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TOPIC HIGHLIGHT

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MicroRNAs as lung cancer biomarkers

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Abstract

Lung cancer is the leading cause of cancer mortality worldwide. Its high mortality is due to the poor prognosis of the disease caused by a late disease presentation, tumor heterogeneities within histological subtypes, and the relatively limited understanding of tumor biology. Importantly, lung cancer histological subgroups respond differently to some chemotherapeutic substances and side effects of some therapies appear to vary between subgroups. Biomarkers able to stratify for the subtype of lung cancer, prognosticate the course of disease, or predict the response to treatment are in high demand. In the last decade, microRNAs (miRNAs), measured in resected tumor samples or in fine needle aspirate samples have emerged as biomarkers for tumor diagnosis, prognosis and prediction of response to treatment, due to the ease of their detection and in their extreme specificity. Moreover, miRNAs present in sputum, in plasma, in serum or in whole blood have increasingly been explored in the last five years as less invasive biomarkers for the early detection of cancers. In this review we cover the increasing amounts of data that have accumulated in the last ten years on the use of miRNAs as lung cancer biomarkers.

Key words: Lung cancer; Non-small cell lung carcinomas; MicroRNAs; Biomarkers

Core tip: Biomarkers able to stratify for the subtype of lung cancer, prognosticate the course of disease, or predict the response to treatment are in high demand. In the last decade, microRNAs (miRNAs), measured in resected tumor samples have emerged as biomarkers, due to the ease of their detection and in their extreme specificity. Moreover, miRNAs present in sputum, in plasma, in serum or in whole blood have increasingly been explored in the last five years as less invasive biomarkers for the early detection of cancers.

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INTRODUCTION

Lung cancer is the leading cause of cancer mortality worldwide[1]. About 270000 individuals were predicted to die of lung cancer in the European Union in 2013[2]. This high mortality is due to the poor prognosis of the disease caused by a late disease presentation, tumour heterogeneities within histological subtypes, and the relatively limited understanding of tumour biology. Most lung cancer patients are diagnosed at an advanced stage of disease, and, although a small subsets of these patients can be treated with new drugs offering improved survival and reasonable quality of life, the majority of patients can only be treated with palliative chemotherapy. Overall survival remains poor, and many patients die within a few months

of diagnosis.

Lung cancer is not one, but rather a group of diseases: small cell lung carcinomas (SCLCs) are high grade neuroendocrine tumours (NET), metastasize earlier and are initially more chemosensitive than the so called non-small cell lung carcinomas (NSCLC). Over the past two decades it has become clear that NSCLC itself is a clinically and biologically heterogeneous group of lung cancers, and should not be treated as a single disease entity. The two main subgroups of NSCLC are adenocarcinoma (AD) and squamous cell carcinoma (SCC), with a remaining third class of carcinomas devoid of histological features of adeno- or squamous- differentiation, named LCC (large cell carcinoma)[3]. The appearance of these tumours at light microscopy differs substantially, suggesting that their aetiology and biology differ as well. Importantly, the histological subgroups of NSCLC respond differently to some chemotherapeutic substances[4-7] and side effects of some therapies appear to vary between subgroups[8].

Beside SCLC, NETs include a spectrum of tumors from the low-grade typical carcinoid (TC) and intermediate-grade atypical carcinoid (AC) to the high-grade large-cell NE carcinoma (LCNEC). The distinction between these various entities may be sometimes difficult on histological grounds, but is of great therapeutic relevance.

Biomarkers able to stratify for the subtype of lung cancer, prognosticate the course of disease, or predict the response to treatment are in increasing demand. Lung cancer subtyping has traditionally relied on the histopathological observation of resected specimens, bronchoscopic biopsies, fine needle aspirations or sputum, which represent samples with decreasing invasiveness for the patient, but also of increasing challenge for the pathologist, as proportionally fewer tumour cells are captured[9-11]. Recently, the introduction of several immunohistochemical markers has rendered lung cancer subtyping more accurate and clinically useful[12].

In the last decade, microRNAs (miRNAs), measured either from tumour samples or in biofluids, have emerged as biomarkers for tumor diagnosis, prognosis and prediction of response to treatment. In the following pages we will review the increasing amounts of data that have accumulated in the last ten years on the use of miRNAs as lung cancer biomarkers.

MIRNAS AND CANCER

microRNAs (miRNAs or miRs) are small non-coding single-stranded RNAs, 19-25 nucleotides (nt) in length, acting as negative regulators of gene expression at the post-transcriptional level. More than 1000 miRNAs are transcribed from miRNA genes in the human genome. A single miRNA is able to modulate hundreds of downstream genes by recognizing complementary sequences in the 3'UTRs of their target mRNAs. It has been estimated that in humans about 30% of messenger RNAs (mRNAs) are under miRNA regulation, but this percentage is likely to grow in the future, as studies have shown that miRNAs can also bind target sequences located in the 5' UTR or in the open reading frame (ORF)[13]. The biological functions of miRNAs are diverse and include several key cellular processes, such as differentiation, proliferation, cellular development, cell death and metabolism.

In a seminal paper the Croce laboratory showed in 2002 that the genes for miR-15 and miR-16 are deleted or down-regulated in the majority of chronic lymphocytic leukaemia cases[14]. Scott Hammond, Gregory Hannon and collaborators demonstrated that miRNAs can modulate tumour formation and implicated the mir-17-92 cluster as a potential human oncogene in another fundamental paper[15], in which they demonstrated that enforced expression of the mir-17-92 cluster acts with c-myc expression to accelerate tumour development in a mouse B-cell lymphoma model. Since then, evidences have accumulated to indicate that miRNAs play a role in the onset and progression of several human cancers[16]. The transcription or processing of some miRNAs is altered in neoplastic tissues, in respect to their normal counterparts. miRNAs whose levels increase in tumors are referred to as oncogenic miRNAs ("onco-miRs"), sometimes even if there is no evidence for their causative role in tumorigenesis. On the other hand, miRNAs down-regulated in cancer are considered tumor suppressors. From the mechanistic point of view, it is important to understand how these variations may contribute to tumor progression.

In 2005, the Horvitz and Golub labs demonstrated the potential for miRNAs as diagnostic tumor markers, when they were able to indicate the tumor embryonic origin using miRNA expression profiles, successfully classifying poorly differentiated tumors, among which lung tumors[17]. Subsequently, the Croce lab performed a large-scale miRNome analysis on 540 samples including lung, breast, stomach, prostate, colon, and pancreatic tumors, and identified a solid cancer miRNA signature composed by a large portion of over-expressed miRNAs. While some miRNAs were commonly dys-regulated in the six cancer types, several other miRNAs were associated to a particular type of cancer[18]. The utility of miRNAs levels as diagnostic and prognostic biomarkers became clear already from these first studies[19]. Moreover, the effectiveness of miRNAs as biomarkers for tracing the tissue of origin of cancers of unknown primary origin was demonstrated by Rosenfeld and colleagues[20], who constructed a tissue classifier based on the measurement of 48 miRNAs on a microarray, to identify the tissue origin of metastatic tumors. These results were translated into a qRT-PCR platform, to develop a diagnostic test for the identification of tumour tissue origin[21]. The classifier has been further implemented in a second-generation custom microarray based on the measurement of 64 miRNAs[22] the uselfuness of which as a diagnostic tool was very recently confirmed[23].

A different 47-miRNA signature for the identification

of cancers with unknown primary tissue-of-origin was identified by other authors using a different microarray platform[24].

MIRNAS AND LUNG CANCER

As far as lung cancers are concerned, the role of miRNAs in lung carcinogenesis was indicated as early as 2004, when the Croce lab demonstrated that more than half of the miRNA genes then known were located in cancer-associated genomic regions or in fragile sites and that several miRNAs located in this deleted regions have low expression levels in lung cancer cell lines as well as in chronic lymphocytic leukaemia samples[25]. In the same year, Takamizawa and colleagues reported reduced expression of the let-7 microRNA in human NSCLC lung cancers[26], followed by several independent studies[27-29]. The let-7 family was later shown to have an onco-suppressor activity in NSCLC tumor development in mice xenografts[30]. One of the consequences of let-7a downregulation in lung cancer has been demonstrated to be the upregulation of RAS protein[27]. A single nucleotide polymorphism (SNP) in a let-7 complementary site of KRAS mRNA was found to be associated with increased risk of NSCLC in moderate smokers[31]. Based on *in vitro* experiments and analyses of patient samples the authors concluded that this SNP alters the ability of let-7 to regulate translation of KRAS, leading to overexpression of KRAS and increased lung cancer risk.

Other miRNAs may also interact with RAS. For instance, Wang *et al*[32] found that miR-451 is downregulated in NSCLC, and that low expression correlated with poor survival. The authors were able to show that miR-451 inhibits the expression of ras-related protein 14 (RAB14), suggesting that lower expression of miR-451 may allow this oncogene to escape regulation.

The oncogenic miR-17-92 cluster is markedly overexpressed in lung cancers, especially with SCLC histology and enhances cell proliferation *in vitro*, therefore possibly playing a role in the development of lung cancers[33]. On the other hand, deletion of the miR-17-92 cluster, in mice, is lethal and causes lung and lymphoid cell developmental defects[34].

The tumor suppressor protein p53 is mutated in a large number of lung cancer cell lines and tumour specimens from patients with lung cancer[35,36]. There is growing evidence that p53 regulates the expression of several miRNAs[37-42]. p53 directly regulates the expression of miR-34 family members, and the upregulation of these miRNAs result in the downregulation of genes associated with cell cycle control[37] and promotion of apoptosis[40] in cultured lung cancer cells. Further miRNAs, including miR-125a, have more recently also been linked to p53-regulated apoptosis in lung cancer cells[41].

METHODS FOR THE QUANTIFICATION OF MIRNAS

The main advantage of the use of miRNAs as biomarkers resides in the ease of their detection and in their extreme specificity. miRNAs are stable molecules well preserved in formalin fixed, paraffin embedded tissues (FFPE) as well as in fresh snap-frozen specimens, unlike larger RNA molecules as mRNAs[43].

A range of methods has been used for the isolation and profiling of miRNAs. Purification of total RNA is obtained either through several commercially available column filtration protocols, implemented to guarantee recovery of miRNAs, or via the extraction of RNA by variously named "Tri-reagents" (acid phenol in combination with guanidinium-thiocyanate and chloroform), also available from vendors. Given that the interest is focused on the quantification of specific miRNAs in different conditions, the method of choice should exclude any bias in the purification of miRNAs from the samples. Importantly, the Kim laboratory has recently reported that, differently from what everybody in the field has thought for decades, in Tri-reagents-based RNA purification protocols, short structured miRNAs with low GC content are lost when a small number of cells are used[44]. The finding raises warning flags about comparisons of miRNA levels between populations of cells at different densities.

Sequencing-, microarray- and quantitative reverse-transcription polymerase chain reaction (qRT-PCR)-based methods are currently used for miRNA profiling.

Next generation sequencing (NGS)[45], providing accurate and sensitive miRNAs measurements, allows the identification of miRNAs differently expressed in tumor samples and matched healthy tissue. Furthermore, NGS enables scientists to discover novel miRNAs, as opposed to microarrays and qRT-PCR methods, which only can detect already known miRNAs. NGS techniques are rapidly evolving in power and multiplexing capacities, but they remain expensive and labor-consuming, both in sample preparation and data analysis.

The majority of miRNA profiling studies have been carried out so far using microarrays and have provided signatures consisting in few to several (5-30) distinct miRNAs[46]. With time, it has become clear in the field that the use of microarrays for miRNA profiling presents with major problems of cross-hybridisation between members of miRNA families and discrepancies in comparing results obtained with different microarray platforms[47-59]. A common strategy is to validate the microarray data by qRT-PCR, which warrantees high sensitivity and specificity.

Additionally, the analysis of a great number of genes including ones whose changes are not directly or indirectly associated with cancer, in clinical settings is not necessary. Hence, the large amount of data obtained by microarray and NGS profiling needs to be transposed into clinical trials by developing an easily performed and serviceable assay that can analyze the cancer-specific miRNAs for cancer diagnosis and prognosis. Such an analysis has been so far relying on qRT-PCR assays.

Several methods for the analysis of miRNAs by qRT-PCR have been devised[60] and companies are providing

multiwell plate-based qRT-PCR assays that promise to substitute microarrays in the high-throughput profiling of miRNAs. Additionally, qRT-PCR is the most easily performed and cost-effective technique and it is therefore the method of choice when it comes to measuring the levels of the restricted number of miRNA biomarkers in cancer samples, in the clinical setting.

qRT-PCR measures the relative levels of variable target miRNAs in comparison with one or more stably expressed reference genes (sometimes called housekeeping or endogenous control genes). This normalization is required to allow for variability in RNA quantity and quality and/or in the efficiency of cDNA synthesis. Despite miRNAs having been intensively studied in cancer research in the last years, suitable reference genes for relative quantification of miRNA levels in qRT-PCR assays have not been satisfyingly identified. Ribosomal RNAs (rRNAs) have been used as a reference RNA in miRNA studies[26,61]. However, concerns have been raised regarding the use of rRNAs in normalization, as they can be expressed at much higher levels than the target RNA, making it challenging to quantify an rRNA and a rare transcript in the same RNA dilution. Moreover, there is evidence of rRNA deregulation in apoptosis[62] and cancer[63]. The most commonly used reference RNA in miRNA qRT-PCR experiments is the small nuclear RNA U6 (U6 snRNA)[55,64]. However, using U6 snRNA to normalise miRNA levels is controversial because being much bigger in size (106-107 nt) it might differ from miRNAs with respect to efficiency of its extraction, reverse transcription and PCR amplification. U6 snRNA has been analysed as a reference gene for miRNA studies in several papers, and most of them came to the conclusion that the variance of U6 expression across tissues is high, therefore making U6 not a suitable reference gene for miRNA quantification[65-67]. Further reference genes commonly used used are snoRNAs but they too might be dys-regulated in cancer[68]. Several authors have suggested to use as reference a combination of miRNAs whose levels do not vary in the specific tumor tissue under investigation. For example, in fresh-frozen lung cancer samples, Peltier and Latham[65] suggest the use of a combination of miR-191 and miR-103.

The use of unvalidated and different reference genes makes it difficult to compare papers describing miRNA-expression cancer profiles and might represent one of the major reasons for the discrepancies in published studies regarding differentially expressed miRNAs in specific cancers.

A recent addition to the detection methods for miRNAs is droplet digital PCR (dPCR)[69], a method especially useful for low abundance miRNAs. In droplet dPCR, single cDNA molecules are partitioned evenly among hundreds of individual droplets in which they are amplified to generate binary calls. With this method an absolute readout of total DNA copy number can be obtained, avoiding the need for an endogenous reference gene.

MIRNAS FROM RESECTED SPECIMENS

MiRNAs as biomarkers for the diagnosis of lung cancer and for stratifying lung cancer subtypes

In 2005, the Horvitz and Golub labs used a bead-based flow cytometric miRNA expression method to identify complex profiles consisting of approximately a hundred of dys-regulated miRNAs, able to classify 11 different tumor types, among which lung tumors[17]. Subsequently, the Croce lab used a custom-made oligonucleotide miRNA microarray to compare lung carcinomas to normal tissue, and identified a group of three downregulated and 35 upregulated miRNAs. Among these, miR-21 was commonly up-regulated in the six cancer types analyzed (lung, breast, stomach, prostate, colon, and pancreatic tumors) and miR-17-5p, miR-128b, miR-155, miR-191 and miR-199a-1 were up-regulated in at least other two cancer types[18].

In 2006 Yanaihara *et al*[28] compared the miRNAs expression profiles in 104 pairs of lung cancer tissues and corresponding non-cancerous lung tissue, by the same custom-made oligonucleotide miRNA microarray used by Volinia and colleagues. They identified a unique profile made of 43 differently expressed miRNAs (Table 1) allowing the distinction of lung cancer from the non-cancerous lung tissue. Of the 15 upregulated and 28 downregulated miRNAs, miR-21 and miR-205 are located in a chromosomal region amplified and miR-32, miR-126-5p and miR-126-3p in a region deleted in lung cancers, respectively. The authors next validated the microarray results by a solution hybridization detection method and by qRT-PCR, confirming that miR-21 and miR-205 are frequently up-regulated and miR-126-5p is often downregulated in lung cancer tissues when compared with the corresponding noncancerous lung tissues.

Comparison analyses between ADC *vs* noncancerous tissues and SCC *vs* noncancerous tissues revealed 17 and 16 miRNAs with statistically different expression, respectively (Table 1). Six miRNAs (miR-21, miR-155, miR-191, mir-126-5p, miR-210 and miR-224) were shared in both histological types of NSCLC. Yanaihara and colleagues also directly compared the two most common histological types of NSCLC, identifying two miRNAs (miR-99b and miR-102) that were higher in ADC and 4 miRNAs (miR-202, miR-203, miR-205 and the precursor of miR-204) that were higher in SCC. However, the authors do not explore further the issue of distinguishing ADCs from SCCs, in this paper.

Decreased expression of miR-107, miR-185 and let7a, and the overexpression of miR-31a, was observed by qRT-PCR in lung cancer tissues and cell lines, compared to normal lung tissue[70]. Landi *et al*[71] reported 34 miRNAs that significantly differentiated SCCs from ADs in male smoker patients, of which 2 were downregulated and 32 upregulated in AD *vs* SCC. Raponi *et al*[72] used Ambion microarrays to profile total RNA from 61 SCC samples and 10 matched normal lung samples and identified 15 miRNAs that were differentially expressed

Table 1 MiRNAs from resected samples as biomarkers for the diagnosis of lung cancer

MiRNA	Scope	Sample	Ref.
mir-21, mir-191, mir-210, mir-155, mir-205, mir-24-2, mir-212, mir-214, mir-17-3p, mir-106a, mir-197, mir-192, mir-146, mir-203, mir-150, (UP) mir-126-5p, mir-143, mir-192-prec, mir-224, mir-126, mir-30a-5p, mir-140, mir-9, mir-124a-1, mir-218-2, mir-95, mir-145, mir-198, mir-216-prec, mir-219-1, mir-125a-prec, mir-26a-1-prec, mir-199b-prec, let-7a-2-prec, mir-27b, mir-32, mir-29b-2, mir-220, mir-33, mir-181c-prec, mir-101-1, mir-124a-3, mir-125a (DOWN)	Lung cancer *vs* normal	Solid (Not specified)	[28]
mir-21, mir-191, mir-155, mir-210, mir-24-2 (UP) mir-126-5p, mir-126-3p, mir-219-1, mir-95, mir-192-prec, mir-220, mir-216-prec, mir-204-prec, mir-188, mir-198, mir-145, mir-224 (DOWN)	ADs *vs* normal	Solid (Not specified)	[28]
mir-205, mir-191, mir-210, mir-17-3p, mir-203, mir-155, mir-21, mir-214, mir-212, mir-197 (UP) mir-224, mir-126*, mir-140, mir-29b, mir-143, mir-30a-5p (DOWN)	SCC *vs* normal	Solid (Not specified)	[28]
miR-31 (UP) miR-107, miR-185, let-7a (DOWN)	Lung cancer tissue *vs* normal	Solid (Not specified)	[70]
miR-26a, let-7g, let-7f, miR-98, miR-29a, let-7c, miR-30b, let-7i, let-7b, miR-29b, miR-26b, let-7a, miR-146b-5p, miR-195, miR-29c, miR-30d, miR-20a, miR-17, miR-19b, miR-106a, miR-16, let-7d, miR-106b, miR-181a, miR-498, miR-103, miR-107, miR-191, mir-663, miR-491-5p, let-7e, mir-654-5p (UP) miR-453, miR-509-3p (DOWN)	AD *vs* SCC; male smokers patients	Solid, formalin-fixed, paraffin-embedded	[71]
miR-17-5p, miR-20a, miR-20b, miR-93, miR-106a, miR-106b, miR-182, miR-183, miR-200a, miR-200c, miR-203, miR-210, miR-224 (UP) miR-125a, let7e (DOWN)	SCC *vs* normal	Solid, Snap-frozen	[72]
miR-30a, miR-140-3p, miR-182, miR-210, miR-486-5p	Stage I-III *vs* normal	Solid, Snap-frozen	[73]
miR-182, miR-200c, miR-141, miR-375, miR-7, miR-429, miR-200a, miR-370, miR-200b, miR-382 (UP) miR-126, miR-451, miR-195, miR-486-5p, miR-214, miR-199a-5p (DOWN)	Primary lung tumors *vs* metastases	Solid, formalin-fixed, paraffin-embedded	[74]
miR-205, miR-21 (relative expression)	AD *vs* SCC	Solid, formalin-fixed, paraffin-embedded	[75]
miR-21, miR-155 (UP)	LCNECs and SCLCs *vs* TCs and ACs	Solid, formalin-fixed, paraffin-embedded	[79]
miR-205, miR-27a, miR-29a, miR-29b, miR-34a (DOWN in NSCLC) miR-25, miR-375 (UP in NSCLC)	SCLC *vs* NSCLC	Solid, formalin-fixed, paraffin-embedded	[80]
miR-29a, miR-29b, miR-34a, miR-375 (DOWN in SQ) miR-205, miR-25, miR-27a (UP in SQ)	SCC *vs* AD	Solid, formalin-fixed, paraffin-embedded	[80]
miR-7, miR-21, miR-29b, miR-106a, miR-125a-5p, miR-129-3p, miR-205, miR-375 (relative expression)	Carcinoid, SCLC, and squamous and nonsquamous NSCLC	Solid, Fresh Biopsy	[81]
miR-21, miR-155, miR-7 (UP)	Tumor *vs* normal	Solid, fine-needle aspirate (FNA)	[82]
miR-21, miR-155 (UP)	NSCLC *vs* normal	Sputum	[96]
miR-205, miR-210, miR-708 (relative expression)	SCC *vs* normal	Sputum	[97]
miR-21, miR-200b, miR-375 and miR-486 (relative expression)	AD *vs* Normal	Sputum	[98]
miR-31, miR-210 (relative expression)	Stage I NSCLC *vs* normal	Sputum	[99]
miR-31, miR-210 (Relative Expression) + computed tomography	Stage I NSCLC *vs* normal	Sputum	[100]

AD: Adenocarcinoma; SCC: Squamous cell carcinoma; LCNEC: Large cell neuroendocrine carcinoma; SCLC: Small cell lung carcinoma; TC: Typical carcinoid; AC: Atypical carcinoid; NSCLC: Non-small cell lung cancer.

between normal lung and SCC (Table 1). Two of these miRNAs were down-regulated in SCCs (miR-125a and let7e) while the remaining 13 miRNAs were upregulated (miR-17-5p, miR-20a, miR-20b, miR-93, miR-106a, miR-106b, miR-182, miR-183, miR-200a, miR-200c, miR-203, miR-210, miR-224). More recently, a 5-miRNA classifier was identified by microarray analysis(miR-30a, miR-140-3p, miR-182, miR-210, miR-486-5p,) that could distinguish stage I-III SCC from normal lung tissues[73]. This classifier had an accuracy of 94,1% in a training cohort (34 patients) and 96,2% in a test cohort (26 patients).

A panel of 16 miRNAs has been reported to differentiate between primary lung tumors and metastases to the lung of various origin (Table 1)[74]. This miRNA profile was identified using microRNA microarray data generated from FFPE samples, and was confirmed by qRT-PCR. The panel includes miR-182, which was most strongly over-expressed in the lung primary tumors, and miR-126, which was over-expressed in the metastatic tumors.

Researchers have also aimed at finding one or few miRNAs that can be used as a convenient tool for lung cancers diagnosis. Lebanony *et al*[75] used a microarray to measure miRNA levels in AD and SCC FFPE samples, and verified their findings by qRT-PCR. They identified miR-205 as a highly specific marker for SCC, when combined with the measured miR21 levels. The finding was confirmed by other papers[76,77]. Moreover, an algorithm for accurate classification of NSCLC cases, diagnosed as LCC on purely morphologic grounds, was proposed by integrating immunohistochemical markers (Δnp63, DSC3, and napsin A) with miR-205 and miR-21 measurement[78].

Evaluating by qRT-PCR FPPE specimens from NETs, Lee *et al*[79] found that the levels of miR-21 and

miR-155 were significantly higher in high-grade NET carcinomas (LCNECs and SCLCs) than in carcinoid tumors (TCs and ACs).

Two microRNA panels yielded high diagnostic accuracy in discriminating SCLC from NSCLC (miR-29a and miR-375) and in differentiating SCC from AD (miR-205 and miR-34a) in FFPE surgical lung specimens[80]. Moreover, the same microRNA panels accurately differentiated SCLC from NSCLC and SCC from AD in bronchial brushing specimens.

Gilad *et al*[81] reported a single assay for the classification of the four main types of lung cancer (Table 1): carcinoid, SCLC, and squamous and nonsquamous NSCLC, based on the expression of eight miRNAs (miR-7, miR-21, miR-29b, miR-106a, miR-125a-5p, miR-129-3p, miR-205, miR-375) The assay was effective both on resected and on cytologic (fine-needle aspiration (FNA) and bronchial brushing and washing) lung cancer samples.

A recent study has also evaluated miRNAs in FNA NSCLC biopsies[82]. miR-21, miR-155, and miR-7 showed a higher level in tumoral FNA than in normal FNA specimens, while let7a showed a lower level. A direct comparison of FNAs with resected specimens from the same patients indicated that the measured miRNAs had the same trend in the two types of specimens.

MiRNAs as biomarkers for lung cancers prognosis

Reduced expression of the let-7 family has been correlated with poor postoperative survival in NSCLC[26]. In a later study, AD patients with high expression of either mir-155, mir-17-3p, mir-106a, mir-93, or mir-21 and low expression of either let-7a-2, let-7b, or mir-145 were found to have a significantly worse prognosis (Table 2)[28]. Overexpression of the precursor of miR-155 and reduced expression of let-7-a was especially predictive of poor survival.

Analyzing frozen resected specimens from NSCLC patients, Yu *et al*[83] identified a five-microRNA signature that can predict the survival and relapse of patients with lung cancer (Table 2). Two of these miRNAs (miR-221 and let-7a) were protective (*i.e.*, their down-regulation correlated with poor survival and high relapse probability), while the other three (miR-137, miR-182-3p, miR-372) were risky, and their up-regulation was predictive of poor survival and high relapse probability. The authors also demonstrated that miR-221, miR-137, miR-182-3p and miR-372 can alter the invasive ability of lung cancer cells in culture.

In the already described work by Raponi *et al*[72]. Twenty miRNAs were identified as having a significant association with overall survival in lung SCC patients (Table 2). Among these miRNAs, miR-146b alone was found to have the strongest prediction accuracy as the group with high miR-146b expression had significantly worse overall survival.

The p53-dependent miR-34 family was observed to be down-regulated in surgically resected NSCLC tumor samples compared with normal tissue, and low levels of miR-34a expression were correlated with a high probability of relapse[84]. MicroRNA expression profiles were also identified that may predict recurrence of localized stage I NSCLC after surgical resection[85]. These profiles included mir-124-5p, mir-146b-3p, mir-200b-5p, mir-30c-1-3p, mir-510, mir-585, mir-630, mir-657 and mir-708 (Table 2).

High miR-16 levels, measured by qRT-PCR in resected NSCLC samples, were reported as a prognostic factor for poor disease-free survival and poor overall survival[86]. Low miR-145 and high miR-367 are associated with shorter time to relapse (TTR) in resected NSCLC[87]. Noteworthy, p53 regulates miR-145 expression, which, in turn, inhibits the translation of SRY-related HMG box (SOX)2 and octamer-binding transcription factor (OCT)4. These transcription factors control the expression of the miR-302-367 cluster.

In a panel of 27 miRNAs which were observed by microarray analysis to be deregulated greater than two-fold in NSCLC resected samples compared to normal lung tissue, Gao *et al*[88] identified three miRNAs whose levels (confirmed by qRT-PCR) were related to clinicopathologic characteristics or patient prognosis: low levels of miR-143 were significantly correlated with smoking status, high miR-21 expression and low miR-181a expression were associated with poor survival.

The lower expression level of a 5-miRNA signature (miR-25, miR-34c-5p, miR-191, let-7e, and miR-34a) correlated with poor overall survival among SCC patients (Table 2)[71] and high expression of miR-31 was associated with poor survival in Chinese SCC patients[73].

Eight miRNAs were confirmed to be significantly differentially expressed in NSCLC FFPE samples from patients with brain metastases compared with patients without brain metastases (Table 2)[89]. In particular, in this work, the over-expression of miR-328 and miR-330-3p was indicated as a marker for patients at risk for brain metastases, and a role for miR-328 in conferring migratory potential to NSCLC cells was suggested.

MiRNAs as biomarkers to predict response to therapy in lung cancer

miRNAs have also been used as biomarkers predictive of patient's response to therapy. miR-21 expression was significantly increased in platinum-based chemotherapy-resistant NSCLC patients and increased miR-21 expression was associated with the shorter disease-free survival[90]. A single nucleotide polymorphism in *miR-196a-2* gene was reported to be associated with severe toxicity after platinum-based chemotherapy of advanced NSCLC patients in a Chinese population[91].

Recent therapeutic advances for the treatment of NCLSC include the use of epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) including gefitinib and erlotinib. MiRNA-128b was shown to directly regulate EGFR translation and miR-128b LOH was found to be frequent in NSCLC samples and cor-

Table 2 MiRNAs as biomarkers for the prognosis and to predict response to therapy

miRNA	Experiment	Scope	Sample	Ref.
let-7a family (DOWN)	lung cancer tissue *vs* normal	NSCLC poor postoperative survival	Solid, (not specified)	[26]
mir-155, mir-17-3p, mir-106a, mir-93, mir-21 (UP) let-7a-2, let-7b (DOWN)	AD *vs* SCC	AD poor survival	Solid, (not specified)	[28]
miR-221, let-7a (DOWN) miR-137, miR-182-3p, miR-372 (UP)	NSCLC *vs* normal	NSCLC poor survival	Solid, snap frozen	[83]
miR-146b, miR-191, miR-155, miR-15a, miR-511, miR-100, miR-10a, miR-21, miR-126 (UP) miR-206, miR-299-3p, miR-122a, miR-513, miR-184, miR-453, miR-379, miR-202, miR-494, miR-432, miR-370 (DOWN)	SCC *vs* normal	SCC Overall Survival	Solid, snap frozen	[72]
miR-34a (DOWN)	NSCLC *vs* normal	NSCLC Probability of relapse	Solid, (not specified)	[84]
mir-124-5p, mir-146b-3p, mir-200b-5p, mir-30c-1-3p, mir-510, mir-585, mir-630, mir-657, mir-708 (relative expression)	stage I NSCLC *vs* normal	Stage I NSCLC recurrence	Solid, formalin-fixed, paraffin-embedded (FFPE)	[64]
miR-16 (UP)	NSCLC *vs* normal	NSCLC poor survival	Solid, (not specified)	[86]
miR-143 (DOWN)	Lung cancer tissue *vs* normal	smoking status	Solid, snap frozen	[88]
miR-21 (UP) miR-181a (DOWN)	Lung cancer tissue *vs* normal	NSCLC poor survival	Solid, snap frozen	[88]
miR-25, miR-34c-5p, miR-191, let-7e, miR-34a (DOWN)	AD *vs* SCC	SCC survival	Solid, formalin-fixed, paraffin-embedded (FFPE)	[71]
miR-31 (UP)	SCC *vs* normal	SCC poor survival	Solid, snap frozen	[73]
miR-325, miR-326, miR-328, miR-329-2-pre, miR-330-3p, miR-500a-3p, miR-370, miR-650-pre (UP)	BM − NSCLC *vs* BM + NSCLC	NSCLC, risk for brain metastasis	Solid, formalin-fixed, paraffin-embedded (FFPE)	[89]
miR-21 (UP)		Platinum-based chemotherapy-resistant NSCLC patients	Solid, snap frozen	[90]
miR-196a-2 SNP	Treatment *vs* genotype	Severe toxicity after platinum-based chemotherapy of advanced NSCLC patients	Genomic DNA from peripheral leukocytes	[91]
miR-128b (UP)		NSCLC samples correlated with clinical response and survival following gefitinib treatment	Microdissected primary surgically resected NSCLC tumors	[92]
miR-30b, miR-30c		Regulated by EGFR and hepatocyte growth factor (MET) receptor tyrosine kinase	Solid, Lung tumor tissue samples	[93]
miR-30b, miR-30c		Prognostic predictors in NSCLC patients who underwent first line treatment with TKIs	Solid, formalin-fixed, paraffin-embedded (FFPE)	[94]

NSCLC: Non-small cell lung cancer; AD: Adenocarcinoma; SCC: Squamous cell carcinoma; BM: Brain metastasis.

related significantly with clinical response and survival following gefitinib treatment[92]. miR-30b and miR-30c expression levels, which are regulated by EGFR and hepatocyte growth factor (MET) receptor tyrosine kinase[93] have been reported to be prognostic predictors in NSCLC patients who underwent first line treatment with TKIs[94].

MIRNAS IN SPUTUM AS NON-INVASIVE LUNG CANCER BIOMARKERS

An early diagnosis of cancer remains a challenge and, in this context, it is important to find a sensitive, non-invasive tool to detect early neoplastic changes. One relatively non-invasive source of miRNAs for the diagnosis of lung cancers is sputum[95]. The Jiang laboratory demonstrated that endogenous miRNAs are stably present in sputum specimens. Using qRT-PCR, miR-21 and miR-155 were detected, of which miR-21 was significantly overexpressed in sputum of NSCLC patients as compared with cancer-free subjects[96]. Furthermore, elevated miR-21 expression was more sensitive (70%) than conventional sputum cytology (48%) in diagnosing lung cancer. The same research group defined miRNA signatures for different histologic types of lung cancer in studies of similar design[97,98]. For the diagnosis of SCC, the combination of miR-205, miR-210 and miR-708 yielded 73% sensitivity and 96% specificity. A panel consisting of miR-21, miR-200b, miR-375 and miR-486 produced 81% sensitivity and 92% specificity in discriminating sputum of AD patients from controls. The authors found no association between miRNA expression and stage of lung cancer, suggesting that the miRNA signatures can be used as a tool in the detection of early lung cancer. The same group recently reported that combined quantification of miR-31 and miR-210 copy number by digital PCR in sputum of the cases and controls provided 65.71 % sensitivity and 85.00 % specificity for stage I NSCLC diagnosis[99]. Very recently, the same authors also reported that combining miR-31 and miR-210 detection by qRT-

PCR and Computed Tomography they improved NSCLC diagnosis specificity[100].

In an independent study, a five-miRNA profile (miR-21, miR-143, miR-155, miR-210, miR-372) performed by qRT-PCR on sputum samples detected NSCLC with 83.3% sensitivity and 100% specificity[101].

CIRCULATING MIRNAS

First evidences

The finding that miRNAs have an exceptional stability in several tissues suggested that these tiny molecules were also preserved, detectable and quantifiable in the circulation and in other biofluids.

The first indication of circulating microRNAs with a potential as non-invasive diagnostic biomarkers for diffuse large B-cell lymphoma (DLBCL) and possibly other cancers was found by Lawrie *et al*[102]. These authors were the first to highlight that miRNAs could be reliably detected in serum and they demonstrated that high levels of miR-21, miR-210 and miR-155 could discriminate cancer patients with (DLBCL) from healthy individuals. Plasma miRNAs were observed to be present in a remarkably stable form that is protected from endogenous RNase activity[103]. These papers established the measurement of tumor-derived miRNAs in serum or plasma as an important approach for the blood-based detection of human cancer. Further studies demonstrated that microRNAs are also preserved and detectable in other biofluids such as urine, saliva, cerebro-spinal fluid and amniotic fluid[104] and their composition and concentrations are measurably different among these fluids[105].

On the origin of circulating miRNAs

miRNAs can be released in the circulation by two different pathways: energy-free passive release or active and selective secretion in response to different stimuli. The first process does not need energy, it occurs after cell breakage in pathological conditions such as tissue damage, apoptosis, metastasis or inflammation but it does not play a major role in the generation of circulating miRNAs[106]. miRNAs active secretion, differently from the passive leakage, is a process ATP- and temperature-dependent. It is similar to the release of hormones and cytokines, with or without cell stimulation[106].

Circulating miRNAs can be packaged in microvesicles (MVs) or apoptotic bodies (ABs) or can be found as microvesicle-free miRNAs, associated with various multiprotein or lipoprotein complexes.

MVs are small vesicles derived from cells, generally including microparticles (MPs) and exosomes. These two kinds of MVs have quite different vesicular structures: exosomes (50-90 nm), with an endocytic origin, are released by fusion of multivesicular bodies (MVBs) with the plasma membrane[107]. They have been identified in various body fluids such as blood, urine, malignant ascites, bronchoalveolar lavage fluid, synovial fluid, breast milk and saliva[108]. MPs, on the other hand, are larger than exosomes (> 100 nm diameter) and are shed from plasma membranes[109]. Almost all cell types can release MVs under normal physiological or pathological conditions.

Larger in size than MPs, ABs are generated in response to apoptotic stimuli[110] and implicated in tissue repair and angiogenesis.

It has been demonstrated that MVs and ABs are involved in the transport of circulating miRNAs[111-114]. Gibbings *et al*[115] showed that miRNAs loading into exosomes is not a random event, but it is mediated by proteins as Argonaute protein Ago2, a part of RNA-induced silencing complex.

On the other hand, several studies suggested that a significant fraction of extracellular miRNAs resides outside of vesicles and acts in exosome-independent manner, by its association with RNA-binding proteins including Ago2 and Nucleophosmin 1 (NPM1) or lipoprotein complexes such as high-density lipoprotein (HDL). Arroyo *et al*[116] showed that miRNAs in plasma are predominantly free of exosomes or microvesicles. They demonstrated an association of miRNAs with Ago2 protein, showing that this binding protects and increases the stability of released miRNAs[116]. Turchinovich *et al*[117] confirmed this hypothesis and demonstrated that also additional Argonaute proteins (Ago1, -3, -4) may be associated with cell-free circulating miRNAs. Wang *et al*[118] found that other RNA binding proteins, such as NPM1, can protect miRNAs from degradation, playing a role in the packaging and export of circulating miRNAs. Vickers *et al*[119] in 2011 revealed a potential new role for HDL in gene regulation and intercellular communication, showing that this lipoprotein transports and delivers miRNAs to recipient cells.

Circulating miRNAs as cancer biomarkers

It has been shown that miRNAs present in body fluids can reflect altered physiological conditions, representing new effective biomarkers[104].

A perfect biomarker should have some important features: non-invasivity, specificity, early detection, sensitivity and ease of translatability from model systems to humans.

Proteins used as blood biomarkers (*e.g.*, troponin for cardiovascular conditions, carcinoembryonic antigen (CAE) for various cancers, prostate specific antigen (PSA) for prostate cancer, and aminotransferases for liver function) do not respect all these criteria and are difficult to use in this field. On the contrary, secreted miRNAs have many of these requisites: they are stable in various biofluids, the expression of some miRNAs is specific to tissues or biological stages, and their level can be easily detected by various methods.

However, several challenges have to be overcome in order to successfully usew circulating miRNAs as cancer biomarkers. First, biofluids contain very low amount of RNA, and normal quantification methods are not suitable for these type of samples. Second, it is important

to avoid cellular contamination and hemolysis and third, biofluids contain inhibitors of reverse transcriptase and polymerase enzymes used for miRNAs quantification. All these factors are obstacles to consider when circulating miRNAs are isolated and quantified from biofluids such as plasma or serum. Another major challenge for the analysis of circulating miRNAs is the choice of an appropriate reference gene, since some of the small RNA species frequently used as reference genes (such as U6 RNA) are present in extremely low concentrations in serum and plasma as well as in other biofluids. Moreover, normalization controls used to remove variations and increase the accuracy of miRNAs quantification cannot ensure constant expression under all experimental conditions, underlining the importance of the selection of a proper reference gene.

Chen *et al*[120] reported that U6 and 5S rRNA are degraded in serum samples from lung cancer patients, and miR-16 is inconsistent, choosing to normalize the level of circulating miRNAs to total RNA. In a study on sera from Hepatitis B infected patients and matched controls, snRNA U6-1 is found to have high variability and snRNA U6-2 is not detectable. In this type of samples the combination of miR-26a, miR-221, and miR-22* is recommended as the most stable set of reference genes for circulating miRNAs evaluation[121]. Similarly, U6-2 is inconsistent in serum from gastric cancer patients and healthy controls. In this study, authors recommend the combined use of miR-16 and miR-93 as suitable reference genes[122]. On the contrary, in serum from uro-oncological patients, U6-2 is detectable and rather consistent[123]. snoRNA U44 levels are similar in sera from breast cancer patients and from age-matched healthy women, differently from miR-16 and 5S rRNS that show remarkable variability in the same samples. Surprisingly, snRNA U6-1 serum levels are found consistently higher in breast cancer patients compared to healthy controls, not only confirming that U6 is not an appropriate reference gene, but also indicating an interesting new paradigm in cancer[124]. Finally, Sourvinou and colleagues showed that a combined use of endogenous miR-21 and miR-16 and exogenous cel-miR-39, compensates differences in miRNAs recovery and differences in cDNA synthesis between samples. Using this normalization procedure and miR-21 as a biomarker, it seems possible to clearly discriminate healthy individuals from NSCLC patients[125].

Circulating miRNAs as lung cancer biomarkers

The Jiang laboratory found that miR-155, miR-197 and miR-182 can be potential biomarkers for early detection of lung cancer with 81.33% sensitivity and 86.76% specificity (Table 3). The levels of these miRNAs in plasma of NSCLC patients are elevated compared with healthy controls[126]. The same group demonstrated that another set of plasma miRNAs (miR-21, miR-126, miR-210, and miR-486-5p), had 86.22% sensitivity and 96.55% specificity in distinguishing NSCLC patients from the healthy controls (Table 3). Furthermore, the panel of four miRNAs produced 73.33% sensitivity and 96.55% specificity in identifying stage I NSCLC patients. The miR panel had higher sensitivity (91.67%) in diagnosis of AD compared with SCC (82.35%)[127]. Authors from the Jiang lab recently reported that quantification of the plasma miR-21-5p and miR-335-3p by digital PCR provided 71.8% sensitivity and 80.6% specificity in distinguishing lung cancer patients from cancer-free subjects (Table 3)[128].

Tang *et al*[129] reported that higher plasma miR-21 and miR-155 and lower plasma miR-145 expression levels distinguish lung cancer patients from healthy smokers with 69.4% sensitivity and 78.3% specificity (Table 3). Levels of miR-361-3p and miR-625-3p might have a protective influence on the development of NSCLC, and the quantification of these miRNAs in serum could be useful for the diagnosis of NSCLC, in particular in smokers[130]. A study reported that the expression of miR-146b, miR-221, let-7a, miR-155, miR-17-5p, miR-27a and miR-106a is significantly reduced in sera of NSCLC cases, while miR-29c is significantly increased (Table 3). Unexpectedly, no significant differences were observed in plasma of patients compared with controls[131].

Bianchi *et al*[132] provided an evidence that some serum-circulating miRNAs are important to identify asymptomathic high-risk individuals with early stage lung cancer (Table 3). Between others, they highlighted the importance of let-7 family, members of miR-17-92 cluster, miR-126 and miR-486 in sera of NSCLC patients[132].

Recently, qRT-PCR was used to assess miR-205-5p, miR-205-3p, and miR-21-3p expressions in serum and tissue samples (Table 3)[133]. The relative expressions of miR-205-5p and miR-205-3p were significantly higher in NSCLC tissues compared with cancer-adjacent paired specimens. In the serum, significantly higher miR-205-5p, miR-205-3p, and miR-21-3p relative expressions were observed in the NSCLC group compared with healthy volunteers or patients diagnosed with a benign lung disease (pulmonary tuberculosis, pneumonia, chronic obstructive pulmonary disease, or interstitial pneumonia). The relative expressions of miR-205-5p and miR-21-3p in NSCLC tissues and serum were significantly correlated, while no significant correlation was observed for miR-205-3p. Expressions of miR-205-5p and miR-205-3p in SCC specimens were significantly higher than in lung adenocarcinoma specimens. Similarly, higher serum miR-205-5p and miR-205-3p levels were observed in SCC patients.

MiRNAs expression profile in whole-blood showed that miR-190b, miR-630, miR-942 and miR-1284 are present in a majority of the classifiers generated during the analyses to distinguish lung cancer cases from controls[64]. In a different study, miR-22, miR-24, and miR-34a were found upregulated in RNA extracted from whole blood of NSCLC patients *vs* healthy controls (Table 3)[134].

In a recent paper, Aushev *et al*[135] described a specific panel of miRNAs (miR-205, -19a, -19b, -30b, and -20a)

Table 3 Circulating miRNAs as biomarkers in lung cancer

MiRNA	Function	Scope	Sample	Ref.
miR-155, miR-197, miR-182 (UP)	Diagnostic	Lung cancer patients *vs* healthy controls	Plasma	[126]
miR-21, miR-210, miR-126, miR-486-5p (relative expression)	Diagnostic	NSCLC patients *vs* healthy controls	Plasma	[127]
miR-21–5p (UP) and miR-335–3p (DOWN)	Diagnostic	Lung cancer patients *vs* healthy controls	Plasma	[128]
miR-21, miR-155 (UP), miR-145 (DOWN)	Diagnostic	Lung cancer patient *vs* healthy smokers	Plasma	[129]
miR-361-3p, miR-625* (DOWN)	Diagnostic	Lung cancer patients *vs* healthy controls	Serum	[130]
miR-146b, miR-221, let-7a, miR-155, miR-17-5p, miR-27a and miR-106a (DOWN), miR-29c (UP)	Diagnostic	Early stage NCSLC *vs* healthy controls	Serum	[131]
miR-92a, miR-484, miR-486-5p, miR-328, miR-191, miR-376a, miR-342, miR-331-3p, miR-30c, miR-28-5p, miR-98, miR-17-5p, miR-26b, miR-374, miR-30b, miR-26a, miR-142-3p, miR-103, miR-126, let-7a, let-7d, let-7b, miR-22, miR-148b, miR-139 (DOWN), miR-32, miR-133b, miR-566, miR-432-3p, miR-223, miR-29a, miR-148a, miR-142-5p, miR-140-5p (UP)	Diagnostic	Asymptomatic NSCLC patients *vs* healthy smokers	Serum	[132]
miR-205-5p, miR-205-3p, and miR-21-3p (UP)	Diagnostic	NSCLC patients *vs* benign lung disease and healthy controls	Serum	[133]
miR-190b, miR-630, miR-942 and miR-1284 (relative expression)	Diagnostic	Lung cancer patients *vs* healthy controls	Whole-blood	[85]
miR-22, miR-24, and miR-34a (UP)	Diagnostic	NSCLC patients *vs* healthy controls	Whole-blood	[134]
miR-205, miR-19a, miR-19b, miR-30b, miR-20a (DOWN)	Diagnostic	Patients after lung cancer surgery *vs* healthy controls	Plasma	[135]
miR-7, miR-21, miR-200b, miR-210, miR-219-1, miR-324 (UP), miR-126, miR-451, miR-30a, miR-486 (DOWN)	Diagnostic	NSCLC patients *vs* healthy controls	Plasma	[137]
miR-101, miR-106a miR-126, miR-133a, miR-140-3p, miR-140-5p, miR-142-3p, miR-145, miR-148a, miR-15b, miR-16, miR-17, miR-197, miR-19b, miR-21, miR-221, miR-28-3p, miR-30b, miR-30c, miR-320, miR-451, miR-486-5p, miR-660, and miR-92a (relative expression)	Diagnostic	NSCLC patients *vs* healthy controls	Plasma	[138]
miR-155, miR-197 (UP)	Prognostic	Lung cancer patients with metastasis *vs* patients without metastasis	Plasma	[126]
miR-486, miR-30d, miR-1, miR-499 (relative expression)	Prognostic	NSCLC patients *vs* healthy controls	Serum	[139]
let-7f, miR-30e-3p (DOWN)	Prognostic	NSCLC patients *vs* healthy controls	Plasma	[140]
miR-125b (relative expression)	Prognostic	NSCLC patients *vs* healthy controls	Serum	[141]
miR-21 (UP)	Response to treatment	Platinum chemotherapy-resistant patients *vs* non resistant patients	Plasma	[90]
miR-21 and miR-10b (UP)	Response to treatment	NSCLC patients with EGFR mutation *vs* patients without mutation	Plasma	[142]
miR-22 (UP)	Response to treatment	NSCLC patients *vs* healthy controls	Whole-blood	[134]

NSCLC: Non-small cell lung cancer.

decreasing in plasma of patients after SCC surgery (Table 3). Interestingly, high levels of these miRNA are found in tumor-specific exosomes[135].

Also NGS has been used to depict the differential expression of miRNAs in peripheral blood of lung cancer patients detecting 76 previously unknown miRNAs and 41 novel mature forms of known precursors. In addition, the authors identified 32 annotated and seven unknown miRNAs that were significantly altered in NSCLC patients[136].

A plasma-based 24-miRNA signature classifier with predictive, diagnostic, and prognostic value was described, whose use could reduce the false-positive rate of low-dose computed tomography (LDCT), thus improving the efficacy of lung cancer screening (Table 3)[137,138].

Regarding the potential of circulating miRNAs as prognostic factors, the levels of miR-155 and miR-197 have been found higher in plasma from lung cancer patients with metastasis than in those without metastasis (Table 3)[126].

Moreover, Hu *et al*[139] using NGS described that serum levels of miR-486, miR-30d, miR-1 and miR-499 are significantly associated with overall survival (Table 3). NSCLC patients and healthy controls differ in vesicle-related miRNAs in plasma: let-7f and miR-30e-3p levels decreased in plasma vesicles of NSCLC patients and the expression of these miRNAs is associated with poor outcome (Table 3)[140]. Finally, serum miR-125b may represent a biomarker in NSCLC with an independent prognostic potential for overall survival (Table 3)[141].

Circulating miRNAs have also been explored for their ability to predict response to treatment. miR-21 expression has trends similar in plasma and matched resected specimens and was significantly increased in platinum-based chemotherapy-resistant patients, in which increased miR-21 expression was associated with the shorter disease-free survival (Table 3)[90].

The expression of miR-21 and miR-10b was much higher in plasma samples of patients with NSCLCs with EGFR mutation than without mutation (Table 3)[142]. Patients who had up-regulated miR-21 expression had shorter overall survival, but a better response to gefitinib than patients who had low expression of the microRNA. Additionally, miR-10b is highly expressed in progressive disease compared with complete remission or stable disease.

Franchina *et al*[134] recently reported a correlation between high expression of miR-22 in whole blood and the lack of response in pemetrexed treated NSCLC patients (Table 3).

CONCLUSION

MiRNAs have increasingly been pointed as important players in carcinogenesis and cancer progression, but also as potential diagnostic and prognostic markers.

For the future, circulating miRNAs could open new opportunities in the field of diagnosis and prognosis in various types of human cancers. The difficulties found in traditional therapies, due to insufficient disruption of oncogenic pathways, drug resistance and drug-induced toxicity, require the development of novel therapeutic strategies. The ease, specificity and sensitivity of determining body fluid miRNAs profiles paves the way for several applications and provides hope to accomplish this task. However, from the technical and applicative point of view, there are still several limitations to consider. Further studies are necessary to find the best possible normalization control and to improve the technique, but also to establish panels of miRNAs specific to each type of tumor, taking into account early or advanced cancer stages, response to treatment, patient outcome and recurrence. After the complete validation for several candidates, miRNAs studies could open a new era in cancer treatments, providing improved targeted agents for the cure of patients[143] and constituting the basis for the development of novel therapies.

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