

Acknowledgments

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Table of contents

List of abbreviations.....	2
Abstract.....	5
Resumo	6
miR-21 overexpression in Pulmonary Adenocarcinomas and Squamous Cell Carcinoma.....	7
Introduction	7
Materials and Methods	8
Materials	8
Methods	8
Laser Microdissection.....	8
RNA extraction.....	9
Reverse Transcription	9
Small RNA quantification.....	9
Primers.....	9
Quantitative PCR.....	9
Statistical analysis.....	10
Results	10
Laser Microdissection.....	11
Small RNA quantification.....	12
miRNA expression analysis.....	12
Discussion	16
Prospective studies	21
References	22

List of Abbreviations

3'-UTR - The three prime untranslated region

ADC – Adenocarcinoma

AP-1 - activator protein 1

CDK1 - cyclin-dependent kinase 1

Cq – Quantification cycle

CYP1A1 - cytochrome P450, family 1, subfamily A, polypeptide 1

EGFR – Epidermal Growth Factor Receptor

EGFR-TKI - Epidermal Growth Factor Receptor tyrosine kinase inhibitors

EIF4A - eukaryotic initiation factor 4A

FFPE - formalin-fixed paraffin-embedded

HER-2/neu - Human Epidermal growth factor Receptor 2

MAP4K1 - Mitogen-activated protein kinase kinase kinase kinase 1

LMD – Laser Microdissection

mRNA – messenger RNA

miRNA – microRNA

NSCLC – Non-Small Cell Lung Carcinoma

p16, 21, p53 – protein 16, 21 and 53

PALM - photoactivated localization microscope

PDCD4 - programmed cell death 4

PI3K - phosphoinositide 3-kinase

pre-miRNA – precursor miRNA

pri-miRNA - primary miRNA

PTEN - phosphatase and tensin homolog

qRT-PCR – quantitative Real Time Polymerase Chain Reaction

Ran-GTP - RAS-related Nuclear protein Guanosine-5'-triphosphate dependent

RISC - RNA-induced silencing complex

RNA – Ribonucleic acid

RB – Retinoblastoma protein

SCC – Squamous Cell Carcinoma

TMEM49 - Transmembrane protein 49

TPM1- tropomyosin 1

uPAR - urokinase receptor

Abstract

Introduction: MicroRNA's (miRNA) as class of small cellular RNA's acting agents of the interference pathway lead to silencing their cognate target genes and are differentially expressed in human cancer. The precise role of microRNA's in specific stages of malignant progression, including metastasis, is still unknown.

Objectives: Our goal was to analyse the expression profile of miR-21 in lung cancer.

Materials and Methods: For that purpose, a microRNA qRT-PCR was performed in 7 pulmonary adenocarcinomas and 5 squamous cell carcinomas and respective metastasis in order to try to understand its role in tumorigenesis.

Results and Discussion: We found an increase in miR-21 expression in primary tumour and metastases in pulmonary adenocarcinomas when compared with miR-21 overexpression in squamous cell carcinoma. Despite the small sample studied, further investigation may indicate therapeutic and prognostic relevance of this determination as previous studies suggest that miR-21 functions as an oncogene and has a role in tumorigenesis through regulation of tumour suppressor genes.

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Keywords: miRNA's; metastatic lung cancer, gene targets, miR-21, qRT-PCR

Resumo

Introdução: os microRNA's (miRNA) são uma classe de pequenos RNA's celulares que actuam na via de interferência e conduzem ao silenciamento de determinados genes-alvo, exprimindo-se de forma distinta no cancro humano.

O papel preciso dos microRNA's nos estadios da progressão tumoral, incluindo a metastização, é ainda desconhecido.

Objectivos: consistiu na pesquisa do perfil de expressão do miR-21 no cancro do pulmão.

Materiais e Métodos: para este propósito, fez-se uma qRT-PCR dos microRNA em 7 adenocarcinomas, em 5 carcinomas de células epidermóides e nas respectivas metástases, com o objectivo de tentar compreender o seu papel na tumorigénese.

Resultados e Discussão: encontrou-se um aumento da expressão de miR-21 nos tumores primários e metástases nos adenocarcinomas pulmonares, quando comparados com a sobre-expressão nos carcinomas de células epidermóides. Apesar do número reduzido de amostras estudadas, investigações futuras podem mostrar a importância terapêutica e prognóstica desta descoberta, dado que estudos anteriores sugerem que o miR-21 se comporta como um oncogene e tem um papel na tumorigénese, através da regulação de genes supressores tumorais.

Palavras-chave: miRNA's; metastatic lung cancer, gene targets, miR-21, qRT-PCR

miR-21 overexpression in Pulmonary Adenocarcinomas and Squamous Cell Carcinoma

Introduction

Lung cancer is the leading cause of cancer deaths worldwide. The main risk factor continues to be tobacco smoke in a genetic and epigenetic context not entirely understood [Travis *et al.*, (2004)]. The systematic analysis of mRNA and protein expression levels among thousands of genes will contribute to define the molecular machinery and process of lung carcinogenesis [Yanaihara *et al.*, (2006)].

Defects in tumour suppressor genes p53 and RB/p16 are common. Oncogenes c-myc, K-ras, EGFR and HER-2/neu are also involved with therapeutic influences [Yanaihara *et al.*, (2006)].

The existence of familiar clusters such as polymorphisms of CYP1A1 of P450 cytochrome suggests a hereditary genetic susceptibility.

Although much information has been gathered, a growing list of reports has been suggesting that miRNA's could play a crucial role in cancer progression and metastization.

Micro-RNA's (miRNA's) are a class of non-coding RNA molecules 17-24 nucleotides in length and are initially express as a bigger precursor (pri-miRNA) which is processed by RNase III (Drosha) and its cofactor Pasha, generating a precursor: pre-miRNA [Yaguang *et al.* (2007)]. This molecule has 60 to 70 nucleotides and a stem-loop to be transported to the cytoplasm by an exportin-5 in a Ran-GTP consuming process. Once in the cytoplasm the stem-loops are broken by the RNase III Dicer, resulting in miRNA with 17 to 24 nucleotides. The mature miRNA's are a double chain of RNA with imperfect complementarity called miRNA/miRNA*. This entity bonds to a protein (Argonaut 2) as part of RISC. Trough this

complex miRNA's control the genetic expression post-transcriptionally by hybridization with cognate mRNA's in the non-translated region (3'-UTR). This results in inhibition of genetic expression by repressing mRNA translation or through its degradation [Kong et al. (2009)].

The expression pattern of a particular miRNA, miR-21, was searched in bronchial-pulmonary carcinomas.

Materials and methods

Materials

Sections from 7 pulmonary adenocarcinomas and 5 squamous cell carcinomas were selected from FFPE blocks together with normal counterpart, concerning pulmonary parenchyma and bronchial axes, and lymph node metastasis.

These cases were collected between 2006-2009 from the Pathology Archives (Hospitais da Universidade de Coimbra): 7 adenocarcinomas – 4 women and 3 men with a median age of 61 years old and 5 squamous cell carcinomas – 5 men with a median age of 69 years old.

Methods

Microdissection (MD) laser

We used microcroscope PALM (Carl Zeiss, Jena, Germany) a high precision and contact free laser microdissection system with laser catapulting to capture excised samples in microcentrifuge tubes. It was used in order to isolate and extract only neoplastic cells from the primary tumour and from the lymph node metastasis (8 µm) and normal counterpart lung tissue.

RNA extraction

Total RNA has been extracted by using miRNeasy FFPE Kit (Qiagen, Hilden, Germany) from FFPE tissue sections. It was made using an on-column DNase digestion step to prepare RNA for use with the miScript PCR System (Qiagen, Hilden, Germany), according to the manufacturer's recommendation.

Reverse transcription

miRNA samples were poly-adenylated, elongated and reverse transcribed using "miScript" Reverse Transcription Kit (Qiagen, Hilden, Germany), cDNA was generated from 100 ng of total RNA.

Small RNA quantification

Quantification and quality assessment of small RNA including miRNA fraction was done with the 2100 Bioanalyzer with "Small RNA Assay" (Agilent Technologies, Palo Alto, California, USA).

Primers

"miScript" Primer Assay (Qiagen, Hilden Germany) was used for our specific target mir-21 and for normalization of expression data RU6, as recommended by Qiagen. The primers were synthesized and ordered at Qiagen (Qiagen, Hilden Germany).

Quantitative PCR

qPCR for miRNA was done using miScript SYBR® Green PCR Kit (Qiagen, Hilden, Germany), the pre amplified cDNA was used as the template for the real-time PCR run on

LightCycler ® 480 II (Roche, Basel, Switzerland), according to the manufacturer's recommendations. Negative Control PCR samples were run with no template.

Fold-changes were calculated using Cq method and normalized to the expression of RU6 with qBasePlus ® Software (Biogazelle, Ghent, Belgium). Data normalized according to the following formula: $\Delta Cq = Cq_{(\text{target gene})} - Cq_{(\text{reference target})}$.

Statistical analysis

A comparative statistical analysis was made using SPSS, Wilcoxon Signed Test (SPSS, Inc, Chicago, IL, USA) to compare the miRNA expression between normal lung tissue and malignant tissue, by histological types (SCC vs ADC). The statistical significance was considered p-values < 0,05.

Results

Materials

Twelve pairs of normal lung tissue and tumour were selected from 7 adenocarcinomas and 5 squamous cell carcinomas. As the samples were collected from surgical specimens, these

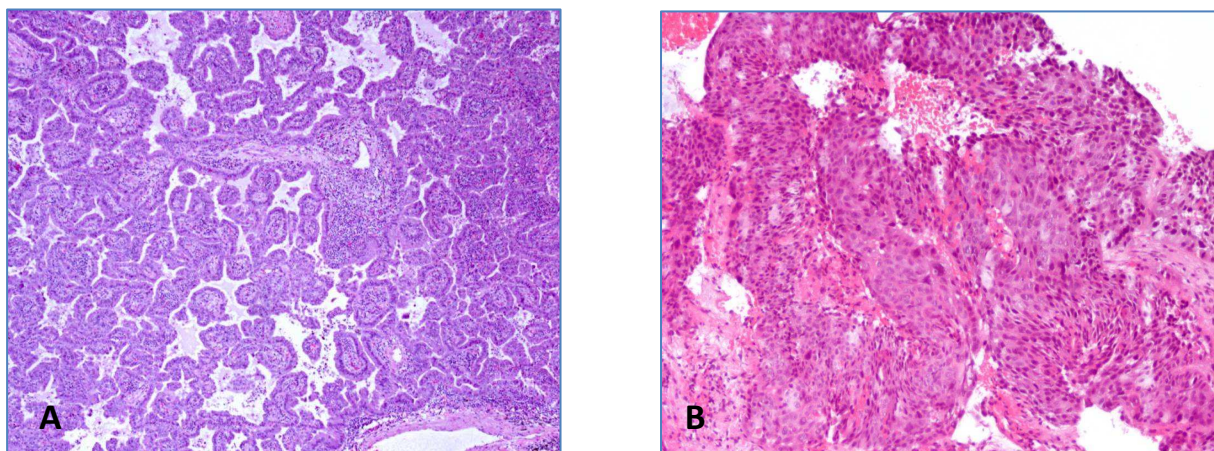


FIGURE 1. A – Adenocarcinoma of the lung – papillary subtype (HE x200), B- Squamous cell carcinoma of the lung (HE x200).

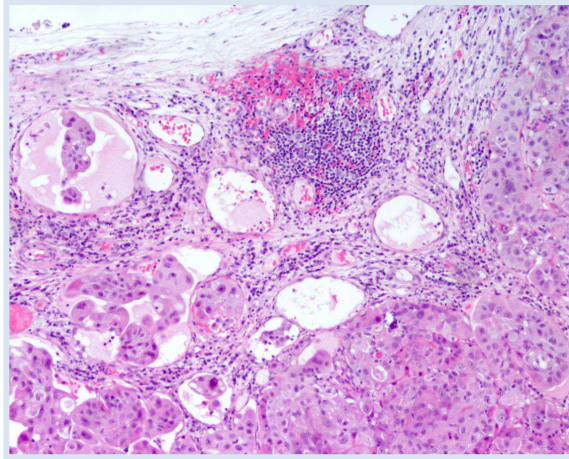


FIGURE 2. Adenocarcinoma: lymph node metastasis (HE x200).

were selected when presenting lymph node metastasis, which were also included in the study (Figures 1A, 1B and 2).

Methods

Laser Microdissection (LMD)

Areas for LMD were selected by a pathologist using PALM microscope (Figure 3A and 3B). All specimens yielded RNA of sufficient quantity and most showed relative integrity.

We compared miR-21 expression of several group pairs as listed in Table I.

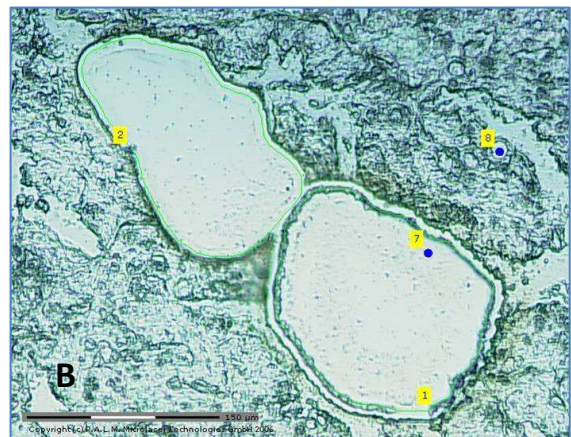
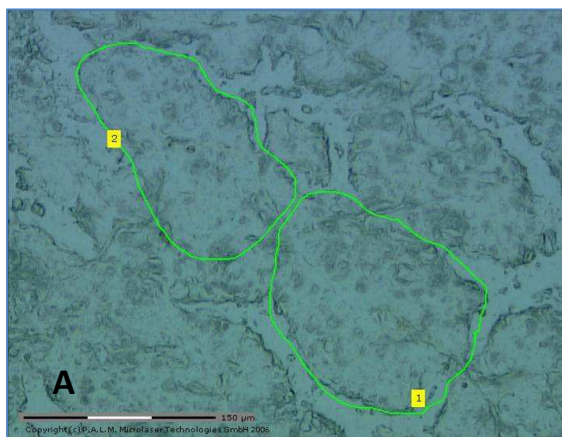


FIGURE 3. PALM images in which extraction of selected lung cells was made from adenocarcinoma of the lung, from a 8µm section (x200).

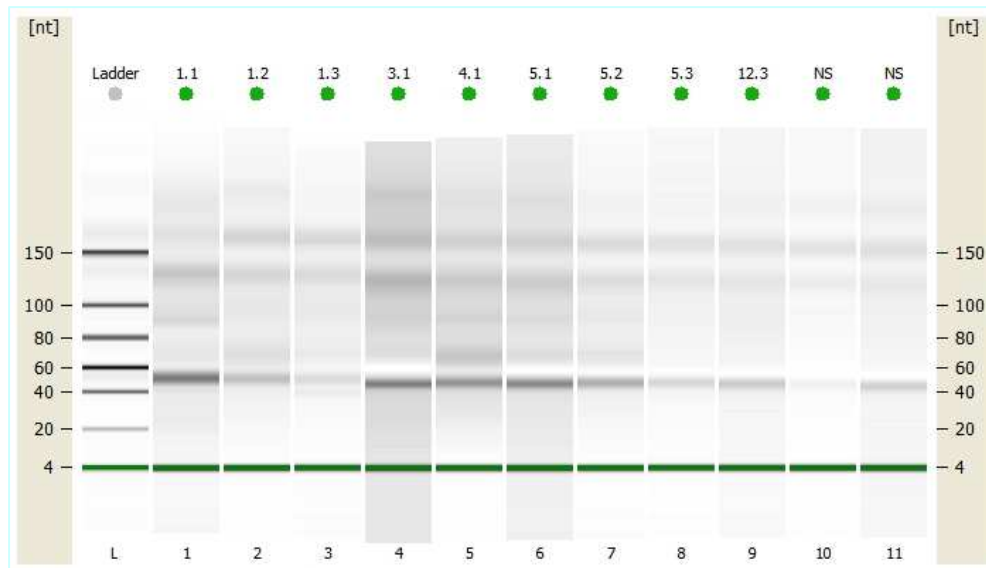


FIGURE 4. An image of Electronic Gel on the small RNA assay from all of our samples. miRNA range from 40 to 60 nt

Small RNA quantification

miRNA quantification using the 2100 Bioanalyser “Small RNA Assay” showed the amount of miRNA contained in our Total RNA samples, with miRNA mean concentration of 333,09 from (127,4 to 705,2) (Figure 4).

Results of miRNA expression analysis

We analyzed the miRU6 and miR-21 expression in 12 pairs of primary lung tissues and corresponding lymphatic metastasis and noncancerous lung tissue. For qRT-PCR analysis an endogenous control miRU6 was used for normalization of miR-21 (Figure 5 and 6).

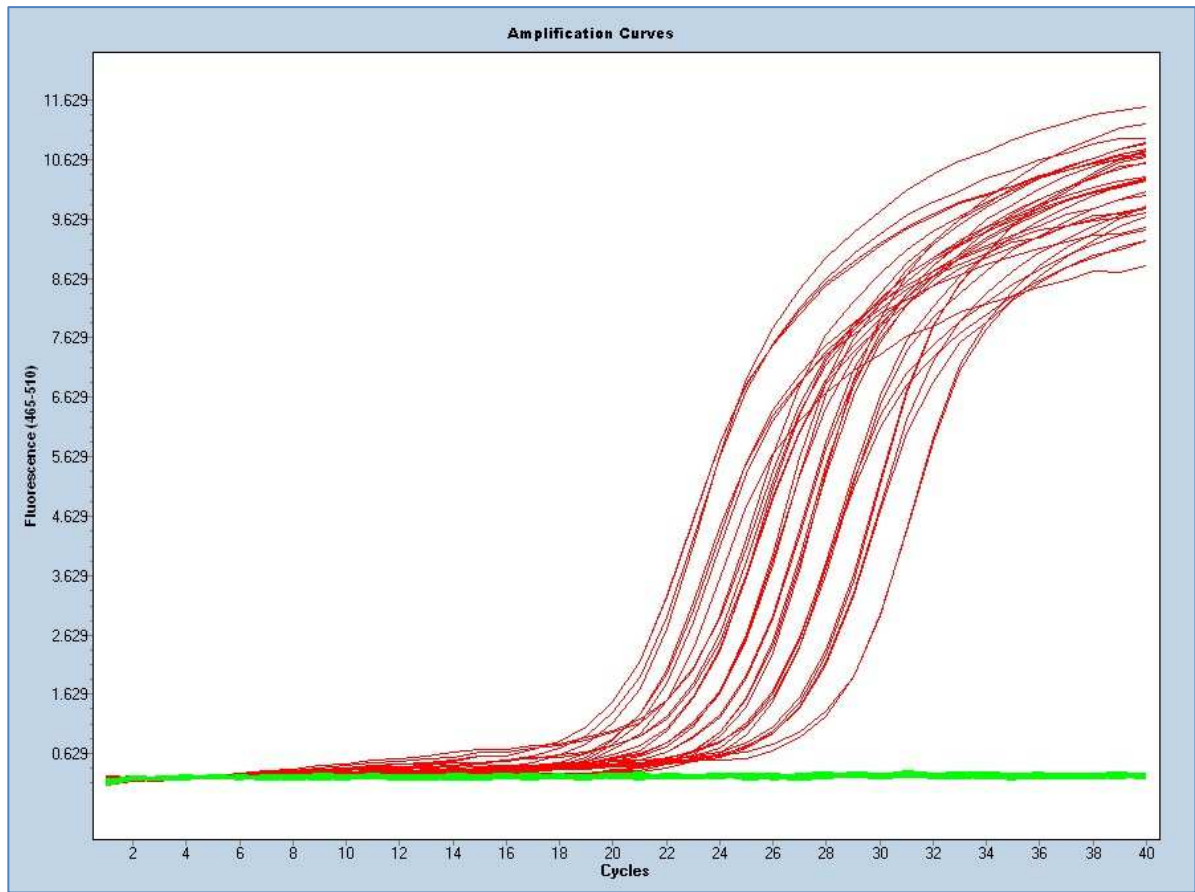


FIGURE 5. Amplification curves for RU6.

These are qPCR results of miRU6 using Cyber Green, with Cq between 20 and 29.

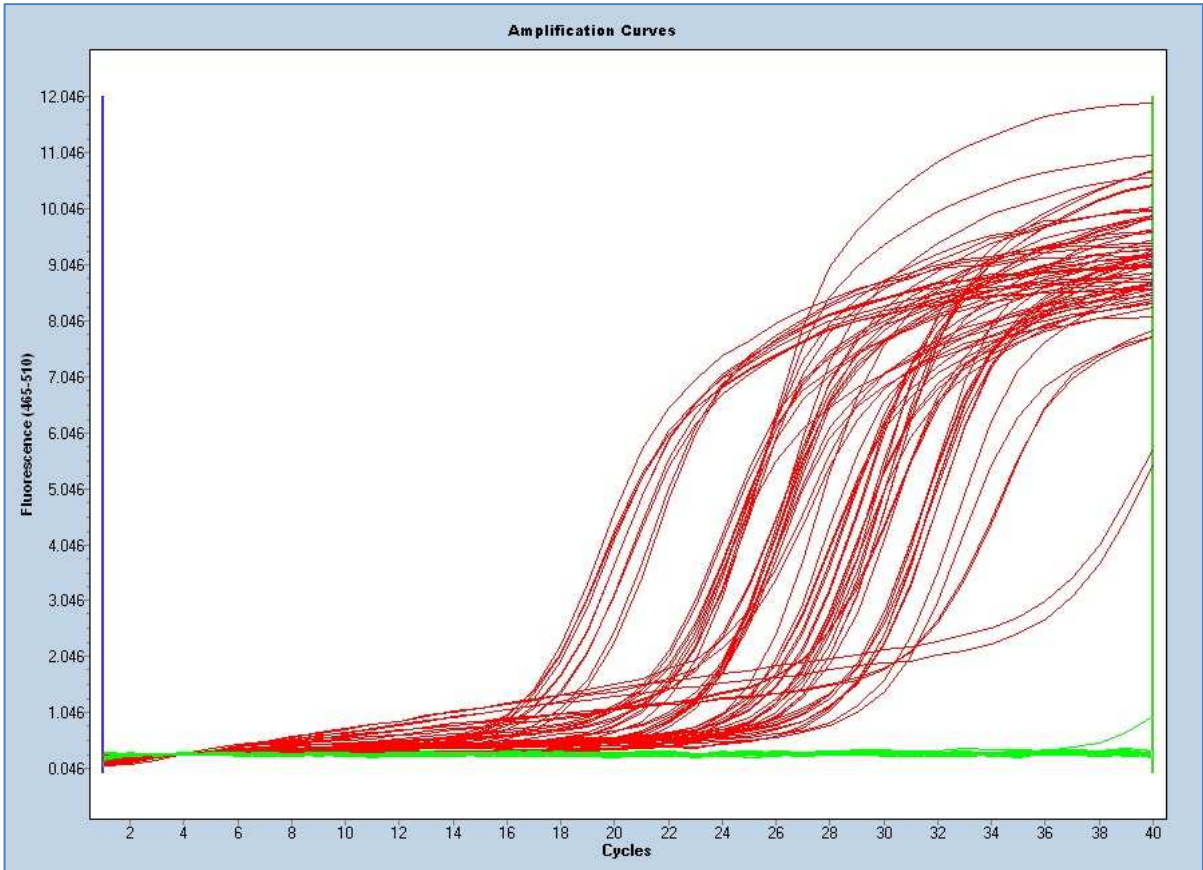


FIGURE 6. Amplification curves for miR-21.
These are qPCR results of miR-21 using Cyber Green, with C_q between 15 and 32.

The results were then analyzed according to the referred statistical tools (Table I).

TABLE I. Comparison analysis of miR-21 in Adenocarcinoma, Squamous Cell Carcinoma, Metastases and Respective Normal Lung

<i>Classification (number)</i>	<i>Total (n)</i>	<i>p value of miR-21</i>
<u>Comparison</u>		
All tumours (12) vs all normal lung(11)	23	0,05
All metastases (10) vs all normal lung (11)	21	0,051
ADC (5) vs normal lung (5)	10	0,225
ADC metastases (4) vs normal lung (5)	9	0,068
SCC (7) vs normal lung (6)	13	0,173
SCC Metastases (6) vs normal lung(6)	12	0,345
p-value was calculated using <i>Wilcoxon Signed Test. For statistical significance $p < 0,05$.</i> ADC – Adenocarcinoma, SCC – Squamous Cell Carcinoma		

After statistical procedures, we established a correlation between the results (Figure 7). The results were significant in relation to the overexpression of miR-21 in the tumour compared to the levels in the normal tissue ($p < 0,05$). The overexpression of miR-21 levels in the metastasis samples in relation to the normal tissue was almost statistical significant ($p = 0,051$).

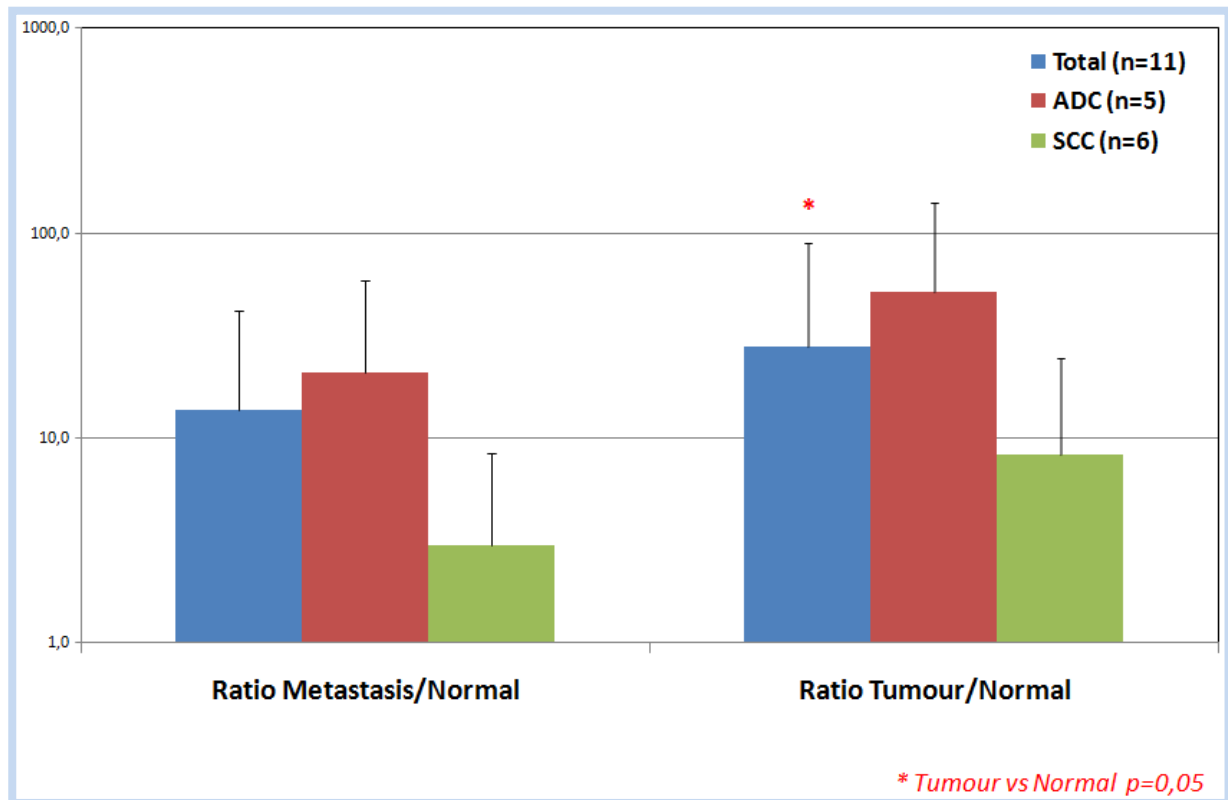


FIGURE 7. Expression profile of miR-21 in NSCLC (logarithmic scale)

These results show an overexpression of miR-21 in the tumour ($p=0.05$) and metastasis ($p=0.051$) tissue in NSCLC.

When histological comparisons are made we observe the same [Adenocarcinoma: Tumour vs Normal ($p=0.225$) and Metastasis vs Normal ($p=0.068$) and Squamous cell carcinoma: Tumour vs Normal ($p=0.173$) and (Metastasis vs Normal ($p=0.345$)).

One Sample of SCC was rejected for lack of statistical data.

Discussion

The role of miRNA's in cancer is being intensively studied. Expression profiling has identified several miRNA's responsible for tumorigenesis processes which correlate with diagnosis, staging, progression, prognosis and response to treatment [Nonn et al. (2009)].

It is thought that approximately 30% of human genes may be regulated by miRNA's and that they are involved in several biological processes: cellular proliferation, cell death, resistance to lipid metabolism stress through genetic expression regulation. These wide roles suggest that miRNA may contribute to several diseases. Indeed many reports of miRNA

expression patterns have demonstrated an aberrant expression in diseases such as cancer, heart disease, diabetes and neurological diseases [Kong *et al.* (2009)].

Most of the times miRNA's are placed in fragile sites (FRA's), which are chromosomal loci more exposed to loss of heterozygosity by amplification, deletions or translocations during tumorigenesis. These new informations suggest miRNA may be a new class of genes involved malignization of human cell lines. There is even data which suggest miRNA's may act as oncogenes or as tumour suppressor genes [Baffa *et al.* (2009)].

The first published report by Ma *et al.* (2007) described the role of miR-10b in breast cancer invasion and metastasis. Following this initial finding, the deregulation of miRNA expression in metastatic human cancer has been demonstrated in other instances too. These data may suggest a simple profiling method to help identify patients with cancer who are likely to develop metastases and/or recurrences.

In lung cancer we also have deregulation of some miRNA's. For instance, the downregulation of let-7a and the overexpression of miR-155 are correlated with worse prognosis [Yanaihara *et al.*, (2006)].

The miRNA's which are underexpressed in malignant lung tissue when compared to normal lung tissue are candidates to tumour suppressor miRNA's. Some examples are miR-34 which plays a role in the inhibition of p53 gene expression. It is also suggested miR-145, miR-142-5p and miR-34c may have anti-neoplastic effects in lung cancer [Liu *et al.* (2009)].

In this study we have compared the expression miR-21 levels in the carcinomas and respective metastasis with the normal lung and respiratory epithelium. This miRNA is known as being an oncogene and it's overexpressed in several human cancers: breast cancer, malignant brain tumours, glioblastomas, Chronic Myelocytic Lymphoma, cervical cancer [Gartel and Kandel, (2008)] and lung cancer [Croce, (2008)].

The global expression of miR-21 was increased in the metastasis and even more in the primary carcinoma cells. This evidence reinforces the notion that miR-21 can be considered as an oncogene in NSCLC.

Previous studies [Zhu et al., (2008)] have shown that miR-21 down-regulate tumour suppressor genes like PTEN, TPM1 (tropomyosin 1) PDCD4 (programmed cell death 4) and maspin. As suppressor genes, they are implicated in cell migration and invasion. The simultaneous repression of these genes may be the pathway for the biological effects of miR-21.

PTEN acts by limiting the activity of phosphoinositide 3-kinase (PI3K) pathway. A decrease in PTEN levels causes an overexpression of PI3K pathway products, including Akt, leading to tumour progression and metastasis.

TPM1 is an actin-binding protein capable of stabilizing microfilaments and of anchorage-independent growth. Since the overexpression of TPM1 suppresses cell invasion it is suggested that this molecule can have a role in it.

PDCD4 is known to be a potential target for miR-21. PDCD4 protein interacts with EIF4A and inhibits protein synthesis. It also suppresses the activation of AP-1-responsive promoters

by c-Jun. Moreover phosphorylation of PDCD4 by Akt has been shown to interfere with the transactivation of the AP-1-responsive promoter by c-Jun. The downregulation of MAP4K1 transcription may also be a consequence of PDCD4 action. In addition to that PDCD4 represses the transcription of the mitosis-promoting factor CDK1/cdc2 through upregulating p21.

Maspin is downregulated possibly through DNA methylation or loss of transcription (by miRNA's for instance). Both PDCD4 and maspin have been implicated in the regulation of uPAR which is a cell invasion and metastasis-promoting factor and a potential prognosis marker. Therefore, downregulation of PDCD4 and maspin by miR-21 may promote tumour invasion and metastasis without affecting cell growth.

Given that a miRNA can target over 100 genes, it's expected that further miR-21 targets will be found. Targeting miR-21 may be a good option to block tumour metastasis. Furthermore, there is significant positive correlation between the activated EGFR signaling pathway and the aberrant up-regulation of miR-21. This can be a possible therapeutic basis for inhibition of miR-21 in lung cancers with EGFR activation (with or without EGFR mutations). Antisense inhibition of miR-21 may improve clinical response to EGFR-TKI therapy [Seike *et al.* (2009)].

Our main goal was achieved by proving that miR-21 was involved in the pathogenesis of primary pulmonary carcinomas. The gene of this particular overexpressed molecule (TMEM49 in 17q23.2) was identified to be located inside a FRA where genomic imbalance in lung cancers has been observed previously with high frequency – FRA17B. As FRA's are preferential sites of translocation, deletion, amplification or integration of exogenous genome,

it's possible that this miRNA may be a target of these genomic alterations [Yanaihara et al., (2006)].

The results obtained show an overexpression of miR-21 in the metastasis ($p=0.051$) and in the tumour ($p< 0,05$). Although there is no statistical significance in the overexpression of the metastasis the p value is very close to the limit, which shows a tendency that must be considered. However, this data has no concern to the histological types of the lung tissue.

When we consider the different histological types – adenocarcinoma and squamous cell carcinoma –the overexpression persisted and was more pronounced in the adenocarcinomas. However, these are preliminary results as there is no statistical significance ($p>0,05$). We recommend the study to go on with more samples.

These results have many implications in the understanding of the tumorigenesis and in a clinical perspective. In fact, miR-21 has been proven to be of prognostic significance in NSCLC [Markou et al. (2008)]. In relation to treatment, miR-21 has shown a significant correlation to higher sensitivity to chemotherapy in NSCLC, ovarian cancer and Glioblastoma. However, the increase of sensitivity was low but maybe enough to be of clinical significance [Blower et al. (2008)].

Although miR-21 has not yet been contemplated, several miRNA's patterns can be used to detect human lung cancers from blood cells. Further research with miR-21 in blood cells could be an interesting approach [Keller et al., (2009)] as the obtained results show that miR-21 is involved in bronchial-pulmonary tumorigenesis.

Prospective studies

As the lung cancer is the leading cause of death by cancer worldwide it is important to study its pathology. In this research we directly compared miR-21 expression from MDL-collected FFPE malignant and normal lung tissue.

The continuation of this study with more samples would achieve statistical significance to reinforce other studies related to therapeutics that can be designed so that the influence of miR-21 in the outcome of lung cancer could be of clinical significance.

A definite correlation understood between miR-21 overexpression and different histological types of bronchial-pulmonary carcinomas and their development and progression will allow knowledge that can influence personalized therapeutics together with blood diagnosis.

References

Travis WD, Brambilla E, Harris CC, and Muller-Hermelink HK, (2004), *Pathology and Genetics of Tumours of the Lung, Pleura, Thymus and Heart*, World Health Organization, 12-15.

Yanaihara N, Caplen N, Bowman E, Masahiro S, Kumamoto K, Yi Ming, Stephens RM, Okamoto A, Yokota J, Tanaka T, Calin GA, Liu Chang-Gong, Croce Carlo M and Harris Curtis C, (2006), *Unique microRNA molecular profiles in lung cancer diagnosis and prognosis*, *Cancer Cell*, 9(3), 189-198.

Yaguang X, Nakajima G, Gavin E, Morris CG, Kudo K, Hayashi K and Ju J, (2007), *Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples*, *RNA*, 13, 1668-1674.

Kong W, Zhao JJ, He L and Cheng JQ, (2009), *Strategies for Profiling MicroRNA Expression*, *Journal of Cellular Physiology*, 218, 22-25.

Nonn L, Vaishnav A, Gallagher L and Gann PH, (2009), *mRNA and micro-RNA expression analysis in laser capture microdissected prostate biopsies: Valuable tool for risk assessment and prevention trials*, *Experimental and Molecular Pathology*, 88, 45-51.

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

Ma L, Teruya-Feldstein J and Weinberg RA, (2007), *Tumour invasion and metastasis initiated by microRNA-10b in breast cancer*, Nature, 449, 682-688.

Liu X, Sempere F, Galimberti F, Freemantle S, Black C, Dragnev KH, Ma Y, Fiering S, Memoli V, Li H, DiRenzo J, Korc M, Cole CN, Bak M, Kauppinen S and Dmitovskiy E, (2009), *Uncovering Growth-Suppressive Micro-RNA in Lung Cancer*, Clinical Cancer Research, 15(4), 1177-1183.

Gartel AL and Kandel ES, (2008) , *miRNAs: Little known mediators of oncogenesis*, Seminars in Cancer Biology, 18(2), 103-110.

Croce CM, (2008), *Oncogenes and Cancer*, New England Journal of Medicine, 358, 502-511.

Zhu S, Whu H, Wu F, Nie D, Sheng S and Mo Y, (2008), *MicroRNA-21 targets tumor suppressor genes in invasion and metastasis*, Cell Research, 18(3), 350-359.

Seike M, Goto A, Okano T, Bowman ED, Schetter AJ, Horikawa I, Mathe EA, Jen J, Yang P, Sugimura H, Gemma A, Kudoh S, Croce CM and Harris CC, (2009), *MiR-21 is an EGFR-regulated anti-apoptotic factor in lung cancer in never-smokers*, Proceedings of the National Academy of Sciences of the United States of America (PNA), 106(29), 12085-12090.

Markou A, Tsaroucha EG, Kaklamanis L, Fotinou M, Georgoulas and Lianidou ES, (2008), *Prognostic Value of Mature MicroRNA-21 and MicroRNA-205 Overexpression in Non-Small Cell Lung Cancer by Quantitative Real-Time RT-PCR*, Clinical Chemistry 54:10, 1696-1704.

Blower PE, Chung JH, Verducci JS, Lin S, Park JK, Dai Z, Liu CG, Schmitt TD, Reinhold WC, Croce CM, Weinstein JN and Sadee W, (2008), *MicroRNAs modulate the chemosensitivity of tumor cells*, *Molecular Cancer Therapeutics*, 7(1), 1-9.

Keller A, Leidinger P, Borries A, Wendschlag A, Wucherpfennig, Scheffler M, Huwer H, Lnhof HP and Meese E, (2009), *miRNAs in lung cancer – Studying complex fingerprints in patient's blood cells by microarray experiments*, *BioMedCentral Cancer*, 9, 353-365.