

# Pathology & Oncology Research

## Distinct angiogenic microRNA-mRNA expression profiles among subtypes of lung adenocarcinoma. --Manuscript Draft--

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<b>Abstract:</b>	<p>Adenocarcinoma (ADC) represents the most common histological type of non-small cell lung cancer (NSCLC), with a heterogeneous pattern of growth classified as lepidic, acinar, papillary, solid, and micropapillary. For ADC patients there are few available therapeutic options and a valuable therapeutic strategy is represented by angiogenesis inhibitors; however, new reliable biomarkers to identify patients with benefit from anti-angiogenic drugs are needed.</p> <p>We designed a panel of sixteen miRNAs together with six their mRNA target involved in the angiogenesis pathway and expression analysis was performed by the nCounter System® (NanoString Technologies) in 88 ADC patients: 29 were predominantly lepidic (33%), 26 solid (29.5%), 22 acinar (25%), and for 11 patients the prevalent pattern was papillary (12.5%).</p> <p>When we compared mRNA expression levels with the different histological ADC subtypes we found a significant higher expression of VEGF in papillary and solid than in other subtypes (<math>p=0.008</math>). Among 16 miRNAs that target the angiogenic mRNA, 4 were significantly downregulated in papillary/solid compared to other groups.</p> <p>Our data suggest a distinct angiogenic miRNA-mRNA expression profile among the subtypes of ADC, with a putative clinical application to stratify patients for anti-angiogenic drugs. Moreover, the regulation of angiogenic mRNA factors by miRNAs could provide a novel therapeutic approach based on their expression pattern specific for distinct ADC subtypes. Further studies are needed in a larger cohort of patients to confirm our results.</p>

<b>Suggested Reviewers:</b>	B Murer Expertise in lung pathology.
	CE Comin Expertise in lung pathology.

**Title page****Distinct angiogenic microRNA-mRNA expression profiles among subtypes of lung adenocarcinoma**

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**Running title:** microRNA and angiogenesis in lung adenocarcinoma subtypes.

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Adenocarcinoma (ADC) represents the most common histological type of non-small cell lung cancer (NSCLC), with a heterogeneous pattern of growth classified as lepidic, acinar, papillary, solid, and micropapillary. For ADC patients there are few available therapeutic options and a valuable therapeutic strategy is represented by angiogenesis inhibitors; however, new reliable biomarkers to identify patients with benefit from anti-angiogenic drugs are needed.

We designed a panel of sixteen miRNAs together with six their mRNA target involved in the angiogenesis pathway and expression analysis was performed by the nCounter System® (NanoString Technologies) in 88 ADC patients: 29 were predominantly lepidic (33%), 26 solid (29.5%), 22 acinar (25%), and for 11 patients the prevalent pattern was papillary (12.5%).

When we compared mRNA expression levels with the different histological ADC subtypes we found a significant higher expression of *VEGF* in papillary and solid than in other subtypes ( $p=0.008$ ). Among 16 miRNAs that target the angiogenic mRNA, 4 were significantly downregulated in papillary/solid compared to other groups.

Our data suggest a distinct angiogenic miRNA-mRNA expression profile among the subtypes of ADC, with a putative clinical application to stratify patients for anti-angiogenetic drugs. Moreover, the regulation of angiogenic mRNA factors by miRNAs could provide a novel therapeutic approach based on their expression pattern specific for distinct ADC subtypes. Further studies are needed in a larger cohort of patients to confirm our results.

**Keywords:** microRNAs, angiogenesis, lung adenocarcinoma subtypes, VEGF.

## Introduction

Lung cancer is the leading cause of cancer related mortality in both men and women, and adenocarcinoma (ADC) is the most common histological type of lung cancer [1]. The reclassification proposed by the Association for the Study of Lung Cancer (IASLC)/American Thoracic Society (ATS)/European Respiratory Society (ERS) applicable to small biopsies [2, 3], and the new WHO Classification for surgical specimens identified five main subtypes of lung adenocarcinoma, but few and controversial data exist concerning the true impact of the different prevalent patterns [4-7].

Despite recent advances in treatment, prognosis of patients with lung cancer remains poor, with 5-year overall survival of approximately 15% [8]. Proangiogenic pathways have been established as important and effective therapeutic targets because they are essential for lung tumour growth, progression and metastasis [9]. It is a highly complex process involving a network of autocrine and paracrine signaling pathways within the tumour and surrounding stromal cells. The key proteins involved in angiogenesis include members of the vascular endothelial growth factor (VEGF) family, which consists of 5 members in mammals: VEGF-A (usually referred to as VEGF, the primary growth factor associated with vessel formation), VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PDGF) [10]. VEGF binds to a family of receptor tyrosine kinases called VEGF receptors (VEGFRs), including VEGFR-1 or FLT1, VEGFR-2 or KDR (the dominant VEGFR in angiogenic signaling with VEGF), and VEGFR-3 or FLT4, and causes dimerization of the tyrosine kinase domain. Targeting tumor angiogenesis has been approached through two primary methods: monoclonal antibodies that block VEGFR binding or small molecule tyrosine kinase inhibitors (TKIs) that inhibit the downstream VEGFR mediated signaling [11]. Bevacizumab and ramucirumab, monoclonal antibodies targeting VEGF and the VEGFRs, respectively, have each led to improvements in overall survival (OS) for non-small cell lung cancer (NSCLC) when added to standard first and second line chemotherapy, respectively [12]. Currently, the only anti-angiogenic agent approved for use in NSCLC is bevacizumab (approved in 2006; Avastin®; Genentech Inc., San Francisco, CA, USA), but treatment with antiangiogenic drugs for a period of time often causes some adverse event and treatment failure. To date, differently to what occurs for other targeted agents, such as gefitinib, there are neither factors predictive of response to treatment with bevacizumab or data regarding differences in angiogenic pathways among ADC subgroups. MicroRNAs (miRNAs) are a class of small noncoding RNA that regulate the expression of many target gene via mRNA degradation or translation inhibition [13]. Despite the accumulating evidence linking miRNAs to lung carcinogenesis, very little is known about the expression level of these small RNAs in relation to angiogenesis in different subtype of lung adenocarcinoma.

In this study we analyzed simultaneously the expression level of sixteen miRNAs, selected using the miRNA target prediction tools, and six angiogenic mRNA angiogenic factors in a series of 88 adenocarcinoma lung patients using a

high sensitive technique such as Nanostring Technologies, with particular attention to differences in the histotypes subgroups.

## **Materials and Methods**

### *Patients and tumor characteristics*

A total of 88 NSCLC patients who underwent surgical resection at the Unit of Thoracic Surgery in the Department of Surgical, Medical, Molecular Pathology and Critical Area at Pisa University between 2004 and 2013 were retrospectively selected. All samples were formalin fixed and paraffin embedded (FFPE) for microscopic examination, and the histological diagnoses were formulated according to the World Health Organization classifications [3]. The most representative paraffin blocks of the tumor tissues were selected for the molecular analysis for each case. The clinicopathological characteristics data were collected for all patients.

### *RNA isolation*

According to the manufacturer's instructions, three to five paraffin sections with a thickness of 5µm per sample were utilized to total RNA isolation, including miRNAs after standard deparaffinization and manual macrodissection of the area with the prevalent adenocarcinoma pattern, using the miRNeasy FFPE kit (QiagenInc, Hilden, Germany). RNA samples, after quality and quantity evaluation using a NanoDrop ND-1000 spectrophotometer, were stored at -80°C until used in the experiments.

### *NanoString Custom Panel*

The NanoString nCounter mirGE assay kit was used to test the miRNAs-mRNAs expression profile. This assay allows to study miRNAs and associated gene transcripts to simultaneously measure expression levels of both mRNAs and miRNAs in a single reaction from a single sample. The nCounter custom code set used in this study was designed and synthesized by NanoString Technologies (Seattle, WA, USA). It consists of reporter and capture probe pairs specific for 16 miRNAs and 6 genes that were reported in Table 1. Five potential reference genes (CLTC, GUSB, TUBB, PGK1 and HPRT1) were also included in the CodeSet for biological normalization purposes. In total, 100-150 ng of RNA was used for the nCounter miRNA sample preparation reactions. All sample preparations were performed in accordance with the manufacturer instructions (NanoString Technologies, Seattle, WA, USA). The small RNA molecule were ligated with a specific DNA tag onto the 3' end of each mature miRNA. After hybridization and the removal of excess capture and reporter probes, counts of digital reports were performed on the nCounter digital analyzer according to the manufacturer's protocol.

Using the nSolver Software 2.5 (NanoString Technologies, Seattle, WA, USA), raw NanoString counts for each gene were subjected to a technical normalization taking positive and negative probes into account. After this procedure a

biological normalization using reference genes was performed. All samples with a scaling factor outside of 0.3-3 for technical normalization and 0.1-10 for biological normalization, were excluded from further analysis.

#### *Statistical analysis*

Once mRNA raw data were normalized, differential expression was tested applying Mann-Whitney U test with linearity correction using JMP10 software (SAS) in order to investigate the association between miRNAs-mRNAs expression and clinic-pathological parameters. Two-tailed p value <0.05 was considered significant.



## Results

### *Patients and tumor characteristics*

In total, 80–90% of surgically resected lung ADCs consist of a mixture of histopathological subtypes; in 2015, the International Association for the Study of LungCancer (IASLC), American Thoracic Society (ATS) and EuropeanRespiratory Society (ERS) proposed a novel international multidisciplinary classification system for lung ADC, which classifies patients according to the predominant structural morphology observed in ADC [3].

This study was conducted in 88 patients with lung adenocarcinoma (56 males and 32 females), with a median age at diagnosis of 54.5 years (range: 30-81, mean: 58.9). Patients under median years old (including 50) were defined as the younger group and patients above 54.5 years old were defined as the older group. The adenocarcinomas were all invasive; 40 stages I (17 IA, and 23 IB), 22 stages II (13 IIA, and 9 IIB), 24 stages III-IV (23 IIIA, 1 IIIB, and 2 IV) were identified. As regards histological classification, different histologic subtypes of adenocarcinoma were recognised: the most common histologic subtype was predominant lepidic (29/88, 33%), solid (26/88, 29.5%), acinar (22/88, 25%), and papillar (11/88, 12.5%) variants. There were 61 G1-G2 tumor grading (3 G1, and 58 G2), and 27 G3 cases.

### *Gene and microRNA expression profiling*

Gene and microRNA expression profiling using NanoString technology was performed on 88 selected FFPE samples using a custom NanoString panel (Table 1). The raw data were normalized in two steps: the first was based on positive and negative controls and the second was based on housekeeping gene expression counts. After normalization, 5 samples were excluded from further statistical analysis because the normalization factor was outside of the selected range.

The unsupervised clustering analysis by Pearson correlation confirmed the regulation of mRNA targeted by predicted microRNAs. As shown in Figure 1, high levels (in red) of miRNAs were associated with low (in green) expression of mRNA targets and viceversa.

### *Distinct angiogenic microRNA-mRNA expression profiles among subtypes of lung adenocarcinoma*

We analyzed the association between *VEGF* expression and the main clinicopathological characteristics; interestingly, comparing mRNA expression levels between ADC prevalent patterns, we found a significant higher level of *VEGF* in

papillary and solid than in acinar and lepidic ADC patterns ( $p=0.008$ ), as shown in Table 2. *VEGF* mean expression was similar in acinar ( $518.41\pm97.5$ ) and lepidic prevalent pattern ( $524.35\pm84.9$ ), as well as solid ( $787.87\pm89.7$ ) and papillar ( $725.94\pm137$ ) subtype both showed high *VEGF* mean. Figure 2 showed *VEGF* digital count among lung adenocarcinoma grouped subtypes. Regarding the other angiogenic factors/receptors (*VEGFRs*, *PDGFRa* and *PDGFRb*) expression level in relation to different ADC prevalent pattern, only *PDGFRb* reached a statistical significance ( $p=0.007$ ) (data not shown).

Regarding microRNAs expression and the relationship with their target angiogenic genes, high miR-30c, miR-144, miR-145, and miR-342-5p expression was significantly associated with *VEGF* downregulation ( $p=0.008$ ,  $p=0.03$ ,  $p=0.01$ , and  $p=0.008$ , respectively), as shown in Figure 3 and in Figure 4.

Interestingly, when we compared the above mentioned miRNAs expression in relation to the different ADC patterns, we found they were differentially expressed among subtypes and their expression profile was precisely opposite to the trend of *VEGF* (Figure 5).

## Discussion

Nowadays, beyond VEGF there are a number of alternative antiangiogenic agents directed against several components of angiogenesis signalling pathway (among which VEGFR1/2/3, PDGFRa and PDGFRb) that are investigated in clinical trials [14, 15]; however, after demonstrating progression free benefits, they have frequently failed to translate into significant improvement in overall survival, especially with a single mechanism anti-angiogenic agent [16]. In this context, there is a need for additional translational research to identify predictive biomarkers for anti-angiogenic therapy. VEGF is the most effective pro-angiogenesis factor, acting directly on endothelial cells to induce endothelial cell proliferation, migration, survival, and finally angiogenesis, which facilitates tumor growth [17]. From a clinical point of view, targeting tumor angiogenesis has been approached through two primary methods, monoclonal antibodies that block VEGF-vascular endothelial growth factor receptor (VEGFR) binding or small molecule tyrosine kinase inhibitors (TKIs) that inhibit the downstream VEGFR mediated signaling. A current challenge, therefore, is to identify of clinically relevant biomarkers, which will allow for selecting the subset of patients who benefit from the treatment and predict drug response.

Moreover, miRNAs regulates a great diversity of mRNAs involved in various biological processes [18]. By regulating a great diversity of mRNAs, miRNAs are involved in gene functioning during various biological processes, such as proliferation, apoptosis, differentiation, and carcinogenesis [19, 20]. In recent years, miRNAs have been shown to regulate angiogenesis through directly targeting signaling protein or angiogenic mRNA factors [21-25]. For example, miR-15/16 and miR-221/222 suppress tumor-induced vasculature formation by targeting VEGF, c-kit, and eNOS mRNAs [26, 27]. On the other hand, miR-17-92, let-7, and miR-210 positively regulate tumor angiogenesis by inhibiting genes encoding endogenous angiogenesis inhibitors [28-31]. Although some recent studies implicate miRNAs in the regulation of various aspects of angiogenesis [32, 33], there are few published data concerning the role of miRNA in angiogenesis in NSCLC patients [34-38].

Moreover, most of lung adenocarcinomas show mixed pattern, but the predominant pattern is taken into account for the impact on overall survival (OS) and disease-free survival (DFS). Solid and micropapillary patterns are associated with recurrence and with worsening [39], while lepidic predominant component is more frequent in patients without recurrence and with favourable prognosis. Most importantly, specific miRNA profiles could be useful in the subclassification of adenocarcinomas as well as the selection of the prevalent pattern best treatable with anti-angiogenic agents. Our data suggested a new insight into a distinct angiogenic miRNA-mRNA expression profile among the different lung ADC subtypes. The higher level of VEGF in association with the lower expression of several miRNAs directly targeting angiogenic factors in papillary/solid than in lepidic/acinar subtypes could represent an useful tool to

stratify patients who can effectively treated with bevacizumab. The regulation of angiogenic mRNA factors by miRNAs could provide a novel therapeutic approach based on their expression pattern specific for distinct ADC subtypes. Our preliminary results could represent a starting point to identify biomarkers that could promote the selection of patients who might benefit from antiangiogenic treatment; further studies are needed in a larger cohort of patients to confirm our results and to investigate whether different rates of response to treatment are observed among patients stratified according to the proposed biomarkers.

**Compliance with Ethical Standards:****Funding**

Not applicable.

**Disclosure of potential conflicts of interest**

The authors declare that they have no conflict of interest.

**Authors' contributions**

Laura Boldrini, Mirella Giordano and Gabriella Fontanini conceived and designed the experiments; Mirella Giordano performed the experiments; Laura Boldrini wrote the paper; Gabriella Fontanini diagnosed lung cancer; Franca Melfi, and Marco Lucchi, performed lung surgery.

**Research involving human participants**

Our study was conducted in accordance with the ethical standards of our institutional research committee and with the 1964 Helsinki declaration.

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## Figure legends

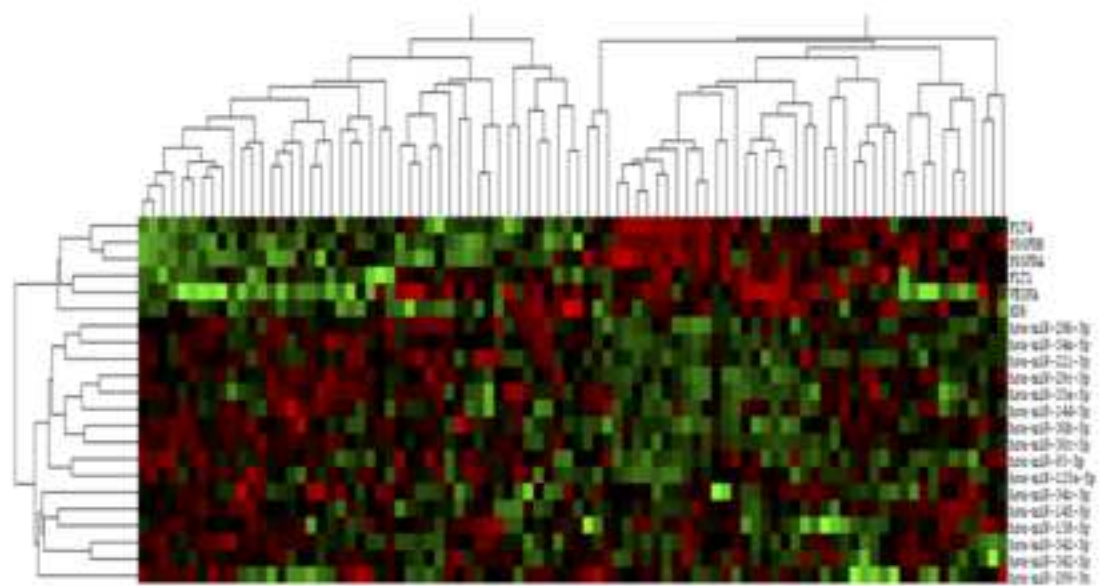
**Figure 1:** The unsupervised clustering analysis by Pearson correlation confirmed the regulation of angiogenic mRNA targeted by predicted microRNAs. High levels (in red) of miRNAs were associated with low (in green) expression of mRNA targets and viceversa.

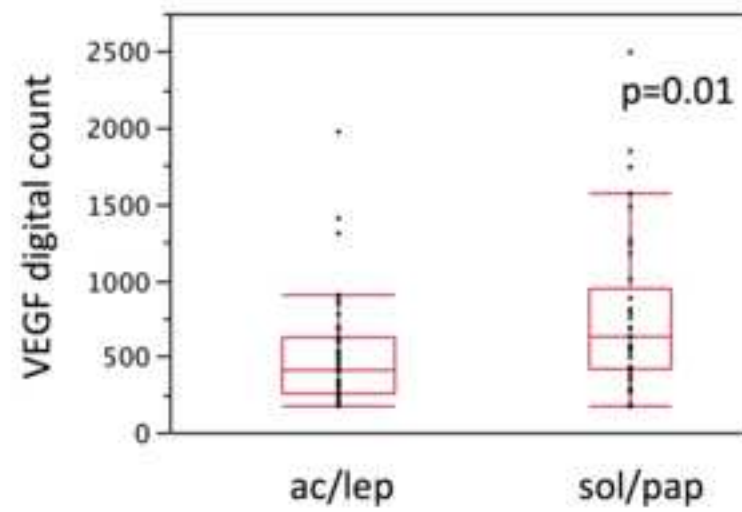
**Figure 2:** VEGF digital count among lung adenocarcinoma grouped subtypes.

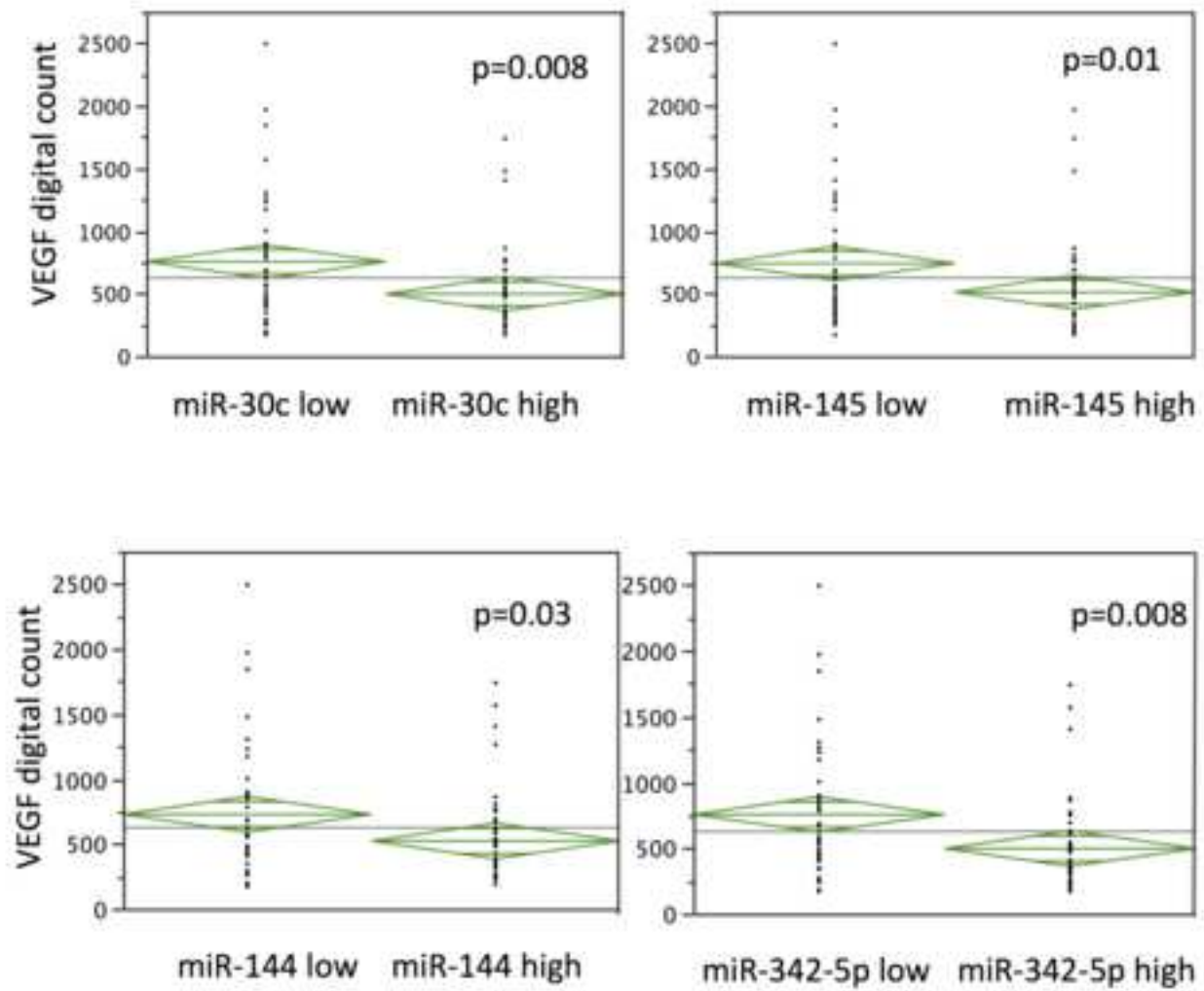
**Figure 3:** VEGF digital count in relation to miR-30c, miR-144, miR-145, and miR-342-5p expression level. Error bars on the bar charts represent the standard deviations.

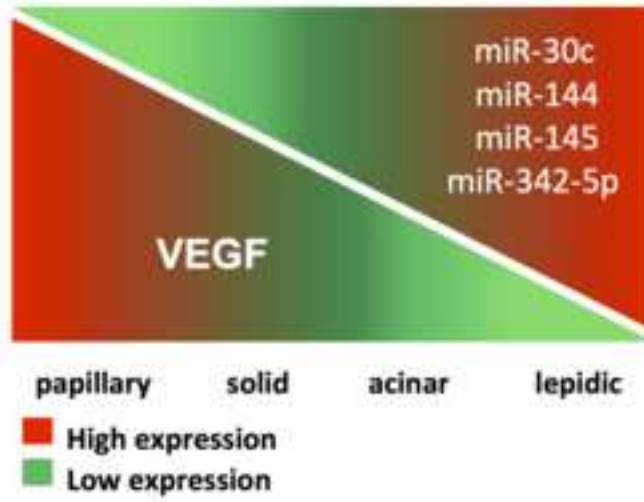
**Figure 4:** Opposite trend for VEGF digital count and miR-30c, miR-144, miR-145, and miR-342-5p expression level.

**Figure 5:** MiR-30c, miR-144, miR-145, and miR-342-5p expression level in relation to the different ADC prevalent patterns. Error bars on the bar charts represent the standard deviations.

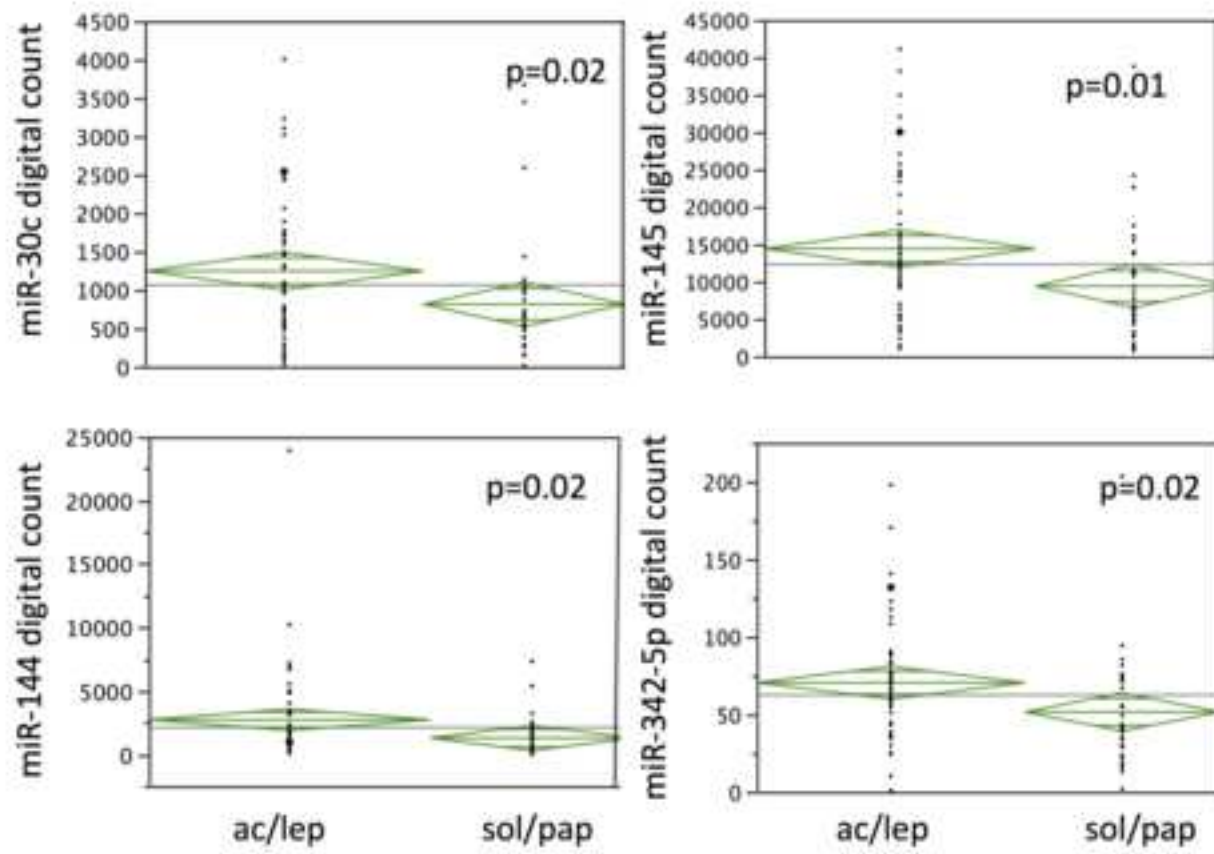












**Table 1** Our nCounter custom code set (angiogenesis related genes/miRNAs)

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**Gene/miR**

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VEGF-A (VEGF)  
VEGFR1 (FLT1)  
VEGFR2 (KDR)  
VEGFR3 (FLT4)  
PDGFRa  
PDGFRb  
hsa-miR-29b-3p  
hsa-miR-34a-5p  
hsa-miR-221-3p  
hsa-miR-29c-3p  
hsa-miR-33a-5p  
hsa-miR-144-3p  
hsa-miR-30b-5p  
hsa-miR-30c-3p  
hsa-miR-93-5p  
hsa-miR-125a-3p  
hsa-miR-34c-3p  
hsa-miR-145-5p  
hsa-miR-138-5p  
hsa-miR-342-3p  
hsa-miR-342-5p  
hsa-miR-299-3p

**Table 2 VEGF expression in 88 lung adenocarcinoma patients**

Variables	VEGF level		p
	low	high	
Age			0.39
Young ( $\leq 54.5$ years)	24	20	
Old ( $> 54.5$ years)	20	24	
Gender			0.99
Male	28	28	
Female	16	16	
Adenocarcinoma prevalent pattern			<b>0.008</b>
Lepidic	19	10	
Solid	9	17	
Acinar	14	8	
Papillar	2	9	
Tumor grading			0.11
G1-G2	34	27	
G3	10	17	
Stage			0.61
I	21	19	
II	9	13	
III-IV	14	12	

Pisa, November 19<sup>th</sup>, 2018

Dear

Editor-in-Chief,

We wish to submit to you the manuscript titled "Distinct angiogenic microRNA-mRNA expression profiles among subtypes of lung adenocarcinoma", which was authored by myself, Giordano, Melfi, Lucchi, and Fontanini for your kind consideration for publication in *Pathology & Oncology Research*. We performed expression profiling using NanoString technology for six angiogenic genes and sixteen miRNAs in 88 lung adenocarcinoma patients. Our data present novel insights into a distinct angiogenic miRNA-mRNA expression profile among the subtypes of adenocarcinoma, with a potential use to novel therapeutic approach and to stratify patients who can effectively treated with anti-angiogenic factors. The manuscript conforms to the journal's policies. The article is original, is not under consideration elsewhere and has not been previously published. All authors have approved the manuscript for submission and they have no conflict interests to declare.

We hope that you find our data interesting and consider the manuscript for publication in *Pathology & Oncology Research*.

Thank you very much for your kind attention.

Sincerely yours,

Laura Boldrini

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