MicroRNA Signatures in Tumor Tissue Related to Angiogenesis in Non-Small Cell Lung Cancer

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Abstract

Background: Angiogenesis is regarded as a hallmark in cancer development, and anti-angiogenic treatment is presently used in non-small cell lung cancer (NSCLC) patients. MicroRNAs (miRs) are small non-coding, endogenous, single stranded RNAs that regulate gene expression. In this study we aimed to identify significantly altered miRs related to angiogenesis in NSCLC.

Methods: From a large cohort of 335 NSCLC patients, paraffin-embedded samples from 10 patients with a short disease specific survival (DSS), 10 with a long DSS and 10 normal controls were analyzed. The miRs were quantified by microarray hybridization and selected miRs were validated by real-time qPCR. The impacts of different pathways, including angiogenesis, were evaluated by gene set enrichment analysis (GSEA) derived from Protein ANalysis THrough Evolutionary Relationship (PANTHER). One of the most interesting candidate markers, miR-155, was validated by in situ hybridization (ISH) in the total cohort (n = 335) and correlation analyses with several well-known angiogenic markers were done.

Results: 128 miRs were significantly up- or down-regulated; normal versus long DSS (n = 68) and/or normal versus short DSS (n = 63) and/or long versus short DSS (n = 37). The pathway analysis indicates angiogenesis-related miRs to be involved in NSCLC. There were strong significant correlations between the array hybridization and qPCR validation data. The significantly altered angiogenesis-related miRs of high interest were miR-21, miR-106a, miR-126, miR-155, miR-182, miR-210 and miR-424. miR-155 correlated significantly with fibroblast growth factor 2 (FGF2) in the total cohort (r = 0.17, P = 0.002), though most prominent in the subgroup with nodal metastasis (r = 0.34, P<0.001).

Conclusions: Several angiogenesis-related miRs are significantly altered in NSCLC. Further studies to understand their biological functions and explore their clinical relevance are warranted.

Introduction

Lung cancer is the number one cause of cancer related mortality in both men and women, and approximately 80% are non-small cell lung cancer (NSCLC) [1]. Despite emerging improvements in early diagnosis and treatment modalities, the overall 5-year survival is at meager 15%. TNM (Tumor, Nodes, Metastasis) stage has been regarded the most important prognostic variable as survival is at meager 15%. TNM (Tumor, Nodes, Metastasis) stage has been regarded the most important prognostic variable as survival is at meager 15%. 

MicroRNAs (miRs) are small non-coding, endogenous, single stranded RNAs that regulate gene expression [6–9]. By regulating the gene expression at a posttranscriptional level, miRs have a large impact on a wide variety of pathways, including different pathways related to cancer development. Several studies have explored the deregulation of different miRs in NSCLC and their potential oncogenic and tumor suppressor functions as well as their prognostic impact [10–20].

Some miRs appear to be involved in angiogenesis [21–24]. Angiogenesis is a process of new blood vessel formation from pre-existing ones, and plays a key role in tumor development [25]. Angiogenic inhibitors are used in NSCLC treatment. The monoclonal antibody bevacizumab in combination with chemotherapy is FDA approved as a treatment option in metastatic non-squamous NSCLC patients, and many tyrosine
kinase inhibitors targeting angiogenic pathways are promising [3,26].

The first evidence showing miRs in the regulation of angiogenesis came from Dicer knockout mice. Dicer is a critical enzyme in miR synthesis. The Dicer-deficit mice died early during development due to the thinning of vascular walls and severe disorganization of the network of blood vessels [27]. Besides, another murine study showed that a subset of miRs altered during the angiogenic switch became oppositely regulated in response to anti-angiogenic treatment [23].

In a large unselected NSCLC cohort we have previously studied the prognostic impact of several key angiogenic growth factors and receptors as well as the prognostic role of miR-155 and miR-126 by in situ hybridization [28–37]. Herein, we evaluated the miR expression profiles in NSCLC by using tissue from selected patients from this cohort. We aimed to identify interesting angiogenesis-related miR candidates for further potential large-scale and in-depth studies.

Results

Patients

Demographic, clinical, and histopathological variables in the patient groups (long DSS, short DSS and controls) are shown in Table 1. The median DSS were 8.0 (range 6.5–9.9) months and 160.5 (range 60.5–184.3) months in the low and high DSS group, respectively. There were four adenocarcinomas and six squamous cell carcinomas in each group.

Micro RNA expression analysis

Out of 281 miRs, Figure 1 shows the number and distribution of 128 differentially expressed miRs significant across different comparisons in the study (P<0.1 adjusted for false discovery rate, FDR). The respective -up and down-regulated (≥1,1) miRs are presented in Table S1. miR-182 was the only miR altered in all three combinations; normal versus short DSS, normal versus long DSS and short versus long DSS. A Principal component analysis

Table 1. Characteristics of 20 non-small cell lung cancer (NSCLC) patients and ten normal controls; NSCLC_01-10 with a short disease-specific survival (DSS), NSCLC_11-20 with a long DSS and 10 normal controls (Norm_01-10).

| Patient ID | Histology | DSS (months) | Status | Stage | T | N | M | Differentiation | Age | Sex |
|------------|-----------|--------------|--------|-------|---|---|---|-----------------|-----|-----|
| NSCLC_01   | AC        | 6.5          | LC Dead| Ila   | 2a| 1| 0 | Moderate        | 53  | M   |
| NSCLC_02   | AC        | 7.0          | LC Dead| Ila   | 2a| 1| 0 | Poor            | 73  | M   |
| NSCLC_03   | SCC       | 7.3          | LC Dead| Illa  | 1b| 2| 0 | Poor            | 62  | M   |
| NSCLC_04   | AC        | 7.4          | LC Dead| Ila   | 1b| 1| 0 | Moderate        | 74  | M   |
| NSCLC_05   | AC        | 7.6          | LC Dead| Ilb   | 3 | 0| 0 | Poor            | 54  | M   |
| NSCLC_06   | SCC       | 8.4          | LC Dead| Ilb   | 3 | 0| 0 | Well            | 53  | M   |
| NSCLC_07   | SCC       | 8.4          | LC Dead| Ilb   | 3 | 0| 0 | Poor            | 74  | M   |
| NSCLC_08   | SCC       | 9.3          | LC Dead| Ila   | 2a| 1| 0 | Poor            | 74  | M   |
| NSCLC_09   | SCC       | 9.6          | LC Dead| Ila   | 3 | 0| 0 | Poor            | 68  | F   |
| NSCLC_10   | SCC       | 9.9          | LC Dead| Ila   | 3 | 0| 0 | Poor            | 70  | F   |
| NSCLC_11   | SCC       | 147.8        | Alive  | Ila   | 2a| 1| 0 | Moderate        | 53  | F   |
| NSCLC_12   | AC        | 74.1         | Alive  | Ila   | 2b| 0| 0 | Moderate        | 75  | M   |
| NSCLC_13   | AC        | 60.5         | Alive  | la    | 1b| 0| 0 | Moderate        | 75  | F   |
| NSCLC_14   | SCC       | 147.8        | Alive  | Ila   | 2b| 0| 0 | Moderate        | 60  | M   |
| NSCLC_15   | SCC       | 163.8        | Alive  | Illa  | 3 | 1| 0 | Poor            | 68  | M   |
| NSCLC_16   | AC        | 170.5        | Alive  | Ila   | 2b| 0| 0 | Moderate        | 47  | M   |
| NSCLC_17   | SCC       | 158.7        | Non-LC Dead | Ib  | 2a| 0| 0 | Moderate        | 69  | M   |
| NSCLC_18   | AC        | 177.4        | Alive  | Ilb   | 3 | 0| 0 | Moderate        | 64  | M   |
| NSCLC_19   | SCC       | 184.3        | Non-LC Dead | Ia  | 1b| 0| 0 | Moderate        | 61  | M   |
| NSCLC_20   | SCC       | 162.3        | Alive  | Ia    | 1b| 0| 0 | Moderate        | 53  | M   |
| *NORM_01   | AC        | 6.5          | LC Dead| Ila   | 2a| 1| 0 | Moderate        | 53  | M   |
| *NORM_02   | AC        | 7.0          | LC Dead| Ila   | 2a| 1| 0 | Poor            | 73  | M   |
| *NORM_03   | SCC       | 8.4          | LC Dead| Ila   | 2a| 1| 0 | Well            | 53  | M   |
| *NORM_04   | SCC       | 147.8        | Alive  | Ila   | 2a| 1| 0 | Moderate        | 53  | F   |
| *NORM_05   | AC        | 74.1         | Alive  | Ila   | 2b| 0| 0 | Moderate        | 75  | M   |
| *NORM_06   | AC        | 60.5         | Alive  | la    | 1b| 0| 0 | Moderate        | 75  | F   |
| *NORM_07   | SCC       | 163.8        | Alive  | Illa  | 3 | 1| 0 | Poor            | 68  | M   |
| *NORM_08   | AC        | 170.5        | Alive  | Ila   | 2b| 0| 0 | Moderate        | 47  | M   |
| *NORM_09   | AC        | 177.4        | Alive  | Ila   | 3 | 0| 0 | Moderate        | 64  | M   |
| *NORM_10   | SCC       | 184.3        | Non-LC Dead | Ia  | 1b| 0| 0 | Moderate        | 61  | M   |

Abbreviations: SCC, squamous cell carcinoma; AC, adenocarcinoma; DSS, lung cancer related; T, tumor; N, nodal status; M, metastasis.

*Samples from normal lung tissue sites in ten NSCLC patients (NSCLC_01, NSCLC_02, NSCLC_06, NSCLC_11, NSCLC_12, NSCLC_13, NSCLC_15, NSCLC_16, NSCLC_18 and NSCLC_19) were included in the study (NORM_01 - NORM_10).

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Correlation analysis between angiogenic markers and miR-155

We have previously published on the prognostic impact of miR-155 in our large (335 patients) NSCLC population [37]. In this study we explored the correlation between miR-155 and several well-known angiogenic markers. The same cut-off values as previously published were used for FGF2 and miR-155 [33,37]. Figure S1 shows in situ hybridization (ISH) analysis of NSCLC representing strong and weak intensities for tumor cell miR-155 expression as well as negative and positive controls. Table 5 shows the correlation between angiogenic markers (vascular endothelial growth factor - A (VEGF-A), platelet derived factor – B (PDGF-B), HIF-1α and fibroblast growth factor 2 (FGF2)) and miR-155. miR-155 correlated significantly with FGF2 in the total cohort (r = 0.17, P = 0.002), though most prominent in the subgroup with nodal metastasis (r = 0.34, P<0.001). The crosstabs in Table 6 and Table 7 show the correlation and distribution of high and low expression of FGF2 and miR-155.

Discussion

To our knowledge, this is the first NSCLC miR expression profiling study showing a gene set connected to angiogenesis-related miRs with the highest impact in pathway analysis. Consistent with previously published studies we observe significant differences in miR profiles between normal and tumor tissue and between samples from patients with a poor versus a favorable DSS. Several of these significant altered miRs are associated with angiogenesis and are interesting candidate markers for further evaluation. By validating one of these markers, miR-155, in a cohort of 335 NSCLC patients, we find this miR for the first time to be significantly associated with the well-known angiogenic marker FGF2.

The major weaknesses of the study in the search for novel candidate markers are the relatively heterogeneous study population and the low number of patients included in the analysis. Many of the results presented herein are therefore only borderline significant (or some only showing a trend) and further validation in a larger NSCLC set, as done for miR-155, is a prerequisite for firmer conclusions. This is especially the case when comparing significant differences between tumor tissues (long versus short survival) as there, in general, are larger differences between tumor tissues and normal controls. The fold changes are in average higher and the p-values stronger in Table 2 (tumor tissue versus normal controls) compared with Table 3 (long versus short survival). From a clinical point of view, at least when it comes to miRs as potential prognostic or predictive markers, miRs with altered expression between short versus long survival group are of special interest. We have therefore explored the literature on four angiogenesis related miRs from Table 3 (miR-106a, miR-155, miR-182 and miR-424) and validated miR-155 in a large set of NSCLC patients. In addition we wanted to highlight the potential impact of miR-21, miR-126 and miR-210 from Table 2 as there are convincing reports indicating these miRs to have potential impact in angiogenesis. Besides, we have previously shown miR-126 to have prognostic impact in our NSCLC cohort [38].

Strengthening our results are strong and significant correlations between the microarray hybridization data and the same miRs validated by qPCR. Further, the altered miR expressions in tumor versus normal tissue are to a large degree consistent with previously published NSCLC miR studies [15–17,20] and as discussed in the following there are constantly new literature added, supporting our candidate miRs to be involved in angiogenesis.
Few studies have explored the impact of pathways related to miR gene targets in NSCLC. The interpretation of such analyses is not straightforward as the results are highly dependent on which target genes are linked to the miRs and which miRs are connected to the different pathways. In addition, miRs are often multifunctional and the same miR may target diverse genes in different pathways, and a gene may be targeted by several miRs. Although not statistically significant, the GSEA ranged angiogenesis as the most important pathway in our NSCLC samples. There are several novel reports connecting specific miRs to angiogenesis supporting our findings [23,24,39–42]. Besides, Raponi et al. have shown that the angiogenesis-related fibroblast growth factor (FGF) pathway had a significant gene enrichment in squamous NSCLC [17].

In a comprehensive murine study, Olson et al. identified specific miR expression signatures associated with steps in tumorigenesis and different hallmarks of cancer, including angiogenesis [23]. Angiogenesis–inhibiting signature miRs were evaluated by qPCR technique and defined as those miRs significantly altered in normal versus sunitinib-treated primary tumors by ≥1.3 fold change [23]. Sunitinib is a tyrosine kinase inhibitor targeting vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor (PDGFR) and c-Kit. In accordance with our qPCR validation results, PCR data in general often show an increased fold change compared with hybridization data. Many of the angiogenesis–related miRs observed by Olson and coworkers are also significantly altered in our material [25]. Among the most interesting angiogenesis-related miRs significantly altered in both studies we find miR-126, miR-155, and miR-21 and miR-424.

miR-126 has recently been reviewed as an important player in angiogenesis [43] and Wang et al. showed in a murine model that it enhances the proangiogenic actions of VEGF and FGF by repression the expression of Sprd-1, an intracellular inhibitor of angiogenic signaling [44]. miR-126 has also been located within the epidermal growth factor-like domain 7 (EGFL7) gene. EGFL7 may have a major role in angiogenesis by promoting VEGF signaling and vascular integrity [45]. Moreover, we have previously reported that miR-126 expression is significantly associated with VEGF-A in NSCLC [38]. Further, we observed that the prognostic impact of miR-126 is related to histological subtypes and nodal status. In addition the expression and roles of miR-126 may be different in various malignancies [23].

miR-155 is one of the miRs most consistently involved in neoplastic disease [46], but to our knowledge, not been related to

### Table 2. Ten each of the differentially expressed miRs that are up- or down-regulated the most ranged by fold change (long survival versus normal control and short survival versus normal control).

|                      | Long survival versus normal | Short survival versus normal |
|----------------------|-----------------------------|-----------------------------|
|                      | miR | Fold change | P-adjusted* | miR | Fold change | P-adjusted* |
| **Up-regulated**     |     |             |             |     |             |             |
| miR-205              | 8.75 | 0.0273      |             | miR-1308 | 3.92 | 0.0117 |
| miR-21               | 3.68 | 0.0090      |             | miR-1308 | 3.92 | 0.0094 |
| miR-1038             | 2.89 | 0.0532      |             | miR-182  | 3.32 | 0.0008 |
| miR-93               | 2.11 | 0.0443      |             | miR-31   | 2.87 | 0.0362 |
| miR-1274a            | 1.99 | 0.0067      |             | miR-205  | 2.75 | 0.2917 |
| miR-182              | 1.80 | 0.0693      |             | miR-193b | 2.31 | 0.0062 |
| miR-708              | 1.75 | 0.0039      |             | miR-1259 | 2.28 | 0.0008 |
| miR-210              | 1.74 | 0.0070      |             | miR-93   | 2.23 | 0.0337 |
| miR-1259             | 1.64 | 0.0427      |             | miR-106a | 2.23 | 0.0933 |
| miR-106b             | 1.62 | 0.1644      |             | miR-183  | 2.20 | 0.0304 |
| miR-451              | 5.74 | 0.0427      |             | miR-451  | 5.85 | 0.0397 |
| **Down-regulated**   |     |             |             |     |             |             |
| miR-126              | 4.53 | 0.0005      |             | miR-126  | 4.09 | 0.0008 |
| miR-30a              | 3.14 | 0.00006     |             | miR-30a  | 2.81 | 0.0003 |
| miR-30b              | 3.07 | 0.0124      |             | miR-140-3p | 2.68 | 0.0035 |
| miR-30c              | 2.45 | 0.0035      |             | miR-143  | 2.33 | 0.0787 |
| miR-140-3p           | 2.45 | 0.0070      |             | miR-126* | 2.28 | 0.0035 |
| miR-126*             | 2.41 | 0.0016      |             | miR-145  | 2.22 | 0.0070 |
| miR-145              | 2.28 | 0.0061      |             | miR-30b  | 2.07 | 0.0958 |
| let-7a               | 2.25 | 0.0480      |             | miR-29c  | 2.01 | 0.0934 |
| miR-125a-5p          | 2.19 | 0.00001     |             | miR-30d  | 1.89 | 0.0200 |

*P-adjusted; corrected for false discovery rate (FDR).

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Figure 2. Principal component analysis (PCA) and partial least square analysis (PLS) on different NSCLC patient samples. (A) Two-dimensional PCA of miRs, derived from 20 patients with NSCLC and 10 tissue samples from normal lung tissue, showing separation of the two sample groups. (B) The plot depicts components 1 and 2 of PLS model which used survival time as a scoring criteria. The analysis clearly separates the tissue sample groups for short and long survival NSCLC patients. All samples are colour coded according to group: Black: Normal patient samples; green: Samples from patients with short survival; red: Samples from patients with long survival.
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overexpression of miR-21 in DU145 cells increased the expression
et al., miR-21 was overexpressed by transfecting pre-miR-21 into
highly expressed in endothelial cells [24]. In a recent study by Liu
angiogenic treatment [23]. miR-21 has also been found to be
reports [15,17,20] and the observed down-regulation after anti-
vascular parameters as VEGF-A, PDGF-B, HIF-1α and FGF2.
Interestingly, we observed, for the first time, miR-155 to be
significantly associated with FGF2 with the highest impact in the
NSCLC subgroup. Though FGF2 has several functions, it is
important physiological role in post-ischemic angiogenesis. In
contrast, Nakshima et al. reported down-regulation of miR-424 to
contribute to the abnormal angiogenesis via MEK1 and cyclin E1
in senile hemangioma, demonstrating the potential tissue and stage
specific impact of the same miR [50]. In our study, miR-424 was
not altered when comparing tumor with normal tissue, but was
significantly up-regulated in tissue from patients with poor
prognosis compared to tissue from patients with a favorable
prognosis (Table 3). There is to our knowledge no published study
which has explored the prognostic impact of miR-424 in cancer.
However, as there is a significant miR-424 up-regulation in the
poor prognostic group it would be of interest to further evaluate
this marker in a large scale NSCLC study.

Another significant up-regulated marker in our material is miR-
210 (Table 2), which previously has been related to angiogenesis
[41,51,52]. miR-210 up-regulation is believed to be a crucial
element of endothelial cell response to hypoxia and therefore
potentially important in tumor angiogenesis. This miR has
recently been reviewed by Devlin et al. [51], concluding that
miR-210 is a robust target of hypoxia-inducible factor and that its
overexpression has been detected in a variety of cardiovascular
diseases and solid tumors. In NSCLC cell lines, miR-210 is
reported to mediate mitochondrial alterations associated with
modulation of HIF-1 activity [52]. However, much of the
functional and prognostic impact of miR-210 in NSCLC still
remains unresolved.

miR-182 is included in the angiogenesis pathway in the GSEA,
partly because fibroblast growth factor receptor substrate 2 (FRS2)
is one of its target genes. FRS2 is a major regulator of the
fibroblast growth factor pathway that we and others have shown to
be important in NSCLC progression, potentially by stimulating
angiogenesis [33,53]. Interestingly, miR-182 was the only miR
significantly altered in all three combinations as shown in the
Venn diagram (Figure 1). Its up-regulation in tumor (both poor
and favorable prognostic group, Table 2) when compared to
normal tissue, is consistent with the previous study by Raponi et al.
[17]. Moreover, miR-182 is significantly up-regulated in short
dSS versus long dSS patients (Table 3). However, Barchack et al.
observed higher miR-182 expression in primary tumors than in
the metastatic lung tumors, indicating that miR-182 expression
can reach a peak in early stage NSCLC.

We observe the same trend for miR-106a, which has been
shown to be up-regulated during hypoxia in breast and colon
cancer cell lines [40]. As previously reported, miR-106a was up-
regulated in NSCLC [16,17,20]. We also observe its expression to
be significantly augmented in tumors versus normal controls
(Table 2). As for miR-182, miR-106a was further increased in
tissue from patients with a short survival rather than long survival
(Table 3).

As expected, many miRs known to be associated with
angiogenesis were not significantly altered in our study. Cluster

Table 3. Ten each of the differentially expressed miRs that are up- or down-regulated the most ranged by fold change (short survival versus long survival).

| miR          | Fold change | P-adjusted* |
|--------------|-------------|-------------|
| **Up-regulated** |             |             |
| miR-31       | 2.00        | 0.161       |
| miR-182      | 1.85        | 0.084       |
| miR-106a     | 1.73        | 0.245       |
| miR-183      | 1.72        | 0.094       |
| let-7a       | 1.68        | 0.194       |
| miR-151-5p   | 1.64        | 0.093       |
| miR-138-1*   | 1.61        | 0.073       |
| miR-98       | 1.56        | 0.089       |
| miR-424      | 1.53        | 0.044       |
| miR-193b     | 1.50        | 0.178       |
| miR-205      | 3.16        | 0.232       |
| miR-142-3p   | 1.96        | 0.084       |
| miR-557      | 1.65        | 0.084       |
| miR-720      | 1.52        | 0.078       |
| miR-378      | 1.44        | 0.070       |
| miR-708      | 1.41        | 0.075       |
| miR-29c      | 1.39        | 0.076       |
| miR-552      | 1.35        | 0.084       |
| miR-27a*     | 1.32        | 0.035       |
| miR-155      | 1.31        | 0.095       |

*P-adjusted; corrected for false discovery rate (FDR).
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Table 4. Impacts of the different pathways evaluated by Gene Set Enrichment Analysis (GSEA) derived from Protein Analysis THrough Evolutionary Relationship (PANTHER).

| GS     | Size | Pathway                                                                 | NES   | NOM p-value | FDR  |
|--------|------|-------------------------------------------------------------------------|-------|-------------|------|
| P00005 | 31   | Angiogenesis                                                            | 2.00  | 0.00        | 0    |
| P00027 | 17   | Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway | 2.00  | 0.00        | 0    |
| P00004 | 42   | Alzheimer disease-presenilin pathway                                    | 2.00  | 0.00        | 0    |
| P00047 | 37   | PDGF signaling pathway                                                  | 2.00  | 0.00        | 0    |
| P00029 | 37   | Huntington disease                                                      | 2.00  | 0.00        | 0    |
| P00059 | 21   | p53 pathway                                                             | 2.00  | 0.00        | 0    |
| P00006 | 28   | Apoptosis signaling pathway                                             | 2.00  | 0.00        | 0    |
| P00048 | 18   | PI3 kinase pathway                                                      | 2.00  | 0.00        | 0    |
| P00031 | 45   | Inflammation mediated by chemokine and cytokine signaling pathway        | 2.00  | 0.00        | 0    |
| P00019 | 19   | Endothelin mediated pathway                                             | 2.00  | 0.00        | 0    |
| P00036 | 35   | Interleukin signaling pathway                                           | 2.00  | 0.00        | 0    |
| P00057 | 62   | Wnt signaling pathway                                                   | 2.00  | 0.00        | 0    |
| P00052 | 44   | TGF-beta signaling pathway                                              | 2.00  | 0.00        | 0    |
| P00034 | 43   | Integrin signalling pathway                                             | 2.00  | 0.00        | 0    |
| P00012 | 40   | Cadherin signalling pathway                                             | 2.00  | 0.00        | 0    |
| P00046 | 22   | Oxidative stress response                                               | 2.00  | 0.00        | 0    |
| P00026 | 33   | Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway | 2.00  | 0.00        | 0    |
| P04398 | 28   | p53 pathway feedback loops 2                                            | 2.00  | 0.00        | 0    |
| P00016 | 23   | Cytoskeletal regulation by Rho GTPase                                   | 2.00  | 0.00        | 0    |
| P00060 | 44   | Ubiquitin proteasome pathway                                            | 2.00  | 0.00        | 0    |
| P00007 | 16   | Axon guidance mediated by semaphorins                                   | 2.00  | 0.00        | 0    |

GS, gene set; Size, numbers of miRs included; NES, nominal enrichment score; NOM p-value, nominal p-value; FDR, false discovery rate.

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Figure 3. Heat map showing expression of 31 microRNAs (miRs) included in angiogenesis pathway gene set. The difference in miR expression between tumor samples from NSCLC patients with long (L) and short (S) survival is shown. miR expression values are shown in a spectrum where down-regulated is blue and up-regulated is red.

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miR-17-92 (miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-19b, miR-20a and miR92-1), let-7, let-7f, miR-27b, miR-15b, miR-16, miR-92a, miR-99a, miR-130a, miR-214, miR-221, miR-222 and miR-296 as well as some of the miRs presented in the heat map (Figure 3), were not significantly altered or they exhibited only minor fold changes [24,39,41,42]. This lack of significant alterations may be due to false negative results as there are few patients in each group. Furthermore, as many miRs appear to be tissue and stage specific, some of these miRs may alternatively exert an impact limited to subgroups of resected NSCLC or may be important in other malignancies.

Angiogenic inhibitors in combination with chemotherapy are established as NSCLC treatment, but a further knowledge on biology, effectors mechanisms and prognostic and predictive markers are warranted. miRs are stable and potentially measurable in both tissue and serum. Several miRs are linked to angiogenesis and this study supports the assumption that a number of angiogenesis-related miRs are potentially important in NSCLC progression. We propose miR-21, miR-106a, miR-126, miR-155, miR-182, miR-210 and miR-424 to be among the most interesting candidates. Since miRs often are stage and tissue specific, large scale studies are warranted to further explore their prognostic and

Figure 4. Scatter plot comparing microarray hybridization (all ten samples in each group) and qPCR data (five selected samples from each group: NSCLC_03, NSCLC_05, NSCLC_08, NSCLC_10, NSCLC_15, NSCLC_16, NSCLC_17, NSCLC_18, NSCLC_19, NSCLC_20, NORM_03, NORM_04, NORM_08, NORM_09 and NORM_10) and 28 miRs according to Table S2. Comparison between microarray hybridization (dLMR, difference in average expression levels between sample groups, log2 scale) and qPCR (ddCP, difference in average expression levels between sample groups, log2 scale) data, correlation coefficient = r (Pearson): (A) low versus normal r = 0.81, P < 0.001; (B) high versus normal r = 0.85, P < 0.001; (C) high versus low r = 0.85, P < 0.001. In red; miR-150.
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predictive impact. Besides the same miR often has multiple target genes and different functions. Therefore functional studies are highly needed to gain better insight into their biological functions.

Materials and Methods

Ethics statement

The study is approved by The National Data Inspection Board, The Regional Committee for Research Ethics and Biobank Board Collection of Tissue. Information and subsequent written consent from patients or next of kin was considered, but as this was a retrospective study with more than half of patients deceased, some more than ten years ago, The Regional Committee for Research Ethics specifically waived the need for consent.

Patients and tissue samples

From a previously well-described large NSCLC cohort [30], twenty formalin-fixed and paraffin-embedded (FFPE) tumor tissue samples were obtained from patients diagnosed with stage I-IIIA at the University Hospital of Northern Norway (UNN) and Nordland Central Hospital (NLCH). These were ten patients with a short disease-specific survival (DSS) (NSCLC_01 - NSCLC_10), and ten patients with long DSS (NSCLC_11 - NSCLC_20). In addition, samples from normal lung tissue sites in ten NSCLC patients (NSCLC_01, NSCLC_02, NSCLC_06, NSCLC_11, NSCLC_12, NSCLC_13, NSCLC_15, NSCLC_16, NSCLC_18 and NSCLC_19) were included in the study (NORM_01 - NORM_10). Four sample cores, each 0.6 mm in diameter, from viable tumor areas from each tumor and normal specimen were taken and pooled prior to RNA isolation in order to obtain a representative sample of each patient. Long and short DSS groups were stratified in order to get equal distribution of gender and histology. The tumor specimens were stored at the Departments of Pathology at UNN and NLCH, staged according to The International Union Against Cancer (UICC) TNM classification, and histologically subtyped according to the World Health Organization guidelines [54,55]. All patient material was reviewed by two independent and trained pathologists (SAS and KAS). None of the patients received chemo- or radiotherapy prior to surgery. Complete patient data was obtained from medical records, including demographic, clinical, treatment and outcome data.

RNA isolation

RNA was isolated from the collected core samples by using the Recover All™ Total Nucleic Acid Isolation Kit for FFPE Tissues (Ambion, Austin, TX), according to the manufacturer’s instructions, with some minor adjustments. Xylene treatment was increased from 3 min to 8 min, and protease treatment was increased from 3 hrs to approximately 20 hrs. RNA quality and quantity was assessed by using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and further verified by an Agilent 2100 Bioanalyzer profile.

Microarray procedures

Total RNA (700 ng) from sample and reference was labelled with Hy3™ and Hy5™ fluorescent label using the miCURY™ LNA Array power labelling kit (Exiqon, Vedbaek, Denmark) following the procedure described by the manufacturer. The Hy3™-labelled samples and a Hy5™-labelled reference RNA sample (containing an equal aliquot of all RNA species included in this study) were mixed pair-wise and hybridized to the miCURY™ LNA Array microarray slides below 2.0 ppb) in order to prevent potential bleaching of the fluorescent dyes. The miCURY™ LNA Array microarray slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent technologies Inc., USA) and the image analysis was carried out using the ImaGene 8.0 software (BioDiscovery Inc., USA). The quantified signals were background corrected with offset value 10 and normalized using the global Lowess (Locally Weighted Scatterplot Smoothing) regression algorithm [56].

Database submission of microarray data

The microarray data were prepared according to minimum information about a microarray experiment (MIAME) recom-

### Table 5. Correlations analyses between angiogenic markers and miR-155 expression in NSCLC patients.

| miR-155 | Total cohort (n = 335) | N0 (n = 232) | N+ (n = 103) |
|---------|-----------------------|-------------|-------------|
| VEGF-A  | r = 0.09, P = 0.11     | r = 0.05, P = 0.50 | r = 0.18, P = 0.07 |
| PDGF-B  | r = 0.04, P = 0.49     | r = 0.06, P = 0.37 | r = 0.01, P = 0.96 |
| HIF-1α  | r = 0.02, P = 0.68     | r = 0.02, P = 0.75 | r = 0.01, P = 0.27 |
| FGF2    | r = 0.17, P = 0.002    | r = 0.06, P = 0.35 | r = 0.34, P < 0.001 |

Abbreviations: VEGF-A, vascular endothelial growth factor-A; PDGF-B, platelet derived factor -B; HIF-1α, hypoxia inducible factor 1α; FGF2, fibroblast growth factor 2; N0, lymph node negative patients; N+, lymph node positive patients.

### Table 6. Crosstab showing the correlation between miR-155 and FGF2 in the total cohort.

|          | Low expression | High expression | Total |
|----------|----------------|----------------|-------|
| FGF2     | 156            | 137            | 293   |
| High expression | 6             | 21             | 27    |
| Total    | 162            | 158            | 320   |

Spearman correlation, r = 0.17, P = 0.002.

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### Table 7. Crosstab showing the correlation between miR-155 and FGF2 in patients with lymph node metastasis (N+).

|          | Low expression | High expression | Total |
|----------|----------------|----------------|-------|
| FGF2     | 48             | 37             | 85    |
| High expression | 1             | 13             | 14    |
| Total    | 49             | 50             | 99    |

Spearman correlation, r = 0.34, P<0.001.

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Pathogenic Angiogenesis-related miRs in NSCLC
miRCURY LNATM Universal RT miR PCR, Polyadenylation PCR reactions according to the protocol for miRCURY LNATM triplicates. cDNA was diluted 100

Data analysis and statistical analysis

Quantification of mature miRNAs by real-time qPCR

One or several of the following criteria were used when selecting miRs for PCR validation: significant P-value, high fold change and/or angiogenesis-related miRs. Five samples from each group with sufficient tumor miR remaining after the array analysis were used in the PCR validation. Out of the 41 tested miRs, 20 miRs were detected in 7 or more samples and analysed. The stability of the miRNAs detected in all samples was tested by Exiqon using the Normfinder software. miR-423-5p was one of the recommended reference miRNAs together with miR-150, miR-423-3p and miR-300. miR-300 was excluded due to very low levels detected. Further Normfinder found large variation in miR-150, however miR-423-5p was found to be the most stable miRNA in the data set and consequently used as reference. All real-time qPCR experiments were performed by Exiqon, Vedbaek, Denmark. 10 ng RNA was reverse transcribed in 10 µl reactions using the miRCURY LNA™ Universal RT miR PCR, Polyadenylation and cDNA synthesis kit (Exiqon); each sample was processed in triplicates. cDNA was diluted 100× and 4 ul was used in 10 ul PCR reactions according to the protocol for miRCURY LNA™ Universal RT miR PCR; each miR was assayed once by qPCR in triplicate cDNA. The amplification was performed in a Light-Cycle® 480 Real-Time PCR System (Roche) in 384 well plates. LinRegPCR (version 11.5) software was used to determine the qPCR amplification efficiency. The average amplification efficiency was used to correct the Raw Cp values.

Pathway analysis

The impacts of the different pathways were evaluated by Gene Set Enrichment Analysis (GSEA) derived from Protein ANalysis THrough Evolutionary Relationship (PANTHER). The PANTHER classification system is a unique resource that classifies genes by their functions, using published scientific experimental evidence and evolutionary relationships to predict function even in the absence of direct experimental evidence. The gene set was taken from the MirDB (http://www.mirdb.org) (http://rnajournal.cshlp.org/content/14/6/1012.full) database who has compiled gene lists from pathways curated in the PANTHER database. A full list is available at http://mirdb.org/cgi-bin/pathway.cgi. miRNA annotation is based on miRBase version 15 (http://www.mirbase.org/). GSEA analysis was carried out using the R statistical language (http://www.r-project.org) and the GSEA package for R available at http://www.broadinstitute.org/gsea/downloads.jsp.

Data analysis and statistical analysis

The data were analyzed using Linear Models and Empirical Bayes Methods for Assessing Differential Expression in Microarray experiments (LIMMA) [58], and significance was determined at the 0.1 level corrected for false discovery rate (FDR) using the method of Benjamini & Hochberg [59]. Principal component analysis (PCA) and Partial least squares (PLS) were carried out on the data in order to visualize the data structure and look for potential outlier samples [60].

The enrichment score (ES) reflects the degree to which a gene set is overrepresented at the top or bottom of a ranked list of genes. Normalized enrichment scores (NES) are used to compare analysis results across gene sets. GSEA accounts for differences in gene set size and in correlations between gene sets and the expression dataset [61]. The false discovery rate (FDR) is the estimated probability that a gene set with a given NES represents a false positive finding. The nominal p-value estimates the statistical significance of the enrichment score for a single gene set.

Pearson's correlation coefficient, r, was used to describe the association between array data and results from qPCR. Pearson Chi-Square and Fisher Exact Test were used to correlate angiogenic markers with miR-153.

Supporting Information

Figure S1 In situ hybridization (ISH) analysis of NSCLC representing strong and weak intensities for tumor cell miR-155 expression. Negative (scramble-miR) and positive (U6) controls from the same tissue area are shown. Strong miR-155 staining (A) with corresponding negative (B) and positive (E) controls to the left. Weak miR-153 staining (D) with corresponding negative (C) and positive (F) controls to the right. ISH positive signals (miR-155 and U6) stain blue, while nuclei stain red. (TIF)

Table S1 The 128 significant -up and down-regulated (−1, 1) miRs are presented with FDR adjusted p-values and respective fold changes (log2 scale). (XLS)

Table S2 Comparison between the array and the qPCR data in validation set. The differences in average expression levels between the sample groups in the array study are shown in columns 2–4 (dLMR values = log2 scale). The differences in average expression levels between the sample groups in the qPCR study are shown in columns 5–7 (dCP values = log2 scale). Positive numbers >1 are shown in red (= fold change >2), negative numbers <−1 (= fold change <−2). (DOC)

Author Contributions

Conceived and designed the experiments: TD KL KE TB SA HS KA-S SA-S RMB L-TB. Performed the experiments: TD KL KE TB SA HS KA-S SA-S. Analyzed the data: TD CGF. Contributed reagents/materials/analysis tools: TD CGF KL KE TB SA HS KA-S SA-S RMB L-TB. Performed the experiments: TD KL KE TB SA HS KA-S SA-S. Wrote the paper: TD CGF KE TB SA HS KA-S SA-S RMB L-TB.

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