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miR-126 and miR-126* Expression in Pulmonary Carcinomas: Future important markers of carcinogenesis

Sofia Pereira Constantino Romano

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Professora Doutora Lina Carvalho (Faculdade de Medicina, Universidade de Coimbra) e da Professora Doutora Emília Duarte (Faculdade de Ciências e Tecnologia, Universidade de Coimbra)

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Agradecimentos

Gostaria de agradecer a todos os que me ajudaram e apoiaram durante este ano:

À Professora Doutora Lina Carvalho e à Dra. Maria Reis Silva, pela oportunidade de aprofundar os meus conhecimentos e pela orientação durante este projecto.

A todos os elementos do Instituto de Anatomia Patológica, pela indispensável colaboração.

Ao Dr. Paulo Santos e à Doutora Luísa Cortes, pela constante disponibilidade para me ajudarem.

À família e amigos, pelo apoio insubstituível e paciência infinita.

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Abbreviations

5-aza-dC	5-aza-2'-deoxycytidine
ADAM9	ADAM metallopeptidase domain 9
ADC	adenocarcinoma
Ago2	Argonaute 2
BA	bronchioloalveolar
BCL6	B-cell lymphoma 6
bp	base-pairs
C. elegans	*Caenorhabditis elegans*
CpG	cytosine-phosphate-guanine
CRK	v-crk sarcoma virus CT10 oncogene homolog
DGCR8	DiGeorge syndrome critical region gene 8
DNMT	DNA methyltrasnferase
EDTA	Ethylenediamine tetraacetic acid
EGFL7	epidermal growth factor-like domain 7
EMT	ephitelial-mesenchimal transition
FBXO33	F-box protein 33
H&E	haematoxylin and eosin
HDAC4	histone deacetylase 4
HOXC8	homeobox C8
IRS-1	insulin receptor substrate 1
KANK2	KN motif and ankyrin repeat domains 2
let-7	lethal-7
lin-4	abnormal cell lineage 4
LNM	lymph node metastasis
MGMT	O-6-methylguanine-DNA methyltransferase
mRNA	messenger RNA
MS-PCR	methylation-specific polymerase chain reaction
NSCLC	non-small cell lung cancer
nt	nucleotide
PCR	polymerase chain reaction
PIK3R2	phosphoinositide-3-kinase, regulatory subunit, polypeptide 2 p85 beta

PLK2	polo-like kinase 2
PP	pulmonary parenchyma
pre-microRNA	precursor microRNA
pri-miRNA	primary microRNAs
PTPN9	protein tyrosine phosphatase, non-receptor type 9
RB	retinoblastoma
RGS3	regulator of G-protein signaling 3
RISC	RNA-induced-silencing complex
RNA Pol	RNA polymerase
SCC	squamous cell carcinoma
SCL7A5	solute carrier family 7 member 5
SCLC	small cell lung cancer
SLC45A3	solute carrier family 45 member 3
TIMP-3	TIMP metallopeptidase inhibitor 3
TRBP	Tar RNA binding protein
TRU	terminal respiratory unit
UTR	untranslated region
VEGFA	vascular endothelial growth factor A

Abstract

Squamous cell carcinoma and adenocarcinoma are the main types of pulmonary malignant epithelial tumours and are the main cause of death from cancer. Considering the increasing incidence of these pathologies in advanced stages prone to targeted therapy, unravelling the molecular mechanisms underlying their development and characterising the differences between the two histological types becomes essential. microRNAs have been recently identified as important regulators of gene expression at the post-transcriptional level, playing a key role in tumourigenesis. miR-126 and miR-126* are two of those regulators and several studies confirm their importance in cancer. However, a detailed characterization of these microRNAs in pulmonary carcinoma is lacking.

In this study, we performed laser microdissection of squamous cell carcinoma and adenocarcinoma samples, as well as of matched pulmonary parenchyma and lymph node metastasis from surgical specimens to evaluate the expression of miR-126 and miR-126* by real-time PCR.

miR-126 and miR-126* presented lower expression in primary pulmonary carcinomas and lymph node metastasis when matched with pulmonary parenchyma. Primary carcinomas and lymph node metastasis presented similar expression, as well as tumours of patients with and without lymph node metastasis. Adenocarcinomas from female patients presented lower levels of miR-126 and miR-126* than the cases of male patients. In male gender, squamous cell carcinomas presented lower levels than adenocarcinomas. In addition, pulmonary parenchyma from male patients with squamous cell carcinoma and from female patients with adenocarcinoma presented higher expression than the pulmonary parenchyma of male patients with

adenocarcinoma. These results support a role for these microRNAs in pulmonary adenocarcinoma and squamous cell carcinoma carcinogenesis and progression from early stages and suggest that they could be used as biomarkers to distinguish between the histological types and for an early detection of adenocarcinomas.

An attempt of characterising the methylation pattern of miR-126/miR-126* host gene promoter by MS-PCR was made. However, adequate primer design for this particular region presented some constraints. Other alternatives should be therefore considered for this type of analysis.

Key words: squamous cell carcinoma, adenocarcinoma, miR-126, miR-126*

Resumo

O carcinoma epidermóide e o adenocarcinoma são os principais tipos de tumores epiteliais malignos e são a principal causa de morte por cancro. Tendo em conta a crescente incidência destas patologias em estádios avançados propensos a terapia dirigida, torna-se essencial perceber os mecanismos moleculares subjacentes ao seu desenvolvimento e caracterizar as diferenças entre os dois tipos histológicos. Os microRNAs foram recentemente identificados como importantes reguladores da expressão génica ao nível pós-transcricional, desempenhando um papel chave na tumorigénese. O miR-126 e o miR-126* são dois destes regulares e vários estudos confirmam a sua importância no cancro. Contudo, não existe uma caracterização detalhada destes microRNAs nos carcinomas pulmonares.

Neste estudo realizou-se microdissecção a laser de amostras de peças cirúrgicas de carcinoma epidermóide e adenocarcinoma, assim como do parênquima pulmonar e metástases dos gânglios linfáticos correspondentes, e avaliou-se a expressão do miR-126 e do miR-126* por PCR em tempo real.

O miR-126 e o miR-126* apresentaram menor expressão nos carcinomas pulmonares primários e nas metástases dos gânglios linfáticos do que no parênquima pulmonar correspondente. Os carcinomas primários e as metástases dos gânglios linfáticos apresentaram expressão semelhante, assim como os tumores de doentes com e sem metástases dos gânglios linfáticos. Os adenocarcinomas de doentes do género feminino apresentaram menores níveis de miR-126 e miR-126* do que os dos doentes do género masculino. No género masculino, os carcinomas epidermóides apresentaram menores níves do que os adenocarcinomas. Além disso, o parênquima pulmonar das peças cirúrgicas de doentes do género masculino com carcinoma epidermóide e de

doentes do género feminino com adenocarcinoma apresentou maior expressão do que os de doentes do género masculino com adenocarcinoma. Estes resultados apontam para um papel destes microRNAs na carcinogénese de adenocarcinoma e carcinoma epidermóide pulmonares e progressão em fases iniciais e sugerem que estes possam ser utilizados como biomarcadores para distinguir os dois tipos histológicos e para uma detecção precoce dos adenocarcinomas.

Tentou-se caracterizar o padrão de metilação do promotor do gene hospedeiro dos miR-126/miR-126* por MS-PCR. Contudo, o desenho de *primers* adequados para esta região em particular apresentou algumas restrições. Assim, outras alternativas devem ser consideradas para este tipo de análise.

Palavras-chave: carcinoma epidermóide, adenocarcinoma, miR-126, miR-126*

Introduction

CANCER

Cancer is one of the leading causes of death worldwide. Its incidence has been increasing and it is now the cause of more than 10% of deaths. The number of cases of cancer is predicted to increase even further and it is estimated that 12 million people will die from it in 2030 [1]. Considering this high incidence and mortality and the problems still prevalent regarding therapy and relapse, cancer has been a major focus in health research. However, this is a complex and highly ramified disease. Hence, many of the underlying principles remain to be elucidated and their connections established.

Cancer hallmarks

The normal cell pattern of division and death is tightly controlled in order to ensure that cell proliferation only takes place when necessary. Cancer arises when this regulation fails and uncontrolled proliferation and inappropriate survival of damaged cells occur, which results in tumour formation.

There are several pathways involved in the regulation of these processes and consequently several components whose dysregulation may promote tumourigenesis. Notwithstanding, it is widely accepted that the wide range of modifications underlying cancer development can be grouped in eight main categories, the hallmarks of cancer, proposed by Hanahan and Weinberg. Although their acquisition might differ both mechanistically and chronologically, all cancers are believed to share these traits: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion to programmed cell death, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, energy metabolism reprogramming and evasion to immune

destruction. The disruption of the defences against each of these characteristics has a partial contribution in cancer development. It has been proposed that the acquisition of cancer hallmarks is made possible by two enabling characteristics: genome instability and mutation and tumour-promoting inflammation [2].

As pointed out by Hanahan and Weinberg, angiogenesis is a central step in carcinogenesis. It consists on the development of new blood vessels from pre-existing vessels and is fundamental for the tumour to grow, since it allows the necessary supply of nutrients and oxygen to the tumoural cells. The process of angiogenesis is regulated by the balance between pro- and anti-angiogenic factors. When this balance is altered towards angiogenesis, an "angiogenic switch" is said to occur. This event leads a dormant avascularized hyperplasia to become a vascularized tumour and eventually to metastasize [3]. The angiogenic switch can be triggered by several signals, such as metabolic stress, mechanical stress and immune response, as well as genetic mutations [4].

Metastases, a central feature of many tumours, originate from a multistage process that leads to the spread of malignant cells from primary tumours to distant sites. Tumour cells become metastatic by a series of events, in which epithelial-mesenchimal transition (EMT) plays a central role. In this process, epithelial cells acquire genomic and functional characteristics resembling those of mesenchymal cells. After EMT, these cells travel through the vasculature and proliferate in other tissues, from microscopic growths (micrometastases) to macroscopic secondary tumours (macrometastases), either in lymph nodes and organs as the lungs, liver, bones and brain [5].

Signalling pathways

Cancer arises due to the disruption of the normal functioning of several signalling pathways (Fig. 1). These pathways consist of complex webs of components, from membrane signal receptors that activate membrane or cytoplasmic intermediary proteins, promoting signal propagation, to downstream components that translocate to the nucleus and alter gene expression. Signalling pathways are involved in every cell process, from cell cycle control to DNA damage repair and apoptosis. Their alteration can therefore lead to one or more of the cancer hallmarks and promote tumour formation [2].

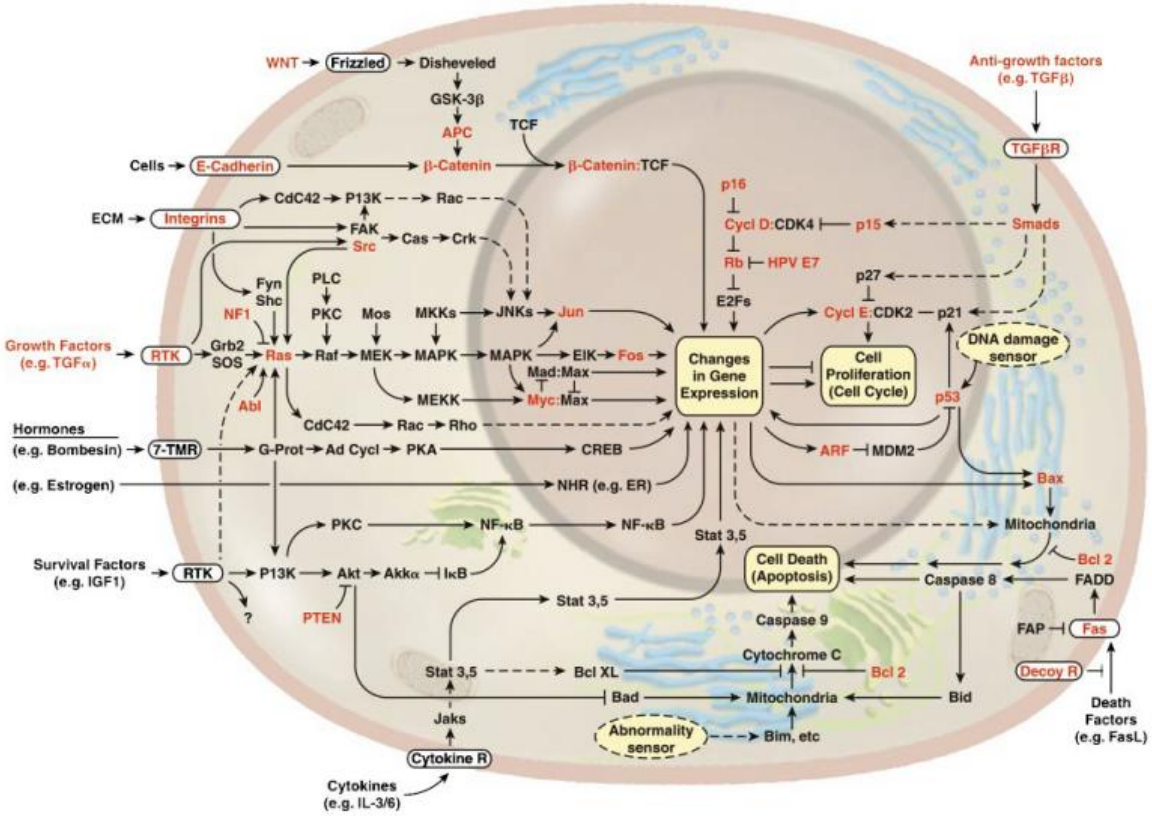

Figure 1. Signalling pathways involved in cancer. Cell functioning and fate are controlled by a complex web of signalling pathways that respond to several stimuli through membrane receptors. These receptors allow signal transduction to intracellular components and eventually nuclear effects at the gene expression regulation level. Different stimuli activate different pathways, thus leading to specific effects in cancer [2].

Methylation

Methylation of DNA is an epigenetic process, that is, a process that changes DNA without altering the nucleotide sequence. It consists on the transfer of a methyl group to a cytosine ring, a reaction catalyzed by DNA methyltransferases (DNMTs) that leads to gene silencing. In mammals, it occurs at cytosines located 5' of a guanosine (cytosine-phosphate-guanine (CpG) dinucleotides). Regions with high CpG content are known as CpG islands and locate mainly in the proximal promoter regions of genes. Methylation is known to play a central role on embryonic development, genomic imprinting and inactivation of genes on the X chromosome [6].

As a function in normal processes, methylation also plays an important role in some pathological conditions, such as cancer. Tumoural cells usually present abnormal patterns of DNA methylation, with hypermethylated CpG islands in gene promoters and hypomethylation of gene coding-sequences. Promoter hypermethylation has been extensively related to tumourigenic features. For instance, methylation of *TIMP metallopeptidase inhibitor 3* (*TIMP-3*), which encodes a protease inhibitor, leads to tissue invasion in cancer [7]. Also, hypermethylation patterns can be used as diagnostic markers. Herman and colleagues have shown that different types of tumours present specific hypermethylation patterns, which can be useful for diagnosis [8]. A central feature of this kind of alteration is that they are reversible, therefore being potential therapeutic targets.

Oncogenes and tumour suppressor genes

Genes involved in cancer can be divided into two main categories: oncogenes, cancer-promoting genes, and tumour suppressor genes, whose activity contributes to

prevention of carcinogenesis. In normal conditions, these genes are usually switched off and on, respectively. However, the activation of oncogenes and the inactivation of tumour suppressor genes can lead to drastic changes in the signalling pathways and originate conditions that promote the alterations underlying the hallmarks of cancer, thus leading to tumour formation [2].

Although the focus on these genes used to concern mainly protein-coding ones, it has been recently discovered that some non-coding RNA genes can also act as oncogenes or tumour suppressor genes, playing an important role in cancer. These genes codify small molecules of RNA, called microRNAs, which present an extensive function in gene expression regulation and whose significance in cancer has been acknowledged as more studies in this area are carried out [9].

MICRORNAS

microRNAs constitute a family of approximately 22-nucleotide (nt) small RNAs with a crucial role on gene expression regulation at the post-transcriptional level, by complementary binding to target messenger RNA (mRNA) molecules [10].

microRNAs were first identified in *Caenorhabditis elegans* during a genetic screening on which it was found that mutations in gene *lin-4* (*abnormal cell lineage 4*) lead to disruption of temporal regulation in larval development [11]. The characterization of this gene as a microRNA gene was made later by Ambros and colleagues, who described that the transcript contained sequences complementary to regions of the 3' untranslated region (UTR) of other gene mRNA. It was then suggested that *lin-4* could be a member of a new class of regulatory genes that would act by originating small RNA products, antisense to their targets [12]. This was followed by

the identification of many other microRNAs in *C. elegans*. Posterior studies identified microRNAs also in plants and other animals, including humans, and established their importance in several processes such as cell differentiation, proliferation, apoptosis, metabolism and morphogenesis [13] [14].

microRNA genomics and biogenesis

The human genome is predicted to contain hundreds of microRNA genes. Several are located in intergenic regions but the majority is placed within transcription units, mainly in introns of protein-coding genes [15]. Many microRNAs present the same orientation as the host gene, which enables coupling of microRNA expression with its host gene and so microRNA and mRNA present similar expression profiles. This could be a way of acquiring a more efficient expression but could also reflect a genetic interaction between the microRNA and its host [16]. Actually, it has been shown that microRNAs residing within protein-coding genes are processed from capped, polyadenylated transcripts, which are characteristics of messenger RNA, and that the transcript can then originate either microRNA or mRNA, depending on its further processing [17]. Furthermore, a microRNA has been found that localizes within an intron of a protein coding gene and which is expressed in stress conditions, targeting the mRNA resulting from its host gene [18].

microRNAs are transcribed as large RNA precursors and then undergo a complex multistep processing (Fig.2) that leads to a double-stranded RNA of approximately 22 nt with a 2 nt 3' overhang in both strands, the microRNA:microRNA* duplex. Afterwards, the two strands undergo different processes: one of them gets associated to a multiprotein complex named RNA-induced-

silencing complex (RISC) and will be responsible for the post-transcriptional regulation, whereas the microRNA* strand (also called passenger strand) is degraded [19].

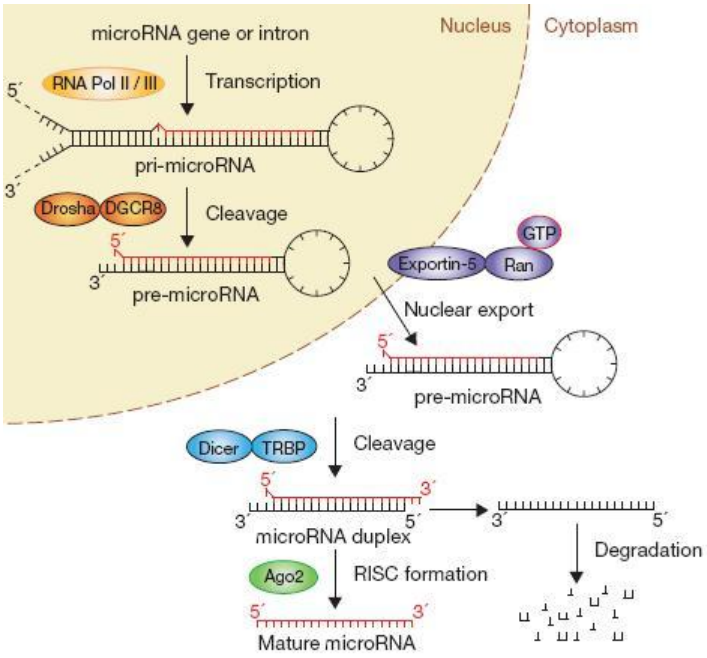

Figure 2. microRNA biogenesis. In the nucleus, microRNAs are transcribed by RNA Pol II or III in a long precursor presenting a hairpin stem, a terminal loop and a 2-nt overhang at both 3' and 5' ends. This precursor molecule, called pri-microRNA, is cleaved twice, first in the nucleus by the Drosha/DGCR8 complex, originating pre-microRNA and, after translocation to the cytoplasm through exportin-5-Ran-GTP, by the Dicer/TRBP complex. This second cleavage yields a microRNA duplex: one strand is incorporated into RISC and originates the mature microRNA and the other is usually degraded. RNA Pol – RNA polymerase; pri-microRNA – primary microRNA; DGCR8 – DiGeorge syndrome critical region gene 8; pre-microRNA – precursor microRNA; Ran – RAS-related nuclear protein; GTP – guanosine triphosphate; TRBP – Tar RNA binding protein; Ago2 – argonaute 2 (adapted from [19]).

The process underlying microRNA:microRNA* dissociation has not been fully elucidated yet. Several reports have identified different helicases intervening in this event [20] [21], but there are also evidence that these kind of proteins might not be required [22]. The fact that only one strand of the duplex associates with RISC raises a question about the mechanism by which the distinction between them is made. Two

studies revealed that the different fates of each strand are determined by the thermodynamic stability of the base pairs at the 5' ends: the strand with the less stable 5' end is incorporated in RISC and the other strand is degraded [23]. This model implies that when the stability of both 5' ends is similar, such distinction cannot be made; in this case, both strands would be equally likely to be incorporated. It has been proposed that the shared activity of both strands regulates the activity of targets with similar complementarity to either of them [24]. However, the incorporation of one of the strands instead of the other could also be tissue- and developmental-specific [25]. One way this could be regulated is by editing of one of the strands. For instance, pri-microRNA editing, in which adenosine are converted to inosine by deamination, have shown to occur and alter the thermodynamic properties of the duplex, potentially leading to higher levels of the microRNA* strand [26].

microRNA activity

microRNA post-transcriptional control of gene expression occurs through two main processes: endonucleolytic mRNA cleavage, less common in animals, and translation prevention without mRNA degradation. This control relies on microRNA complementarity to the target mRNA molecules and the properties of the microRNA-mRNA binding determine the mechanism by which the regulation takes place. When microRNAs present perfect or high complementarity to the protein-coding sequence of a mRNA, the binding leads to mRNA cleavage by RISC. However, more commonly the microRNA molecule only presents partial complementarity to the target molecule, usually to regions in the mRNA 3' UTR. In this case, the binding of RISC microRNA to

its target usually only prevents mRNA translation but not its degradation. Thus the levels of mRNA are maintained and only the protein levels decrease [27].

mRNA targeting

In an attempt to ascertain the principles guiding microRNA-mRNA target binding, several studies have been performed that allowed the identification of some general rules of this recognition.

microRNAs usually bind to the 3' UTR of the target mRNA, although some also bind to the coding sequence [27]. According to Cohen and colleagues, microRNA target sites can be divided in two broad categories: 5' dominant sites and 3' compensatory sites, generally corresponding to the two main types of gene expression regulation by microRNAs (mRNA degradation and translation inhibition, respectively). Targets with 5' dominant sites base-pair well to the 5' end of the microRNA, mainly to the seed (nucleotides 2-7) and targets with 3' compensatory sites present weak 5' base-pairing and highly depend on compensatory pairing to the 3' end of the microRNA [28]. Another central characteristic in microRNA-mRNA binding is the target site multiplicity: the presence of several binding sites for a microRNA in a mRNA molecule has been shown to increase the regulation efficacy [29].

Even though these characteristics have long been considered central to the microRNA-mRNA recognition, accumulating evidence points to several exceptions and reveals the lack of knowledge still prevailing on this issue. For example, it has been shown that the "seed rule" (importance of the seed region in microRNA-mRNA binding) is not as general as previously thought [30] and that high complementarity between the seed region of a microRNA and mRNA may not be sufficient to promote

their interaction [31]. In addition, in spite of the greater significance of the 3' UTR comparatively to the 5' UTR that is found in the microRNAs targets described so far, artificial constructs with target sites in the 5' UTR presented equally efficient transcription as the one seen in the equivalent microRNA-3' UTR interaction [32].

Although much is still to uncover about this subject, it is clear that microRNAs have the capacity to regulate the expression of many of the genes in an organism. Due to the characteristics of target recognition, one microRNA can affect the expression of hundreds of genes [33] [34] and it is estimated that 30% of the protein coding genes are under microRNA regulation [34]. In spite of the difficulties concerning microRNA-target interaction elucidation and the imprecision of the general principles so far recognized, the establishment of some general rules widely observed, even if not applicable to a subset of microRNAs, provided a way for determining potential microRNA targets by bioinformatic predictive approaches. Several bioinformatic tools have been developed that make these predictions according to different criteria, such as seed pair stringency, site number and overall pairing stability, among others [35]. These tools have been deeply valuable in the prediction of thousands of potential microRNA targets, some of which were later validated [36] [37]. Nonetheless, one should keep in mind that owing to the unclear aspects of this subject, and consequently the flaws in the models used to make these predictions, it is likely that many microRNA targets are not predicted by such approaches, therefore remaining unknown.

microRNA in cancer

Since high complementarity between a microRNA and its target is not a requirement for microRNA activity, microRNAs may present hundreds of targets and

are predicted to regulate a large part of the human genome [38]. Therefore, it is not surprising that these regulators are involved in countless processes [13] [14] and that all the main cell signalling pathways are microRNA-regulated, namely those that are involved in cancer (Table I) [39]. Accordingly, microRNA impaired expression has been implicated in tumourigenesis and this expression correlates with several cancers. Some microRNA genes can function either as oncogenes or tumour suppressor genes and the microRNAs are therefore called oncomirs [9].

Table I. microRNA involvement in signalling pathways (adapted from [39]).

Target	microRNAs	Biological processes
Transforming growth factor-β		
ACVR2A	miR-15 miR-16	Early embryogenesis
Smad3, Smad4 and Smald5	miR-23b	Liver stem cell differentiation
Hedgehog		
Smoothened	miR-324-5p miR-125b miR-326	Neural stem cell proliferation and medulloblastoma
GLI1	miR-324-5p	Neural stem cell proliferation and medulloblastoma
Receptor tyrosine kinase and mitogen-activated protein kinase		
NRAS, KRAS	let-7	Cancer cell differentiation
Sprouty1	miR-21	Cardiac fibrosis
Receptor tyrosine kinase and AKT		
PI3KR2	miR-126	Angiogenesis
PTEN	miR-21	Hepatocellular cancer
	miR-26a	Glioma
	miR-216a miR-217	Glomerular mesangial cell survival and hypertrophy
Hippo		
LATS	miR-372/373	Testicular germ cell tumour
p53		
TP53	miR-125b	Apoptosis in brain
TP63	miR-203	Keratinocyte differentiation

The first evidence that microRNAs could be involved in cancer came from studies on chronic lymphocytic leukaemia in which it was found that two microRNA genes, *miR-15* and *miR-16*, are located in a region deleted in a high number of cases of this cancer [40]. Further studies confirmed that these regulators are often dysregulated in cancer: a high number of microRNAs are located at fragile sites, minimal regions of loss of heterozygosity, minimal regions of amplification and breakpoint regions, regions that are usually involved in cancer [41]; Dicer is downregulated in lung cancer and its expression correlates with shortened post-operative survival [42]; tumours tend to express lower levels of microRNAs than normal tissues (though some microRNAs are up-regulated) [9].

The alterations in microRNA expression could be potentially useful in cancer diagnosis since it has been shown that specific microRNA profiles constitute signatures that distinguish tumoural from normal tissues and allow the identification of different kinds of cancer [43]. microRNAs are also promising tools for cancer prognosis, as first evidenced by the work of Takahashi and colleagues describing an association between reduced expression of the microRNA lethal-7 (let-7) and shortened post-operative survival in patients with lung cancer [44]. Moreover, it has been suggested that microRNA signature of a tumour reflects its developmental history, since distinct patterns of microRNA expression are present within a single developmental lineage [45].

The importance of microRNA expression in diagnosis and prognosis and its impact on cancer has been further acknowledged with the discovery that this expression can vary not only from normal tissue to primary tumours but also from metastatic to non-metastatic primary tumours and from primary tumours to metastasis. Several studies have shown that some microRNAs are highly expressed in primary tumours that

metastasize whereas others present inversely correlated expression with metastatic behaviour and constitute therefore a way to evaluate the aggressiveness of a tumour [46]. Other microRNAs have been found to be altered in the cells from a primary tumour that acquire metastatic characteristics, comparatively to the other cells in that tumour [47] [48] [49]. One of the first studies identifying this correlation between metastatic potential and microRNA profile approached breast cancer metastasis in lung and bone. Massagué and colleagues proposed that the loss of specific microRNAs would provide a selective advantage for cells destined for metastatic colonization due to altered regulation of metastasis genes, such as those involved in EMT and cell adhesion [50]. This and other reports identified specific microRNAs metastasis signatures and thus revealed the potential of microRNA as prognostic markers for the risk of future metastasis. The role of some microRNAs not only in primary tumours development but also in metastatic progression relies on their capability to regulate several mRNA targets. According to the group of targets of a microRNA, this regulator can act as oncogene and/or as metastasis-promoter (or conversely as tumour-suppressor and/or metastasis-inhibitor).

An important consequence arising from microRNA involvement in cancer is their potential as targets for cancer therapy. Oncogenic microRNAs could be targeted by using complementary molecules, the anti-microRNA oligonucleotides, the antagomirs, that have been shown to effectively inhibit microRNA activity when injected into mice and thus constitute promising therapeutic agents. On the other hand, microRNAs that function as tumour suppressors could be overexpressed through transient expression systems that would administer large quantities of the microRNA. However, these procedures are still under study and their effective clinical potential has not been determined yet [9].

microRNA dysregulation

The processes that lead to microRNA dysregulation, namely in cancer, have been extensively studied and found to be caused by several factors: transcriptional dysregulation, which can occur through the activity of several proteins, such as p53 and c-Myc; germ-line or somatic mutations of microRNA genes; DNA copy number abnormalities, which alter DNA dosage; defects at the biogenesis level owed to dysfunctional or dysregulated proteins involved in this process, such as Dicer; and epigenetic alterations [51]. One of the major epigenetic alterations is methylation, whose association with cancer has already been established. Several microRNAs have been found to be downregulated due to hypermethylation in some cancers. Plus, methylation of some of these has been associated to metastatic potential, since

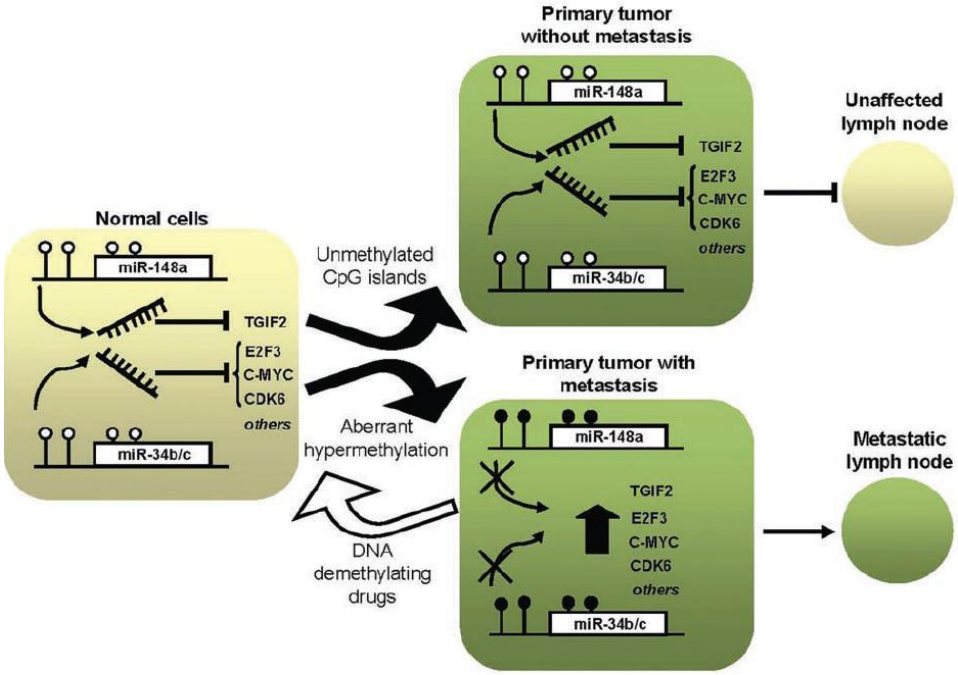

Figure 3. Methylation of microRNA genes specific of metastatic primary tumours. Some microRNAs genes, such as miR-148a and miR-34b/c, present metastasis-specific methylation, being normally expressed in primary tumours that do not metastasize. Consequently, the methylation of these genes highly influences the capacity of a tumour to develop metastasis. DNA demethylationg drugs revert this methylation pattern. White circle, unmethylated CpG; black circle, methylated CpG. [56]

methylation signature was predictive of lymph node metastasis (Fig. 3) [52]. This could have significant consequences at the clinical level, since the expression of some microRNAs has been shown to return to normal levels after treatment with demethylating drugs. This was first shown for miR-127, which is downregulated in prostate, bladder and colon tumours. When treating tumour cell line with 5-aza-2'-deoxycytidine (5-Aza-dC), a DNA demethylating agent, its expression was partially restored and led to reduced levels of one of its targets, B-cell lymphoma 6 (BCL6) [53].

MICRORNAS IN PULMONARY CARCINOMA

Pulmonary carcinoma, commonly called lung cancer, is the leading cause of death from cancer worldwide and has one of the highest incidences, representing 17.8% of cancer deaths and 12.6% of all new cases cancers. This disease presents 5-year survival rates of approximately 10% and is usually diagnosed at an advanced stage [54]. Hence, it becomes important to improve the knowledge about this cancer and optimize diagnosis and prognosis techniques.

Tobacco smoking represents the main cause of pulmonary carcinoma, as supported by both epidemiological and molecular studies. This is due to the presence of several carcinogens and inflammation-related agents. However, only 10%-15% of smokers develop pulmonary carcinoma. Other factors contribute for this disease, which is usually the result of a combination of genetic and other environmental factors, such as air pollution [55].

Pulmonary carcinoma was usually divided into two main groups, small cell lung cancer (SCLC), with a more rapid growth and early development of metastasis, and non-small cell lung cancer (NSCLC), which included squamous cell carcinoma,

adenocarcinoma and large cell carcinoma. This distinction was made mainly for treatment purposes, since SCLC was usually treated with chemotherapy and radiotherapy, whereas NSCLC patients were usually subject to surgery based on staging. However, this division is nowadays considered inadequate because NSCLC showed to be a very heterogeneous group and SCLC is today prone to surgery when in limited stage [54]. Therefore, there is the need of a personalized characterization of the tumours of each group. This becomes of the most importance to choose the adequate treatment, especially considering the opportunities that are now offered by targeted therapies.

The classification of the histological type of pulmonary carcinoma is made according to their microscopic features, using haematoxylin and eosin (H&E)-stained sections. These compounds stain the nucleus and the cytoplasm, respectively, thus allowing a better identification of characteristic features of each type of carcinoma [55].

Pulmonary squamous cell carcinoma and adenocarcinoma

Squamous cell carcinoma and adenocarcinoma are the most prevalent types of lung carcinoma (25% and 40%, respectively) [54].

Squamous cell carcinoma is a malignant epithelial tumour that is characterized by the presence of cellular keratinization and/or intercellular bridges (Fig. 4A). It arises mainly from bronchial epithelium after metaplasia, is highly associated with tobacco smoking (90% of the cases) and is more predominant in males; it may also show isolated and bizarre-shaped cells forming groups that are separated by fibrous stroma in less differentiated cases. Cells at the periphery of the nests are usually smaller, whereas

those found centrally are larger, with more cytoplasm, and present more keratinization and intercellular bridges [54] [55].

Adenocarcinoma is a malignant epithelial tumour with glandular, acinar or pappilary differentiation with or without epithelial mucin (Fig. 4B) [56]. It is the most common histological subtype of pulmonary carcinoma nowadays and also develops mainly in smokers. However, this is the histological type with a more strong correlation with non-smokers, particularly women. Adenocarcinoma is characterized by large cells, which may be single or arranged in clusters, originating the referred patterns. Cells are cyanophilic and more translucent in comparison with squamous cell carcinoma and present single and eccentric nuclei and prominent nucleoli. The characteristics of chromatin correlate to the degree of differentiation of the tumour: it is granular and evenly dispersed in highly-differentiated tumours, whereas it presents irregular distribution or hyperchromatin in less differentiated adenocarcinomas [54] [55].

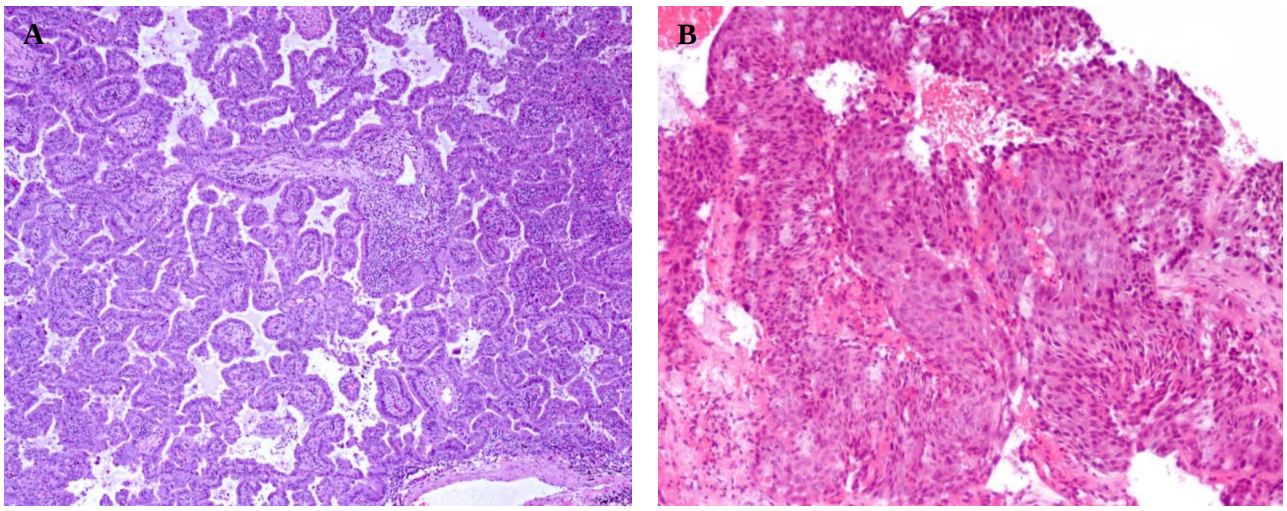

Figure 4. (A) Adenocarcinoma and **(B)** squamous cell carcinoma of the lung. H&E staining, 200x.

Pulmonary carcinoma staging is done according to the TNM system [57], which describes general characteristics of the primary tumour (T), such as size and

organ invasion, and the status of regional lymph nodes (N) and distant metastases (M). Based on this system, the stage of the tumour can then be classified into four major subgroups (I-IV). This staging system reflects the 5-year survival rates of lung cancer patients and is thus useful for prognosis and to determine the best treatment option for each patient.

Methylation in pulmonary carcinoma

Methylation is an important event in pulmonary carcinoma. Several genes have been described as being hypermethylated and are associated to different functions that are impaired in this pathology, such as DNA repair (*O-6-methylguanine-DNA methyltransferase* - *MGMT*) and cell cycle control (*retinoblastoma* - *RB*). In fact, methylation has been shown to be an early event in the development of this type of cancer and to be related to the progression and aggressiveness of lung cancer [58]. This is the case of *p16*, a cell cycle control gene, which has been shown to become hypermethylated in rats by exposure to radiation, heavy metals and tobacco carcinogens [59]. The silencing of this gene increased from cell hyperplasia to more advanced stages of the disease, in rats and in human tissue samples [60]. Minna and colleagues showed that this silencing mechanism presents a very high percentage in lung tumours and is likely to be the main process of gene silencing in this type of cancer [61]. Besides, *p16* promoter methylation presents differences between squamous cell carcinoma and adenocarcinoma and is associated with smoking habits and short post-surgery survival [62]. Other genes have also been described to be more frequently methylated in one of the histological types of than in the other [63] [64].

The value of therapeutic strategies targeting this silencing mechanism has already been approached in several studies. For instance, Baylin and colleagues have shown that disruption of *DNMT1* in mice leads to a 50% decrease in tobacco carcinogen-induced lung cancer. In *DNMT1* wild-type mice, treatment with the demethylating agent 5-aza-dC decreases tumour incidence by 30% [65]. These studies highlight the importance of clarifying the methylation mechanisms in pulmonary carcinoma, as well as the methylation patterns and their molecular consequences.

microRNA expression

microRNA expression has proved to be very useful in pulmonary carcinoma studies, for both diagnosis and prognosis. Furthermore, several molecular aspects of microRNA activity in pulmonary carcinoma have already been elucidated.

One of the first microRNAs to be identified, let-7, was found to be downregulated in lung cancer and related to shortened post-operative survival [44]. Besides, this was the first oncomir that was shown to regulate the expression of an oncogene, *RAS*. Slack and colleagues reported that this gene contains let-7 complementary sites in its 3' UTR and that low expression of let-7 correlated to higher levels of RAS [66]. This microRNA was later shown to reduce tumour growth in mouse models of lung cancer, confirming its relevance in this pathology and providing evidence that it could be a therapeutic target [67].

Some microRNA profiles have been identified that allow discriminating tumour from normal tissues in lung and different histological types, as well as predict survival [68]. For instance, miR-205 has been used to distinguish squamous from non-

squamous lung carcinomas with a 96% sensitivity and a 90% specificity [69], clearly showing the potential of microRNAs in diagnosis.

Several microRNAs present altered expression in pulmonary carcinoma and a link has already been established between these alterations and most of the processes involved in tumourigenesis and metastasis formation: regulation of growth factor receptors (e.g. miR-128b and let-7), regulation of proto-oncogenes *RAS* and *MYC* (let-7 and miR-98), telomere maintenance (miR-200 family), apoptosis (miR-29 and miR-34 family), angiogenesis (miR-126) and invasion (miR-200 family) [70]. The overall dysregulation of such processes due to microRNA altered expression affects the controlled cell growth, the regulation of apoptosis, genetic stability and promotes cell invasiveness and formation of new vessels that allow tumour propagation. A link between microRNAs and lung cancer metastasis has also been established: miR-148a and miR-9-3 genes have been shown to be hypermethylated in lung tumours, which was associated to the presence of lymph node metastasis [52].

miR-126 and miR-126*

miR-126 and miR-126* are intronic microRNAs processed from the same precursor pre-microRNA. They locate in intron 7 of the *epidermal growth factor-like domain 7* (*EGFL7*) gene. (Fig. 5) [71]. miR-126 has been extensively studied, but little information is available on its complement miR-126*.

EGFL7 is a 30 kDa protein highly expressed in the vascular endothelium during embryonic development. Upon birth, its expression decreases dramatically and remains restricted mainly to sites of active vascular remodelling and in a small subset of vessels in highly vascularized organs, such as the lung, heart and kidney. Although the

functions of this protein have not been fully understood yet, EGFL7 is believed to be involved in vasculogenesis. In addition, a role in angiogenesis sprouting has also been suggested [72]. Interestingly, recent studies support the hypothesis that many of the functions previously attributed to EGFL7 based on knock-down/out studies may in fact be a reflection of the loss of miR-126 [73]. Kuo and colleagues generated two mouse lines, one with an EGFL7 protein deletion and one with a miR-126 deletion. *EGFL7*$^{-/-}$ mice were phenotipically normal and born at the expected frequency, whereas miR-126$^{-/-}$ mice presented 50% of embryonic lethality and vascular defects that had previously been attributed to EGFL7 loss. These defects comprise vascular leakage and haemorrhaging, which indicate an important role of miR-126 in angiogenesis.

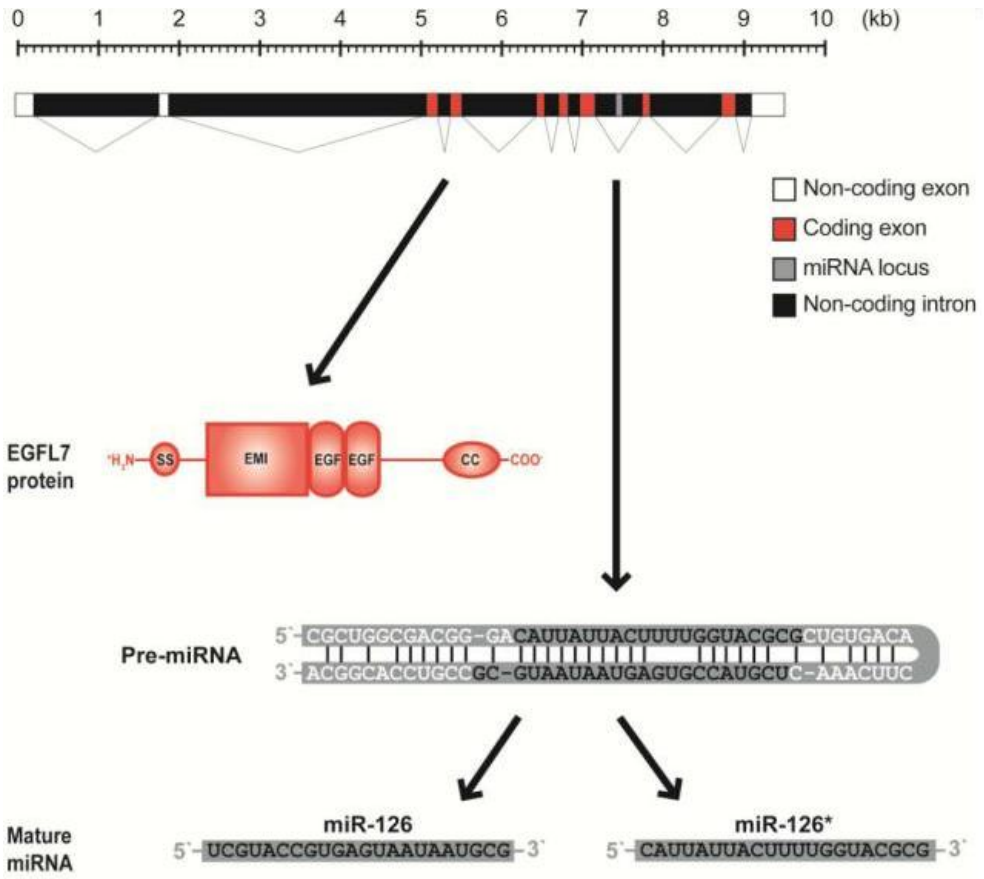

Figure 5. *EGFL7* gene. This gene contains 9 exons that originate the EGFL7 protein. However, its intron 7 also constitutes an important part of the gene, by originating a pre-microRNA. This pre-microRNA can then be processed and form two different microRNAs, miR-126 and miR-126* (adapted from [69]).

mir-126 expression is downregulated in several human cancers [74] [75] [76] and together with miR-335 it was one the first microRNAs to be described as a metastasis-suppressor in breast cancer. In this type of cancer, low miR-126 expression correlates with poor metastasis-free survival and its restoration leads to suppression of tumour growth and metastasis [50]. In gastric cancer, its expression has been shown to be associated to clinicopathological parameters, such as tumour size, lymph node metastasis, local invasion and TNM stage [75]. In lung cancer, both mir-126 and mir-126* have been reported to present low expression [68]. Overexpression of miR-126 in tumourigenic cell lines has shown to inhibit cell growth by inducing cell cycle arrest in G0/G1 phase, migration, adhesion and invasion, and it lead to tumour size reduction *in vivo* [75] [77] [78]. Although miR-126* effects have not been so widely approached, its overexpression in a prostate cancer cell line was shown to decreased cell migration and invasion [79]. These studies also identified some miR-126 and miR-126* targets that are involved in various tumourigenic processes, as well as molecules that regulate these microRNAs (Fig. 6).

One of the mechanisms underlying miR-126 downregulation is host gene methylation. *EGFL7* promoter has been shown to be hypermethylated in bladder and prostate tumour and tumour cell lines. When treating these cell lines with 5-Aza-dC, miR-126 expression was partially restored [74].

The importance of both miR-126 and miR-126* in cancer, namely pulmonary carcinoma, is thus quite supported by several studies. Nonetheless, there is still much to uncover on this issue.

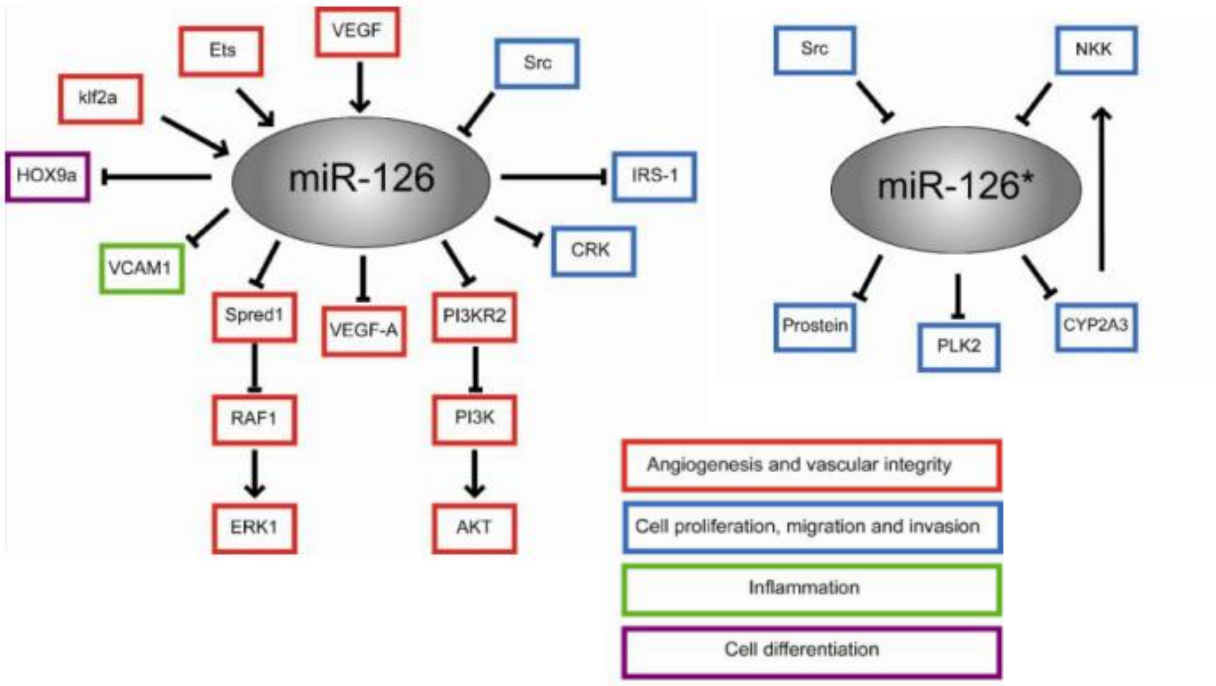

Figure 6. miR-126 and miR-126 functions and regulation. miR-126 has been associated to several cellular processes. So far, targets/modulators related to angiogenesis, cell proliferation, migration and invasion, inflammation and cell differentiation have been identified. miR-126* has been less studied, but a link has also been established between this microRNA and tumourigenic cell properties. (adapted from [69].)

OBJECTIVES

miR-126 and miR-126* downregulation in pulmonary carcinoma has already been reported. However, information is lacking regarding many aspects of their role in pathology and the mechanisms underlying their dysregulation.

The main objective of this project was to characterize miR-126 and miR-126* expression in patient samples of pulmonary squamous carcinoma and adenocarcinoma. We intended to compare their expression in pulmonary parenchyma, primary tumour and lymph node metastasis and detect differences according to clinicopathological parameters (TNM stage, age, gender and histological type). At the same type, we compared the expression of miR-126 and miR-126* in between.

The second objective of this project approached to the mechanism of microRNA dysregulation and comprised the study of the methylation pattern of miR-126 and miR-126* host gene, *EGFL7*.

Matherials and Methods

Tissue samples

This study comprised 37 formalin-fixed paraffin-embedded (FFPE) samples of bronchial-pulmonary carcinomas, collected from the Archives of the Anatomical Pathology of the Service of Pathology of the *Hospitais da Universidade de Coimbra*, surgically-resected between 2005 and 2010. These samples comprised 10 adenocarcinomas and 9 squamous cell carcinomas without lymph node metastasis, as well as 10 adenocarcinomas and 8 squamous cell carcinomas already with hillum and/or mediastinal lymph node metastasis. Data was collected regarding clinical and pathological information of each patient from the Pathology Registry (Table II). For each sample, at least two tumoural sections were selected after H&E staining, in order to gather the appropriate representative tumoural fields that allowed WHO 2004 lung cancer tumour classification [54]. Representative pulmonary parenchyma distant from each carcinoma and lymph node metastasis (when metastasis were present) were also identified by a pathologist.

miR-126 and miR-126* target prediction

Target prediction was made with three softwares: miRanda (http://www.microrna.org/), PicTar (http://pictar.mdc-berlin.de/) and TargetScan 5.2 (http://targetscan.org/). For miR-126* target prediction with TargetScan 5.2, nucleotides 2-8 were used. This sequence was obtained from miRBase (http://www.mirbase.org/). Information obtained from these softwares was then crossed and only targets with higher scores and predicted by at least two of the softwares were considered (Table III).

Table II. Tissue samples and clinicopathological data.

	Sample	Differentiation/Pattern	TNM	Gender	Age (years)
SCC without LNM	1	well differentiated	pT2 N0 Mx	M	84
	2	well differentiated	pT1 N0 Mx	M	43
	3	well differentiated	pT1 N0 Mx	M	64
	4	well differentiated	pT2 N0 Mx	M	65
	5	well differentiated	pT2 N0 Mx	M	65
	6	well differentiated	pT2 N0 Mx	M	74
	7	well differentiated	pT2 N0 Mx	M	58
	8	well differentiated	pT3 N0 Mx	M	66
	9	well differentiated	pT1 N0 Mx	F	71
SCC with LNM	10	well differentiated	pT2 N1 Mx	M	64
	11	well differentiated	pT2 N2 Mx	M	48
	12	Poorly differentiated	pT1 N1 Mx	M	62
	13	well differentiated	pT2 N1 Mx	M	69
	14	well differentiated	pT1 N1 Mx	M	65
	15	well differentiated	pT2 N1 Mx	M	65
	16	well differentiated	pT2 N1 Mx	M	68
	17	well differentiated	pT1 N1 Mx	M	72
ADC without LNM	18	solid (acinar)	pT4 N0 Mx	F	60
	19	papillary (BA)	pT2 N0 Mx	M	71
	20	acinar (BA, papillary)	p T2 N0 Mx	F	75
	21	papillary (micropapillary, acinar, BA)	p T2 N0 Mx	M	73
	22	acinar (mucinous BA)	p T2 N0 Mx	M	48
	23	acinar	pT1 N0 Mx	M	75
	24	acinar	pT1 N0 Mx	F	64
	25	acinar (solid with mucin)	pT2 N0 Mx	F	49
	26	microacinar (solid)	pT2 N0 Mx	F	59
	27	acinar (solid)	pT1 N0 Mx	M	60
ADC with LNM	28	acinar (micropappilary, BA)	pT1 N2 Mx	M	63
	29	pappilary	pT2 N1 Mx	M	59
	30	solid (acinar, micropappilary)	pT2 N1 Mx	F	77
	31	micropappilary (microglandular)	pT2 N2 Mx	F	51
	32	acinar (microacinar, solid)	pT1 N1 Mx	M	61
	33	acinar (microacinar, micropapillary)	pT2 N1 Mx	F	70
	34	acinar (papillary)	pT2 N2 Mx	M	68
	35	acinar (BA, micropapillary)	pT2 N1 Mx	F	77
	36	acinar (BA, papillary)	pT2 N1 Mx	F	44
	37	papillary	pT1 N1 Mx	F	62

SCC – squamous cell carcinoma; LNM – lymph node metastasis; ADC – adenocarcinoma; BA - bronchioloalveolar

Table III. miR-126 and miR-126* target prediction. Targets predicted by miRanda, PicTar and TargetScan. Putative targets listed were predicted by at least 2 of the softwares.

microRNA	Putative target	Predicted by		
		miRanda	PicTar	TargetScan
miR-126	PTPN9	x	x	x
	KANK2	x		x
	PLXNB2	x		x
	ADAM9	x		x
	PLK2		x	x
	CRK		x	x
	RGS3		x	x
	FBXO33		x	x
	SLC7A5		x	x
miR-126*	HOXC8	x		x
	ESRRG	x		x
	MAP3K2	x		x
	AKNA		x	x
	KIAA0355	x		x
	MEF2D		x	x
	ARID1A		x	x
	FGF7		x	x
	GRID1	x		x
	MFAP4	x		x
	HDAC4		x	x
	MAT2B		x	x
	RFX4	x		x
	HOXA13	x		x
	PPARGC1A		x	x

Laser microdissection

MembraneSlide 1.0 Pen slides with a polyethylene naphtalate membrane (Carl Zeiss MicroImaging GmbH, Jena, Germany) were used for laser microdissection. The slides, highly absorptive in the UV-A range, were irradiated with UV light for 20 minutes to make the membrane more hydrophilic and thus promote tissue adherence. 8 μm-cuts of tissue were made on a Leica 2235 microtome (Leica Microsystems, Nussloch, Germany) previously washed with RNase Zap (Sigma Aldrich, St. Louis, MO, USA). Cuts were placed on water at 60 ºC and then transferred to membrane slides, which were kept on the oven at 30 ºC until they were dry. The slides were stored at -20 ºC until they were used for laser microdissection. Prior to laser microdissection, sample cuts were deparaffinized by a 2-minute passage in xylene and a 1-minute passage in pure ethanol (both reagents from Merck, Darmstadt, Germany).

Laser microdissection was performed with PALM Laser Microdissection System (Carl Zeiss MicroImaging) (Fig. 7), which consists on an inverted optical microscope coupled to a video system and to a UV-A laser beam. Images of the tissue were visualized on the microscope and the areas of interest were selected using the *PALM RoboMover and Navigator software*. In order to correctly identify the regions of interest and prevent the removal of unwanted types of cells, an H&E slide from the same sample was visualized during the procedure. Cutting was performed at 200x amplification. Tissue fragments were catapulted to the adhesive cap of an eppendorf (Adhesive Cap 500 clear, Carl Zeiss MicroImaging). For primary tumours and lymph node metastasis, an area of approximately 2,000,000 μm^2 was removed, whereas for

pulmonary parenchyma this was increased to 4,000,000 μm^2, since the tissue had a lower cell density. After microdissection, samples were stored at -20 ºC.

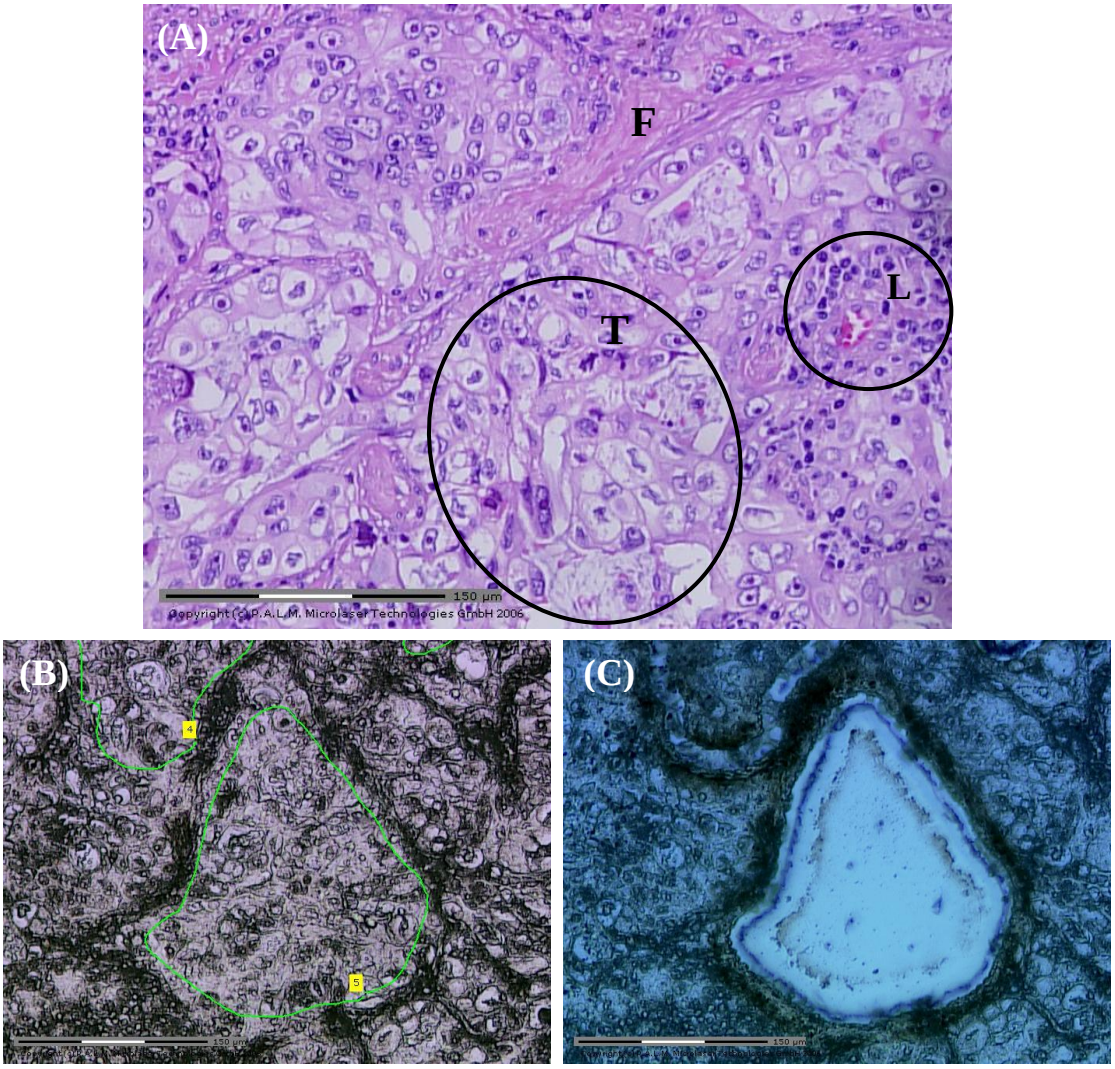

Figure 7. Laser microdissection. Laser microdissection of a sample of adenocarcinoma, using PALM Laser Microdissection System, 200x magnification. (A) H&E-stained slide, used to confirm the tumourigenic and non-tumourigenic areas in the sample. Polyethylene naphtalate-membrane slide from the same sample, after (B) selecting the tumour area and (C) laser ablation and tissue collection. T – tumour cells; F – fibroblasts; L – lymphocytes.

RNA extraction

RNA extraction from microdissected tissue was performed using miRNeasy FFPE Kit (Qiagen, Valencia, CA, USA). The steps of paraffin removal were disregarded, since it had been accomplished prior to microdissection. The remaining procedure was performed according to the manufacturer's protocol "Copurification of total RNA and miRNA from FFPE tissue sections". RNA was eluted in 17 µl of RNase-free water. All the material and equipment used that were not from the kit was washed with RNase Zap.

Real-time Polymerase Chain Reaction (Real-time PCR)

Extracted RNA was reverse transcribed to cDNA using miScrip Reverse Transcription Kit (Qiagen). 15 µl of template RNA form each sample were used. Reversion transcription was performed on a MyCycler™ thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). cDNA was stored at -20 ºC until real-time PCR was performed. Expression of miR-126 and miR-126* was evaluated by real-time PCR using miScript SYBR® Green PCR Kit, following the protocol "Real-Time PCR for Detection of miRNA or Noncoding RNA" (Qiagen) and using RUB6 snRNA for normalization. Primers were purchased from Qiagen and reconstituted with TE , pH 8.0 (10 mM Tris-Cl pH 8.0; 1 mM EDTA, reagents from Sigma Aldrich, St. Louis, MO, USA and Roche Applied Science, Indianapolis, IN, USA respectively). Reactions were performed on 96-well plates, using 2 µl of template cDNA. Each set of primers was used on a different plate and reactions were performed in duplicate, using two wells without DNA as negative controls. Since more than one plate was needed for each

microRNA and normalizer, two samples were repeated from one plate to the other, for inter-run calibration. After dispensing the reaction mix into the wells, the plates were covered with a coverslip and briefly centrifuged. Real-time PCR was then performed on LightCycler® 480 II (Roche, Basel, Switzerland) using LightCycler® 480 Software release 1.5.0. 40 cycles of amplification were performed. Cq was calculated using the Second Derivative Maximum Method. These data were then analyzed using qBasePlus® Software (Biogazelle, Ghent, Belgium), performing relative quantification according to the formula $\Delta Cq = Cq_{microRNA} - Cq_{RUB6}$.

Statistical analysis

Statistical analysis was performed with Graph Pad Prism 5 software. Statistical significance of differences in microRNA expression was assessed by: Friedman test followed by Dunn's Multiple or Wilcoxon signed rank test when comparing matched tissues and Mann Whitney test when comparing tissues of unpaired samples. $p<0.05$ was considered significant.

METHYLATION ANALYSIS OF *EGFL7* GENE PROMOTER

Manual microdissection

Manual microdissection was performed on a Leica RM 2135 microtome. Areas to be studied were marked on the paraffin block using the corresponding H&E slide for identification of the fields and 10 μm cuts were made (5-25 cuts, depending on the size

of the fragment). Distant lung parenchyma, primary tumours and lymph node metastasis samples were microdissected to follow the appropriate technical procedures.

DNA extraction

DNA was extracted with QIAamp DNA MiniKit (Qiagen). Prior to extraction, samples were deparaffinized following the protocol "Isolation of genomic DNA from paraffin-embedded tissue". However, instead of adding 1200 µl of xylene/ethanol and then centrifuge, these steps were divided into two, adding only 600 µl each time. DNA extraction was then performed according to the protocol "DNA Purification from Tissues" and following the manufacturer's instructions. The optional centrifugation for DNA purification was performed. For the elution step, DNA was incubated with 50 µl AE for 5 min before centrifugation. After extraction, DNA samples were quantified and their purity determined using GeneQuant *pro* (Biochrom, Cambridge, UK). Absorbance at 260 nm was used to calculate the concentration of DNA in the samples and the ratio 260/280 was used to evaluate purity. DNA was then stored at -20 ºC until bisulfite conversion.

Bisulfite conversion

DNA samples were treated with sodium bisulfite. Bisulfite conversion and subsequent DNA recovery was performed using EpiTect Bisulfite Kit (Qiagen), according to the Protocol "Sodium Bisulfite Conversion of Unmethylated Cytosines in DNA Isolated from FFPE Tissue Samples". For each reaction, 200 ng of DNA were used. At the recovery step, DNA was eluted in 40 µl.

Methylation-specific Polymerase Chain Reaction (MS-PCR)

EGFL7 promoter sequence was obtained from Transcriptional Regulatory Element Database (http://rulai.cshl.edu/cgi-bin/TRED/). Primers for MS-PCR were then designed using 2 softwares, MethPrimer (http://www.urogene.org/methprimer/) (Fig. 8; Table IV) and MethylPrimer Express (Applied Biosystems). CpG island prediction (GC≥50%) was used for primer design. Primers were designed for the first CpG island. Set number 5 was selected for MS-PCR.

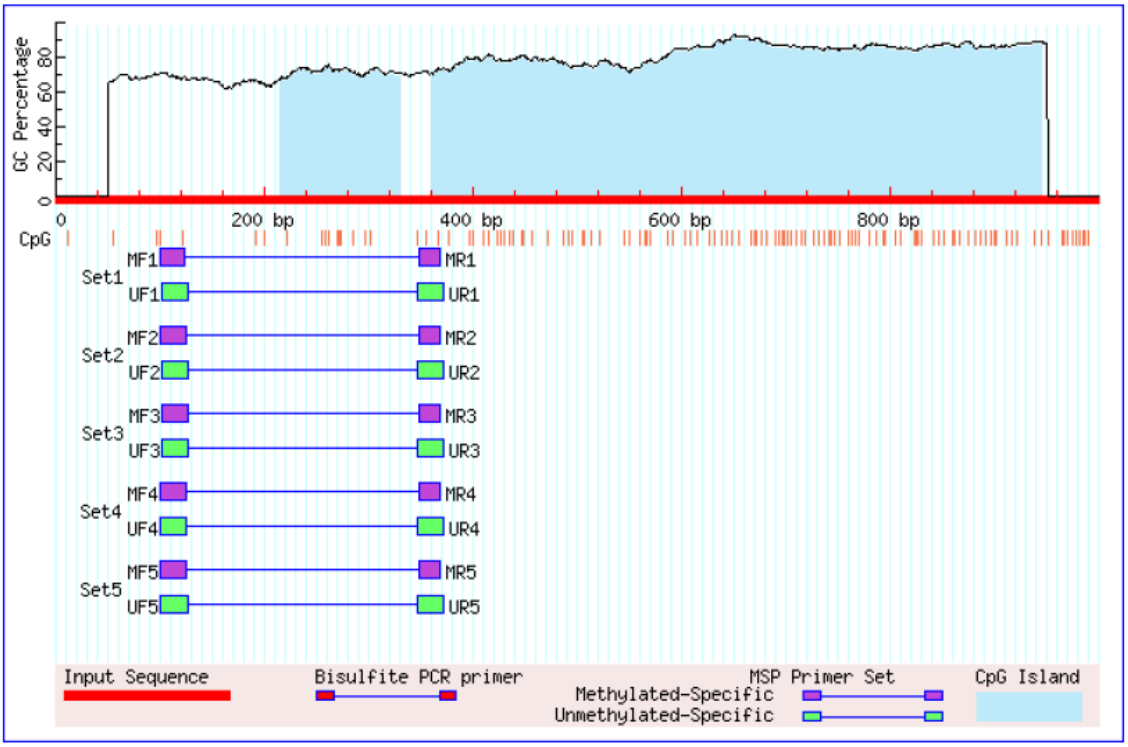

Figure 8. **Promoter region of *EGFL7***. Image obtained on MethPrimer software. This promoter presents 2 CpG islands (blue). Five sets of primers were obtained for MS-PCR.

Controls for MS-PCR were obtained from Qiagen, consisting in a methylated and an unmethylated sequence, both treated with sodium bisulfite (EpiTect Control

DNA, methylated, and EpiTect Control DNA, unmethylated). Primers were obtained from TIB Molbiol (Berlin, Germany).

Table IV. Primer design. Sets of primers obtained with MethPrimer software.

Set	Primer	Tm	GC%	Sequence
1	Mehylated Forward	59.53	36	GGCGTAGGGGTTTTTAATTTTAATAC
	Methylated Reverse	59.68	52	GCCCACACTAACGAAACTACG
	Unmethylated Forward	54.72	24	TGTAGGGGTTTTTAATTTAATATGT
	Unmethylated Reverse	59.00	36	AAACACCCACACTAACAAAACTACA
2	Mehylated Forward	59.53	36	GGCGTAGGGGTTTTTAATTTAATAC
	Methylated Reverse	59.68	52	GCCCACACTAACGAAACTACG
	Unmethylated Forward	55.51	27	GTGTAGGGGTTTTTAATTTAATATGT
	Unmethylated Reverse	59.00	36	AAACACCCACACTAACAAAACTACA
3	Mehylated Forward	58.16	36	CGTAGGGGTTTTTAATTTAATACGT
	Methylated Reverse	59.68	52	GCCCACACTAACGAAACTACG
	Unmethylated Forward	54.72	24	TGTAGGGGTTTTTAATTTAATATGT
	Unmethylated Reverse	59.00	36	AAACACCCACACTAACAAAACTACA
4	Mehylated Forward	56.72	33	GCGTAGGGGTTTTTAATTTAATAC
	Methylated Reverse	59.68	52	GCCCACACTAACGAAACTACG
	Unmethylated Forward	54.72	24	TGTAGGGGTTTTTAATTTAATATGT
	Unmethylated Reverse	59.00	36	AAACACCCACACTAACAAAACTACA
5	Mehylated Forward	59.53	36	GGCCTAGGGGTTTTTAATTTAATAC
	Methylated Reverse	59.68	52	GCCCACACTAACGAAACTACG
	Unmethylated Forward	58.32	30	GGTGTAGGGGTTTTTAATTTAATATGT
	Unmethylated Reverse	59.00	36	AAACACCCACACTAACAAAACTACA

PCR was performed using a master mix with 3.3x NH_4, 2mM or 3mM of Mg^{2+} (Bioline, London, UK), 16.6% glycerol (Merck), 300 µg/mL cresol red (Sigma Aldrich, St. Louis, MO, USA), 600 µM of each nucleotide and 0.4 U/ µl of Taq DNA polymerase (Bioline). Concentrations of 50 nM, 300 nM or 900 nM of each primer were tested. PCR conditions: initial denaturation for 30 s at 95 ºC, amplification of 65 cycles of 10 min at 95 ºC, 30 s at 60 ºC and 30 s at 72 ºC and final extension for 10 minutes at 72 ºC. An alternative approach comprising a pre-amplification step of 20 cycles

followed by 45 cycles was also tested, as well as different annealing temperatures (from 58 ºC to 65 ºC). PCR reactions were performed on a MyCycler™ thermal cycler or a MJ Mini™ Gradient thermal cycler (Bio-Rad Laboratories). PCR products were visualized in an ethidium bromide-stained 2% agarose gel with TBE (ethidium bromide from Sigma Aldrich, agarose fom Roche Applied Systems and TBE from Bio-Rad Laboratories).

Results

miR-126 and miR-126* expression in pulmonary squamous cell carcinoma

In order to evaluate the role and diagnostic potential of miR-126 squamous cell carcinoma, we determined its relative expression in pulmonary parenchyma and primary tumours of samples without lymph node metastasis (Fig. 9A). miR-126 was found to

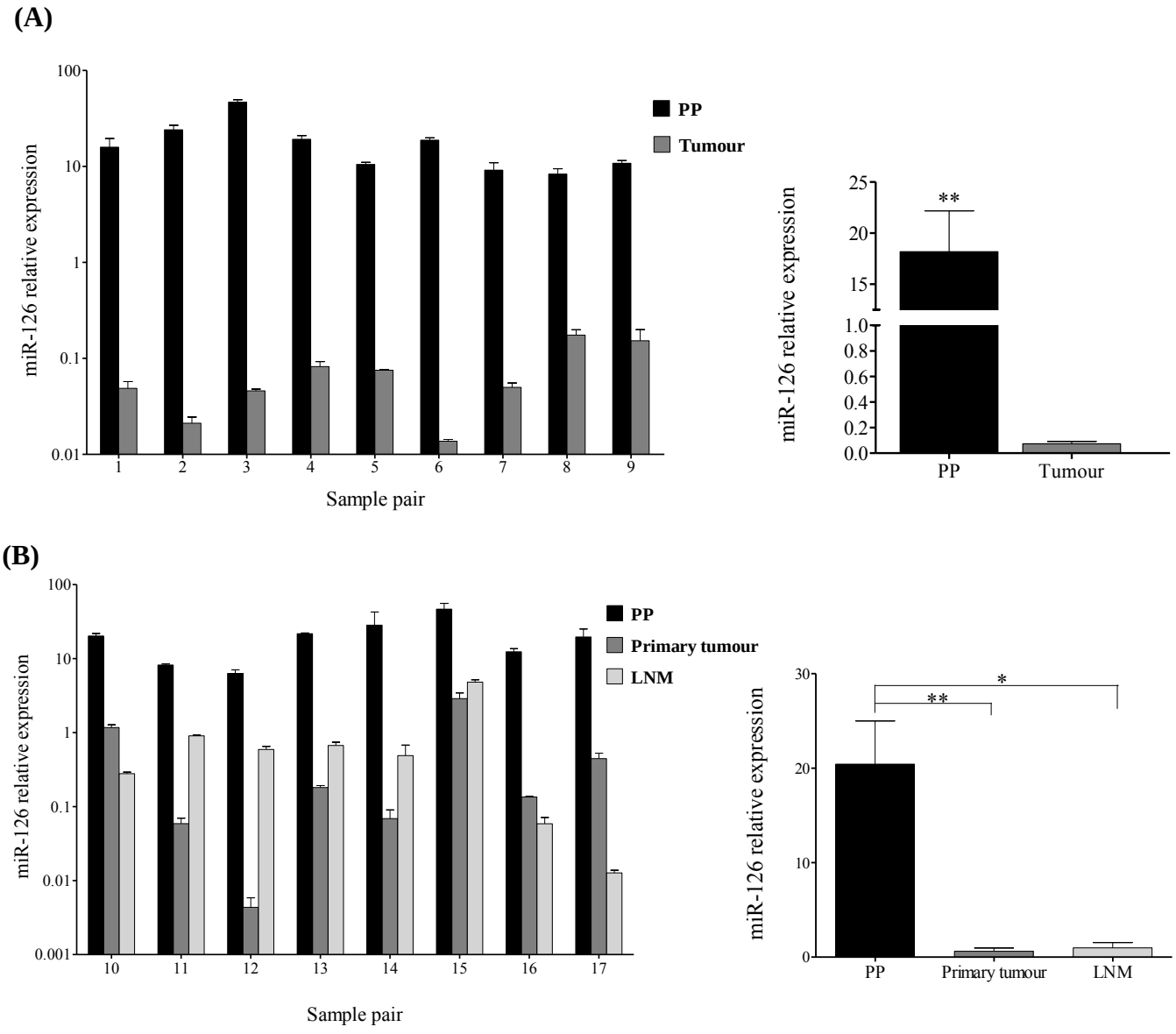

Figure 9. miR-126 expression in squamous cell carcinoma samples. (A) Expression in pulmonary parenchyma and tumour of samples without lymph node metastasis. **(B)** Expression in pulmonary parenchyma, primary tumour and lymph node metastasis. The data on the graphs in the left represent the mean value of duplicate measurements by Real-Time PCR and standard deviation (Y axis in logarithmic scale). Expression was normalized using RNUB6. The data of the graphs in the right represent mean ± SEM. (** $p<0.01$ and * $p<0.05$, for matched samples). PP – pulmonary parenchyma; LNM – lymph node metastasis

present a lower expression in all tumour samples comparatively to matched pulmonary parenchyma, a difference that was highly significant. A similar analysis was performed for samples presenting lymph node metastasis and miR-126 expression and this type of cells was also evaluated (Fig. 9B). Consistently to what was observed for the samples without lymph node metastasis, all primary tumours showed a decreased miR-126 expression comparatively to pulmonary parenchyma. Lymph node metastasis also had lower levels of miR-126 than matched non-tumour tissue across all samples. However, the expression of miR-126 in primary tumours and matched lymph node metastasis did not present such a clear pattern: whereas for some samples its expression was lower in primary tumours, for others the opposite situation was observed. No significant differences were found between the two types of tissue. For further evaluation of the role of miR-126 in metastatic potential, its expression was compared in primary tumours of samples with and without lymph node metastasis (Fig. 10). No significant differences were found between these carcinomas (p=0.1139).

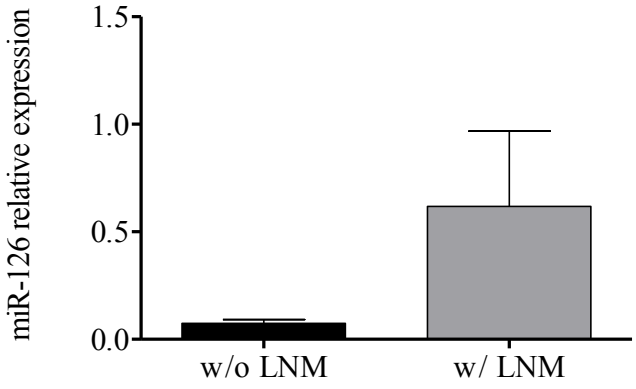

Figure 10. miR-126 expression in squamous cell carcinoma with and without lymph node metastasis. Bars represent mean ± SEM. w/o LNM – without lymph node metastasis; w/ LNM – with lymph node metastasis

Similarly to the analysis made for miR-126, the expression of miR-126* was also evaluated in the different types of tissue of samples without (Fig. 11A) and with lymph node metastasis (Fig. 11B). As observed for its complement, miR-126* expression was found to be decreased in all primary tumours comparatively to matched

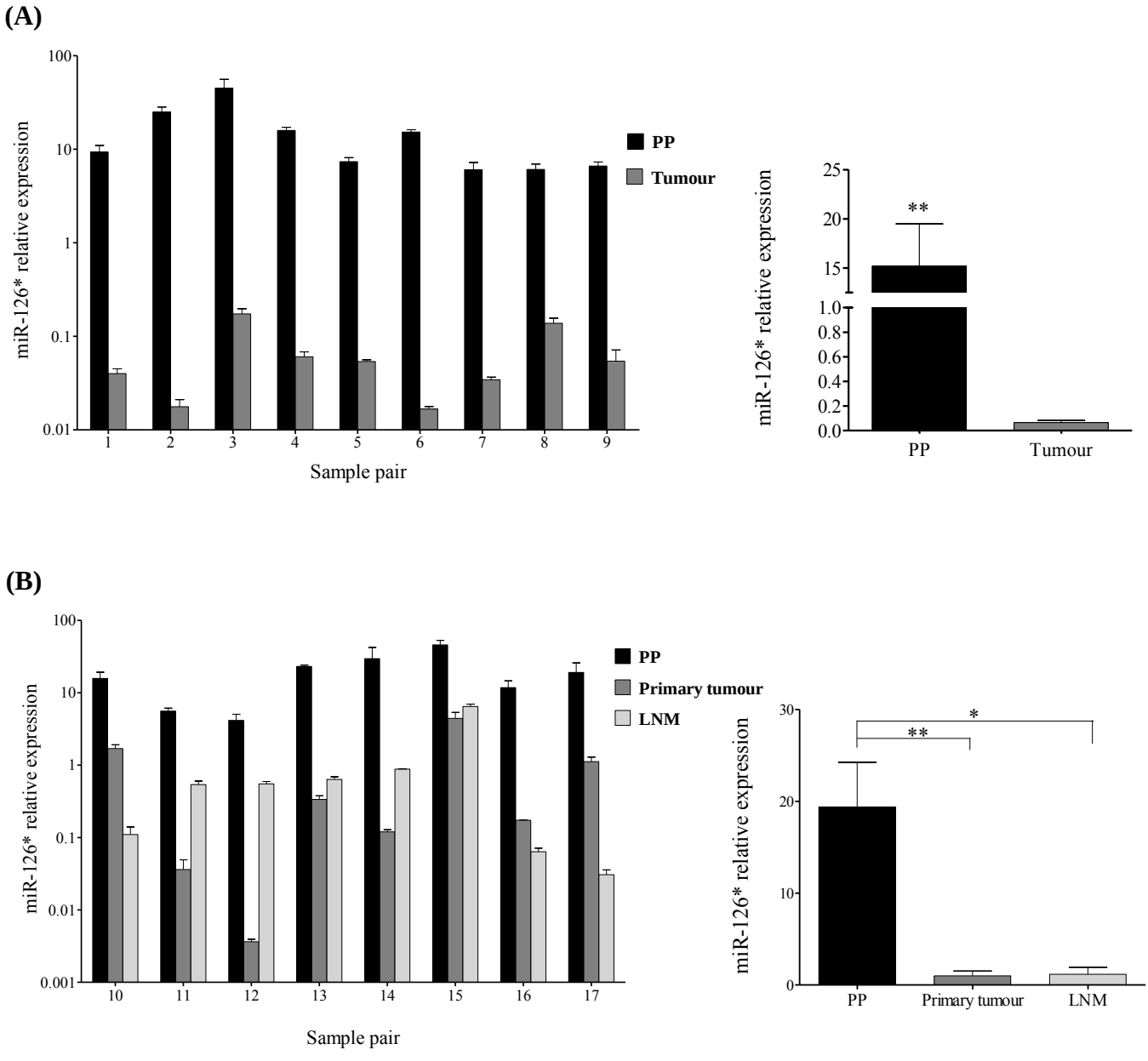

Figure 11. miR-126* expression in squamous cell carcinoma samples. (A) Expression in pulmonary parenchyma and tumour of samples without LNM. **(B)** Expression in pulmonary parenchyma, primary tumour and LNM. The data on the graphs in the left represent the mean value of duplicate measurements by Real-Time PCR and standard deviation (Y axis in logarithmic scale). Expression was normalized using RNUB6. The data of the graphs in the right represent mean ± SEM. (** $p<0.01$ and * $p<0.05$, for matched samples). PP – pulmonary parenchyma; LNM – lymph node metastasis

pulmonary parenchyma, the same being verified in lymph node metastasis. Also resembling what was observed for miR-126, miR-126* had no consistent alterations in lymph node metastasis when compared to primary tumours and no significant differences were detected between carcinomas with and without lymph node metastasis (p=0.0927) (Fig.12).

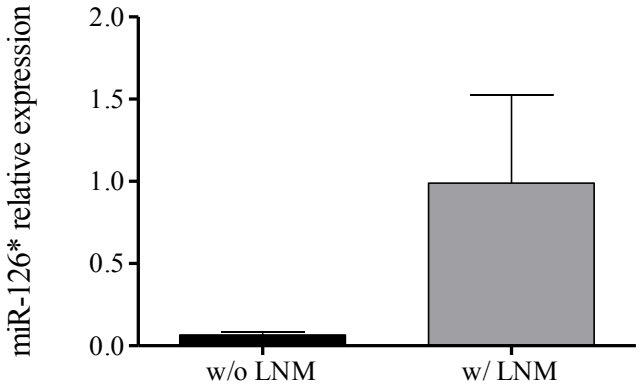

Figure 12. miR-126* expression in squamous cell carcinomas with and without lymph node metastasis. Bars represent mean ± SEM. w/o LNM – without lymph node metastasis; w/ LNM – with lymph node metastasis

Expression of miR-126 and miR-126* was also evaluated according to the age of the patients (equal or inferior to 70 years/superior to 70 years) but no significant differences were found (p=0.8651 for miR-126 and p=0.6919 for miR-126*). Gender differences were not analysed due to reduced number of female patients.

miR-126 and miR-126* expression in pulmonary adenocarcinoma

The parameters considered in squamous cell carcinoma were also studied in adenocarcinoma for both miR-126 and miR-126*. miR-126 expression was lower in all

samples of tumours without lymph node metastasis relatively to pulmonary parenchyma (Fig. 13A) and this decrease was highly significant. This was also verified in samples

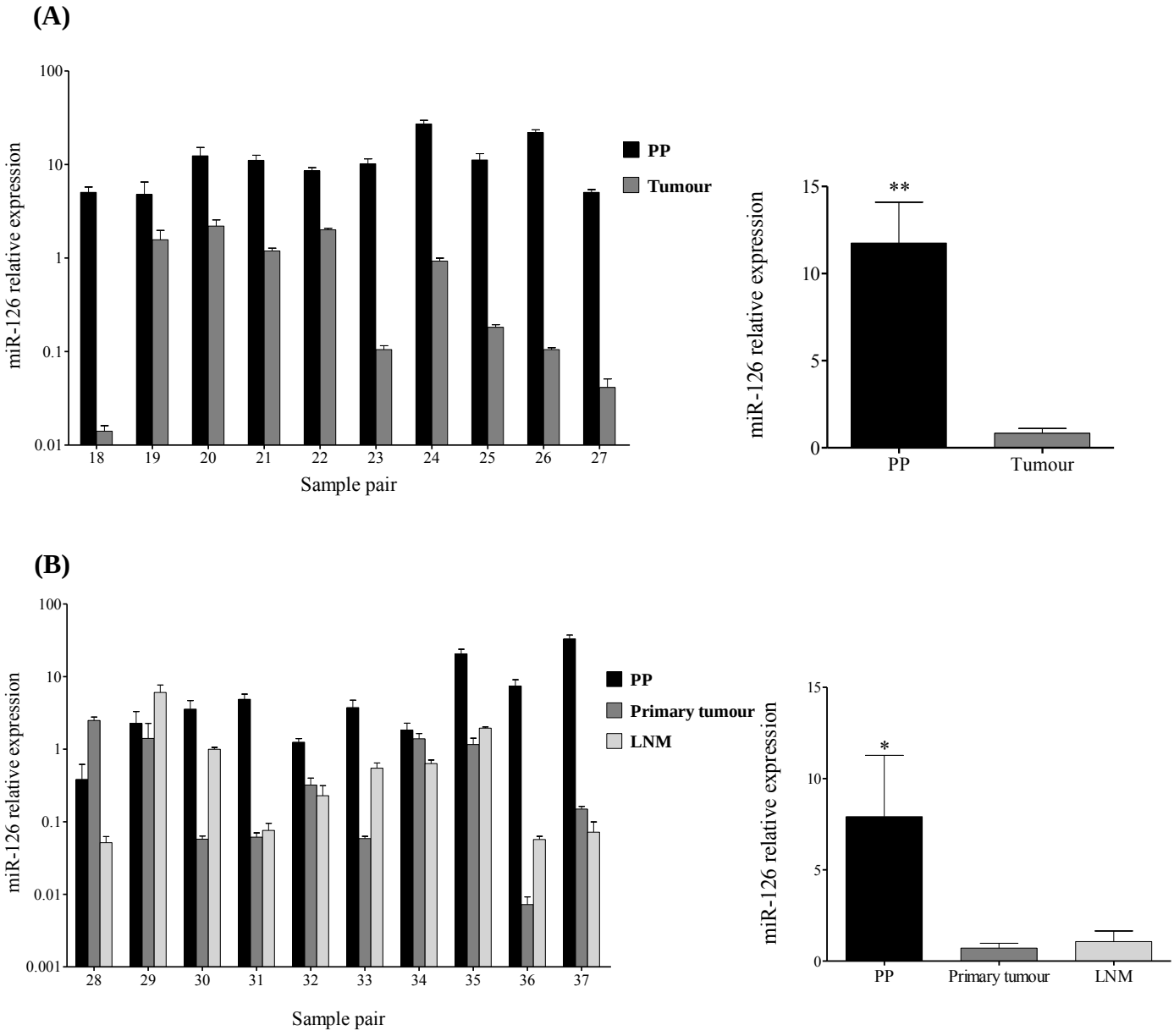

Figure 13. miR-126 expression in adenocarcinoma. (A) Expression in pulmonary parenchyma (normal) and tumour of samples without lymph node metastasis. **(B)** Expression in pulmonary parenchyma (normal), primary tumour and lymph node metastasis (LNM). The data on the graphs in the left represent the mean value of duplicate measurements by Real-Time PCR and standard deviation (Y axis in logarithmic scale). Expression was normalized using RNUB6. The data of the graphs in the right represent mean ± SEM. (** $p<0.01$ and * $p<0.05$, for matched samples). PP – pulmonary parenchyma; LNM – lymph node metastasis

with lymph node metastasis, although one of them presented higher miR-126 expression in the tumour (Fig. 13B). Lymph node metastasis presented a wide variation of miR-126 expression, as observed for squamous cell carcinoma, and significant differences were only found relatively to matched parenchyma (Fig. 13B). Primary tumours from patients with and without metastasis also presented no differences in miR-126 expression (Fig. 14). The same results were obtained for miR-126* when comparing their expression in the different types of tissue (Fig. 15 and Fig. 16).

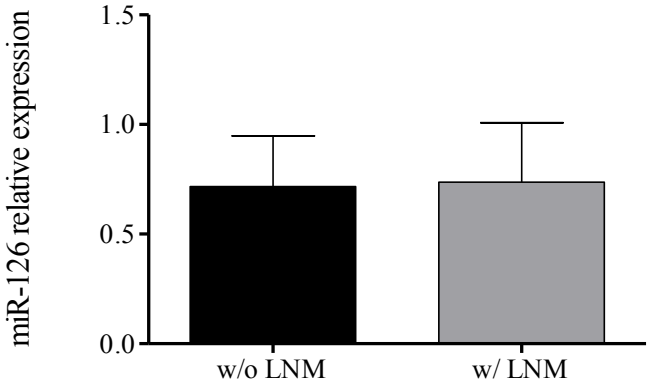

Figure 14. miR-126 expression in adenocarcinomas with and without lymph node metastasis. Bars represent mean ± SEM. w/o LNM – without lymph node metastasis; w/ LNM – with lymph node metastasis

No differences were found according to age (p=0.3641 for miR-126 and p=0.4833 for miR-126*). However, when comparing the expression of miR-126 in tumours from female and male patients, a more pronounced decrease was found to be associated to female gender (Fig. 17). This was not significant for miR-126* when not taking the expression in parenchyma in consideration. Nonetheless, the difference was almost significant (p=0.0575).

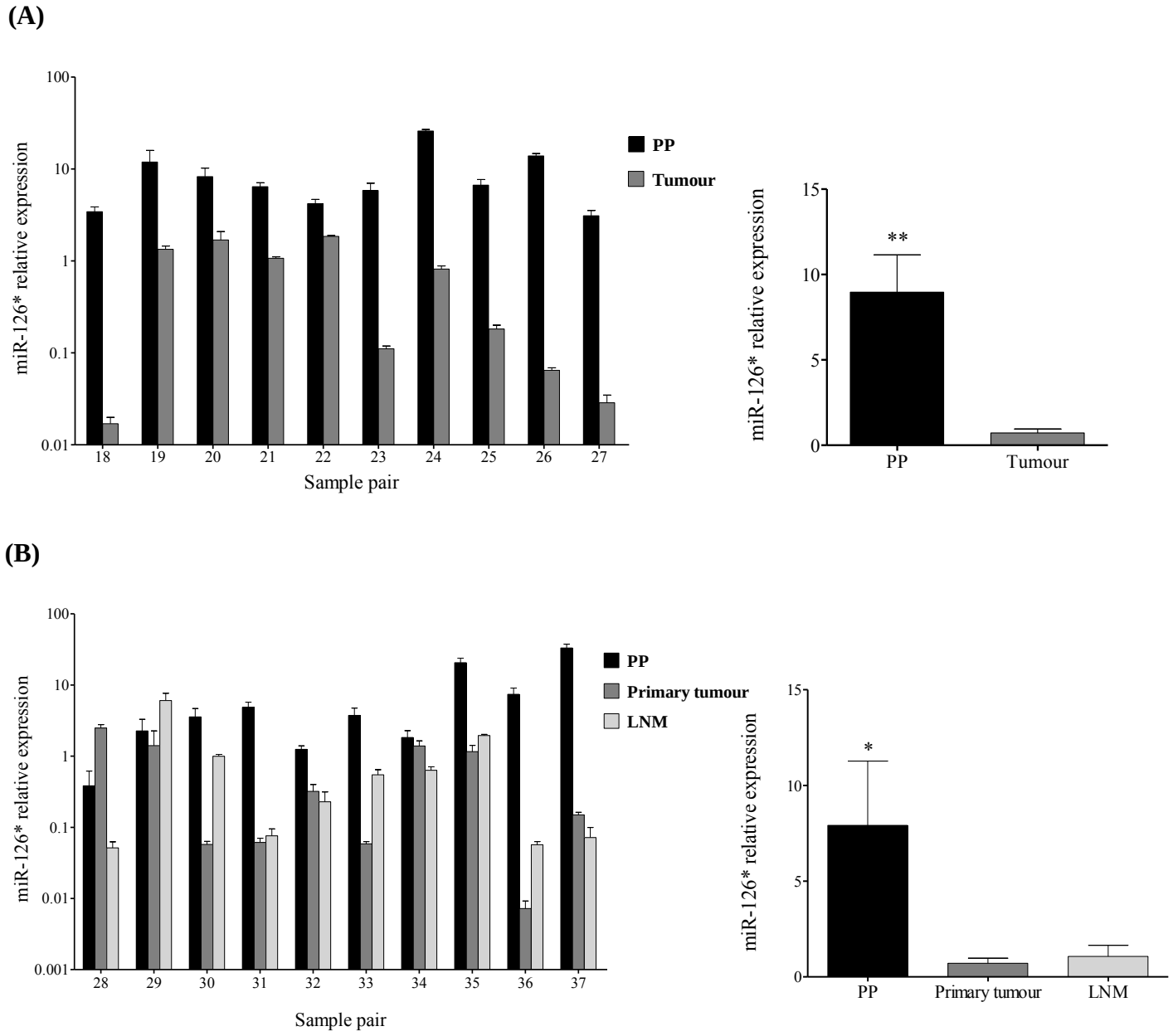

Figure 15. miR-126* expression in adenocarcinoma samples. (A) Expression in pulmonary parenchyma and tumour of samples without LNM. **(B)** Expression in pulmonary parenchyma, primary tumour and LNM. The data on the graphs in the left represent the mean value of duplicate measurements by Real-Time PCR and standard deviation (Y axis in logarithmic scale). Expression was normalized using RNUB6. The data of the graphs in the right represent mean ± SEM. (** $p<0.01$ and * $p<0.05$, for matched samples). PP – pulmonary parenchyma; LNM – lymph node metastasis

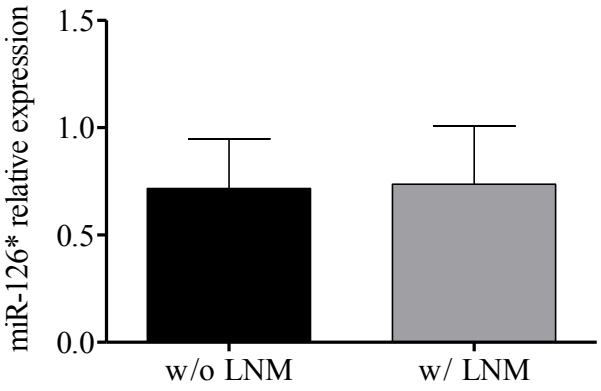

Figure 16. miR-126* expression in adenocarcinomas with and without lymph node metastasis. Bars represent mean ± SEM. w/o LNM – without lymph node metastasis; w/ LNM – with lymph node metastasis

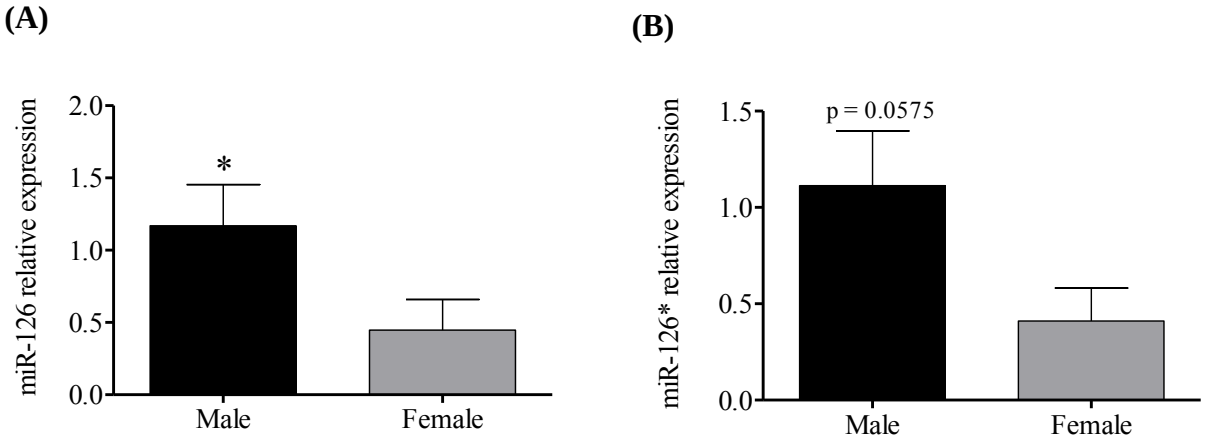

Figure 17. Adenocarcinomas according to gender. Expression of **(A)** miR-126 and **(B)** miR-126* in adenocarcinomas from female and male patients. Bars represent mean ± SEM. (* $p<0.05$)

Differences in miR-126 and miR-126* expression between squamous cell carcinoma and adenocarcinoma

Since adenocarcinomas and squamous cell carcinomas are very different types of carcinoma, we compared the expression of miR-126 and miR-126* in the primary

tumours of these two histological types. This analysis was made only for male gender. miR-126 and miR-126* presented lower expression in squamous cell carcinomas than in adenocarcinomas (Fig. 18). We then compared the samples of pulmonary parenchyma according to three groups: male patients with squamous cell carcinoma, male patients with adenocarcinoma and female patients with adenocarcinoma. Pulmonary parenchyma of male patients with adenocarcinoma presented a lower expression of both miR-126 and miR-126* than the two other groups (Fig. 19).

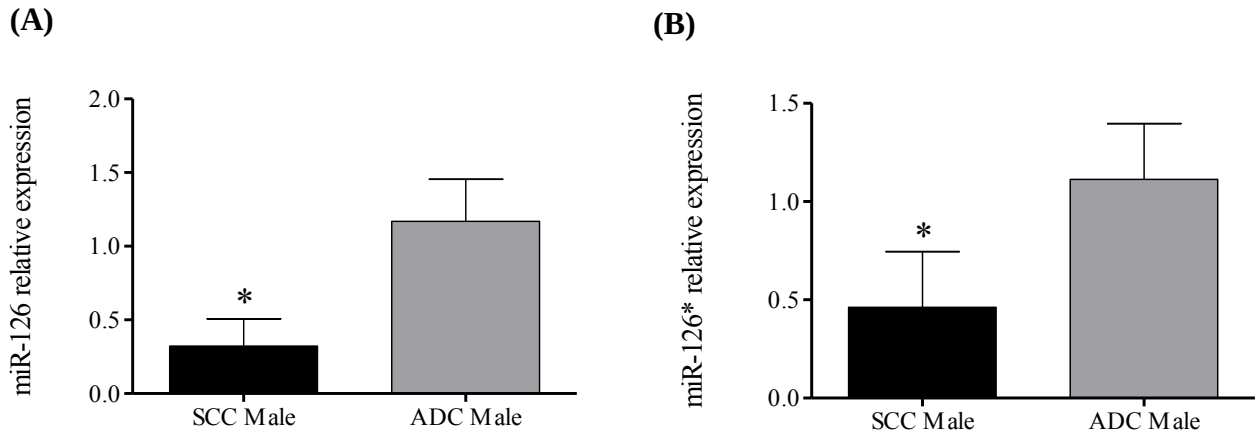

Figure 18. Tumours from male patients according to histological type. (A) Expression of miR-126 and **(B)** miR-126* in tumours. Bars represent mean ± SEM. (* $p<0.05$) SCC – squamous cell carcinoma; ADC – adenocarcinoma.

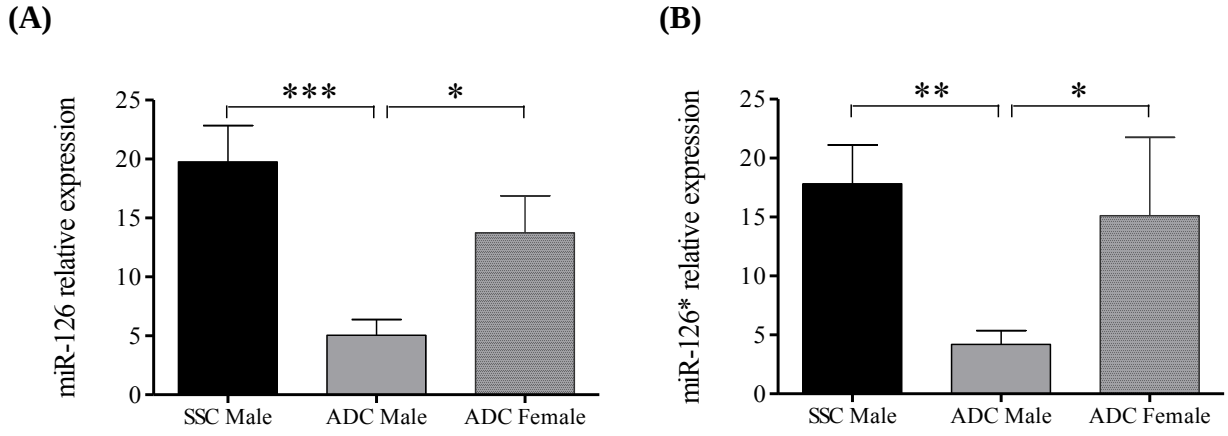

Figure 19. Pulmonary parenchyma according to gender and histological type. (A) Expression of miR-126 and **(B)** miR-126* in pulmonary parenchyma. Bars represent mean ± SEM. (*** $p<0.001$, ** $p<0.01$ and * $p<0.05$) SCC – squamous cell carcinoma; ADC – adenocarcinoma.

Compared expression of miR-126 and miR-126*

Since miR-126 and miR-126* are processed from the same precursor, we made a comparative analysis of their expression considering five types of tissue: pulmonary parenchyma, squamous cell carcinoma primary tumour, adenocarcinoma primary tumour, lymph node metastasis of squamous cell carcinoma and lymph node metastasis of adenocarcinoma (Fig. 20). No significant differences were found in any of these tissues.

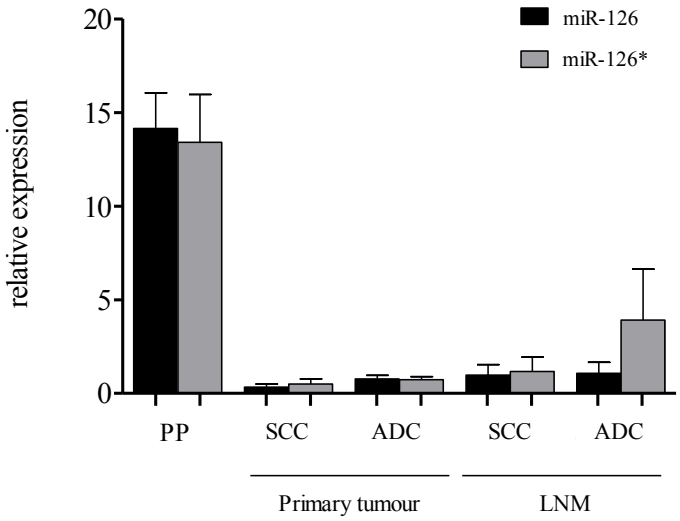

Figure 20. Compared expression of miR-126 and miR-126*. Expression in pulmonary parenchyma (PP), and primary tumour and lymph node metastasis (LNM) of squamous cell carcinoma (SCC) and adenocarcinoma (ADC). Comparison was made considering the matched values of miR-126 and miR-126* for each case. Bars represent mean ± SEM.

EGFL7 promoter methylation

PCR was performed using the primers designed with MethPrimer software. However, we did not succeed on amplifying the control for the unmethylated sequence in any of the conditions tested.

Discussion

Squamous cell carcinoma and adenocarcinoma are the most prevalent types of lung cancer (25% and 40%, respectively). Although they have been previously categorized in the same broad group of non-small cell lung cancer, this classification is nowadays not considered to be significant or useful, since the carcinomas of this group present several differences both at the molecular and histological levels and regarding therapy [54]. Realizing that squamous cell carcinomas and adenocarcinomas are so different brings the need of a more deep and detailed characterization in order to elucidate the mechanism underlying their development and the potential clinical implications of such studies.

microRNAs are a broad family of small regulatory RNA molecules that act at the post-transcriptional level and whose importance in carcinogenesis has been recently discovered. In the last years they have been a major focus of cancer research and their central role in carcinogenesis is now widely recognized. microRNA alterations in cancer and the molecular consequences have been approached for several types of cancer and tumours at different stages of progression [9] [46] [80]. Lung cancer is no exception and in fact it was one of the first types of cancer for which cancer-related microRNAs have been identified [44]. Several microRNAs present altered expression in lung cancer, directly altering the levels of a wide range of targets. microRNA profiles have been proven to be very useful in lung cancer diagnosis and prognosis, as well as to distinguish different histological types of cancer [70].

miR-126 and miR-126* are two microRNAs that share a common precursor and that have been implicated in lung cancer, with reports of decreased expression in NSCLC [68] [76]. In addition, miR-126 is underexpressed in highly metastatic breast cancer cell lines and its expression has been shown to be lower in the breast tumours of patients that suffered metastatic relapse than in those who did not [50]. However, their

differential expression in adenocarcinoma and squamous cell carcinoma lung cancer has not been reported. Likewise, information is lacking regarding a role of these microRNAs on metastatic potential of lung carcinomas.

A striking characteristic of microRNAs is that total complementarity to the target mRNA is not usually required for silencing to occur. As a result, most microRNAs are predicted to have several targets [38]. This potential to regulate such a wide range of mRNAs makes microRNAs central players in the main cellular processes [39]. However, knowledge on the precise rules that define a mRNA as a microRNA target is still lacking. Consequently, the bioinformatic tools used are still not refined enough as to accurately identify the targets of microRNAs. Although these tools are widely used for target prediction, they often present false positives and thus this information must be experimentally validated [81]. Nonetheless, despite their inaccuracies, bioinformatic approaches have been deeply valuable for predicting the role of microRNAs in cellular regulation. In order to determine whether miR-126 and miR-126* are potential tumour- and metastasis-suppressors in lung cancer, their putative targets were determined using bioinformatic approaches. 3 softwares were used for target prediction: miRanda, PicTar and TargetScan, according to Elton and colleagues [81]. These algorithms are based in different characteristics of microRNAs and targets and therefore predict different binding sites [82]. Three of the predicted targets, protein tyrosine phosphatase, non-receptor type 9 (PTPN9), v-crk sarcoma virus CT10 oncogene homolog (CRK) and solute carrier family 7 member 5 (SCL7A5) have already been confirmed to be direct targets of miR-126 [76] [83] [84]. Polo-like kinase 2 (PLK2) has not been experimentally validated, but its expression inversely correlates to miR-126 expression [85]. Several of the predicted targets are known to be involved in cancer development and progression, such as regulator of G-protein signaling 3 (RGS3),

PLK2 and histone deacetylase 4 (HDAC4) [86] [87] [88]. Plus, some of them have been specifically implicated in lung carcinoma. For instance, ADAM metallopeptidase domain 9 (ADAM9) expression has been found to correlate with NSCLC metastasis to the brain [89] and HOXC8 is involved in lung cancer progression [90]. In addition, both ADAM9 and homeobox C8 (HOXC8) have been shown to alter the expression of angiogenic factors [91] [92]. However, it should be noted that some of the miR-126 targets in this list also present miR-126* binding sites: PTPN9, KN motif and ankyrin domains 2 (KANK2), ADAM9, PLK2, F-box protein 33 (FBXO33) have at least one of these sites, although they present lower scores (predicted by miRanda). It is important to keep in mind that putative targets predicted by only one program and presenting low scores have been experimentally confirmed. Such is the case of solute carrier family 45 member 3 (SLC45A3), for instance, which has been proven to be directly targeted by miR-126* [79]. Thus, it is possible that these can be regulated by both miR and miR* strands. Likewise, it is highly probable that these microRNAs target many other molecules not not predicted with the parameters we considered (for example, phosphoinositide-3-kinase, regulatory subunit, polypeptide 2 p85 beta (PIK3R2), vascular endothelial growth factor A (VEGF-A) and insulin receptor substrate 1 (IRS-1) are validated targets of miR-126) [77] [93] [94].

Taking into account the predicted targets of miR-126 and miR-126* and the reported evidence of their role in angiogenesis and in breast cancer metastasis, we considered that these microRNAs could be potential metastasis-suppressors in pulmonary squamous cell carcinoma and adenocarcinoma. In these carcinomas, lymph node metastasis correlate with survival and are therefore an important prognostic factor [95]. We therefore intended to compare miR-126 and miR-126* expression in (1) pulmonary parenchyma, primary tumour and lymph node metastasis, for both squamous

cell carcinoma and adenocarcinoma, (2) tumours with and without lymph node metastasis, (3) tumours of patients with squamous cell carcinoma and with adenocarcinoma. The elucidation of these parameters could not only contribute for understanding the molecular mechanisms underlying lung cancer progression but also be important for diagnosis and prognosis. By determining differences in miR-126/miR-126* expression in primary tumours and lymph node metastasis, it would be possible to unravel potential layers of gene expression regulation that are altered in these cells and that would help explain the features of the tumour. Comparing their expression in adenocarcinoma and squamous cell carcinoma could also determine their potential as biomarkers.

Tumours are now recognized as highly heterogeneous entities, whose development and progression is strongly influenced by the respective supporting stroma. The tumour microenvironment comprises a broad variety of components in the extracellular matrix such as fibroblasts, immune and inflammatory cells and blood-vessel cells [96]. When characterising a tumour, it is thus important to keep in mind its different components and the interactions they establish. Most studies of microRNA expression in tumours, namely those regarding miR-126 and miR-126*, evaluate expression using the bulk tumoural tissue, without taking in consideration the kind of cells that are collected (whether they are tumoural or stromal cells). In this study, we intended to characterize the expression of these microRNAs exclusively in neoplastic cells. Laser microdissection uses an UV-A laser focused through the microscope to promote ablation of cells and tissue by photofragmentation and represents a reliable and accurate way to collect specific types of cells since it allows the visualization of the tissue under the microscope while selecting the areas to be removed. Therefore, this

technique was used to obtain the cells from pulmonary parenchyma, primary tumours and lymph node metastasis.

Due to their small size, evaluating microRNA expression can be a challenging task and it is important to choose the best technique for each case. Many studies perform microarrays analysis, which allows the study of several microRNAs at once. However, this should be only used to obtain a general view of dysregulated microRNAs. More quantitative methods should be employed to confirm the levels of specific microRNAs [97]. We therefore evaluated the expression of miR-126 and miR-126* by real-time PCR using SYBR Green detection. SYBR Green is a double-stranded DNA-binding agent that fluoresces when bound to DNA. Quantification of the samples can thus be made by measuring fluorescence after each extension step. U6 snRNA (RNUB6, 45 nt) was used as an endogenous control and normalizing. This snRNA is widely used due to its small size, namely in studies on microRNA expression in lung cancer [68] [69].

miR-126 and miR-126* expression in pulmonary squamous cell carcinoma

To evaluate the correlation between miR-126 and miR-126* and the tumourigenic and metastatic potential of squamous cell carcinoma, we compared the microRNA expression in cells from pulmonary parenchyma, primary tumour and lymph node metastasis. The decrease found for miR-126 and miR-126* expression between parenchyma and tumour is in accordance to what had been previously described [68] [76]. However, no differences were found either between primary tumours and matching lymph node metastasis or between primary tumours of samples with and without lymph node metastasis. These results support previous evidence that miR-126 and miR-126* play a role in tumourigenesis but suggest that they are particularly

relevant in the tumourigenesis of primary lung tumour and not in the spread and colonization of other organs. This differs from what has been described for breast cancer [50], which could be explained by the fact that microRNAs can play different roles in different tumours. A possible explanation for that comes from the work of Marks and colleagues, showing that the activity of a microRNA depends on the abundance of its target [98]. Consequently, different environments could lead an mRNA to be a target in a certain type of tumour and not a target in other type.

miR-126 and miR-126* expression in pulmonary adenocarcinoma

The results obtained for miR-126 and miR-126* expression in adenocarcinoma in pulmonary parenchyma, primary tumours and lymph node metastasis resemble those verified for squamous cell carcinoma. Therefore, it seems that these microRNAs are also involved in tumourigenesis in this type of carcinoma but have not a prominent role in metastatic potential. miR-126 and miR-126* expression was also found to be different according to gender, a distinction that was not possible to evaluate for squamous cell carcinoma due to lack of a representative number of samples from both genders. Both microRNAs presented a more decresed expression in the female gender. A deeper knowledge on the molecular mechanism of miR-126 and miR-126 activity could help understand this difference. Similar patterns have been observed for estrogen receptors, for example, which have been proposed to be one of the causes of the differences of adenocarcinoma between genders [99]. It should be noticed that although no differences were found between miR-126 and miR-126* expression, only miR-126* expression was not significantly different in male and female patients when tumour

expression was compared. However, the p-value associated to this difference was close to a significant value.

Differences in miR-126 and miR-126* expression between squamous cell carcinoma and adenocarcinoma

After detecting the decrease of miR-126 and miR-126* in both types of carcinoma, a comparison was made between squamous cell carcinoma and adenocarcinoma. Since a difference between tumours from female and male patients had been previously detected, we performed this analysis according to gender. Only tumours from male patients were analysed, due to the lack of samples of squamous cell carcinomas from female patients. The differences found between squamous cell carcinomas and adenocarcinomas show that these microRNAs are not equally expressed in these types of tumour and could therefore be partly responsible for the different traits they present.

A decreased expression of miR-126 and miR-126* was also found in pulmonary parenchyma from males with adenocarcinoma comparatively to males with squamous cell carcinoma and females with adenocarcinoma. This becomes particularly relevant considering that adenocarcinoma of female patients present a lower expression of these microRNAs. Two conclusions come out from these results. First, taking into account that adenocarcinomas from female patients presented lower expression of miR-126/miR-126* than those from male patients, these microRNAs seem to be important players of carcinogenesis mainly in women. Another very important conclusion regards the use of pulmonary parenchyma as non-tumoural tissue with which tumour tissue is compared. Squamous cell carcinomas usually develop in the bronchial epithelium after epidermoid metaplasia, but adenocarcinomas develop mainly in the terminal respiratory

unit (TRU) and it is thus possible that pulmonary parenchyma that appears histologically normal already presents molecular changes that predispose to carcinogenesis. In fact, our results support that hypothesis, since pulmonary parenchyma from patients with squamous cell carcinomas presented higher expression of both miR-126 and miR-126*. Therefore, miR-126 and miR-126* loss correlates with the development of a predisposing field to adenocarcinoma.

Most studies of miR-126 overexpression in lung cancer cell lines have used adenocarcinoma cells [77] [78] and described a decrease in cell proliferation. Interestingly, a study on which a squamous carcinoma cell line was used, reported that miR-126 had no effect on cell proliferation [76]. However, these results are usually discussed considering miR-126 effects on lung cancer, without making any distinction between squamous cell carcinoma and adenocarcinoma. Considering the results we obtained, it becomes clear the importance of taking this distinction into account. Similarly, studies validating miR-126 targets should be carried on in these two cell lines and not be extrapolated for both histological types.

Compared expression of miR-126 and miR-126*

During processing, microRNAs acquire a duplex structure due to partial self-complementarity. When associating to RISC, this duplex is unwound and one of the strands is selected for target silencing. However, the principles underlying such selection remain elusive. Usually, only one of the strands is used in this process and the other, named miR*, is degraded. Nevertheless, it has become clear that in many cases the other strand can also be used in silencing, although usually on a less extent. This process is differentially regulated across tissues [25].

Although both microRNAs have been studied in lung cancer, most studies approach miR-126 and no extensive studied has been carried comparing the expression of the two microRNAs. We therefore compared the expression of miR-126 and miR-126* in pulmonary parenchyma and in primary tumours and lymph node metastasis from each type of carcinoma. The results obtained suggest that miR-126 and miR-126* are equally express in the lung. Therefore this miR* might also be an important factor on gene regulation in this tissue, as already verified for prostate cancer [79]. The fact that the two microRNAs are underexpressed in cancer and present similar patterns of expression suggests that an event anterior to RISC assembly could be one of the causes for their downregulation. One of such mechanisms could be methylation of the host gene, *EGFL7*.

These results have important implications when considering the targets silenced through pre-miR-126 expression. So far, most studies on the targets of miR-126 used a vector with a precursor form that can originate both microRNAs, miR and miR*, in a biologically active form. It is thus possible that some of the mRNAs identified as miR-126 targets are in fact regulated by miR-126*. Plus, the effects seen when overexpressing the these vectors on tumourigenic cell lines could be partly due to miR-126*. It is therefore important that miR-expressing vectors that originate only one active strand be used when trying to determine specific targets of miR-126 (or miR-126*), instead of vectors expressing the precursor form of the microRNA.

EGFL7 promoter methylation

Hypermethylation of tumour suppressor genes has been proved to be an important event in cancer. This process occurs mainly in the promoters of those genes,

in CpG-enriched regions. The importance of methylation in this carcinogenesis is clear in studies where the use of demethylating drugs leads to partial restoration of expression of genes that are silenced in cancer cells [8]. In lung carcinoma, the methylation of some promoters is associated with prognosis [100]. Several studies have approached microRNA genes methylation and uncovered some expression patterns that could be useful as biomarkers [101].

We therefore intended to characterize adenocarcinoma and squamous cell carcinoma according to miR-126/miR-126* host gene promoter methylation. These microRNAs are processed from the same precursor and thus have the same host gene, *EGFL7*. Esteller and colleagues have shown that it presents in several normal tissues, such as colon and skin [52]. Its methylation status in lung, however, was not studied. Given the acknowledged importance of miR-126 downregulation in lung cancer, it is thus possible that its host gene presents hypermethylation in lung tumour cells. We decided to perform methylation analysis with MS-PCR. This technique is based on sodium bisulfite treatment followed by PCR using 2 sets of primers, one for the methylated sequence and one for the unmethylated sequence. At high temperatures and low pH, sodium bisulfite converts unmethylated cytosines to uracil, leaving methylated residues unmodified. Consequently, after treating a DNA sample with bisulfite, two main sequences can result, according to the methylation pattern. PCR is then performed, using primers for the same region, but one designed for the original sequence and other designed for the sequence with uracil replacing cytosine.

Since the second CpG island would be very difficult to amplify, especially from paraffined samples, due to its size, we designed primers for the first region. Two sets of primers were designed, with 2 softwares. However, the sequences obtained presented low levels of GC content and some poly(T) (especially the primers for the

unmethylated sequence) which makes the PCR reaction more difficult to perform. In fact, when PCR was performed for both methylated and unmethylated controls, no amplification was obtained for the unmethylated sequence. This inefficiency was verified even when changing several PCR parameters, such as annealing temperature, Mg^{2+} and primer concentration and addition of a pre-amplification step. Therefore, we could not proceed with the methylation analysis of the DNA already extracted and bisulfite-converted from microdissected samples. Recently, the occurrence of *EGLF7* methylation in primary tumours of NSCLC has been reported, although in a small percentage of cases [102]. This study was performed with bisulfite sequencing, thus avoiding the issue of designing MS-PCR primers for the CpG region. In fact, the authors also attempted to perform MS-PCR and report its inefficiency due to problems on primer design. We can therefore conclude that for methylation studies of miR-126/miR-126* host gene MS-PCR is not a suitable technique and others should be considered.

Closing Remarks

Conclusions

In this study, miR-126 and miR-126* were found to be downregulated in both pulmonary squamous cell carcinoma and adenocarcinoma, pointing to a role in tumourigenesis, in accordance to what has already been described. However, they do not seem to be involved in metastatic potential of these carcinomas, contrary to what has been found for miR-126 in breast cancer. miR-126 and miR-126* presented a more marked decrease in squamous cell carcinoma, which leads to the hypothesis that they could be partly responsible for the differences between the two histological types and therefore be useful biomarkers. In adenocarcinoma, both microRNAs presented lower expression in female than in male patients. Pulmonary parenchyma from patients with adenocarcinoma presented less expression than from patients with squamous cell carcinoma, suggesting that miR-126 and miR-126* are important in the early steps of adenocarcinoma carcinogenesis. Regardless of the type of tissue (pulmonary parenchyma, squamous cell carcinoma or adenocarcinoma), miR-126 and miR-126* presented similar expression, which suggests that their downregulation occurs at a precursor level and indicates that miR-126* might also play an important role in tumorigenesis. A possible mechanism of downregulation could be methylation of the host gene promoter. However, MS-PCR is not a feasible technique for this particular case, due to difficulties on effective primer design.

Future Perspectives

Most samples used in this study were of stage I or II of TNM stage. Since miR-126 has been described to have different expression according to TNM stage in gastric

cancer, it would be important to perform a similar study for squamous carcinoma and adenocarcinoma of the lung.

Due to the constraints in evaluating the methylation of *EGFL7* using MS-PCR, other techniques should be considered to proceed with this study. A good alternative would be combined bisulfite restriction analysis (COBRA): first, DNA is treated with sodium bisulfite and amplified; restriction enzymes that cleave DNA in bisulfite-sensitive sites are then used. According to the methylation pattern, different fragments are then obtained and can be visualized in a gel. This technique has already been proven to be suitable for *EGFL7* promoter methylation analysis. Therefore, it would allow to evaluate the methylation status in our patient cohort and relate it to the differences we found in miR-126/miR-126* expression according to type of tissue and tumour histological type.

Having confirmed that both miR-126 and miR-126* are downregulated in squamous cell carcinoma and adenocarcinoma, analysing the effects of their overexpression in cell lines would allow a better understanding of their role in these pathologies. It is important to notice that this should carried on in at least two cell lines, one from each of the histological types. In addition, vectors should express the mature form of only one of the microRNAs (miR-126 and miR-126*), since a vector expressing both forms would make it more difficult to determine whether the observed effects are due to miR-126 or miR-126* expression or even a combination of both. After overexpression, cell proliferation, migration and invasion should be measured to assess the effect of these microRNAs in tumourigenic traits.

After studying the effects of the microRNAs in tumourigenic cell lines, it would be interesting to determine some of their targets. In order to do that, a microRNA mimic should be introduced in an adenocarcinoma/squamous carcinoma cell line and

the levels of the putative target should be determined by western blot. A decrease in the levels of the protein would be evidence that the microRNA has effects on its expression, but it would not give information on whether it is a direct or indirect target. That could be determined by using a reporter gene containing the 3' UTR of the putative target, since this is usually the region to which microRNAs bind and one of the parameters considered in bioinformatic predictions. After transfecting a vector with this gene and with the microRNA mimic, a decrease in the levels of the protein of the target would mean that microRNA would be directly exerting its effects. Additional information on that silencing could be obtained by performing real-time PCR to evaluate the levels of the target mRNA. A decrease in mRNA levels would mean that the microRNA would be regulating its expression by promoting its degradation, whereas unaltered levels of mRNA would be consistent with translation prevention.

In this study, laser microdissection was performed in order to specifically collect tumour cells, avoiding cells of the stroma. Given the recognized importance of tumour microenvironment in carcinogenesis, using this procedure to evaluate miR-126 and miR-126* levels in stroma and compare them to tumour cells could provide a more detailed characterization of carcinogenesis of these tumours.

References

[1] World Health Organization, Cancer: http://www.who.int/cancer/en/index.html (17.08.2011).

[2] Hanahan D, Weinberg RA (2011). *Hallmarks of Cancer: The Next Generation.* Cell, 144: 646-674.

[3] Baeriswyl V, Christofori G (2009). *The angiogenic switch in carcinogenesis.* Seminars in Cancer Biology, 19 (5): 329-337.

[4] Carmeliet P, Jain RK (2000). *Angiogenesis in cancer and other diseases.* Nature, 407: 249-257.

[5] Geiger TR, Peeper DS (2009). *Metastasis mechanisms*. Biochimica et Biophysica Acta, 1796: 293-308.

[6] Bird A (2002). *DNA methylation patterns and epigenetic memory*. Genes and Development, 16: 6-21.

[7] Herman JG, Baylin SB (2003). *Gene Silencing in Cancer in Association with Promoter Hypermethylation.* The New England Journal of Medicine, 349 (21): 2042-2054.

[8] Esteller M, Corn PG, Baylin SB, Herman JG (2001). *A Gene Hypermethylation Profile of Human Cancer.* Cancer Research, 61: 3225-3229.

[9] Esquela-Kerscher A, Slack FJ (2006). *Oncomirs - microRNAs with a role in cancer.* Nature Reviews Cancer, 6: 259-269.

[10] He L, Hannon GJ (2004). *MicroRNAs: Small RNAs with a big role in gene regulation.* Nature Reviews Genetics, 5: 522-531.

[11] Chalfie M, Horvitz RH, Sulston JE (1981). *Mutations that lead to reiterations in the cell lineages of* C. elegans*.* Cell, 24: 59-69.

[12] Lee RC, Feinbaum RL, Ambros V (1993). *The C. elegans Heterochronic Gene* lin-4 *Encodes Small RNAs with Antisense Complementarity to* lin-14. Cell, 75: 843-854.

[13] Carrington JC, Ambros V (2003). *Role of MicroRNAs in Plant and Animal Development*. Science, 301: 336-338.

[14] Alvarez-Garcia I, Miska EA (2005). *MicroRNA functions in animal development and human disease.* Development, 132: 4653-4662.

[15] Rodriguez A, Griffiths-Jones S, Ashurst JL (2004). *Identification of Mammalian microRNA Host Genes and Transcription Units.* Genome Research, 14: 1902-1910.

[16] Shomron N, Golan D, Hornstain E (2009). *An Evolutionary Perspective of Animal MicroRNAs and Their Targets.* Journal of Biomedicine and Biotechnology, 2009: 594738.

[17] Cai X, Hagedorn CH, Cullen BR (2004). *Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs.* RNA, 10: 1957-1966.

[18] Rooij EV, Sutherland LB, Qi X, Richardson JA, Hill J, Olson EN (2007). *Control of Stress-Dependent Cardiac Growth and Gene Expression by a MicroRNA.* Science, 316: 575-579.

[19] Winter J, Jung S, Keller S, Gregory RI, Diederichs S (2009). *Many roads to maturity: microRNA biogenesis pathways and their regulation.* Nature Cell Biology, 11: 228-234.

[20] Meister G, Landthaler M, Peters L, Chen PY, Urlaub H, Luhrmann R, Tuschl T (2005). *Identification of Novel Argonaute-Associated Proteins*. Current Biology, 15: 2149-2155.

[21] Salzman DW, Shubert-coleman J, Furneaux H (2007). *P68 RNA Helicase Unwinds the Human let-7 MicroRNA Precursor Duplex and Is Required for let-7-directed Silencing of Gene Expression.* The Journal of Biological Chemistry, 282: 32773-32779.

[22] Diederichs S, Haber DA (2007). *Dual Role for Argonautes in MicroRNA Processing and Posttranscriptional Regulation of MicroRNA Expression*. Cell, 131: 1097-1108.

[23] Khvorova A, Reynolds A, Jayasena SD (2003). *Functional siRNAs and microRNAs Exhibit Strand Bias*. Cell, 115: 209-216.

[24] Hutvagner G (2005). *Small RNA asymmetry in RNAi: Function in RISC assembly and gene regulation.* FEBS Letters, 579: 5850-5857.

[25] Ro S, Park C, Young D, Sanders KM, Yan W (2007). Tissue-dependent paired expression of miRNAs. Nucleic Acids Research, 35 (17): 5944-5953.

[26] Blow MJ, Grocock RJ, Dongen SV, Enright AJ, Dicks E, Futreal PA, Wooster R, Stratton MR (2006). *RNA editing of human microRNAs*. Genome Biology, 7: R27.

[27] Filipowicz W, Bhattacharyya SN, Sonenberg N (2008). *Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?* Nature, 9: 102-114.

[28] Brennecke J, Stark A, Russell RB, Cohen SM (2005). *Principles of MicroRNA–Target Recognition*. PLoS Biology, 3: e85.

[29] Heale BS, Jr OS, Aagaard L, Alluin J, Rossi JJ (2007). *Distance constraints between microRNA target sites dictate efficacy and cooperativity.* Nucleic Acids Research, 35: 2333-2342.

[30] Didiano D, Hobert O (2006). *Perfect seed pairing is not a generally reliable predictor of microRNA-target interactions*. Nature Structural & Molecular Biology, 13: 849-851.

[31] Didiano D, Hobert O (2008). *Molecular architecture of a microRNA-regulated 3′ UTR*. RNA, 14: 1297-1317.

[32] Lytle JR, Yario TA, Steitz JA (2007). *Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5′ UTR as in the 3′ UTR.* Proceedings of the National Academy of Sciences, 104: 9667-9672.

[33] Selbach M, Schwanhausser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N (2008). *Widespread changes in protein synthesis induced by microRNAs*. Nature, 455: 58-63.

[34] Lewis BP, Burge CB, Bartel DP (2005). *Conserved Seed Pairing, Often Flanked by Adenosines, Indicates that Thousands of Human Genes are MicroRNA Targets*. Cell, 120:15-20.

[35] Bartel DP (2009). *MicroRNAs: Targer Recognition and Regulatory Functions.* Cell, 23: 215-233.

[36] Lewis BP, Shih I, Jones-Rhoades MW, Bartel DP, Burge CB (2003*) Prediction of Mammalian MicroRNA Targets.* Cell, 115: 787-798.

[37] Friedman RC, Farh KK, Burge CB, Bartel DP (2009). *Most mammalian mRNAs are conserved targets of microRNAs.* Genome Research, 19: 92-105.

[38] Rajewsky N (2006). *microRNA target predictions in animals*. Nature Genetics, 38(Suppl): S8-S13.

[39] Inui M, Martello G, Piccolo S (2010). *MicroRNA control of signal transduction*. Nature Reviews Molecular Cell Biology, 11: 252-263.

[40] Calin GA, Dumitru CA, Shimizu M, Bichi R, Zuppo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, Rassenti L, Kipps T, Negrini M, Bulltich F, Croce CM (2002). *Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia.* Proceedings of the National Academy of Sciences, 99: 15524-15529.

[41] Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, Shimizu M, Rattan S, Bulrrich F, Negrini M, Croce CM (2004). *Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers*. Proceedings of the National Academy of Sciences, 101: 2999-3004.

[42] Karube Y, Tanaka H, Osada H, Tomida S, Tatematsu Y, Yanagisawa K, Yatabe Y, Takamizawa J, Miyoshi S, Mitsudomi T, Takahashi T (2005). *Reduced expression of Dicer associated with poor prognosis in lung cancer patients.* Cancer Science, 96: 111-115.

[43] Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR (2005). *MicroRNA expression profiles classify human cancers.* Nature, 435: 834-838.

[44] Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, Harano T, Yatabe Y, Nagino Y, Mitsudomi T, Takahashi T (2004). *Reduced Expression of the let-7 MicroRNA in Human Lung Cancers in Association with Shortened Postoperative Survival.* Cancer Research, 64: 3753-3756.

[45] Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR (2005). *MicroRNA expression profiles classify human cancers.* Nature, 435: 834-838.

[46] Ma L, Weinberg RA (2008). *Micromanagers of malignancy: role of microRNAs in regulating metastasis.* Trends in Genetics, 24: 448-456.

[47] Huang Q, Gumireddy K, Schrier M, Sage C, Nagel R, Nair S, Egan DA, Li A, Huang G, Klein-Szanto AJ, Gimotty PA, Coukos G, Zhang L, Puré E, Agami R (2008). *The microRNAs miR-373 and miR-520c promote tumour invasion and metastasis.* Nature Cell Biology, 10: 202-210.

[48] Spahn M, Kneitz S, Scholz C, Stenger N, Rudiger T, Strobel P, Riedmiller H, Kneitz B (2010). *Expression of microRNA-221 is progressively reduced in aggressive prostate*

cancer and metastasis and predicts clinical recurrence. International Journal of Cancer, 127: 394-403.

[49] Baffa R, Fassan M, Volinia S, O'Hara B, Liu CG, Palazzo JP, Gardiman M, Rugge M, Gomella LG, Croce CM, Rosenberg A (2009). *MicroRNA expression profiling of human metastatic cancers identifies cancer gene targets.* Journal of Pathology, 219: 214-221.

[50] Tavazoie SF, Alarcón C, Oskarsson T, Padua D, Wang Q, Bos PD, Gerald WL, Massagué J (2008). *Endogenous human microRNAs that suppress breast cancer metastasis*. Nature, 451: 147-152.

[51] Deng S, Calin GA, Croce CM, Coukos G, Zhang L (2008). *Mechanisms of microRNA deregulation in human cancer.* Cell Cycle, 7: 2643-2646.

[52] Lujambio A, Calin GA, Villanueva A, Ropero S, Blanco D, Montuenga LM, Rossi S, Nicoloso MS, Faller WJ, Gallagher WM, Eccle SA, Croce CM, Esteller M (2008). *A microRNA DNA methylation signature for human cancer metastasis.* Proceedings of the National Academy of Sciences, 105: 13556-13561.

[53] Saito Y, Liang G, Egger G, Friedman JM, Chuang JC, Coetzee GA, Jones PA (2006). *Specific activation of microRNA-127 with downregulation of the proto-oncogene* BCL6 *by chromatin-modifying drugs in human cancer cells*. Cancer Cell, 9: 435-443.

[54] Travis WD, Brambilla E, Muller-Hermelink HK, Harris CC (Eds) (2004). *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Lung, Pleura, Thymus and Heart.* IARC Press: Lyon.

[55] Weitberg AB (Eds) (2002). *Cancer of the Lung: From Molecular Biology to Treatment Guidelines.* Humana Press: Totowa, New Jersey.

[56] Travis WD, Brambilla E, Noguchi M, et al. (2011). *International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society international multidisciplinary classification of lung adenocarcinoma.* Journal of Thoracic Oncology, 6:244–285.

[57] Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A (Eds) (2010). *AJCC cancer staging manual (7th ed).* New York, NY: Springer.

[58] Digel W, Lübbert M (2005). *DNA methylation disturbances as novel therapeutic target in lung cancer: Preclinical and clinical results.* Critical Reviews in Oncology/Hematology, 55: 1-11.

[59] Swafford DS, Middleton SK, Palmisano WA, Nikula KJ, Tesfaigzi J, Baylin SB, Herman JG, Belinsky SA (1997). *Frequent aberrant methylation of* $p16^{INK4\alpha}$ *in primary rat lung tumours.* Molecular and Cellular Biology, 17 (3): 1366-1374.

[60] Belinsky SA, Nikula KJ, Palmisano WA, Michels R, Saccomano G, Gabrielson E, Baylin SB, Herman JG (1998). *Aberrant methylation of* $p16^{INK4\alpha}$ *is an early event in lung cancer and a potential biomarker for early diagnosis.* Proceedings of the National Academy of Sciences, 95: 11891-11896.

[61] Zöchbauer-Müller S, Fong KM, Virmani AK, Geradts J, Gazdar AF, Minna JD (2001). *Aberrant Promoter Methylation of Multiple Genes in Non-Small Cell Lung Cancers*. Cancer Research, 61: 249-255.

[62] Kim D, Nelson HH, Wiencke JK, Zheng S, Christiani DC, Wain JC, Mark EJ, Kelsey KT (2001). $p16^{INK4\alpha}$ *and Histology-specific Methylation of CpG Islands by Exposure to Tobacco Smoke in Non-Small Cell Lung Cancer.* Cancer Research, 61: 3419-3424.

[63] Yanagawa N, Tamura G, Oizumi H, Takahashi N, Shimazaki Y, Motoyama T (2003). *Promoter hypermethylation of tumour suppressor and tumour-related genes in non-small cell lung cancers.* Cancer Science, 94 (7): 589-592.

[64] Jin M, Kawakami K, Fukui Y, Tsukioka S, Oda M, Watanabe G, Takechi T, Oka T, Minamoto T (2009). *Different histological types of non-small cell lung cancer have distinct folate and DNA methylation levels.* Cancer Science, 100 (12): 2325-2330.

[65] Belinsky SA, Klinge DM, Stidley CA, Issa JP, Herman JG, March TH, Baylin SB (2003). *Inhibition of DNA Methylation and Histone Deacetylation Prevents Murine Lung Cancer.* Cancer Research, 63: 7089-7093.

[66] Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D, Slack FJ (2005). *RAS is regulated by the let-7 microRNA family.* Cell, 120: 635-647.

[67] Esquela-Kerscher A, Trang P, Wiggins JF, Patrawala L, Cheng A, Ford L (2008). *The let-7 microRNA reduces tumour growth in mouse models of lung cancer.* Cell Cycle, 7: 759-764.

[68] Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, Stephens RM, Okamoto A, Yokota J, Tanaka T, Calin GA, Liu C, Croce CM, Harris CC (2006). *Unique microRNA molecular profiles in lung cancer diagnosis and prognosis.* Cancer Cell, 9: 189-198.

[69] Lebanony D, Benjamin H, Gilad S, Ezagouri M, Dov A, Ashkenazi K, Gefen N, Izraeli S, Rechavi G, Pass H, Nonaka D, Li J, Spector Y, Rosenfeld N, Chajut A, Cohen D, Aharonov R, Mansukhani M (2009). *Diagnostic Assay Based on hsa-miR-205 Expression Distinguishes Squamous From Nonsquamous Non–Small-Cell Lung Carcinoma.* Journal of Clinical Oncology, 27: 2030-2037.

[70] Du L, Pertsemlidis A (2010). *microRNAs and lung cancer: tumours and 22-mers.* Cancer Metastasis Reviews, 29: 109-122.

[71] Meister J, Schmidt M (2010). *miR-126 and miR-126*: New Players in Cancer.* The Scientific World Journal, 10: 2090-2100.

[72] Nikolic I, Plate K, Schmidt MHH (2010). *EGFL7 meets microRNA-126: an angiogenesis alliance.* Journal of Angiogenesis Research, 2: 9.

[73] Kuhnert F, Mancuso MR, Hampton J, Stankunas K, Asano T, Chen C, Kuo CJ (2008). *Attribution of vascular phenotypes of the murine* Egfl7 *locus to the microRNA* miR-126. Development, 135: 3989-3993.

[74] Saito Y, Friedman JM, Chihara Y, Egger G, Chuang JC, Liang G (2009). *Epigenetic therapy upregulates the tumour suppressor microRNA-126 and its host gene* EGFL7 *in human cancer cells.* Biochemical and Biophysical Research Communications, 379: 726-731.

[75] Feng R, Chen X, Yu Y, Su L, Yu B, Li J, Cai Q, Yan M, Liu B, Zhu Z (2010). *miR-126 functions as a tumour suppressor in human gastric cancer.* Cancer Letters, 298: 50-63.

[76] Crawford M, Brawner E, Batte K, Yu L, Hunter MG, Otterson GA, Nuovo G, Marsh CB, Nana-Sinkam SP (2008). *MicroRNA-126 inhibits invasion in non-small cell lung carcinoma cell lines.* Biochemical and Biophysical Research Communications, 373: 607-612.

[77] Liu B, Peng X, Zheng X, Wang J, Qin Y (2009). *MiR-126 restoration down-regulate VEGF and inhibit the growth of lung cancer cell lines* in vitro *and* in vivo. Lung Cancer, 66: 169-175.

[78] Sun Y, Bai Y, Zhang F, Wang Y, Guo Y,, Guo L (2010). *miR-126 inhibits non-small cell lung cancer cells proliferation by targeting EGFL7.* Biochemical and Biophysical Research Communications, 391: 1483-1489.

[79] Musiyenko A, Bitko V, Barik S (2008). *Ectopic expression of miR-126*, an intronic product of the vascular endothelial EGF-like 7 gene, regulates prostein translation and invasiveness of prostate cancer LNCaP cells.* Journal of Molecular Medicine, 86: 313-322.

[80] Olson P, Lu J, Zhang H, Shai A, Chun G, Wang Y, Libutti S, Nakakura EK, Golub TR, Hanahan D (2009*). MicroRNA dynamics in the stages of tumorigenesis correlate hallmark capabilities of cancer.* Genes & Development, 23: 2152-2165.

[81] Kuhn DE, Martin MM, Feldman DS, Terry Jr. AV, Nuovo GJ, Elton TS (2008). *Experimental validation of miRNA targets.* Methods, 44: 47-54.

[82] Witkos TM, Koscianska E, Krzyzosiak WJ (2011). *Practical Aspects of microRNA Target Prediction.* Current Molecular Medicine, 11: 93-109.

[83] Huang X, Gschweng E, Handel BV, Cheng D, Mikkola HKA, Witte ON (2011). *Regulated expression of microRNAs-126/126* inhibits erythropoiesis from human embryonic stem cells.* Blood, 117 (7): 2157-2165.

[84] Miko E, Margitai Z, Czimmerer Z, Várkony I, Dezso, Lányi A, Bacsó Z, Scholtz B (2011). *miR-126 inhibits proliferation of small cell lung cancer cells by targeting SLC7A5.* FEBS Letters, 585: 1191-1196.

[85] Cammarata G, Augugliaro L, Salemi D, Agueli C, La Rosa M, Dagnino L, Civiletto G, Messana F, Marfia A, Bica MG, Cascio L, Floridia PM, Mineo AM, Russo M, Fabbiano F, Santoro A (2010). *Differential expression of specific microRNA and their targets in acute myeloid leukemia.* American Journal of Hematology, 85 (5): 331-339.

[86] Tatenhorst L, Senner V, Puttman S, Werner P (2004). *Regulators of G-Protein Signaling 3 and 4 (RGS3, RGS4) Are Associated with Glioma Cell Motility.* Journal of Neuropathology & Experimental Neurology, 63 (3): 210-222.

[87] Matthew EM, Hart LS, Astrinidis A, Navaraj A, Doloff NG, Dicker DT, Henske EP, El-Deiry WS (2009). The p53 target Plk2 interacts with TSC proteins impacting mTOR signaling, tumor growth and chemosensitivity under hypoxic conditions. Cell Cycle, 8 (24): 4168-4175.

[88] Wilson AJ, Byun D, Nasser S, Murray LB, Ayyanar K, Arango D, Figueroa M, Melnick A, Kao GD, Augenlicht LH, Mariadason JM (2008). *HDAC4 Promotes Growth of Colon Cancer Cells via Repression of p21.* Molecular Biology of the Cell, 19: 4062-4075.

[89] Plowright L, Harrington KJ, Pandha HS, Morgan R (2009). *HOX transcription factors are potential therapeutic targets in non-small-cell lung cancer (targeting HOX genes in lung cancer).* British Journal of Cancer, 100: 470-475.

[90] Shintani Y, Higashiyama S, Ohta M, Hirabayashi H, Yamamoto S, Yoshimasu T, Matsuda H, Matsuura N (2004). *Overexpression of ADAM9 in Non-Small Cell Lung Cancer Correlates with Brain Metastasis.* Cancer Research, 64: 4190-4196.

[91] Peduto L (2009). *ADAM9 as a Potential Target Molecule in Cancer.* Current Pharmaceutical Design, 15 (20): 2282-2287.

[92] Lei H, Juan AH, Kim M, Ruddle FH (2006). *Identification of a* Hoxc8-*regtranscriptional network in mouse embryo fibroblast cells.* Proceedings of the National Academy of Sciences, 103 (27): 10305-10309.

[93] Guo C, Sah JF, Beard L, Willson JKV, Markowitz SD, Guda K (2008). *The Noncoding RNA, miR-126, Suppresses the Growth of Neoplastic Cells by Targeting Phosphatidylinositol 3-Kinase Signaling and Is Frequently Lost in Colon Cancers.* Genes, Chromossomes & Cancer, 47: 939:946.

[94] Zhang J, Du Y, Lin Y, Chen Y, Yang L, Wang H, Ma D (2008). *The cell growth suppressor, mir-126, targets IRS-1.* Biochemical and Biophysical Research Communications, 377: 136-140.

[95] Kubuschok B, Passlick B, Izbicki JR, Thetter O, Pantel K (1999). *Disseminated Tumor Cells in Lymph Nodes as a Determinant for Survival in Surgically Resected Non-Small-Cell Lung Cancer.* Journal of Clinical Oncology, 17: 19-24.

[96] Mueller MM, Fusenig NE (2004). *Friends or Foes – Bipolar Effects of the Tumour Stroma in Cancer.* Nature Reviews Cancer, 4: 839-849.

[97] Rooij EV (2011). *The Art of MicroRNA Research.* Circulation Research, 108: 219-234.

[98] Arvey A, Larsson E, Sander C, Leslie CS, Marks DS (2010). Target mRNA abundance dilutes microRNA and siRNA activity. Molecular Systems Biology, 6: 363.

[99] Dougherty SM, Mazhawidza W, Bohn AR, Robinson KA, Mattingly KA, Blankenship KA, Huff MO, McGregor WG, Klinge CM (2006). *Gender difference in the activity but not expression of estrogen receptors α and β in human lung adenocarcinoma cells.* Endocrine-Related Cancer, 13: 113-134.

[100] Brock MV, Hooker CM, Ota-Machida E, Han Y, Guo M, Ames S, Glöckner S, Piantadosi S, Gabrielson E, Pridham G, Pelosky K, Belinsky SA, Yang SC, Baylin SB, Herman JG (2008). *DNA Methylation Markers and Early Recurrence in Stage I Lung Cancer.* The New England Journal of Medicine, 358 (11): 1118-1128.

[101] Kunej T, Godnic I, Ferdin J, Horvat S, Dovc P, Calin GA (2011). *Epigenetic regulation of microRNAs in cancer: An integrated review of literature.* Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis.

[102] Watanabe K, Emoto N, Hamano E, Sunohara M, Kawakami M, Kage H, Kitano K, Nakajima J, Goto A, Fukayama M, Nagase T, Yatomi Y, Ohishi N, Takai D (2011). *Genome structure-based screening identified epigenetically silenced microRNA associated with invasiveness in non-small cell lung cancer.* International Journal of Cancer.