Independent and Tissue Specific Prognostic Impact of miR-126 in Non-Small Cell Lung Cancer: Co-expression with Vascular Endothelial Growth Factor-A Predicts Poor Survival

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TITLE PAGE

Independent and Tissue Specific Prognostic Impact of miR-126 in Non-Small Cell Lung Cancer: Co-expression with Vascular Endothelial Growth Factor-A Predicts Poor Survival

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Key Words: NSCLC, miR-126, angiogenesis, prognostic factor, *in situ* hybridization

Running Title: Prognostic Impact of miR-126 in NSCLC

ABSTRACT

Purpose: Angiogenesis is pivotal in tumor development. Vascular endothelial growth factor-A (VEGF-A) is considered one of the most important angiogenic factors, but lately several microRNAs (miRs) have been associated with vascular development. miR-126 has been related to tumor angiogenesis and in the regulation of VEGF-A. We aimed to investigate the prognostic impact of miR-126 and its co-expression with VEGF-A in non-small cell lung cancer (NSCLC) patients.

Patients and methods: Tumor tissue samples from 335 resected stage I to IIIA NSCLC patients were obtained and tissue microarrays (TMAs) were constructed with four cores from each tumor specimen. VEGF-A expression was evaluated by immunohistochemistry and *in situ* hybridization was used to evaluate the expression of miR-126.

Results: In the total material, miR-126 was a significant negative prognostic factor in both univariate (P=0.005) and multivariate analyses (HR 1.6, 95% CI 1.1–2.5, P=0.028). Stratified by histology, miR-126 was only significant in squamous cell carcinomas (univariate; P<0.001, multivariate; HR 2.6, CI95% 1.5–4.5, P<0.001). Stratified by lymph node status, miR-126 was significant only in the lymph node positive subgroup (univariate; P<0.001, multivariate; HR 3.4, CI95%1.8–6.3, P<0.001). High miR-126 expression correlated significantly with high VEGF-A expression (P=0.037). The co-expression of miR-126 and VEGF-A had a significant prognostic impact (P=0.002), with 5-year survival rates of 68%, 51% and 42% for low/low (n=150), mixed combinations (n=129) and high/high (n=35) expression, respectively.

Conclusion: miR-126 is a strong and independent negative prognostic factor in NSCLC, and its prognostic impact appears related primarily to histology and nodal status.

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths in both women and men, and approximately 80% of the patients have non-small cell lung cancer (NSCLC). Because lung cancer is a deadly disease with a 5-year survival rate of less than 15%, a prognostic assessment of these patients is essential for treatment stratification.[1,2]

MicroRNA (miRNAs or miRs) are regulatory, non-coding RNAs about 21-23 nucleotides in length and are expressed at specific stages of tissue development, and have large-scale effects on the expression of a variety of genes at a post-transcriptional level. miRs are involved in many biological and pathological processes, and are emerging as highly tissue-specific biomarkers.[3,4]

Angiogenesis is defined as the growth of endothelial sprouts of pre-existing post-capillary venules and regarded as a hallmark in cancer development.[5,6] Evidence is emerging that miRs are important players in endothelial cell biology and tumor angiogenesis.[5,7] Dynamic changes in miR expression in response to growth factor stimulation or hypoxia imply that miRs are an integral component of the angiogenic program.[7]

miR-126, a key positive regulator, promotes angiogenesis in response to angiogenic growth factors, such as vascular endothelial growth factor-A (VEGF-A). This is done by repressing negative regulators of signal transduction pathways.[7] In this context miR-126 work as an oncogene, but several studies have shown that miR-126 is down-regulated in different malignancies and is a potential tumor suppressor.[8-11] miR-126 has also been shown to be down-regulated in lung cancer and *in vitro* and murine NSCLC studies have indicated that miR-126 restoration may down-regulate VEGF-A.[12-14]

These contradictory results may indicate that miR-126 have several functions and is highly tissue specific. NSCLC classification according to histology and nodal status are two of the most important determinants for NSCLC treatment strategies.[15,16] However, a

considerable variability in prognosis has been observed for subsets of patients with the same histological or clinical features. Consequently, the clinical incorporation of predictive and prognostic molecular biomarkers together with traditional cancer staging should improve the management of patients with NSCLC.

Considering the previous conflicting results regarding impact of miR-126, our hypothesis is that miR-126 may have different prognostic impacts within NSCLC subgroups. In an unselected NSCLC cohort of 335 patients,[17] we aimed to explore possible prognostic roles by miR-126 in all NSCLC cases and subgroups according to histology and nodal status, using *in situ* hybridization and a high throughput TMA platform. Further, we examined the correlations between VEGF-A and miR-126 and the prognostic impacts of their co-expression.

PATIENTS AND METHODS

Patients and Clinical Samples

Primary tumor tissues from anonymized patients diagnosed with NSCLC pathologic stage I to IIIA at the University Hospital of North Norway (UNN) and Nordland Central Hospital (NLCH) from 1990 through 2004 were used in this retrospective study. In total, 371 patients were registered from the hospital database. Of these, 36 patients were excluded from the study due to: (i) Radiotherapy or chemotherapy prior to surgery (n = 10); (ii) Other malignancy within five years prior to NSCLC diagnosis (n = 13); (iii) Inadequate paraffin-embedded fixed tissue blocks (n = 13). Adjuvant chemotherapy was not introduced in Norway during this period (1990 – 2004). Thus, 335 patients with complete medical records and adequate paraffin-embedded tissue blocks were eligible.

This report includes follow-up data as of November 30, 2008. The median follow-up of survivors was 86 (range 48-216) months. The tumors were staged according to the new 7th edition of TNM in Lung Cancer and histologically subtyped and graded according to the World Health Organization guidelines.[16,18] Regarding N-status, ipsilateral peribronchial or hilar nodes and intrapulmonary nodes are defined as N1, while N2 includes ipsilateral mediastinal or subcarinal nodes. The term N+ (lymph node metastasis present) includes both N1 and N2. The National Data Inspection Board and The Regional Committee for Research Ethics approved the study.

Microarray construction

All lung cancer cases were histologically reviewed by two pathologists (S.A.S. and K.A.S.) and the most representative areas of viable tumor cells were carefully selected. The TMAs were assembled using a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD). The Detailed methodology has been previously reported.[17] Briefly, we used a 0.6 mm

diameter stylet, and the study specimens were routinely sampled with four replicate core samples (different areas) of tumor tissue. In addition, normal lung tissue localized distant from the primary tumor, and one slide with normal lung tissue samples from 20 patients without a cancer diagnosis, were stained. Multiple 4-µm sections were cut with a Micron microtome (HM355S) and used for in situ hybridization analysis.

In Situ Hybridization (ISH)

The method used for in situ hybridization is based on a protocol developed by Nuovo et al,[19] with minor adjustments.

Double-DIG labeled (Exiqon, Vedbaek, Denmark) miRCURY LNA detection probes were used for visualization of the miRNA hsa-miR-126 and included a scrambled probe as negative control and U6 as a positive control.

Briefly, we placed 4 µm sections from the TMA blocks in a heater at 59 °C over night to make the cores attach to the slide. Sections were deparaffinised with xylene (2x5 min), rehydrated with ethanol (100 – 50 – 25% for 5 min each), and treated with DEPC water for 1 min. Protease treatment was performed with pepsin solution (Dako, Glostrup, Denmark) at 1.3mg/ml at 37°C for 50 min, and re-fixated sections for 10 min in 4% PFA. Hybridization reaction of the LNA-probe was carried out in a Hybrite (Abbott Laboratories, IL) at 60 °C for 5 min and 37 °C over night (12-18h). Immunological detection was done with anti-DIG/alkaline phosphate conjugate (Enzo Diagnostics, NY) in a heater at 37 °C for 30 min and color reaction with NBT/BCIP (Enzo Diagnostics, NY) at 37 °C for 15-30 min, depending on when background coloring started to appear on the negative control (scrambled probe). The slides were then counterstained with nuclear fast red (Enzo Diagnostics, NY) to visualize the nuclei, before the slides finally were mounted.

Immunohistochemistry (IHC)

The detailed VEGF-A (rabbit polyclonal; RB-1678; Neomarkers, CA; 1:10) IHC procedure has been previously published.[17,20] All TMA stainings were done in one single experiment.

Scoring of ISH and IHC

The ARIOL imaging system (Genetix, San Jose, CA) was used to scan the TMA slides of ISH staining. The slides were loaded in the automated loader (Applied Imaging SL 50) and specimens were scanned at low (1.25×) and high resolution (20×) using the Olympus BX 61 microscope with an automated platform (Prior). Representative and viable tissue sections were scored manually and semiquantitatively for cytoplasmic staining on a computer screen. The dominant staining intensity in tumor cells was scored as: 0 = negative; 1 = weak; 2 = intermediate; 3 = strong (Figure 1). In case of disagreement (score discrepancy > 1), the slides were re-examined and a consensus was reached by the observers. In most cores there was a mixture of stromal cells and tumor cells. By morphological criteria only tumor cells were scored for staining intensity.

All samples were anonymized and independently scored by one experienced pathologist and one technician (S.W.S. and K.L.). When assessing a variable for a given core, the observers were blinded to the scores of the other observer and to outcome. Mean score for each case was calculated from all four cores and both examiners. High expression of miR-126 in tumor cells was defined as a mean score ≥ 2. For VEGF-A, the same cut-off value as previously published was used.[17,21]

Statistical Methods

All statistical analyses were done using the statistical package SPSS (Chicago, IL), version 17. The Chi-square test and Fishers Exact test were used to examine the association between molecular marker expression and various clinicopathological parameters. The ISH scores from each observer were compared for interobserver reliability by use of a two-way random effect model with absolute agreement definition. The intraclass correlation coefficient (reliability coefficient) was obtained from these results. Plots of the disease-specific survival (DSS) according to marker expression were drawn using the Kaplan-Meier method, and statistical significance between survival curves was assessed by the log rank test. DSS was determined from the date of surgery to the time of lung cancer death. The multivariate analysis was carried out using the Cox proportional hazards model. Only variables of significant value from the univariate analysis were entered into the Cox regression analysis. The significance level used was $P < 0.05$.

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RESULTS

Clinocopathologic Variables

Demographic, clinical, and histopathologic variables are shown in Table 1. The median age was 67 (range, 28-85) years and the majority of patients were male (76%). The NSCLC tumors comprised 191 squamous cell carcinomas (SCCs), 113 adenocarcinomas (ACs) and 31 large-cell carcinomas (LCCs). There were 232 lymph node negative patients, while 103 patients had lymph node metastasis. Due to nodal metastasis or non-radical surgical margins, 59 (18%) patients received postoperative radiotherapy.

Interobserver Variability

Interobserver scoring agreement was tested for miR-126. The scoring agreement was good ($r = 0.91$, $P < 0.001$).

Expression of miR-126 and Correlations

miR-126 was expressed in the cytoplasm of most neoplastic tumor cells and to some lesser extent expressed in the cytoplasm of normal epithelial cells in lung tissue. Based on morphological criteria, inflammatory cells (macrophages, lymphocytes, granulocytes and plasma cells), pneumocytes and fibroblasts, normal as well as tumor associated, showed variable and in general slightly reduced cytoplasmic expression compared to tumor cells.

There were no significant correlations between miR-126 expression levels and any of the clinicopathological variables except for histology ($P = 0.039$). There was a higher frequency of high miR-126 expression in SCCs (22.7%) compared to ACs (11.7%) and LCCs (10.7%) ($P = 0.039$). There was a weak, but significant, correlation between miR-126 and VEGF-A ($r = 0.12$, $\kappa = 0.10$, $P = 0.037$). In low respective high miR-126 expression group, the percentage of high VEGF-A expression was 41% and 57%.

Univariate Analysis

Survival analyses according to clinicopathological variables are shown Table 1. Performance status (P = 0.013), histological differentiation (P < 0.001), surgical procedure (P < 0.004), pathological stage (P < 0.001), T-stage (P < 0.001), N-stage (P < 0.001) and vascular infiltration (P < 0.001) were all significant prognostic indicators for DSS. In the total material, miR-126 expression was a significant negative prognostic marker (Figure 2A, P = 0.005).

Stratified by histology, high miR-126 expression was a significant negative prognostic marker in SCCs (Figure 2B, P < 0.001), but not in ACs (Figure 2C, P = 0.61) or LCCs (P = 0.65). Stratified by nodal status, high miR-126 expression was significant associated with a poor prognosis in lymph node positive patients (Figure 2E, P<0.001), but not in lymph node negative patients (Figure 2D, P = 0.39).

Multivariate Cox Proportional Hazard Analysis

In the total material (Table 2), performance status (P = 0.027), pathological T-stage (P = 0.001), N-stage (P < 0.001), histological differentiation (P = 0.038), vascular infiltration (P = 0.01) and miR-126 (P = 0.028) were all independent prognostic factors.

Stratified by histology, miR-126 was an independent negative prognostic factor (HR 2.6, CI95% 1.5–4.5, P < 0.001) in SCCs. Stratified by lymph node status, miR-126 was independently associated with a dismal prognosis (HR 3.4, CI95%1.8–6.3, P<0.001) in lymph node positive patients.

Co-expression VEGF-A and miR-126

We have previously presented the prognostic impact of VEGF-A in the same tumor material.[17] Herein, we have used the same cut-off value, but because of a survival update in 2008 the follow-up time is prolonged. According to the tumor cell VEGF-A expression, the 5-

year survival rates were 48% (high expression, n =142) versus 66% (low expression, n =192) (P= 0.001) after the survival update.

The co-expression of miR-126 and VEGF-A had a significant (P=0.002) prognostic impact in the univariate analysis (Figure 3) as low/low (n=150), mixed (n=129) and high/high (n=35) expression resulted in 5-year survival rates of 68%, 51% and 42%, respectively. In the multivariate analysis, these co-expressions of VEGF-A and miR-126 also had an independent prognostic impact (P=0.018). For patients with high VEGF-A/high miR-126 expression, HR was 2.2, (CI95% 1.3-3.8, P=0.005) compared to patients who had low VEGF-A/low miR-126 expression.

DISCUSSION

We present the first large-scale study combining high-throughput TMA and *in situ* hybridization to evaluate the prognostic impact of miR-126 expression. In this unselected population of surgically resected NSCLC patients, high miR-126 expression is an independent negative prognostic factor in the total cohort. However, the prognostic impact is strongly related to those with SCCs or regional nodal metastasis. Moreover, there was a weak, although significant association between VEGF-A and miR-126 expression, and the co-expression of VEGF-A and miR-126 was a strong independent negative prognostic factor.

miRNAs are well preserved in formalin-fixed tissue, making them attractive candidates for use in routinely processed tissue materials.[22,23] Most of the previous studies on miRNA expression were done on microarrays using RNA extracted from human cancer tissue samples, containing a mixture of neoplastic tumor cells and tumor related stromal cells. A major advantage of *in situ* hybridization is to precisely identify positive signals at the cellular level. For instance, recent data have demonstrated that some miRNAs had high expression levels in stromal cells but not in tumor cells.[24] Using RNA extracts from whole tumors, this finding would easily be missed. As miR-126 often is down-regulated in tumor cells compared to stromal tissue, using RNA extracts may have led to erroneous results for this marker.

To our knowledge, this is the first study to explore the prognostic impact of miR-126 in a larger cancer cohort. miR-126 has been found down-regulated in several malignancies such as cervical[8], breast[9], colorectal[10], and gastric cancer[11] and was perceived a tumor suppressor, although, the prognostic impact was not addressed in these studies. Also in NSCLC, miR-126 has been interpreted as a tumor suppressor.[12-14] In a combined *in vitro* and *in vivo* murine NSCLC study, Liu et al concluded that miR-126 inhibits VEGF-A expression. It should be noted that adenocarcinoma lung cancer cell lines were used in this study. Interestingly, we observe a significantly higher frequency of high miR-126 expression in

SCCs when compared to ACs and LCCs, and the marker is a strong negative independent prognostic factor for the SCCs subgroup only. It is noteworthy that treatment responses and side effects from novel therapies have lately been correlated to NSCLC histology subgroups, and ACs and SSCs are increasingly recognized as different diseases instead of one. Regarding anti-angiogenic treatment in NSCLC, the VEGF monoclonal antibody, bevacizumab, is only given to non-SCCs due to the risk of fatal bleeding in SCCs.[25]

Supporting our findings, miR-126 is also found to be an important positive stimulator of angiogenesis.[5,26] However, its angiogenic function has so far been mostly studied in the endothelial cells. In a comprehensive murine study, Wang et al observed miR-126 as an important player in angiogenesis. VEGF and fibroblast growth factor (FGF) binding to their respective receptors on endothelial cells leads to activation of the MAP kinase signaling pathway, which culminates in the nucleus to stimulate the transcription of genes involved in angiogenesis. miR-126 repress the expression of Spred-1, a negative regulator of Ras/MAP kinase signaling. Thus, loss of miR-126 function diminishes MAP kinase signaling in response to VEGF and FGF, whereas gain of miR-126 function enhances angiogenic signaling.[5,26]

We observed a weak but significant correlation between high VEGF-A and high miR-126 expression in the tumor cells, indicating that a similar connection may be present in the cancer cells. However, as there were several patients with high VEGF-A expression without high mir-126 expression, it indicates that miR-126 is only one of many potential VEGF-A regulators. Nevertheless, the co-expression of VEGF-A and miR-126 is independently associated with a poor prognosis.

In the clinic, valid prognostic markers in the subpopulation of N+ patients are warranted. Among lymph node positive patients, high miR-126 expression was a strong independent negative prognostic factor. Indeed, 19 out of 21 N+ patients with high miR-126

expression had a lung cancer related death within 5-years after diagnosis. Further studies are needed to address the question why miR-126 has such a strong prognostic impact in this subgroup.

In conclusion, microRNAs are well preserved in formalin-fixed tissue, making them ideal candidates for investigation in routinely processed surgical specimens. miR-126 may stimulate angiogenesis through VEGF-A release from the tumor cells, but its prognostic impact seems to be highly tissue specific. miR-126 is a strong and independent prognostic factor in the total NSCLC cohort, but when stratified the prognostic impact is highly associated with SCCs patients or those with nodal metastasis. This may have significant clinical implications as new treatment strategies are becoming more individualized.

Table 1. Prognostic Clinicopathologic Variables as Predictors for Disease-Specific Survival in 335 NSCLC Patients (Univariate Analyses; Log-rank Test).

Characteristic	Patients (n)	Patients (%)	Median survival (months)	5-Year survival (%)	P
Age					0.34
≤ 65 years	156	47	83	55	
> 65 years	179	53	NR	60	
Sex					0.20
Female	82	25	190	63	
Male	253	75	83	56	
Smoking					0.23
Never	15	5	19	43	
Current	215	64	NR	60	
Former	105	31	71	54	
Performance status					**0.013**
ECOG 0	197	59	NR	63	
ECOG 1	120	36	64	52	
ECOG 2	18	5	25	33	
Weight loss					0.71
< 10%	303	90	127	58	
> 10%	32	10	98	57	
Histology					0.08
SCC	191	57	NR	66	
Adenocarcinoma	113	34	54	45	
LCC	31	9	98	56	
Differentiation					**< 0.001**
Poor	138	41	47	47	
Moderate	144	43	190	64	
Well	53	16	NR	68	
Surgical procedure					**0.004**
Lobectomy + Wedge*	243	73	190	61	
Pneumonectomy	92	27	37	47	
Pathological stage					**< 0.001**
I	157	47	190	71	
II	136	41	61	51	
IIIa	42	12	17	23	
Tumor status					**< 0.001**
1	85	25	190	74	
2	188	56	84	57	
3	62	19	25	36	
Nodal status					**< 0.001**
0	232	69	190	66	
1	76	23	35	43	
2	27	8	18	18	
Surgical margins					0.29
Free	307	92	190	58	
Not free	28	8	47	47	
Vascular infiltration					**< 0.001**
No	284	85	190	58	
Yes	51	15	27	32	

NR, not reached
* Wedge, n = 10
Abbreviations: SCC; squamous cell carcinoma; LCC, large-cell carcinoma; BAC, bronchioalveolar carcinoma

Table 2. Results of the Cox regression analysis summarizing significant independent prognostic factors in the total material.

Factor	HR	95% CI	P
Tumor status			.001*
1	1.00		
2	1.48	0.90 – 2.42	.12
3	2.65	1.53 – 4.61	.001
Nodal status			<.001*
0	1.00		
1	1.99	1.31 – 3.01	.001
2	3.36	1.95 - 5.79	<.001
Differentiation			0.038*
Poor	1.00		
Moderate	0.64	0.43 – 0.94	0.023
Well	0.58	0.31 – 1.09	0.088
Performance status			.027*
Normal	1.00		
Slightly reduced	1.66	1.13 – 2.43	.009
In bed < 50%	1.63	0.75 – 3.55	.22
Vascular infiltration			
No	1.00		
Yes	1.85	1.16 – 2.96	.010
miR-126 Tumor			
Low	1.00		
High	1.62	1.05 – 2.49	.028

* Overall significance as a prognostic factor

LEGENDS OF FIGURES

Fig. 1: *In situ* hybridization (ISH) analysis of NSCLC representing strong and weak intensities for tumor cell miR-126 expression. Negative (scramble-miR) and positive (U6) controls from the same tissue area are shown. Strong miR-126 staining (A) with corresponding negative (C) and positive (E) controls to the left. Weak miR-126 staining (B) with corresponding negative (D) and positive (F) controls to the right. ISH positive signals (miR-126 and U6) stain blue, while nuclei stain red.

Fig. 2: Disease-specific survival curves according to miR-126 expression in: (A) The total material; (B) Squamous cell carcinomas (SCCs); (C) Adenocarcinomas (ACs); (D) Lymph node positive patients; (E) Lymph node negative patients.

Fig. 3: Disease-specific survival curves according to the co-expression of VEGF-A and miR-126 in 335 resected NSCLC.

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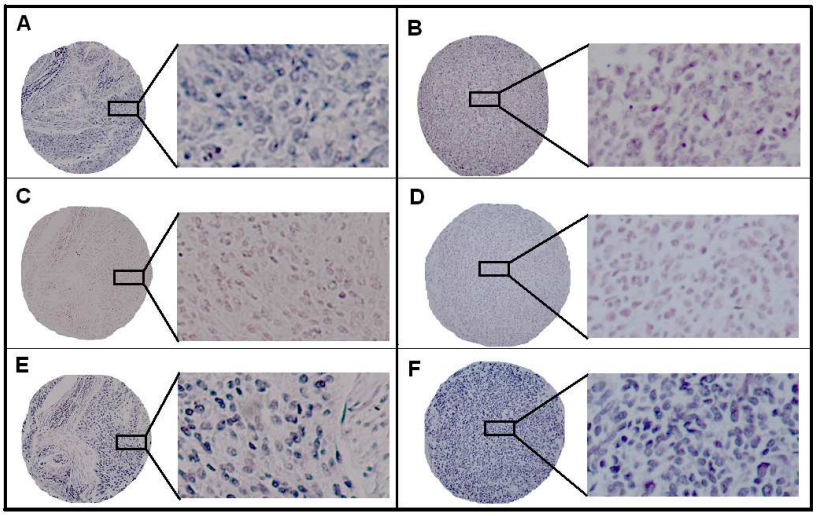
Figure 1



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336x287mm (96 x 96 DPI)

Figure 2

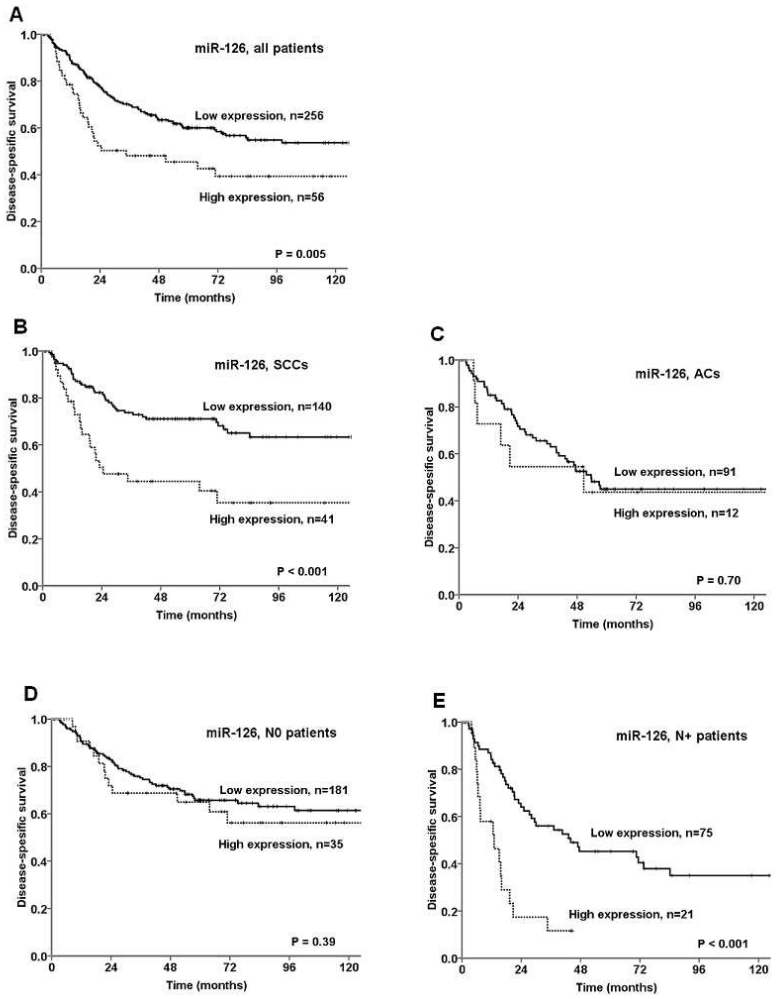

Fig. 2: Disease-specific survival curves according to miR-126 expression in: (A) The total material; (B) Squamous cell carcinomas (SCCs); (C) Adenocarcinomas (ACs); (D) Lymph node positive patients; (E) Lymph node negative patients.
220x320mm (96 x 96 DPI)

Figure 3

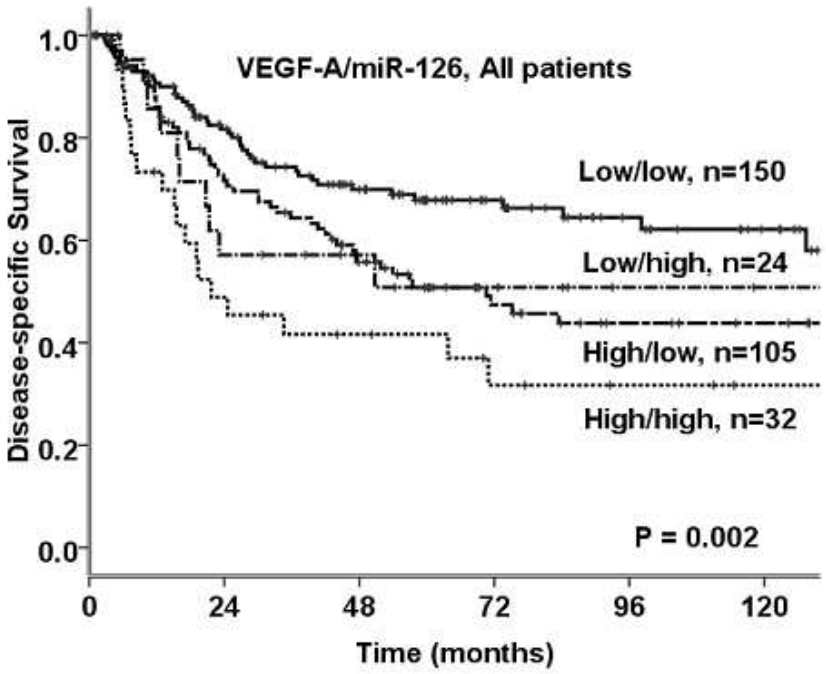

Fig. 3: Disease-specific survival curves according to the co-expression of VEGF-A and miR-126 in 335 resected NSCLC.
145x134mm (96 x 96 DPI)