrum” methodologies, e.g., chip array or low-density array. The most promising miRNAs were then validated with qRT-PCR methods and almost all studies identified a panel of miRNAs rather than a single miRNA that showed good diagnostic accuracy in discriminating between cancer patients and healthy individuals (Table 1).

One of the first studies to focus on the diagnostic accuracy of miRNA expression in serum was carried out by Bianchi et al. [53] who evaluated a panel of 361 miRNAs in patients from the COSMOS trial [88]. The authors showed that a 34-miRNA signature was capable of discriminating between healthy individuals and patients with adenocarcinoma (ADC) or squamous cell carcinoma (SCC). In particular, an AUC ROC value of 0.89, with 71% sensitivity and 90% specificity, was obtained in the testing set in which 30 healthy donors were compared with 22 ADC and 12 SCC patients. Moreover, the diagnostic accuracy increased and reached an AUC ROC of 0.94 when a comparison was made between healthy donors and only SCC patients. Using a more restricted panel of 10 miRNAs, Chen et al. [55] obtained higher diagnostic accuracy. In their validating set comprising 110 healthy donors and 200 NSCLC patients, they obtained an AUC ROC value of 0.972. Similar results were obtained by Hennessey et al. [52] using only two miRNAs (miR-15b and miR-27b). They began their study by evaluating 328 miRNAs in a group of 20 healthy donors and 16 NSCLC patients, but subsequently restricted the analysis to 26 miRNAs in 75 healthy donors and 55 NSCLC cases. Although different miRNA pairs showed good diagnostic accuracy, the best pair was miR-15b and miR-27b, which showed absolute specificity and 84% sensitivity. Numerous other studies have also analyzed the diagnostic potential of miRNAs in plasma (Table 1). Recently, Sozzi et al. hypothesized the potential for using miRNAs in screening programs [4]. The authors considered patients enrolled onto the MILD clinical trial [89] and identified a miRNA signature classifier (MSC) which, when used together with low dose computed tomography (LDCT), increased screening sensitivity to 98%, with a false-positive rate of only 35%. Furthermore, the false-positive rate of LDCT was reduced more than fivefold when patients double-positive for MSC and LDCT were taken into consideration.
Table 1. Studies on circulating miRNAs as biomarkers for lung cancer.

| Ref. | Case Series | Biological Material | Methodologies | Normalization | Promising miRNAs | Diagnostic Potential |
|------|-------------|---------------------|---------------|---------------|------------------|----------------------|
| [11] | 75 HD 152 NSCLC | Serum | Solexa deep sequencing/qRT-PCR | Total RNA | miR-25, miR-223 | - |
| [52] | 75 HD 55 NSCLC | Serum | qRT-PCR | - | miR-15b, miR-27b pair | Sens = 100% Spec = 84% PPV = 82% NPV = 100% AUC = 0.98 |
| [53] | 39 HD 25 ADC (Training set) | Serum | qRT-PCR | miR-197, miR-19b, miR-24, miR-146, miR-15b, miR-19a | Panel of 34 miRNAs | Sens = 69% Spec = 84% AUC = 0.92 |
| [54] | 30 HD 34 NSCLC (Testing set) | Serum | qRT-PCR | - | miR-16, miR-518a-5p, miR-574-5p, miR-593, miR-663, miR-718, miR-1228, miR-1972, miR-2114 | Sens = 71% Spec = 90% AUC = 0.89 |
| [55] | 6 HD 8 NSCLC | Serum | qRT-PCR | Serum volume | miR-222, miR-199a-5p, miR-320, miR-20a, miR-24, miR-223, miR-25, miR-152, miR-145, miR-221 | AUC = 0.97 |
| [56] | 110 HD 200 NSCLC | Serum | qRT-PCR | RNU6B, miR-39 | miR-1254, miR-574-5p | Sens = 82% Spec = 77% AUC = 0.77 |
| [56] | 11 HD 11 NSCLC (Training set) | Serum | qRT-PCR | - | miR-1254, miR-574-5p | Sens = 73% Spec = 71% AUC = 0.75 |
| [56] | 31 HD 22 NSCLC (Validating set) | Serum | qRT-PCR | - | miR-1254, miR-574-5p | Sens = 73% Spec = 71% AUC = 0.75 |
Table 1. Cont.

| Ref. | Case Series | Biological Material | Methodologies | Normalization | Promising miRNAs | Diagnostic Potential |
|------|-------------|---------------------|---------------|---------------|-------------------|----------------------|
| [57] | 40 HD 40 NSCLC (ADC) | Serum | qRT-PCR | - | miR-30c, miR-616, miR-146b-3p, miR-566, miR-550, miR-939, miR-339-5p, miR-656 | AUC of different miRNAs: miR-30c = 0.74, miR-616 = 0.81, miR-146b-3p = 0.71, miR-566 = 0.79, miR-550 = 0.72, miR-939 = 0.82, miR-339-5p = 0.60, miR-656 = 0.60 |
| [58] | 30 HD 20 benign diseases 97 NSCLC | Serum | qRT-PCR | RNU6B, miR-1233 | miR-361-3p, miR-625 | AUC miR-361-3p = 0.86, AUC miR-625 = 0.77 |
| [59] | 50 HD 82 NSCLC pre-surgery 10 NSCLC post surgery | Serum | qRT-PCR | miR-16 | miR-21, miR-205, miR-30d, miR-24 | AUC miR-21 = 0.70, AUC miR-205 = 0.81, AUC miR-30d = 0.76, AUC miR-24 = 0.86 |
| [60] | 110 HD 193 NSCLC | Serum | qRT-PCR | - | miR-125b | Sens = 78% Spec = 66% AUC = 0.79 |
| [61] | 68 HD 74 lung cancer | Plasma | qRT-PCR | - | miR-155, miR-197, miR-182 | Sens = 81.3% Spec = 86.8% AUC = 0.90 |
| [63] | 29 HD 58 NSCLC | Plasma | qRT-PCR | miR-16 | miR-21, miR-126, miR-210, miR-486-5p | Sens = 86.2% Spec = 96.6% AUC = 0.93 |
| [64] | 48 HD 78 NSCLC | Plasma vesicles | qRT-PCR | miR-142-3p, miR-30b | let-7f, miR-20b, miR-30e-3p | - |
| [62] | 80 BSN 76 MSN | Plasma | qRT-PCR | miR-16 | miR-21, miR-210, miR-486-5p | Sens = 76.3% Spec = 85% AUC = 0.86 |
Table 1. Cont.

| Ref. | Case Series | Biological Material | Methodologies | Normalization | Promising miRNAs | Diagnostic Potential |
|------|-------------|---------------------|---------------|---------------|------------------|---------------------|
| [65] | 60 HD 62 NSCLC (Training set) | Plasma | qRT-PCR | RNU6B | miR-21, miR-145, miR-155 | Sens = 69.4% Spec = 78.3% AUC = 0.85 |
|      | 32 HD 34 NSCLC (Validation set) |          |          |          |                  | Sens = 76.5% Spec = 81.3% AUC = 0.87 |
| [66] | 46 HD 54 NSCLC | Plasma | qRT-PCR | miR-16 | miR-21, miR-486 | Sens = 87% Spec = 86.5% AUC = 0.90 |
|      |          | EBC    | qRT-PCR | miR-16 |                  | Sens = 60% Spec = 71.1% AUC = 0.68 |
| [67] | 10 HD 16 NSCLC | Plasma | qRT-PCR | miRNA ratio | 16-miRNA ratio | Sens = 75% Spec = 100% AUC = 0.88 |
| [68] | 30 HD 63 NSCLC | Plasma | qRT-PCR | miR-16 | miR-21 | Sens = 76.2% Spec = 70% AUC = 0.78 |
| [69] | 20 HD 62 NSCLC | Serum  | qRT-PCR | RNU6B | miR-126, miR-183 |                          |
| [70] | 220 HD 220 NSCLC | Serum and plasma | qRT-PCR | C. elegans miRs (cel-miR-54, cel-miR-238) | miR-146b, miR-221, let-7a, miR-155, miR-17-5p, miR-27a, miR-106a, miR-29c | AUC = 0.60 |
| [4]  | 870 disease-free individuals at screening 69 NSCLC | Plasma | qRT-PCR | miRNA ratio | miRNA signature classifier | Sens = 87% Spec = 81% |
| [71] | 38 HD 36 NSCLC | Plasma | qRT-PCR and digital PCR | - | miR-21-5p, miR-335-3p | Sens = 71.8% Spec = 80.6% AUC = 0.86 |
| [72] | 30 HD 35 NSCLC | Whole blood | qRT-PCR | RNU6B | let-7a | AUC = 0.95 |
Table 1. Cont.

| Ref. | Case Series | Biological Material | Methodologies | Normalization | Promising miRNAs | Diagnostic Potential |
|------|-------------|---------------------|---------------|---------------|------------------|---------------------|
| [73] | 91 NMLD 137 NSCLC (Training set) | Whole blood | Array | - | 29-miRNA signature | Sens = 91%  
Spec = 80%  
AUC = 0.92 |
|      | 17 NMLD 38 NSCLC (Validation set) | Whole blood | Array | - | 24-miRNA signature | Sens = 92.5%  
Spec = 98.1% |
| [74] | 19 HD 17 NSCLC | Whole blood | Array | - | 250-miRNA signature | NSCLC vs COPD  
Sens = 92%  
Spec = 89% |
| [75] | 23 HD 22 NSCLC | Whole blood | Array and qRT-PCR | - | miR-190b, miR-630, miR-942, miR-1284 | TSP method:  
Sens = 91%  
Spec = 100%  
SVM method:  
Sens = 88%  
Spec = 89% |
| [76] | 24 HD 86 NSCLC | Whole blood | qRT-PCR | RNU38B, RNU58A | miR-328 | Sens = 70%  
Spec = 83%  
AUC = 0.82 |
| [77] | 26 HD 64 NSCLC | Whole blood | qRT-PCR | RNU6B | miR-143, miR-150 | AUC miR-143 = 0.89  
AUC miR-150 = 0.83 |
| [87] | 10 HD 10 granuloma 10 ADC | Exosome | qRT-PCR | let-7a | miR-378a, miR-379, miR-139-5p, miR-200b-5p (screening test)  
miR-151a-5p, miR-30a-3p, miR-200b-5p, miR-629, miR-100, miR154-3p (diagnostic test) | Screening test  
Sens = 97.5%  
Spec = 72%  
AUC = 0.91  
Diagnostic test  
Sens = 96%  
Spec = 60%  
AUC = 0.76 |
Table 1. Cont.

| Ref. | Case Series | Biological Material | Methodologies | Normalization | Promising miRNAs | Diagnostic Potential |
|------|-------------|---------------------|---------------|---------------|------------------|---------------------|
| [79] | 17 HD 23 NSCLC | Sputum | qRT-PCR | RNU6B | miR-21 | Sens = 69.7% Spec = 100% AUC = 0.90 |
| [80] | 36 HD 36 ADC (Training set) | Sputum | qRT-PCR | RNU6B | miR-21, miR-486, miR-375, miR-200b | Sens = 80.6% Spec = 91.7% AUC = 0.90 |
| [80] | 58 HD 64 ADC (Validation set) | Sputum | qRT-PCR | RNU6B | miR-21, miR-486, miR-375, miR-200b | Sens = 70.3% Spec = 80.0% AUC = 0.83 |
| [81] | 48 HD 48 SCC (Training set) | Sputum | qRT-PCR | RNU6B | miR-205, miR-210, miR-708 | Sens = 73% Spec = 96% AUC = 0.87 |
| [81] | 55 HD 67 SCC (Validation set) | Sputum | qRT-PCR | RNU6B | miR-205, miR-210, miR-708 | Sens = 72% Spec = 95% |
| [82] | 6 HD 24 NSCLC | Sputum | qRT-PCR | RNU6B | miR-21, miR-155, miR-210, miR-143, miR-372 | Sens = 83.3% Spec = 100% |
| [83] | 68 HD 66 NSCLC (Training set) | Sputum | qRT-PCR | RNU6B | miR-31, miR-210 | Sens = 65.2% Spec = 89.7% AUC = 0.83 |
| [84] | 73 HD 64 NSCLC (Validation set) | Sputum | qRT-PCR | RNU6B | miR-31, miR-210 | Sens = 64.1% Spec = 89.2% |
| [84] | 40 HD 35 NSCLC | Sputum | qRT-PCR | RNU6B | miR-31, miR-210 | Sens = 65.7% Spec = 85.00% AUC = 0.86 |

HD: healthy donor; NSCLC: non small cell lung cancer; BSN: benign solitary nodule; MSN: malignant solitary nodule; EBC: exhalate breath condensate; NMLD: non malignant lung disease; ADC: adenocarcinoma; COPD: chronic obstructive pulmonary disease; TSP: top scoring pairs; SVM: support vector machines; SCC: squamous cell carcinoma; qRT-PCR: qualitative real-time polymerase chain reaction; Sens: sensitivity; Spec: specificity.
Other studies have aimed to identify miRNAs in whole peripheral blood that are capable of distinguishing between NSCLC patients and healthy donors. This would not only permit miRNAs released by the tumor to be evaluated, but also those expressed by blood cells, which could reflect specific inflammatory or immune-modulatory processes that occur during carcinogenesis. One of the first studies in this area was conducted by Showe et al. [73] who identified a 29-miRNA signature capable of discriminating between 137 patients with NSCLC and 91 patients with non-malignant lung diseases. Sensitivity and specificity were 91% and 80% in the training set and 76% and 82% in the validating set, respectively. Subsequent studies identified other miRNA signatures showing high diagnostic accuracy [74,75].

Research has also focused on the diagnostic potential of miRNAs analyzed in sputum. As before, some studies initially evaluated a large panel of miRNAs by microarray and then validated the most promising miRNAs by RT-PCR [80,81]. Yu et al. [80] identified a panel of four miRNAs that discriminated between healthy donors and patients with ADC. They also demonstrated that three other miRNAs were capable of distinguishing healthy donors from patients with SCC [81]. More recently, the same group showed the high diagnostic accuracy of only two miRNAs in discriminating between healthy donors and patients with either ADC or SCC [82,83] (Table 1).

7. Conclusions and Future Prospects

Robustness and reproducibility are key requisites for the clinical implementation of biomarkers. Although qRT-PCR based multiplex assays for the detection of miRNAs are straightforward and robust, they are still hampered by a lack of agreement about the normalization approach and the use of an adequate internal control. There is still no general consensus in the literature regarding the best method to use and a standardized protocol would help to guarantee the reproducibility of results on different biological samples (blood, serum or plasma and sputum) obtained by non-invasive methods. Moreover, although the majority of studies conducted to date confirmed their own results through a training and validating set, almost all were based on small case series which reduced the diagnostic power of the biomarkers.

In conclusion, although the future of miRNAs as non-invasive diagnostic markers for the early detection of NSCLC looks promising, a number of important issues, i.e., protocol standardization, choice of normalization factors, and definition of the best biological samples to use for this type of analysis, need to be addressed.

Acknowledgments

The authors thank Prof. Rosella Silvestrini for her invaluable scientific contribution and Ursula Elbling for editing the manuscript.

Conflict of Interest

The authors declare no conflict of interest.
References

1. National Lung Screening Trial Research Team; Aberle, D.R.; Berg, C.D.; Black, W.C.; Church, T.R.; Fagerstrom, R.M.; Galen, B.; Gareen, I.F.; Gatsonis, C.; Goldin, J.; et al. The National Lung Screening Trial: Overview and Study Design. *Radiology* 2011, 258, 243–253.

2. Jemal, A.; Siegel, R.; Xu, J.; Ward, E. Cancer Statistics, 2010. *CA Cancer. J. Clin.* 2010, 60, 277–300.

3. Ulivi, P.; Silvestrini, R. Role of Quantitative and Qualitative Characteristics of Free Circulating DNA in the Management of Patients with Non-Small Cell Lung Cancer. *Cell. Oncol. (Dordr)* 2013, 36, 439–448.

4. Sozzi, G.; Boeri, M.; Rossi, M.; Verri, C.; Suatoni, P.; Bravi, F.; Roz, L.; Conte, D.; Grassi, M.; Sverzellati, N.; *et al.* Clinical Utility of a Plasma-Based miRNA Signature Classifier within Computed Tomography Lung Cancer Screening: A Correlative MILD Trial Study. *J. Clin. Oncol.* 2014, doi:10.1200/JCO.2013.50.4357.

5. Markopoulou, S.; Nikolaidis, G.; Liloglou, T. DNA Methylation Biomarkers in Biological Fluids for Early Detection of Respiratory Tract Cancer. *Clin. Chem. Lab. Med.* 2012, 50, 1723–1731.

6. Fleischhacker, M.; Dietrich, D.; Liebenberg, V.; Field, J.K.; Schmidt, B. The Role of DNA Methylation as Biomarkers in the Clinical Management of Lung Cancer. *Expert Rev. Respir. Med.* 2013, 7, 363–383.

7. Ulivi, P.; Mercatali, L.; Casoni, G.L.; Scarpi, E.; Bucchi, L.; Silvestrini, R.; Sanna, S.; Monteverde, M.; Amadori, D.; Poletti, V.; *et al.* Multiple Marker Detection in Peripheral Blood for NSCLC Diagnosis. *PLoS One* 2013, 8, e57401.

8. Quintans, J.S.; Antoniolli, A.R.; Onofre, F.M.; Onofre, A.S. Detection of Lung Cancer using Multiple Genetic Markers—A Systematic Review. *Diagn. Cytopathol.* 2013, 41, 834–842.

9. Andriani, F.; Conte, D.; Mastrangelo, T.; Leon, M.; Ratcliffe, C.; Roz, L.; Pelosi, G.; Goldstraw, P.; Sozzi, G.; Pastorino, U. Detecting Lung Cancer in Plasma with the use of Multiple Genetic Markers. *Int. J. Cancer* 2004, 108, 91–96.

10. Redova, M.; Sana, J.; Slaby, O. Circulating miRNAs as New Blood-Based Biomarkers for Solid Cancers. *Future Oncol.* 2013, 9, 387–402.

11. Chen, X.; Ba, Y.; Ma, L.; Cai, X.; Yin, Y.; Wang, K.; Guo, J.; Zhang, Y.; Chen, J.; Guo, X.; *et al.* Characterization of microRNAs in Serum: A Novel Class of Biomarkers for Diagnosis of Cancer and Other Diseases. *Cell Res.* 2008, 18, 997–1006.

12. Bartel, D.P. MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell* 2004, 116, 281–297.

13. Ambros, V. The Functions of Animal microRNAs. *Nature* 2004, 431, 350–355.

14. Krol, J.; Loedige, I.; Filipowicz, W. The Widespread Regulation of microRNA Biogenesis, Function and Decay. *Nat. Rev. Genet.* 2010, 11, 597–610.

15. Garzon, R.; Marcucci, G.; Croce, C.M. Targeting microRNAs in Cancer: Rationale, Strategies and Challenges. *Nat. Rev. Drug Discov.* 2010, 9, 775–789.

16. Kasinski, A.L.; Slack, F.J. Epigenetics and Genetics. MicroRNAs En Route to the Clinic: Progress in Validating and Targeting microRNAs for Cancer Therapy. *Nat. Rev. Cancer* 2011, 11, 849–864.
17. Lin, P.Y.; Yu, S.L.; Yang, P.C. MicroRNA in Lung Cancer. *Br. J. Cancer* **2010**, *103*, 1144–1148.
18. Murakami, Y.; Yasuda, T.; Saigo, K.; Urashima, T.; Toyoda, H.; Okanoue, T.; Shimotohno, K. Comprehensive Analysis of microRNA Expression Patterns in Hepatocellular Carcinoma and Non-Tumorous Tissues. *Oncogene* **2006**, *25*, 2537–2545.
19. Iorio, M.V.; Ferracini, M.; Liu, C.G.; Veronese, A.; Spizzo, R.; Sabbioni, S.; Magri, E.; Pedriali, M.; Fabbri, M.; Campiglio, M.; *et al.* MicroRNA Gene Expression Deregulation in Human Breast Cancer. *Cancer Res.* **2005**, *65*, 7065–7070.
20. Roldo, C.; Missiaglia, E.; Hagan, J.P.; Falconi, M.; Capelli, P.; Bersani, S.; Calin, G.A.; Volinia, S.; Liu, C.G.; Scarpa, A.; *et al.* MicroRNA Expression Abnormalities in Pancreatic Endocrine and Acinar Tumors are Associated with Distinctive Pathologic Features and Clinical Behavior. *J. Clin. Oncol.* **2006**, *24*, 4677–4684.
21. Chen, W.; Tang, Z.; Sun, Y.; Zhang, Y.; Wang, X.; Shen, Z.; Liu, F.; Qin, X. MiRNA Expression Profile in Primary Gastric Cancers and Paired Lymph Node Metastases Indicates that miR-10a Plays a Role in Metastasis from Primary Gastric Cancer to Lymph Nodes. *Exp. Ther. Med.* **2012**, *3*, 351–356.
22. Porkka, K.P.; Pfeiffer, M.J.; Waltering, K.K.; Vessella, R.L.; Tammela, T.L.; Visakorpi, T. MicroRNA Expression Profiling in Prostate Cancer. *Cancer Res.* **2007**, *67*, 6130–6135.
23. Wang, Q.; Wang, S.; Wang, H.; Li, P.; Ma, Z. MicroRNAs: Novel Biomarkers for Lung Cancer Diagnosis, Prediction and Treatment. *Exp. Biol. Med. (Maywood)* **2012**, *237*, 227–235.
24. Madhavan, D.; Cuk, K.; Burwinkel, B.; Yang, R. Cancer Diagnosis and Prognosis Decoded by Blood-Based Circulating microRNA Signatures. *Front. Genet.* **2013**, *4*, 116.
25. Lawrie, C.H.; Gal, S.; Dunlop, H.M.; Pushkaran, B.; Liggins, A.P.; Pulford, K.; Banham, A.H.; Pezzella, F.; Boulwood, J.; Wainscoat, J.S.; *et al.* Detection of Elevated Levels of Tumour-Associated microRNAs in Serum of Patients with Diffuse Large B-Cell Lymphoma. *Br. J. Haematol.* **2008**, *141*, 672–675.
26. Mitchell, P.S.; Parkin, R.K.; Kroh, E.M.; Fritz, B.R.; Wyman, S.K.; Pogosova-Agadjanyan, E.L.; Peterson, A.; Noteboom, J.; O’Briant, K.C.; Allen, A.; *et al.* Circulating microRNAs as Stable Blood-Based Markers for Cancer Detection. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 10513–10518.
27. Zen, K.; Zhang, C.Y. Circulating microRNAs: A Novel Class of Biomarkers to Diagnose and Monitor Human Cancers. *Med. Res. Rev.* **2012**, *32*, 326–348.
28. Fevrier, B.; Raposo, G. Exosomes: Endosomal-Derived Vesicles Shipping Extracellular Messages. *Curr. Opin. Cell Biol.* **2004**, *16*, 415–421.
29. Lodes, M.J.; Caraballo, M.; Suciu, D.; Munro, S.; Kumar, A.; Anderson, B. Detection of Cancer with Serum miRNAs on an Oligonucleotide Microarray. *PLoS One* **2009**, *4*, e6229.
30. Gutwein, P.; Stoeck, A.; Riedle, S.; Gast, D.; Runz, S.; Condon, T.P.; Marme, A.; Phong, M.C.; Linderkamp, O.; Skorokhod, A.; *et al.* Cleavage of L1 in Exosomes and Apoptotic Membrane Vesicles Released from Ovarian Carcinoma Cells. *Clin. Cancer Res.* **2005**, *11*, 2492–2501.
31. Vickers, K.C.; Palmisano, B.T.; Shoucri, B.M.; Shamburek, R.D.; Remaley, A.T. MicroRNAs are Transported in Plasma and Delivered to Recipient Cells by High-Density Lipoproteins. *Nat. Cell Biol.* **2011**, *13*, 423–433.
32. Arroyo, J.D.; Chevillet, J.R.; Kroh, E.M.; Ruf, I.K.; Pritchard, C.C.; Gibson, D.F.; Mitchell, P.S.; Bennett, C.F.; Pogosova-Agadjanyan, E.L.; Stirewalt, D.L.; et al. Argonaute2 Complexes Carry a Population of Circulating microRNAs Independent of Vesicles in Human Plasma. Proc. Natl. Acad. Sci. USA 2011, 108, 5003–5008.
33. Li, J.; Smyth, P.; Flavin, R.; Cahill, S.; Denning, K.; Aherne, S.; Guenther, S.M.; O’Leary, J.J.; Sheils, O. Comparison of miRNA Expression Patterns using Total RNA Extracted from Matched Samples of Formalin-Fixed Paraffin-Embedded (FFPE) Cells and Snap Frozen Cells. BMC Biotechnol. 2007, 7, 36.
34. Park, N.J.; Zhou, H.; Elashoff, D.; Henson, B.S.; Kastratovic, D.A.; Abemayor, E.; Wong, D.T. Salivary microRNA: Discovery, Characterization, and Clinical Utility for Oral Cancer Detection. Clin. Cancer Res. 2009, 15, 5473–5477.
35. Gilad, S.; Meiri, E.; Yoge, Y.; Benjamin, S.; Lebanony, D.; Yerushalmi, N.; Benjamin, H.; Kushnir, M.; Cholakh, H.; Melamed, N.; et al. Serum microRNAs are Promising Novel Biomarkers. PLoS One 2008, 3, e3148.
36. Valadi, H.; Ekstrom, K.; Bossios, A.; Sjostrand, M.; Lee, J.J.; Lotvall, J.O. Exosome-Mediated Transfer of mRNAs and microRNAs is a Novel Mechanism of Genetic Exchange between Cells. Nat. Cell Biol. 2007, 9, 654–659.
37. Collino, F.; Deregibus, M.C.; Bruno, S.; Sterpone, L.; Aghemo, G.; Viltono, L.; Tetta, C.; Camussi, G. Microvesicles Derived from Adult Human Bone Marrow and Tissue Specific Mesenchymal Stem Cells Shuttle Selected Pattern of miRNAs. PLoS One 2010, 5, e11803.
38. Zernecke, A.; Bidzhekov, K.; Noels, H.; Shagdarsuren, E.; Gan, L.; Denecke, B.; Hristov, M.; Koppel, T.; Jahantigh, M.N.; Lutgens, E.; et al. Delivery of microRNA-126 by Apoptotic Bodies Induces CXCL12-Dependent Vascular Protection. Sci. Signal. 2009, 2, ra81.
39. Bye, A.; Rosjo, H.; Aspenes, S.T.; Condorelli, G.; Omland, T.; Wisloff, U. Circulating microRNAs and Aerobic Fitness--the HUNT-Study. PLoS One 2013, 8, e57496.
40. Ai, J.; Zhang, R.; Li, Y.; Pu, J.; Lu, Y.; Jiao, J.; Li, K.; Yu, B.; Li, Z.; Wang, R.; et al. Circulating microRNA-1 as a Potential Novel Biomarker for Acute Myocardial Infarction. Biochem. Biophys. Res. Commun. 2010, 391, 73–77.
41. Tijssen, A.J.; Creemers, E.E.; Moerland, P.D.; de Windt, L.J.; van der Wal, A.C.; Kok, W.E.; Pinto, Y.M. MiR423–5p as a Circulating Biomarker for Heart Failure. Circ. Res. 2010, 106, 1035–1039.
42. Ji, X.; Takahashi, R.; Hiura, Y.; Hirokawa, G.; Fukushima, Y.; Iwai, N. Plasma miR-208 as a Biomarker of Myocardial Injury. Clin. Chem. 2009, 55, 1944–1949.
43. Haider, B.A.; Baras, A.S.; McCall, M.N.; Hertel, J.A.; Cornish, T.C.; Halushka, M.K. A Critical Evaluation of microRNA Biomarkers in Non-Neoplastic Disease. PLoS One 2014, 9, e89565.
44. Qi, Y.; Zhu, Z.; Shi, Z.; Ge, Y.; Zhao, K.; Zhou, M.; Cui, L. Dysregulated microRNA Expression in Serum of Non-Vaccinated Children with Varicella. Viruses 2014, 6, 1823–1836.
45. Zhu, Z.; Qi, Y.; Ge, A.; Zhu, Y.; Xu, K.; Ji, H.; Shi, Z.; Cui, L.; Zhou, M. Comprehensive Characterization of Serum MicroRNA Profile in Response to the Emerging Avian Influenza A (H7N9) Virus Infection in Humans. Viruses 2014, 6, 1525–1539.
46. Chen, Q.; Si, Q.; Xiao, S.; Xie, Q.; Lin, J.; Wang, C.; Chen, L.; Chen, Q.; Wang, L. Prognostic Significance of Serum miR-17-5p in Lung Cancer. *Med. Oncol.* 2013, 30, 353.

47. Volinia, S.; Calin, G.A.; Liu, C.G.; Ambs, S.; Cimmino, A.; Petrocca, F.; Visone, R.; Iorio, M.; Roldo, C.; Ferracin, M.; et al. A microRNA Expression Signature of Human Solid Tumors Defines Cancer Gene Targets. *Proc. Natl. Acad. Sci. USA* 2006, 103, 2257–2261.

48. Lu, J.; Getz, G.; Miska, E.A.; Alvarez-Saavedra, E.; Lamb, J.; Peck, D.; Sweet-Cordero, A.; Ebert, B.L.; Mak, R.H.; Ferrando, A.A.; et al. MicroRNA Expression Profiles Classify Human Cancers. *Nature* 2005, 435, 834–838.

49. Lowery, A.J.; Miller, N.; McNeill, R.E.; Kerin, M.J. MicroRNAs as Prognostic Indicators and Therapeutic Targets: Potential Effect on Breast Cancer Management. *Clin. Cancer Res.* 2008, 14, 360–365.

50. Coulouarn, C.; Factor, V.M.; Andersen, J.B.; Durkin, M.E.; Thorgeirsson, S.S. Loss of miR-122 Expression in Liver Cancer Correlates with Suppression of the Hepatic Phenotype and Gain of Metastatic Properties. *Oncogene* 2009, 28, 3526–3536.

51. Xu, J.; Wu, C.; Che, X.; Wang, L.; Yu, D.; Zhang, T.; Huang, L.; Li, H.; Tan, W.; Wang, C.; et al. Circulating microRNAs, miR-21, miR-122, and miR-223, in Patients with Hepatocellular Carcinoma Or Chronic Hepatitis. *Mol. Carcinog.* 2011, 50, 136–142.

52. Hennessey, P.T.; Sanford, T.; Choudhary, A.; Mydlarz, W.W.; Brown, D.; Adai, A.T.; Ochs, M.F.; Ahrendt, S.A.; Mambo, E.; Califano, J.A. Serum microRNA Biomarkers for Detection of Non-Small Cell Lung Cancer. *PLoS One* 2012, 7, e32307.

53. Bianchi, F.; Nicassio, F.; Marzi, M.; Belloni, E.; Dall’olio, V.; Bernard, L.; Pelosi, G.; Maisonneuve, P.; Veronesi, G.; di Fiore, P.P. A Serum Circulating miRNA Diagnostic Test to Identify Asymptomatic High-Risk Individuals with Early Stage Lung Cancer. *EMBO Mol. Med.* 2011, 3, 495–503.

54. Keller, A.; Leidinger, P.; Gislefoss, R.; Haugen, A.; Langseth, H.; Staehtler, P.; Lenhof, H.P.; Meese, E. Stable Serum miRNA Profiles as Potential Tool for Non-Invasive Lung Cancer Diagnosis. *RNA Biol.* 2011, 8, 506–516.

55. Chen, X.; Hu, Z.; Wang, W.; Ba, Y.; Ma, L.; Zhang, C.; Wang, C.; Ren, Z.; Zhao, Y.; Wu, S.; et al. Identification of Ten Serum microRNAs from a Genome-Wide Serum microRNA Expression Profile as Novel Noninvasive Biomarkers for Non-small Cell Lung Cancer Diagnosis. *Int. J. Cancer* 2012, 130, 1620–1628.

56. Foss, K.M.; Sima, C.; Ugolini, D.; Neri, M.; Allen, K.E.; Weiss, G.J. MiR-1254 and miR-574–5p: Serum-Based microRNA Biomarkers for Early-Stage Non-Small Cell Lung Cancer. *J. Thorac. Oncol.* 2011, 6, 482–488.

57. Rani, S.; Gately, K.; Crown, J.; O’Byrne, K.; O’Driscoll, L. Global Analysis of Serum microRNAs as Potential Biomarkers for Lung Adenocarcinoma. *Cancer. Biol. Ther.* 2013, 14, 1104–1112.

58. Roth, C.; Stuckrath, I.; Pantel, K.; Izbicki, J.R.; Tachay, M.; Schwarzenbach, H. Low Levels of Cell-Free Circulating miR-361–3p and miR-625* as Blood-Based Markers for Discriminating Malignant from Benign Lung Tumors. *PLoS One* 2012, 7, e38248.
59. Le, H.B.; Zhu, W.Y.; Chen, D.D.; He, J.Y.; Huang, Y.Y.; Liu, X.G.; Zhang, Y.K. Evaluation of Dynamic Change of Serum miR-21 and miR-24 in Pre- and Post-Operative Lung Carcinoma Patients. *Med. Oncol.* **2012**, *29*, 3190–3197.

60. Yuxia, M.; Zhennan, T.; Wei, Z. Circulating miR-125b is a Novel Biomarker for Screening Non-Small-Cell Lung Cancer and Predicts Poor Prognosis. *J. Cancer Res. Clin. Oncol.* **2012**, *138*, 2045–2050.

61. Zheng, D.; Haddadin, S.; Wang, Y.; Gu, L.Q.; Perry, M.C.; Freter, C.E.; Wang, M.X. Plasma microRNAs as Novel Biomarkers for Early Detection of Lung Cancer. *Int. J. Clin. Exp. Pathol.* **2011**, *4*, 575–586.

62. Shen, J.; Liu, Z.; Todd, N.W.; Zhang, H.; Liao, J.; Yu, L.; Guarnera, M.A.; Li, R.; Cai, L.; Zhan, M.; *et al.* Diagnosis of Lung Cancer in Individuals with Solitary Pulmonary Nodules by Plasma microRNA Biomarkers. *BMC Cancer* **2011**, *11*, 374.

63. Shen, J.; Todd, N.W.; Zhang, H.; Yu, L.; Lingxiao, X.; Mei, Y.; Guarnera, M.; Liao, J.; Chou, A.; Lu, C.L.; *et al.* Plasma microRNAs as Potential Biomarkers for Non-Small-Cell Lung Cancer. *Lab. Invest.* **2011**, *91*, 579–587.

64. Silva, J.; Garcia, V.; Zaballos, A.; Provencio, M.; Lombardia, L.; Almonacid, L.; Garcia, J.M.; Dominguez, G.; Pena, C.; Diaz, R.; *et al.* Vesicle-Related microRNAs in Plasma of Nonsmall Cell Lung Cancer Patients and Correlation with Survival. *Eur. Respir. J.* **2011**, *37*, 617–623.

65. Tang, D.; Shen, Y.; Wang, M.; Yang, R.; Wang, Z.; Sui, A.; Jiao, W.; Wang, Y. Identification of Plasma microRNAs as Novel Noninvasive Biomarkers for Early Detection of Lung Cancer. *Eur. J. Cancer Prev.* **2013**, *22*, 540–548.

66. Mozzoni, P.; Banda, I.; Goldoni, M.; Corradi, M.; Tiseo, M.; Acampa, O.; Balestra, V.; Ampollini, L.; Casalini, A.; Carbognani, P.; *et al.* Plasma and EBC microRNAs as Early Biomarkers of Non-Small-Cell Lung Cancer. *Biomarkers* **2013**, *18*, 679–686.

67. Boeri, M.; Verri, C.; Conte, D.; Roz, L.; Modena, P.; Facchinetti, F.; Calabro, E.; Croce, C.M.; Pastorino, U.; Sozzi, G. MicroRNA Signatures in Tissues and Plasma Predict Development and Prognosis of Computed Tomography Detected Lung Cancer. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 3713–3718.

68. Wei, J.; Gao, W.; Zhu, C.J.; Liu, Y.Q.; Mei, Z.; Cheng, T.; Shu, Y.Q. Identification of Plasma microRNA-21 as a Biomarker for Early Detection and Chemosensitivity of Non-Small Cell Lung Cancer. *Chin. J. Cancer* **2011**, *30*, 407–414.

69. Lin, Q.; Mao, W.; Shu, Y.; Lin, F.; Liu, S.; Shen, H.; Gao, W.; Li, S.; Shen, D. A Cluster of Specified microRNAs in Peripheral Blood as Biomarkers for Metastatic Non-Small-Cell Lung Cancer by Stem-Loop RT-PCR. *J. Cancer Res. Clin. Oncol.* **2012**, *138*, 85–93.

70. Heegaard, N.H.; Schetter, A.J.; Welsh, J.A.; Yoneda, M.; Bowman, E.D.; Harris, C.C. Circulating Micro-RNA Expression Profiles in Early Stage Nonsmall Cell Lung Cancer. *Int. J. Cancer* **2012**, *130*, 1378–1386.

71. Ma, J.; Li, N.; Guarnera, M.; Jiang, F. Quantification of Plasma miRNAs by Digital PCR for Cancer Diagnosis. *Biomark. Insights* **2013**, *8*, 127–136.

72. Jeong, H.C.; Kim, E.K.; Lee, J.H.; Lee, J.M.; Yoo, H.N.; Kim, J.K. Aberrant Expression of Let-7a miRNA in the Blood of Non-Small Cell Lung Cancer Patients. *Mol. Med. Rep.* **2011**, *4*, 383–387.
73. Showe, M.K.; Vachani, A.; Kossenkov, A.V.; Yousef, M.; Nichols, C.; Nikonova, E.V.; Chang, C.; Kucharczuk, J.; Tran, B.; Wakeam, E. et al. Gene Expression Profiles in Peripheral Blood Mononuclear Cells can Distinguish Patients with Non-Small Cell Lung Cancer from Patients with Nonmalignant Lung Disease. *Cancer Res.* **2009**, *69*, 9202–9210.

74. Keller, A.; Leidinger, P.; Borries, A.; Wendschlag, A.; Wucherpfennig, F.; Scheffler, M.; Huwer, H.; Lenhof, H.P.; Meese, E. MiRNAs in Lung Cancer—Studying Complex Fingerprints in Patient's Blood Cells by Microarray Experiments. *BMC Cancer* **2009**, *9*, 353.

75. Leidinger, P.; Keller, A.; Borries, A.; Huwer, H.; Rohling, M.; Huebers, J.; Lenhof, H.P.; Meese, E. Specific Peripheral miRNA Profiles for Distinguishing Lung Cancer from COPD. *Lung Cancer* **2011**, *74*, 41–47.

76. Patnaik, S.K.; Yendamuri, S.; Kannisto, E.; Kucharczuk, J.C.; Singhal, S.; Vachani, A. MicroRNA Expression Profiles of Whole Blood in Lung Adenocarcinoma. *PLoS One* **2012**, *7*, e46045.

77. Ulivi, P.; Foschi, G.; Mengozzi, M.; Scarpi, E.; Silvestrini, R.; Amadori, D.; Zoli, W. Peripheral Blood miR-328 Expression as a Potential Biomarker for the Early Diagnosis of NSCLC. *Int. J. Mol. Sci.* **2013**, *14*, 10332–10342.

78. Zeng, X.L.; Zhang, S.Y.; Zheng, J.F.; Yuan, H.; Wang, Y. Altered miR-143 and miR-150 Expressions in Peripheral Blood Mononuclear Cells for Diagnosis of Non-Small Cell Lung Cancer. *Cytotechnology* **2014**, *64*, 29–38.

79. Xie, Y.; Todd, N.W.; Liu, Z.; Zhan, M.; Fang, H.; Peng, H.; Alattar, M.; Deepak, J.; Stass, S.A.; Jiang, F. Early Detection of Squamous Cell Lung Cancer in Sputum by a Panel of microRNA Markers. *Thoracic Cancer* **2014**, *5*, 116–122.

80. Xing, L.; Todd, N.W.; Yu, L.; Fang, H.; Jiang, F. Analysis of MicroRNAs in Sputum to Improve Computed Tomography for Lung Cancer Diagnosis. *J. Thorac. Oncol.* **2014**, *9*, 33–40.

81. Li, N.; Ma, J.; Guarnera, M.A.; Fang, H.; Cai, L.; Stass, S.A.; Jiang, F. Digital PCR Quantification of Breast Cancer miRNAs in Sputum for Diagnosis of Non-Small Cell Lung Cancer. *J. Cancer Res. Clin. Oncol.* **2014**, *140*, 145–150.

82. Zandberga, E.; Kozirovskis, V.; Abols, A.; Andrejeva, D.; Purkalne, G.; Line, A. Cell-Free microRNAs as Diagnostic, Prognostic, and Predictive Biomarkers for Lung Cancer. *Genes Chromosomes Cancer* **2013**, *52*, 356–369.

83. Brase, J.C.; Wuttig, D.; Kuner, R.; Sultmann, H. Serum microRNAs as Non-Invasive Biomarkers for Cancer. *Mol. Cancer* **2010**, *9*, 306.
87. Cazzoli, R.; Buttitta, F.; di Nicola, M.; Malatesta, S.; Marchetti, A.; Rom, W.N.; Pass, H.I. MicroRNAs Derived from Circulating Exosomes as Noninvasive Biomarkers for Screening and Diagnosing Lung Cancer. *J. Thorac. Oncol.* **2013**, *8*, 1156–1162.

88. Veronesi, G.; Bellomi, M.; Mulshine, J.L.; Pelosi, G.; Scanagatta, P.; Paganelli, G.; Maisonneuve, P.; Preda, L.; Leo, F.; Bertolotti, R.; *et al.* Lung Cancer Screening with Low-Dose Computed Tomography: A Non-Invasive Diagnostic Protocol for Baseline Lung Nodules. *Lung Cancer* **2008**, *61*, 340–349.

89. Pastorino, U.; Rossi, M.; Rosato, V.; Marchiano, A.; Sverzellati, N.; Morosi, C.; Fabbri, A.; Galeone, C.; Negri, E.; Sozzi, G.; *et al.* Annual Or Biennial CT Screening Versus Observation in Heavy Smokers: 5-Year Results of the MILD Trial. *Eur. J. Cancer Prev.* **2012**, *21*, 308–315.

© 2014 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution license (http://creativecommons.org/licenses/by/3.0/).