# MicroRNAs as molecular markers in lung cancer

**Javier Silva[1], Vanesa Garcia[1], Ana Lopez-Gonzalez[2], Mariano Provencio[1]**

[1]*Medical Oncology Department, Hospital Universitario Puerta de Hierro, Majadahonda-Madrid, Spain*
[2]*Medical Oncology Department, Complejo Asistencial Universitario de León, Spain*

## Review Article

## Abstract

Lung cancer is the most common cause of cancer death in the western world for both men and women. Lung cancer appears to be a perfect candidate for a screening program, since it is the number one cancer killer, it has a long preclinical phase, curative treatment for the minority of patients who are diagnosed early and a target population at risk (smokers) and it is also a major economic burden. The earliest approaches to identifying cancer markers were based on preliminary clinical or pathological observations, although molecular biology is a strong candidate for occupying a place among the set of methods. In search of markers, several alterations, such as mutations, loss of heterozygosity, microsatellite instability, DNA methylation, mitochondrial DNA mutations, viral DNA, modified expression of mRNA, miRNA and proteins, and structurally altered proteins have all been analysed. MicroRNAs (miRNA) are small RNA molecules, about 19-25 nucleotides long and encoded in genomes of plants, animals, fungi and viruses. It has been reported that miRNAs may have multiple functions in lung development and that aberrant expression of miRNAs could induce lung tumorigenesis. We review here the role of miRNAs in lung tumorigenesis and also as a novel type of biomarker.

**Keywords**: Lung Cancer, MicroRNA, Biomarker, Tumorigenesis

## Introduction

Lung cancer is the most common cause of cancer death in the western world for both men and women.[1] Each year the number of lung cancer deaths is greater than the number of deaths from breast, prostate and colorectal cancer combined.[2] In 2007, estimates calculated 213,380 new cases of lung cancer and 160,390 deaths, accounting for 15% and 29%, respectively, of all cases of cancer.[2] Clinically, lung cancer can be divided into 2 groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which often have different specific genetic alterations.[3] Approximately 75% of lung tumours are NSCLC, which includes squamous cell carcinoma, adenocarcinoma and large cell carcinoma.[1] SCLC is characterized by its rapid growth, early dissemination and chemosensitivity andradiosensitivity.[4]

Over 80% of lung cancers are attributable to cigarette smoking[5], with a risk directly proportional to consumption. Thus, cessation of smoking remains the single most important intervention for lung cancer prevention: recently some progress has been made by the introduction of smoking bans in public places throughout Europe. Although the exposure to tobacco carcinogens is known to be the main risk factor for lung cancer, only a minority of heavy smokers will develop this disease[6], suggesting environmental or genetic determinants in disease initiation and progression. Thus, inter-individual differences in carcinogen metabolism may play an essential role in the development of this environmental cancer.[7] In addition, with the increasing incidence of non-smoking-related lung cancers, particularly in women, the identification of high-risk groups among non-smokers will be an important challenge.[8]

Whereas screening the general population has been recommended for other common cancers, including breast, colorectal and possibly prostate cancer, no such recommendation currently exists for lung cancer.[9] Only between 10-16% of lung cancer patients survive more than 5 years.[2] The dismal

prognosis of lung cancer is largely related to the fact that this tumour is generally diagnosed at a late stage. However, outcome is significantly better in patients diagnosed early, with the 5-year survival rate of stage 1 lung cancer at 60-70%.[10] Early detection of lung cancer could change disease outcome, although current diagnostic tools are either too costly or not sensitive enough to allow early detection.[11, 12] For all these reasons, it is essential to develop an effective screening regimen for this disease.

Lung cancer appears to be a perfect candidate for a screening program, since it is the number one cancer killer, it has a long preclinical phase, curative treatment for the minority of patients who are diagnosed early and a target population at risk (smokers) and it is also a major economic burden.

## Molecular markers

The progression from pre-neoplasia to cancer is accompanied by the accumulation of genetic and epigenetic alterations. These lead to altered expression patterns and modifications in protein structure and function. The changes can be used as molecular tumour markers, being useful in: a) characterization of a predisposition or genetic susceptibility to suffering neoplasia; b) detection of cancer at early stages or pre-malignant phases; c) the evaluation of disease outcome; d) monitoring of disease progression; and e) determination of response to therapy, so favouring a better choice of therapy for each patient.

Currently, the diagnostic tools for lung carcinoma are: chest X-ray, computed tomography (CT), cytological analysis of sputum and bronchoalveolar lavage fluid, and spiral CT, among others. However, to develop more strategies, diagnosis, prognosis and treatment need to be improved. The goal of clinical and potential molecular markers is to develop a clinical screening test that would be useful and practical in clinical practice, complementing the use of imaging modalities such as spiral CT.

The earliest approaches to identifying cancer markers were based on preliminary clinical or pathological observations[13], although molecular biology is a strong candidate for occupying a place among the set of methods. In search of markers, several alterations, such as mutations, loss of heterozygosity, microsatellite instability, DNA methylation, mitochondrial DNA mutations, viral DNA, modified expression of mRNA, miRNA and proteins, and structurally altered proteins have all been analyzed. High-throughput screening approaches that analyze expression patterns of several genes and proteins have been used to search for cancer-associated molecules.[13] In lung carcinomas several alterations have been found, such as *KRAS* mutated in approximately 30% of cases[14], over-expression of *C-erbB2* (Her-2/neu) or *BCL2* in 25% of cases[14] and *DLC1* (deleted in lung cancer 1) altered in 27% of primary NSCLC.[15]

In clinical practice, there are several molecular tumour markers used for screening in some tumour types, such as serum carcino-embryonic antigen (CEA) in colorectal cancer[16] and prostate-specific antigen (PSA) in prostate cancer.[17] However, these markers are not very sensitive in premalignant or early stages or are not specific to malignancy. For most other types of cancer, such as early-stage lung cancer, there are no molecular markers available in clinical practice.

Although many effective cancer therapies have recently been developed, there are only a few molecular markers that are available at present for determining treatment response. For example, breast cancer cells that express high levels of the tyrosine receptor kinase are more likely to respond to trastuzumab.[18] In lung cancer, the ability to predict responses to chemotherapy or targeted agents is extremely limited and is based on tumour histology alone. Therefore, we are unable to identify *a priori* the right treatment regimen for each tumour. Appropriate molecular markers could predict response to specific treatments. An example is the use of epithelial growth factor tyrosine kinase inhibitors (EGFR-TKIs) in lung cancer.[19] Although more studies are necessary, it has been described that a series of mutations in EGFR makes tumours highly sensitive to this form of therapy. Thus, detection of these mutations could identify a group of patients who derive great benefit from EGFR-TKI therapy.[19] Another example is the use of molecular markers in relation to cisplatin treatment. Only a proportion of lung cancer patients will respond to this chemotherapy treatment, whose adverse side-effects are significant. Cisplatin needs to bind to DNA, creating platinum-DNA adducts, which can be repaired by nucleotide excision. Patients with completely resected NSCLC and ERCC1-negative tumours (enzyme with central role in DNA repair) appear to benefit from adjuvant cisplatin-based chemotherapy, whereas patients with ERCC1-positive tumours do not.[20]

## microRNAs as molecular markers

MicroRNAs (miRNA) are small RNA molecules, about 19-25 nucleotides long and encoded in genomes of plants, animals, fungi and viruses.

miRNAs constitute a highly conserved class of naturally occurring non-coding single-stranded RNA molecules, which function as post-transcriptional negative gene regulators of complementary target mRNAs. Although they may differ in distinct organisms, the basic process involves a transcription of dsRNA that is processed into shorter units that mediate target recognition in a sequence-specific manner (Table 1).[21] It has been calculated that between 74% and 92% of the gene transcripts are probably under miRNA control.[22, 23]

TABLE 1: Main Studies that have investigated miRNA in lung cancer

Yanaihara *et al.* Cancer Cell. 2006
Lu *et al.* Proc Am Thorac Soc. 2008
Fabbri *et al.* Proc Natl Acad Sci. 2007
Inamura *et al.* Lung Cancer. 2007
Yu *et al.* Cancer Cell. 2008
Fagi F. Thorac Surg Clin. 2012
Rabinowits *et al.* Clin Lung Cancer. 2009
Hu *et al.* J Clin Oncol. 2010
Hennessey *et al.* PLoS One. 2012
Bianchi *et al.* E cancer medical science. 2012
Matsubara *et al.* Oncogene. 2007
Shen *et al.* Lab Invest. 2011
Rani *et al.* Cancer Biol. Ther. 2013
Xu *et al.* Clin Transl Oncol. 2013
Zhang *et al.* Med Oncol. 2013

miRNAs are specifically expressed or greatly enriched in a particular organ, implying an organ- or tissue-specific function. Furthermore, expression profiles of miRNAs in mice and human lung are very similar, indicating evolutionary conservation of miRNA expression.[24, 25] It has been reported that lung is one of the tissues with the most abundant expression of miRNA let-7.[26]
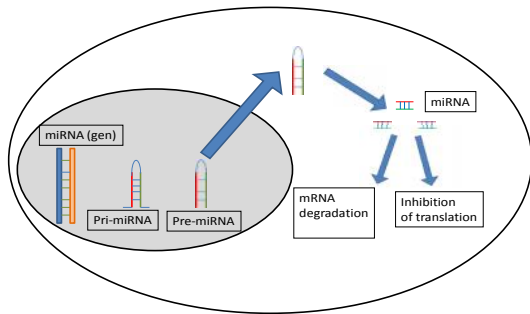


FIG. 1: miRNA function

There are several human diseases, such as spinal muscular atrophy, Parkinson's disease, fragile X mental retardation or DiGeorge syndrome, in which miRNAs or their processing might be involved.[27-29] Among human diseases, it is well-known that miRNAs are aberrantly expressed in numerous human cancers, including colon, breast, ovarian, prostate and lung cancer[30, 31] (Figure 1). Moreover, since a single miRNA may have as many as a few thousand target genes with different biological entities, the data obtained from miRNA profiling may provide information that can be used to classify more accurately the different cancer subtypes described to date. Besides regulating the expression of known oncogenes and tumour suppressors, miRNAs also act as oncogenes and tumour suppressors directly, which provides an apparent connection between the altered expression of miRNAs and cancer development. Thus, miRNA-expression profile of human tumours is closely associated with diagnosis, staging, progression, prognosis and response to treatment.[31-33]

In the case of lung cancer, it has been reported that miRNAs may have multiple functions in lung development and that aberrant expression of miRNAs could induce lung tumorigenesis.[34, 35] Moreover, as mentioned above, the different expression profiles of miRNAs between normal lung and lung cancer has led to their emergence as a novel type of biomarker. This may be helpful for lung cancer diagnosis and therapy using miRNAs as novel therapeutic targets.[36, 37]

The current diagnostic tools available for lung cancer often lack sensitivity and the ability to distinguish different lung cancer subtypes, such as NSCLC and SCLC or adenocarcinoma and squamous cell carcinoma.[38, 39] In this respect, the applicability of miRNA-based biomarkers may improve methodology for both sensitivity and specificity. Differently expressed miR-29 (a, b and c), miR-99b, miR-102, let-7a-2 and let-7f-1 have been used to discriminate histological types of lung carcinomas.[32, 40] As well as data strongly supporting the view that miRNAs work as biomarkers for differentiating lung cancer, the expression of miRNA signatures has been linked to the prognosis of lung cancer. Deregulated expression of the let-7 family has been frequently described in different analyses of miRNA expression signatures in lung cancer. The decrease in the let-7 family is associated with worse prognosis and shortened survival, which supports the view that it has a tumour suppression function.[41-43] Deregulated miRNA has been also related with lymph node metastasis and advanced clinical stage.[44]

Talking about EGFR-TKIs, we know that patients with activating EGFR mutations eventually develop resistance to this drugs. It is thought that a secondary mutation in the EGFR gene, such as T790M, or amplification of the MET proto-oncogene could be implicated. Recently, upregulated miR-214 has been related to acquired resistance to gefitinib, via PTEN/AKT signalling pathway.[45]

# MicroRNAs in plasma from cancer patients

The presence in plasma or serum of circulating nucleic acids (DNA and RNA) released by tumour cells increases the possibility of detecting alterations associated with the tumour and could be used as a tumour marker. Furthermore, as circulating nucleic acids are a source of tumour information obtained through a non-invasive and rapid method, samples could be taken at different times during follow-up to identify residual disease and recurrence at asymptomatic stages.

MiRNAs originating in several tumour tissues enter circulation and can be detected in serum. The fact that plasma contains large amounts of stable miRNAs implies a great potential of serum miRNA profiling as the fingerprint for disease.[46] Regarding the release mechanism of nucleic acids from tumour cells to the bloodstream, we recently reported that a major fraction of these molecules detected in plasma of can-

cer patients are highly protected in tumour-specific microvesicle-like structures.[47] In other studies, cancer-specific miRNAs with high resistance to RNases and harsh conditions were identified in plasma or serum of cancer patients[46, 48-52], showing that the high stability conferred by its particle-associated features makes miRNA levels well suited for being tested as non-invasive cancer biomarkers in patient plasma samples.  Moreover, other studies have suggested that miRNAs contained in tumour vesicles are functional and suppress target mRNAs for signal transduction components within recipient cells.[53]

Recently, our group as well as other research groups have analysed serum miRNA profiles of lung cancer patients to evaluate their potential use in predicting diagnosis and prognosis of disease.[46, 54, 55] Employing Solexa sequencing analysis, the Chen *et al.*[46] identified specific expression patterns of serum miRNAs for lung cancer in the Chinese population. They provided evidence that serum miRNAs contained fingerprints for different cancer types. Most differently expressed miRNAS in lung cancer included miR-205 and miR-206 that were previously involved in tumorigenic processes such as cell division and cell growth. Similarly, Lodes et al. focused their study on the evaluation of miRNA expression profiles in human serum for five types of human tumours, including lung cancer, using a pan-human microRNA high-density microarray. They found that these expression patterns could be used to distinguish between normal and cancer patient samples and, furthermore, that one millilitre of serum contained sufficient miRNAs to detect these expression patterns, without requiring amplification techniques. In a recent study, Hu's group analyzed the differences in levels of serum miRNAs between 30 NSCLC patients with longer survival and 30 patients with shorter survival. Using Solexa sequencing, they found 11 differently altered miRNAs between longer-survival and shorter-survival groups and, moreover, levels of 4 of them (miR-1, miR-30d, miR-486 and miR499) were significantly associated with overall survival.

Recently, a phase I//II biomarker study has been published, in which serum miRNA using PCR was examined from 30 NSCLC patients and 20 healthy controls. A combination of two differentially expressed miRNAs miR-15b and miR-27b was able to discriminate NSCLC from healthy controls.[56]

Combining serum mi-RNA detection and low-dose computed tomography (LD-CT) could be an interesting screening test. Real-time PCR was used to identify the profile of miRNAs extracted from the serum of asymptomatic LD-CT-detected lung adenocarcinoma and normal serum. A multivariate risk-predictor was developed based on 34 miRNA expression levels. When the predictor was tested on an independent cohort of patients with asymptomatic LD-CT detected lung cancer, it displayed an overall accuracy of 80% (sensitivity 71%, specificity 90%), and more important, the risk-predictor was able to distinguish between LD-CT-detected benign nodules and malignant disease.[57] We agree with the relevance of this last point, due to the relative high number of benign lung nodules detected by LD-CT screening.

## Conclusion

miRNAs are frequently altered in many types of tumours, including lung carcinoma. Their expression patterns are associated with the classification and prognosis of these tumours. That miRNAs are stable in plasma from lung cancer patients supports their use as important clinical biomarkers[58, 59, 60] detectable in peripheral blood associated with tumour.

The fact that certain miRNAs have an oncogenic function while others have a role as tumour suppressors is especially relevant in terms of their applicability in anti-tumour therapies. Thus, oncogenic miRNAs would require therapies inhibiting their function, while those therapies for miRNAs with tumour suppressor ability should increase miRNA activity in cancer cells. There are some studies that analyze the potential of miRNA-based therapies in cancer.[61, 62]

In summary, the expression of miRNA signatures in plasma from lung cancer patients can be used as new markers, obtained by a non-invasive method, for disease diagnosis and prognosis. They have higher specificity and sensitivity than analyses used currently.

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