

Angiogenic markers as prognostic factors in non-small cell lung cancer (NSCLC)

**A retrospective translational study on the prognostic
significance of angiogenesis related molecular markers
using immunohistochemistry and *in situ* hybridization on
tissue microarrays**

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LIST OF PAPERS

- Paper I** **Stenvold H, Donnem T, Andersen S, Al-Saad S, Al-Shibli K, Busund LT and Bremnes RM.** Overexpression of Matrix Metalloproteinase-7 and -9 in NSCLC Tumor and Stromal Cells: Correlation with a Favorable Clinical Outcome. *Lung Cancer*. 2012 Feb; 75(2):235-41.
- Paper II** **Stenvold H, Donnem T, Andersen S, Al-Saad S, Valkov A, Pedersen MI, Busund LT and Bremnes RM.** High Tumor Cell Expression of miR-21 in Node Positive NSCLC Predicts a Favorable Clinical Outcome. *BMC Clinical Pathology*. *Submitted*.
- Paper III** **Stenvold H, Donnem T, Andersen S, Al-Saad S, Busund LT and Bremnes RM.** Stage and Tissue-Specific Impact of miR-182 in NSCLC. *BMC Cancer*. *Submitted*.

LIST OF ABBREVIATIONS

AC	= Adenocarcinoma
Akt	= Protein kinase B
ALK	= Anaplastic lymphoma kinase
BAC	= Bronchioloalveolar carcinoma
BCL2	= B-cell lymphoma 2
CAF	= Cancer associated fibroblasts
CTTN	= Cortical actin-associated protein
DIG	= Digoxigenin
DNA	= Deoxyribonucleic acid
DSS	= Disease-specific survival
EGFR	= Epidermal growth factor receptor
EMT	= Endothelial-mesenchymal transition
ERK	= Extracellular regulated kinases
FFPE	= Formalin-fixed paraffin-embedded
FGF	= Fibroblast growth factor
HIF	= Hypoxia induced factor
HR	= Hazard ratio
HUVEC	= Human umbilical vein endothelial cell
IHC	= Immunohistochemistry
ISH	= <i>In situ</i> hybridization
LNA	= Locked nucleic acid
miRNA	= MicroRNA
MITF	= Microphthalmia-associated transcription factor
MMP	= Matrix metalloproteinase
NSCLC	= Non-small cell lung cancer
OS	= Overall survival
PFS	= Progression free survival
PI3K	= Phosphatidylinositol-3-kinase
qRT-PCR	= Quantitative real time polymerase chain reaction
RGS	= Regulator of G-protein signaling
RNA	= Ribonucleic acid
SCC	= Squamous cell carcinoma
SCLC	= Small cell lung cancer
TGF- β	= Transforming growth factor-beta
TKI	= Tyrosine kinase inhibitor
TMA	= Tissue micro array
VEGF	= Vascular endothelial growth factor

1 INTRODUCTION

1.1 Lung Cancer

1.1.1 Epidemiology

Lung cancer is the leading cause of cancer related deaths globally, accounting for 1.4 million deaths worldwide in 2008 (1). Also in Norway is lung cancer is the major cancer killer. In 2010, 1559 men and 1267 women were diagnosed with lung cancer, and it was registered 1275 and 946 lung cancer deaths the same year (2). This accounts for more deaths than for prostate cancer, breast cancer and practically twice the number of deaths from colon cancer. In Norway, six individuals die from lung cancer every day. While the lung cancer incidence rates for men peaked in the mid nineties and have declined the last years, the rates for women have continued to rise (Figure 1).

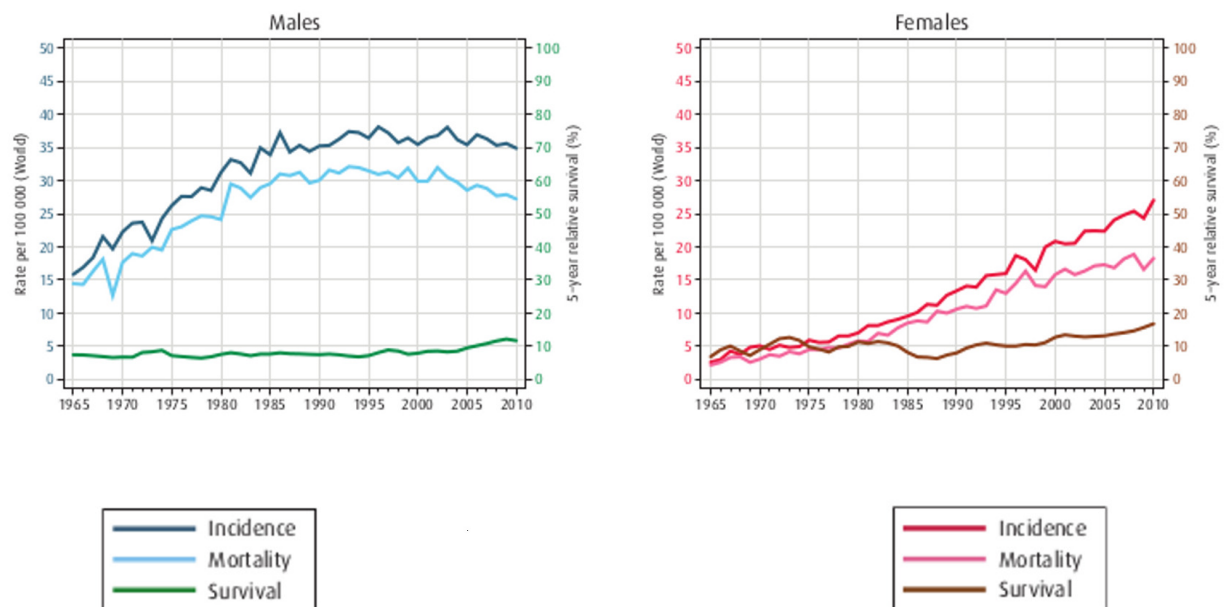


Figure 1: Trends in incidence and mortality rates and 5-year relative survival proportions (adapted from www.kreftregisteret.no; cancer in Norway 2010.)

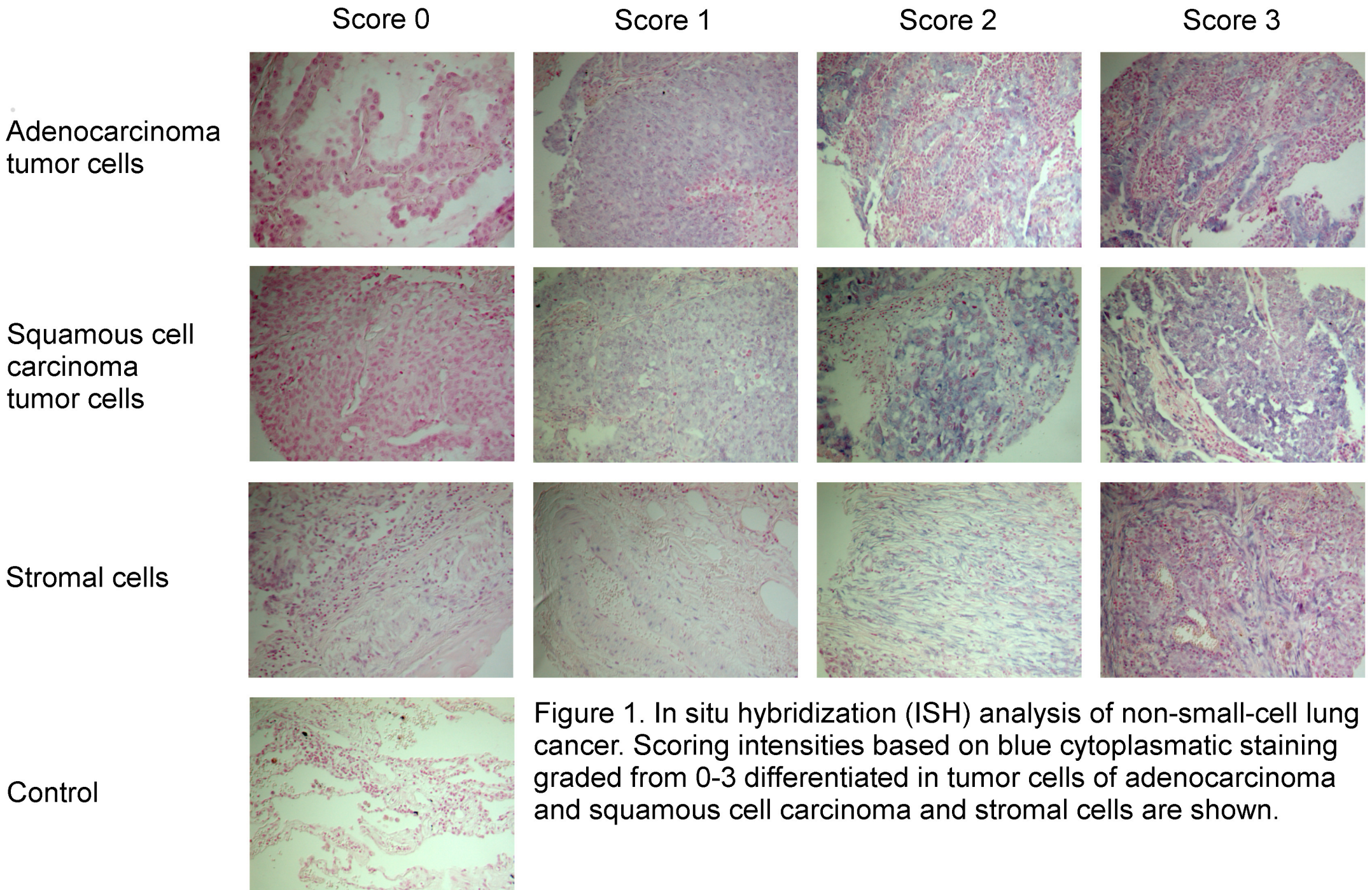


Figure 1. In situ hybridization (ISH) analysis of non-small-cell lung cancer. Scoring intensities based on blue cytoplasmic staining graded from 0-3 differentiated in tumor cells of adenocarcinoma and squamous cell carcinoma and stromal cells are shown.

Worldwide, smoking is believed to account for 80 % of lung cancer cases in men and at least 50 % in women (1). In western countries, 15-20 % of lung cancers are considered to have other causes than smoking. Other known risk factors are asbestos, arsenic, radon and polycyclic aromatic hydrocarbons (1), and from China it is believed that a high fraction of lung cancers are caused by indoor pollution from unventilated coal-fueled stoves (3). In Norway, the incidence rate closely mirrors the smoking habits. There has been a significant decline among male daily smokers since the early seventies, while females maintained their smoking habits until the late nineties before a decline was observed (4)(Figure 2).

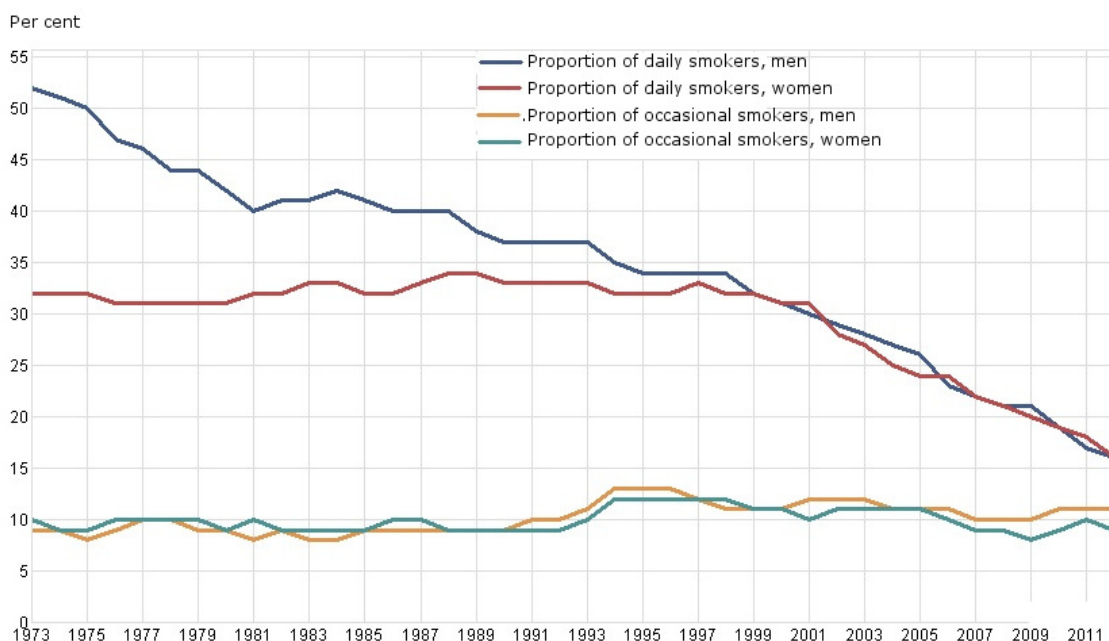


Figure 2: Female and male daily smokers and occasional smokers in Norway 16-74 year-olds. (adapted from www.ssb.no; Statistics Norway 2012.)

1.1.2 Histopathology

Lung cancer is clinically and histopathologically divided into non-small cell lung cancer (NSCLC; 85% of lung cancers) and small cell lung cancer (SCLC; 15% of lung cancers). The largest subgroups of NSCLC are adeno-, squamous cell and large cell

carcinomas. In recent years, adenocarcinomas have become the dominant subgroup, pushing squamous cell carcinoma down to 2nd place (5).

Our database was established in 2005-2006, and the pathology classification was according to the updated work by the World Health Organization from 2004 (6). In 2011, Travis and coworkers released a new, multidisciplinary classification for lung adenocarcinoma. One of the main differences from the 2004 classification is that the term bronchioloalveolar carcinoma (BAC) is replaced by adenocarcinoma in situ (AIS), lepidic predominant adenocarcinoma (LPA) or invasive mucinous adenocarcinoma (7). As we have included the BACs in the adenocarcinoma group when doing the statistics, these changes will have no practical influence on the results of our papers.

To get an exact pathologic diagnosis is essential. There are prognostic differences between histologic subgroups. Moreover, recent years drugs with effects only within certain subgroups with distinct molecular features have been developed. Whereas traditional special stainings and immunohistochemistry were used to differentiate between subgroups of NSCLC, more sophisticated methods is today needed to differentiate between subtypes of interest.

While NSCLC treatment was rather homogenous earlier, today's therapy is becoming more individualized. Pemetrexed and bevacizumab are only used in non-squamous cell carcinomas (8;9). In western countries about 10% of lung adenocarcinomas have mutation in the epidermal growth factor receptor (EGFR). In 1058 Norwegian patients tested, the mutation rate was 11.6% (10). These tumors are associated with a better response to treatment with tyrosine kinase inhibitors (TKIs) (11). 5-7 % of lung adenocarcinomas have a translocation in the anaplastic lymphoma kinase (ALK) gene, which make them more likely to respond to treatment with the ALK/MET inhibitor crizotinib (12). Mutation in the KRAS

gene may also be important to detect. Around 30 % of adenocarcinomas have this mutation, which is associated with resistance to TKI treatment (13;14).

1.1.3 Diagnosis, staging (TNM) and prognosis

Most lung cancer patients have symptoms at the time of diagnosis, which often means that the disease is discovered at an advanced stage beyond the chance of cure. In 2011, the US National Lung Screening Trial (NLST) reported a significant mortality reduction from CT screening of high risk patients (15). However, a screening program for lung cancer has still not started. Results from supplemental studies, and a more optimal selection of high risk groups are warranted before screening programs should be implemented (16).

Chest X-ray is often the primary imaging examination done when lung cancer is suspected, but where there is a strong suspicion a CT of the lungs including the supraclavicular fossa and the upper abdomen with adrenal glands should be done. If a lung tumor is detected on CT scan, bronchoscopy, endobronchial ultrasound or esophageal ultrasound is done for histology or cytology. If unsuccessful, CT guided biopsy is mandatory if the tumor is peripheral. Today PET-CT is often performed to decide the final TNM. PET-CT is important for differentiating benign from malignant masses and for detecting possible distant metastases. Brain MRI is often done to rule out possible brain metastases, especially for small cell lung cancer.

The new TNM classification for lung cancer was published in 2009 by IASCL (17) (Table 1). This classification is important in order to separate patients according to proper risk groups. This is essential in selecting the most appropriate treatment regimen.

Table 1: The seventh edition of TNM classifications and stage groupings (adapted from ref 17)

Stage	Sub-stage	T Category	N Category	M Category	5-year survival
Occult carcinoma		Tx Primary tumor not assessed or proven only by cells	N0 No regional lymph node metastasis	M0 (no distant metastasis)	
Stage 0		Tis carcinoma in situ	N0	M0	
Stage I	IA	T1a Tumor ≤ 2 cm	N0	M0	73%
		T1b Tumor ≤ 3 cm > 2 cm		M0	
	IB	T2a Tumor ≤ 5cm > 3 cm	N0	M0	58%
Stage II	IIA	T1a	N1 metastasis in ipsilateral hilar LN	M0	46%
		T1b	N1		
		T2a	N1		
		T2b Tumor ≤ 7 cm > 5 cm	N0		
	IIB	T2b	N1	M0	36%
		T3 Tumor > 7 cm/involving chest wall, pleura or pericardium/in the main bronchus <2 cm from carina	N0		
Stage III	IIIA	T1	N2 metastasis in ipsilateral mediastinal and/or subcarinal lymph nodes	M0	24%
		T2	N2		
		T3	N1		
		T3	N2		
		T4 Tumor invading mediastinum, heart, great vessels, trachea, esophagus, vertebral body, carina or tumor in another ipsilateral lobe	N0		
		T4	N1		
	IIIB	T4	N2	M0	9%
		Any T	N3 metastasis in contralateral mediastinal, hilar, scalene or supraclavicular LN		
Stage IV	IV	Any T	Any N	M1A pleural or pericardial effusion or separate tumor in contralateral lobe M1B distant metastasis	13%

1.1.4 Treatment of NSCLC

Surgery is the treatment of choice for potentially curable NSCLC patients, and was for a long time the only therapy for this group. During the last decade, adjuvant chemotherapy has become routine in stage II-IIIa patients (18). Today, stage IIIa and IIIB patients are treated with radiochemotherapy (19). Our cohort was treated before introduction of adjuvant chemotherapy and radiochemotherapy. In more advanced stages of the disease, chemotherapy is the main systemic therapy, although targeted drugs have been introduced in specific NSCLC subgroups (see 1.1.4.2).

1.1.4.1 Curable NSCLC

Surgery is the most important treatment modality, and stage I patients are operated with no adjuvant or neoadjuvant treatment. For all stage II and stage IIIa patients (N1 and pN2) disease, surgery and adjuvant chemotherapy is the treatment of choice. pN2 patients should receive thoracic radiation after adjuvant chemotherapy. Four cycles with cisplatin and vinorelbine is the preferred adjuvant regimen (18). Stage IIIa cN2 patients are treated with radiation in combination with chemotherapy (19).

In otherwise resectable tumors in medically inoperable patients, standard treatment is radiation (66-70 Gy) with curative intention, with or without chemotherapy. Postoperative radiotherapy is indicated at unfree surgical margins, and for patients with pN2 or pN3 disease (20).

1.1.4.2 Advanced NSCLC

About 70 % of lung cancer patients are diagnosed when the disease has reached a stage where cure is not possible (21). Some of these patients may not receive any treatment due to severely reduced performance status in addition to well advanced disease, but the majority receives chemotherapy, targeted therapies or radiotherapy treatment with palliative aim.

Platinum doublets, a platinum based drug in combination with vinorelbine, gemcitabine, pemetrexed or docetaxel are the most usual combinations in first line treatment of NSCLC. Chemotherapy in NSCLC is administered as 3-4 cycles with a platinum doublet. In Norway carboplatin and vinorelbine is the regimen of choice based on the efficacy and toxicity profile (22). Similar to other cancers, some studies are suggesting that maintenance therapy may have effect (23). Pemetrexed doublet is used only in patients with non-squamous histology, as it has inferior effect in squamous cell carcinomas when compared with other doublets (9;24). Besides, pemetrexed maintenance, immediately after four platin-based doublet courses, has shown significantly better progression free survival (PFS) and overall survival (OS) (25;26).

In patients with EGFR tyrosin kinase mutation or ALK translocation, tyrosine kinase inhibitors (TKIs) or crizotinib is administered, respectively. For the mentioned genetic alterations, EGFR TKIs have a superior effect in first line therapy, while crizotinib have proven its superiority in second line treatment (27-29).

1.2 Angiogenesis

1.2.1 Angiogenesis

As stated by Folkman in 1971, a tumor can only reach a size of 1-2 mm³ without developing new blood vessels (30). Tumor angiogenesis has throughout the years become one of the central topics in cancer research, and was in the renowned review by Hanahan and Weinberg established as one of the hallmarks of cancer (31). Angiogenesis is the process where blood vessels are formed from preexisting ones, in contrast to vasculogenesis, which is the forming of new vessels as happens in embryogenesis. Blood vessels are normally stable structures, and endothelial cells are among the cells in the body with the slowest turnover. However, when a tumor starts evolving, the need for nutrients and oxygen to the new cells makes development of new vessels necessary, and the turnover of endothelial cells is speeded up. The process where angiogenesis is turned on is called the angiogenic switch (32).

Different signalling molecules are believed to contribute to triggering the shift from a quiescent state to an active angiogenic state (33;34).

One of the most important players in stimulating angiogenesis is the vascular endothelial growth factor (VEGF), and in particular VEGF-A. During the angiogenic switch, the matrix metalloproteinase-9 (MMP-9) is contributing in increasing the bioavailability of VEGF-A. MMP-2 and -7 are also important factors in stimulating angiogenesis. But the complexity of this process is evident, as the same MMPs also play central roles in the production of angiogenesis inhibitors (35).

1.2.2 Biomarkers associated with angiogenesis covered in this thesis

1.2.2.1 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases are a family of zinc-dependent endopeptidases. They are involved in degradation of the extracellular matrix, and also in various other physiological processes including regulation of inflammatory processes, signaling for cell growth and angiogenesis (36;37). MMPs can have both pro- and antiangiogenic properties. They are involved in the “angiogenic switch” in tumors, where VEGF is made available and angiogenesis is stimulated (38;39). But MMPs may also inhibit angiogenesis, as they generate various angiogenesis inhibitors. Three of these inhibitors are endostatin, tumstatin and angiostatin (40-42).

1.2.2.2 MicroRNAs (miRNAs)

MicroRNAs are small non-coding RNAs (19-22 nucleotides). They post-transcriptionally regulate the stability and translation of mRNAs. Today, we know more than 1500 human miRNAs. It is assumed that they regulate approximately 30 % of the genes. miRNAs play an important role in various processes, such as differentiation, proliferation,

apoptosis, angiogenesis, metabolism, development, immunity and stress response (43;44). They are located at sites known to be altered in cancer, and are frequently deregulated (45). miRNAs have become interesting potential therapeutic targets. Novel miRNA targeting agents have not reached clinical trials, but considerable research is going on in this field (46;47).

The mature single-stranded miRNAs are processed from larger double-stranded precursor transcripts with a characteristic hairpin structure. In the nucleus, the first step of sequential cropping of the transcript (pri-miRNA) is carried out by Drosha, a RNase III endonuclease. The resulting 70-90 nucleotide pre-miRNA is transported to the cytoplasm by the export receptor exportin 5. In the cytoplasm another RNase III endonuclease, Dicer, performs cropping of the pre-miRNA resulting in a double-stranded miRNA duplex. One of the strands is loaded into the RNA-induced silencing complex

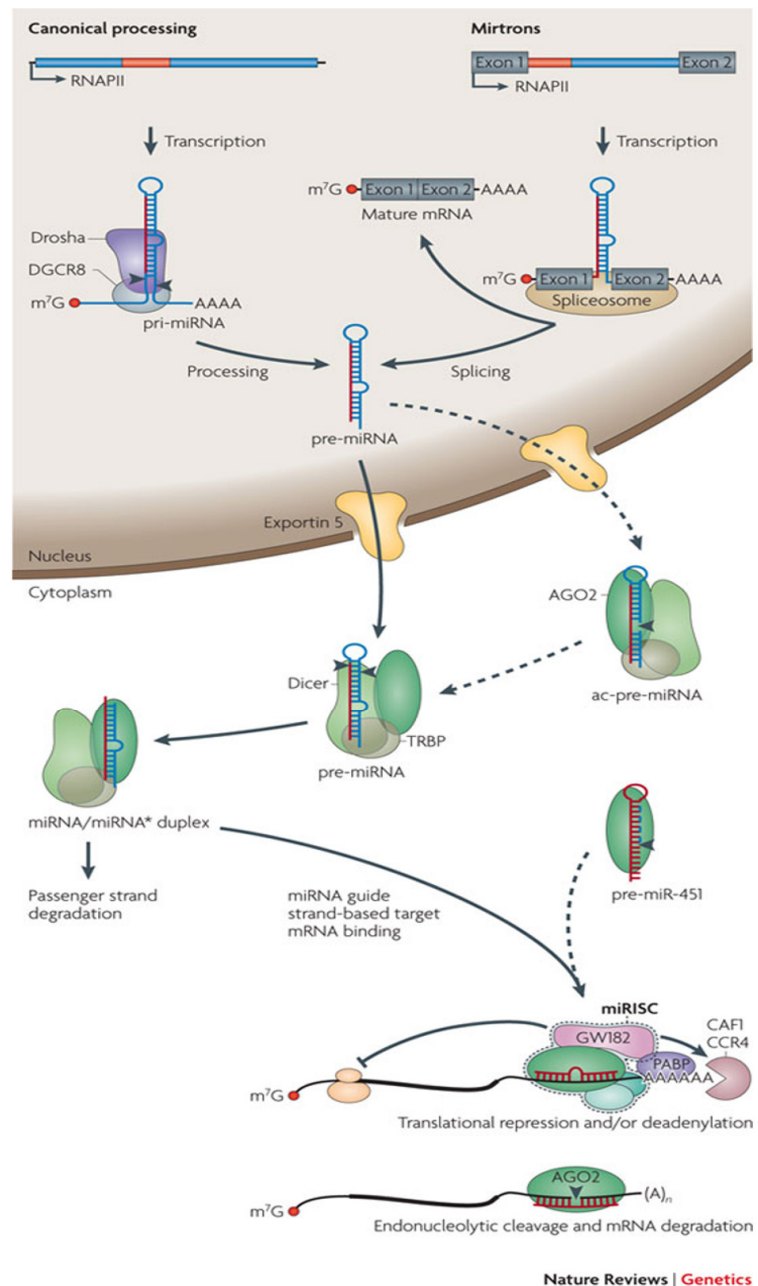


Figure 3: The microRNA biogenesis. Figure adapted from Krol J, Loedige I (46).

(miRISC) while the other strand is degraded. The miRNA is finally base paired with its target mRNA (Figure 3) (48).

MicroRNAs are involved in angiogenesis (49-51). Indications of this were found in a study where Dicer-knockout mice died within 14.5 days of gestation due to lack of angiogenesis. miRNAs have also been found to regulate well known angiogenic factors, such as VEGF, and thereby influencing angiogenesis (49). In a pilot study carried out by our research group, we screened the expression of 281 miRNAs in NSCLC tissues. Herein, pathway analyses showed that the gene set connected to angiogenesis-related miRNAs had the highest impact (52).

1.3 Tumor and stroma

Most research on cancer has been concentrated on the tumor cells alone, but recent years it has become evident that the tumor cells live in a close and dependent relationship with their surroundings, called the tumor stroma. Among other components, the tumor stroma consists of fibroblasts, endothelial cells, pericytes, immune cells and extracellular matrix. When a cancer cell resides in the stromal compartment, it interacts with the surrounding environment. This can make conditions more suitable for tumor growth, as the stroma changes from its usual protective role in cancer development (53). In a growing tumor, there is a continuous paracrine communication between tumor cells and the surrounding stromal cells. Today there is an increasing interest in studying the interaction between tumor cells and their surroundings to understand the dynamics of the growing tumor.

Two of the most interesting cell-types of the tumor stroma are immune cells and cancer associated fibroblasts (CAFs). The latter are activated fibroblasts releasing mediators like growth factors, cytokines and immune modulators (54). The origin of CAFs is still controversial. They may derive from local resident fibroblasts, bone marrow-derived mesenchymal cells, pericytes, smooth muscle cells or even from epithelial cells via epithelial-

mesenchymal transition (EMT). They represent the largest cell population in the tumor stroma (55). CAFs may influence tumor growth by producing growth factors like transforming growth factor-beta (TGF- β), which promotes tumorigenesis (56). Also, angiogenesis may be altered, as CAFs are involved in the PDGF pathway (57). CAFs also contribute to migration and metastasis by modulating the stroma in a paracrine manner through secretion of proteases like MMPs, cathepsins and plasminogen activators (58).

The abundance of inflammatory cells is a known feature of the tumor stroma, especially in NSCLC. An adaptive response of the immune system may create a favorable microenvironment for cancer development (59). Experiments have shown that chronic inflammation promotes carcinogenesis (60).

MicroRNAs play a distinct role in the development of the microenvironment (61). Among other miRNAs, miR-21 is found to contribute to the differentiation of fibroblasts to CAFs (62;63). Several miRNAs are deregulated in CAFs relative to normal fibroblasts. Effects of miRNAs on angiogenesis also show the impact in the stromal compartment of these small molecules. Much is still unknown about the paracrine mediators of the miRNA-mediated response. More knowledge about these mediators will be essential for understanding the crosstalk between tumor cells and their stromal surroundings (61).

2 AIMS OF THESIS

The aim of this thesis was to investigate the prognostic impact of angiogenesis related markers in NSCLC, based on their expression in both tumor cells and tumor related stromal cells.

More specifically we aimed to:

- Investigate the immunohistochemical expression of MMP-2, -7 and -9
- By *in situ* hybridization examine the expression of miR-21 and miR-182
- Explore the prognostic impact of these molecular markers and their relation to other angiogenic markers and known prognostic factors in NSCLC

3 MATERIALS AND METHODS

3.1 Patients

Between 1990 and 2004, 371 patients with pathological stage I to IIIA non-small cell lung cancer were diagnosed at the University Hospital of North Norway and Nordland Central Hospital. Out of 371 patients, 36 were excluded from the study due to radiotherapy or chemotherapy prior to surgery (n = 10), other malignancy within 5 years before NSCLC diagnosis (n = 13) or inadequate paraffin-embedded fixed tissue blocks (n = 13). Thus, 335 patients with complete demographic and clinicopathological data were eligible. (Figure 4).

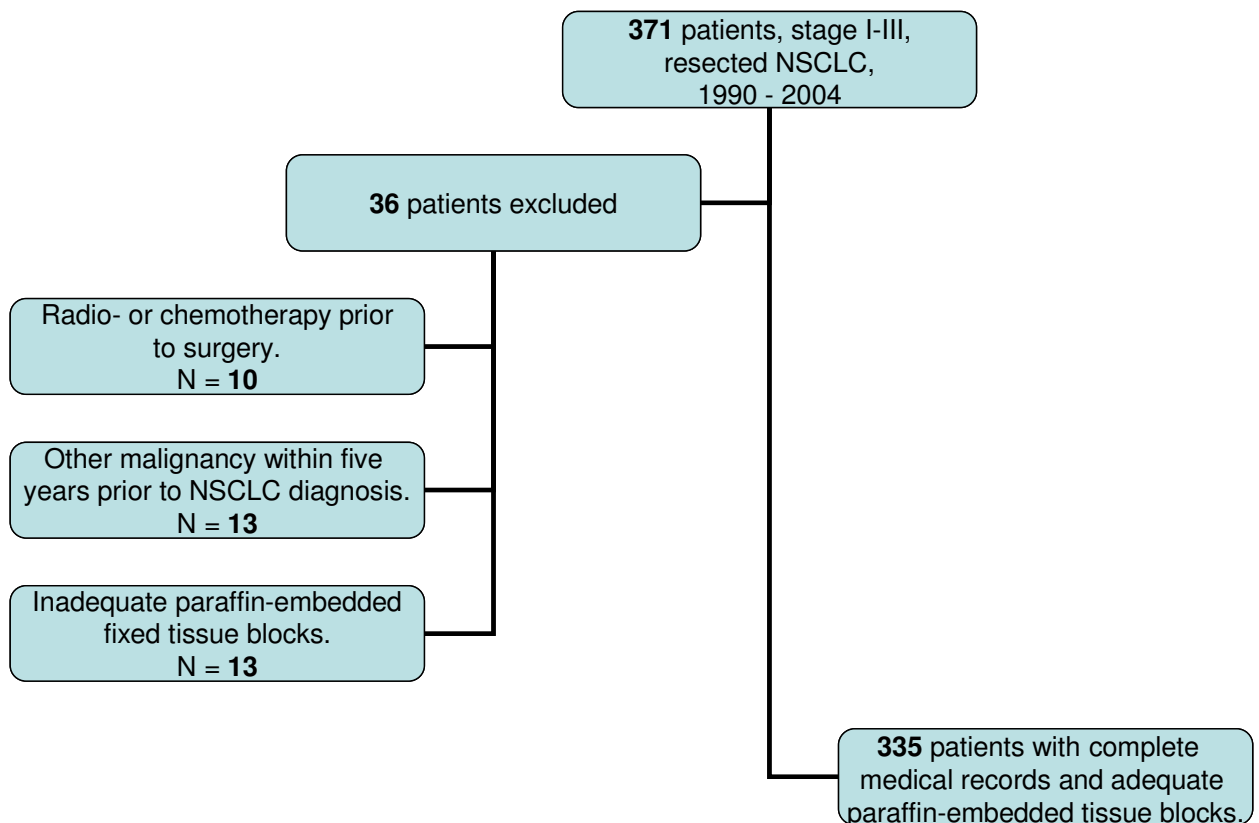


Figure 4: Patient inclusion and exclusion

Demographic, clinical and histopathological variables are shown in Table 2. The most recent (third) disease-specific survival (DSS) update was done in January 2011. The median follow-up time of survivors was 105 months (range 73-204) and the median patient age was 67 years. For the first paper, we used data from the update in November 2008. Based on the latest update in 2011, we have recalculated the results from the first paper. The results remain basically unchanged.

Table 2: Patient characteristics and their variables as predictors for disease-specific survival in 335 NSCLC patients (univariate analyses; log-rank test).

Characteristics	Patients		Median survival	5-year survival	P
	n	(%)	months	%	
Age					
≤ 65 years	156	(47)	98	55	0.42
>65 years	179	(53)	NR	60	
Sex					
Female	82	(24)	190	64	0.22
Male	253	(76)	98	56	
Smoking					
Never	15	(5)	19	43	0.26
Current	215	(64)	NR	60	
Former	105	(31)	84	55	
Performance status					
PS 0	197	(59)	NR	63	0.016
PS 1	120	(36)	64	52	
PS 2	18	(5)	25	33	
Weight loss					
< 10 %	303	(90)	190	58	0.76
> 10 %	32	(10)	98	57	
Histology					
SCC	191	(57)	NR	66	0.028
Adenocarcinoma	113	(34)	54	46	
LCC	31	(9)	98	56	
Differentiation					
Poor	138	(41)	47	47	< 0.001
Moderate	144	(43)	190	65	
Well	53	(16)	NR	68	
Surgical procedure					
Lobectomy + Wedge*	243	(73)	190	62	0.007
Pneumonectomy	92	(27)	37	47	
Pathological stage					
I	157	(47)	NR	61	< 0.001
II	136	(40)	62	51	
IIIa	42	(13)	17	23	
Tumor status					
1	85	(25)	190	75	< 0.001
2	188	(56)	84	57	
3	62	(19)	25	36	
Nodal status					
0	232	(69)	NR	67	< 0.001
1	76	(23)	35	43	
2	27	(8)	18	18	
Surgical margins					
Free	307	(92)	190	59	0.37
Not free	28	(8)	47	48	
Vascular infiltration					
No	284	(85)	190	62	0.001
Yes	51	(15)	27	33	

*Wedge, n = 10

Abbreviations: NR = not reached; PS = performance status; SCC = squamous cell carcinoma, LCC = large-cell carcinoma. Adenocarcinoma including cases with bronchioloalveolar carcinoma.

3.2 Tissue Micro Array

Tissue micro arrays (TMAs) represent an efficient and cost effective way to investigate the molecular profile of a large tissue cohort. In 1986, Battifora introduced the multitumor block for immunohistochemical antibody testing of a large sample of tissue specimen (64). The method was refined, and in 1998 Kononen et al. published what is referred to as the first modern TMA study (65). The technique rapidly became a much used method in molecular profiling studies (66).

On a single slide, cores from hundreds of specimen can be evaluated in one single operation. This method has revolutionized large scale investigations of molecular markers and their biological and prognostic features. Using methods as immunohistochemistry (IHC) or *in situ* hybridization (ISH), it is possible to perform high-throughput marker analyses on DNA, RNA or protein level.

3.2.1 TMA construction

Two pathologists reviewed all lung cancer cases histologically. The most representative paraffin donor blocks were chosen for each case, and then representative areas of tumor and stromal cells were selected and marked on the corresponding hematoxyline and eosine (H/E) slide and sampled for the recipient TMA blocks. With a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD) TMAs were assembled using a 0.6mm thin walled stainless steel biopsy needle. The instrument was used to create holes in the recipient paraffin block to “home” tissue cores from the donor block. Two cores each of tumor cells and stromal cells were biopsied from the donor blocks and transferred to the recipient block. The recipient block was held in a X-Y position guide that was manually adjusted by micrometers. To include the entire 1340 cores of interest plus control cores, eight

tissue array blocks were constructed. Multiple 4- μ m sections were cut with a Micron microtome (HM355S) and stained by specific antibodies for IHC or processed for *in situ* ISH analysis.

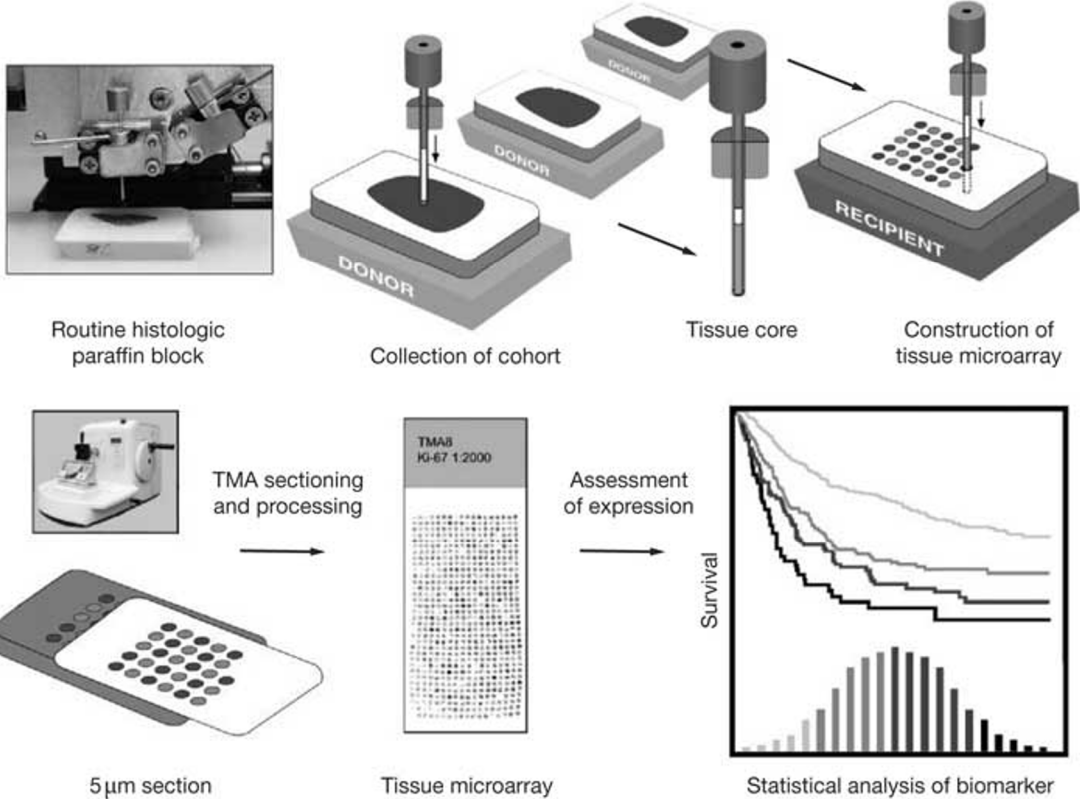


Figure 5: Construction of a microarray. Representative areas from each paraffin-embedded, formalin-fixed donor tumor block are selected. 0.6-2.0 mm in diameter cores are punched and arrayed into a donor block. Sections of the resultant tissue microarray block are cut and transferred to a glass slide for processing of biomarker status by IHC or ISH. Figure adapted from Giltane JM, Rimm DL (67)

3.2.2 TMA – advantages and disadvantages

There are both advantages and disadvantages related to the use of the TMA technology. Table 3 summarizes some of these issues:

Table 3: Advantages and disadvantages with TMA technology

Advantages	Disadvantages
Time saving	Can be technically challenging
Cost saving	Tempting to use otherwise not suited material
Scoring by less trained personnel	Lower accuracy when heterogenous expression
Tissue saving	Variation through the core
Larger study cohorts	Not suited for individual diagnosis
Standardized staining conditions	
Sharing with other institutions	

Compared to using whole sections of tissues, with one slide for every patient, the benefits with the TMA technology are obvious. When the TMA blocks are made, it is time saving for the technician. With our material as an example, eight slides are cut instead of 335, and IHC or ISH can be done on these eight slides in one operation. Processing eight slides instead of 335 will also save significant costs through reduced consumption of antibodies/probes and other reagents and material. It is time saving for the scorer(s), who immediately see the tumor areas or stromal areas in the microscope ready for scoring, and do not each time have to locate suitable areas on a whole section slide.

Concerns have been raised about the representativity of 0.6 mm cores compared to whole section slides. The cores are biopsied from areas which are carefully selected and marked by pathologists, and comparisons have shown a correlation on 90-95% between scoring of TMA cores and whole sections when it comes to evaluation of larger cohorts (68-

70). It is also possible to increase the representativity by increasing the number of cores from each patient (71). However, the TMAs are not well suited for diagnostic purposes, as the cores with diameters from 0.6 mm to 2 mm will not reflect variations in heterogeneous tissues (72).

TMA slides are well suited for exchange between research institutions and laboratories. By exchanging stained slides, it is possible to compare scoring of slides and choice of cut-off values, while unstained slides make it possible to compare IHC or ISH procedures.

Making good TMAs is dependent on dedicated and trained technicians. It is easier to embed and cut traditional blocks than to produce and cut TMAs. It is also important to remember that although it may be tempting to include as many patients as possible because of the features of the TMA technique, it is essential that the tissues have been processed in a similar way. By collecting tissues over a large time span, the chances for alteration in tissue processing techniques increase.

3.3 Immunohistochemistry (IHC)

IHC is a widely adopted method for detecting the expression of a given biological molecule, and it is considered the gold standard for *in situ* protein expression detection in tissue sections. In short, the method allows for the visualization of antigens by sequential application of a specific antibody to the antigen, a secondary antibody to the primary antibody, an enzyme complex and a chromogenic substrate. The enzymatic activation of the chromogen results in a visible reaction product (e.g. color) at the antigen site. The method has the advantage of being relatively inexpensive, it is established in most laboratories, it can be done on archival tissues and it is possible to evaluate expression in cells from different compartments of tissues. It is a multi-step process with potential pitfalls. To get a good result, an experienced technician is warranted. Often adaptations have to be made to find the best

set-up for antigen retrieval, incubation time of antibodies, dilutions, washing time and techniques. The TMA technique gives the advantage of standardization among all cores on the slide compared to staining of multiple whole tissue slides.

3.3.1 Antibodies

There are two principally different groups of antibodies used in IHC: Monoclonal and polyclonal antibodies. Polyclonal antibodies are a heterogeneous mixture of antibodies directed against various epitopes of the same antigen. Polyclonal antibodies are most frequently produced in rabbits, but also other animals are used. They are produced by immunizing the animal with an antigen. Three to eight months later blood is collected, and the antibody is purified. These antibodies can have slightly different affinities and specificities against the antigens.

Monoclonal antibodies are a homogeneous population of immunoglobulin directed against a single epitope of the antigen. They are generated by a single B-cell clone and are therefore immunochemically similar. They are usually produced in rabbits and mice. After achieving a satisfying immune response, B-lymphocytes are isolated and fused with immortal myeloma cell lines. The new isolated cell line can produce antibodies either in a bioreactor system or cells can be injected into the peritoneal cavity of an animal.

There are benefits to both groups of antibodies. The polyclonal antibodies are more robust, and there is a smaller chance for false negative results as the antibody recognizes various epitopes. But at the same time, recognizing more epitopes increases chances for cross-reactivity. Monoclonal antibodies have the advantage of lot-to-lot consistency, since its production depends on an immortal monoclonal cell line and not on the life of the animal as with the polyclonal antibody production. Disadvantages may be weaker signal and a higher chance of false negative results.

3.3.2 IHC procedure

The 4 µm sections were deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval was performed by placing the sections in 0.01 M citrate buffer at pH 6.0 and heating them in microwave oven for 20 min at 450W. The Vectastain elite ABC kit from Vector Laboratories was used for endogen peroxidase blocking. All primary antibodies were incubated overnight at 4°C. The Vectastain kit was then used for detection with secondary antibodies, and the Vector NovaRed Substrate kit was used to visualize the target antigen with a brown color. For each antibody, all the slides were handled in one single operation. As negative staining controls, the primary antibodies were replaced with the primary antibody diluent. Finally, all slides were counterstained with haematoxylin to visualize the nuclei.

The antibodies used in this study were subjected to in-house validation by the manufacturer for IHC analysis on paraffin-embedded material.

Table 4: Antibodies used in paper 1

Antigen	Type	Manufacturer	Catalog #	Dilution
MMP-2	Mouse monoclonal	Abcam	Ab7032	1:15
MMP-7	Rabbit polyclonal	Abcam	Ab4044	1:15
MMP-9	Mouse monoclonal	Abcam	Ab51203	1:100

3.4 In situ hybridization (ISH)

In situ hybridization is a technique that uses labelled complementary DNA or RNA strands to detect a specific DNA or RNA sequence in tissues. ISH is a method where background staining may be a problem. If so, it will be difficult to separate the nucleotide of

interest from the rest of the tissue. There are pitfalls with the ISH method which may cause background staining; a too high concentration of the labelled probe, too long hybridization time, or an inadequate post-hybridization wash may produce staining in areas where there are no nucleotides present. Besides, smaller probes (less than 25 nucleotides) traditionally have a much narrower window of signal to background, and are therefore more likely to produce background staining than longer probes (73). MicroRNAs are small in size, typically from 19-22 nucleotides. For long, the small size made it hard to use ISH as a method for detecting miRs. The problem was solved when the locked nucleic acid (LNA) probe was developed, which fixes the small nucleotide in a three dimensional space and rises the melting temperature for the LNA probe and its complementary miR-sequence substantially.

One of the main advantages using the ISH technique is that it allows us to study the molecular expression in cells in different compartments of tissues. Most previous studies have used polymerase chain reaction (PCR)-techniques to detect miRNAs. With PCR, the miRNAs you detect will originate from a mix of tumor cells and stromal cells. Our research group is among the first to use ISH technique with this purpose, and to our knowledge the very first on NSCLC.

3.4.1 ISH procedure

In situ hybridization was performed following the protocol developed by Exiqon, Vedbaek, Denmark (74). Digoxigenin (DIG) labelled locked nucleic acid (LNA) modified probes from Exiqon for miR-21 (has-miR-21), miR-182 (hsa-miR-182), positive control (U6, hsa/mmu/rno) and negative control (scramble-miR) were used in these papers. Some adjustments were done to get a specific and sensitive detection of miRNA in our sections from formalin-fixed paraffin-embedded (FFPE) TMA blocks.

We placed 4 μm sections of the TMA blocks in a heater at 59°C over night to attach cores to Super Frost Plus slides. Sections were deparaffinised with xylene (3 x 5 min) and

then rehydrated with ethanol solutions (99.9% - 96% - 70%) ending up in PBS, pH 7.4. Proteinase-K (20 µg/ml) (Exiqon, Vedbaek, Denmark) treatment was done in PK-buffer (5mM Tris-HCl, pH 7.5, 1mM EDTA, 1 mM NaCl, autoclaved) at 37°C for 20 min in a HYBrite automated hybridizer (Abbot laboratories, IL, US). After a PBS wash the sections were dehydrated through increasing gradient of ethanol solutions and air-dried. The LNA-probes were denatured by heating to 90°C for 4 min. Hybridization of the LNA-probe miR-182 (100nM) and scramble miR (50nM) control was carried out in the HYBrite automated hybridizer at 50°C for 60 min. The positive control U6 (1nM) was hybridized at 55°C for 60 min. Stringent washes was performed in pre-heated SSC buffers, 1 x 5 min in 5x SSC, 2 x 5 min in 1x SSC and 0,2x SSC. Sections were blocked against unspecific binding in blocking solution from DIG wash and Block Buffer set (Roche, Mannheim, Germany) for 15 min at room temperature (RT). Alkaline phosphatase (AP)-conjugated anti-DIG (Roche) 1:800 was incubated for 60 min at RT for immunologic detection. After PBS-T wash the substrate enzymatic reaction was carried out with NBT/BCIP (Roche) at 30°C in the hybridizer for 120 min. The reaction was stopped with a 2 x 5 min wash in KTBT buffer (50mM Tris-HCl, 150mM NaCl, 10mM KCl). Sections were counter stained with nuclear fast red (WALDECK, ZE-012-250) at RT for 1 min and then rinsed in tap water. Dehydration followed through increasing gradient of ethanol solutions and finally mounting with Histokitt mounting medium (Assistant-Histokitt, 1025/250).

3.5 Scoring

In paper 1 and 3, scoring was performed by light microscopy where representative and viable tissue sections were scored semiquantitatively for cytoplasmic staining. The dominant staining intensity in both tumor and stromal cells was scored as: 0 = negative; 1 = weak; 2 = intermediate; 3 = strong. The cell density of stroma was scored as: 1 = less than 25% positive cells; 2 = between 25% and 50% positive cells; 3 = more than 50% positive cells. In paper 2,

the ARIOL imaging system (Genetix, San Jose, CA) was used, and the cores were scored on a computer screen following loading and scanning of the slides. Tumor cells were scored as described for paper 1 and 3, while stromal cells were given a score from 0-3 based on both staining intensity and cell density.

All samples were anonymized and independently scored by two pathologists in paper 1 and 2, and one pathologist and one oncologist in paper 3. In a previous paper from our group, the interobserver scoring agreement in this material was calculated for two molecular markers (VEGF-C and VEGFR-3). The mean correlation coefficient (r) was 0.95 (range 0.93-0.98) (75).

Mean score for duplicate cores from each individual was calculated separately in tumor and stroma. Regarding paper 2, up to 4 cores was scored by each pathologist, because tumor cells if possible, were scored also in “stromal” cores and vice versa. High expression in tumor cells was defined as score >0 (miR-182), ≥ 0.5 (miR-21) and ≥ 2 (MMP-2, MMP-7, MMP-9). Stromal expression in MMP-2 and MMP-9 was calculated by summarizing intensity score (0-3) and density score (1-3). In miR-21, one score (0-3) was based on both intensity and density. High expression in stroma was defined as score >0 (mir-21), ≥ 3.5 (MMP-2) and ≥ 4.5 (MMP-9).

3.6 Cut-off values

The expression of the biomarkers in our studies varies over a continuous scale, and is not a matter of negative or positive expression. The choice of a cut-off point is therefore an important issue. To standardize cut-off values is difficult, due to variations in methods including differences in tissue preparation, antigen retrieval, and assessment of positive staining. Using the mean value is an approach employed in many studies, but is not necessarily the best option. By using the mean value, there is a risk that biologically important

information is lost leading to false negative results (type 2 errors). On the other hand, the chance for false positive results decreases (type 1 errors), and the results may be easier to reproduce. In our studies, we have used cut-offs which gave the most difference in DSS between the subgroups while maintaining large enough subgroups. By this approach we expect to identify the biologically significant cut-offs. It would be of great value to get an independent validation of our results, and we now try to do this by expanding our own material (using specimen from one of the institutions as a validation set). We have also initiated collaboration with another lung cancer research group to establish a validation set.

3.7 Controls and limitations

Regarding the IHC-procedure, both reagent and tissue controls were used. Negative reagent control was performed by replacing the primary antibody with a primary antibody diluent to rule out staining without the antibody. The positive control was done by staining tissues with a known expression pattern for the actual antibodies. This was done in lung cancer tissues, normal lung and in tissues from other organs. For the ISH procedure, negative control was done by using a miRNA-probe which is not complementary to any known human miRNA, a so called scramble-miR. For positive control, a U6 probe was used.

To ensure that an antibody is specific, a western blot is done to confirm binding of a protein with the expected size. This procedure is normally done by the manufacturer of the antibody, and we have trusted their documentation. However, we have in our group lately performed some western blot procedures on selected antibodies to ensure that they are of proper quality.

Another concern is that tissue storage over several years may affect the results. The oldest tissue blocks are dated back to 1990. Archival blocks dating back 20-40 years are considered adequate for evaluation provided initial fixation in 4% buffered formalin (70). We

did not experience any systematic variation between older and newer blocks when examining the slides

3.8 Statistical analysis

Sample size was estimated with survival as the primary endpoint. At least a 50% increase in hazard ratio resulting from the presence of a specific marker was assumed to represent a clinically significant effect. The 5-year DSS for patients with resected NSCLC is about 60%, and the frequency of a given level of a specific marker is typically about 35%. Analyzing the primary endpoint in a proportional hazard regression with a specific marker at a specific level as a dichotomous independent variable, 300 subjects are necessary to achieve a power of 80% at an alpha of 5% (PASS 2002, Number Cruncher Statistical Systems, Kaysville, Utah, USA). This estimate does not take into account the testing of multiple markers in the actual analysis, and can only serve as a rough indication of the number of needed subjects.

The statistical analyses were done using the package versions 17.0 and 19.0 from SPSS (Chicago, IL). In all three papers, the Chi-square test and Fishers Exact test were used to examine the association between molecular marker expression and various clinicopathological parameters. Univariate analyses were done using the Kaplan-Meier method, and the statistical significance between survival curves was assessed by the log-rank test. Disease-specific survival was determined from the date of surgery to the time of lung cancer specific death. The Cox proportional hazards model was used to assess the independent value of different pretreatment variables on survival, in the presence of other variables. Only variables of significant value from the univariate analysis were entered into the Cox regression analysis. Probability for stepwise entry and removal was set at .05 and .10, respectively. A $p < 0.05$ was considered statistically significant for all analyses.

4 MAIN RESULTS

4.1 Paper 1 (MMP-2, -7 and -9)

This study aimed to explore the prognostic impact of MMP-2, -7 and -9 in tumor cells as well as in stromal cells of resected NSCLC tumors. MMP-2 and -9 were scored in both tumor and stromal cells, while MMP-7 was only possible to score in tumor cells.

4.1.1 Correlations

There was a strong correlation between high tumor cell MMP-2 expression and high stromal MMP-2 expression ($r = 0.409$, $P < 0.001$). Between molecular markers and clinicopathological variables, we found a moderate correlation between age > 65 years and both tumor cell and stromal cell expression of MMP-2 ($r = 0.263$, $P < 0.001$; $r = 0.313$, $P < 0.001$, respectively).

4.1.2 Univariate analyses

MMP-2 expression was not significantly associated with DSS in tumor or stroma, but there was a tendency towards a better survival at high stromal MMP-2 expression ($P = 0.053$). High MMP-7 tumor cell expression was significantly associated with a favorable DSS ($P = 0.029$). Also, patients with high stromal MMP-9 expression had a significantly better prognosis ($P = 0.001$).

4.1.3 Multivariate analyses

High tumor cell MMP-7 expression was an independent positive prognostic factor for DSS (HR 0.63, CI 95% 0.43 – 0.93). Also, high stromal MMP-9 expression was independently associated with a better prognosis (HR 0.52, CI 95% 0.34 – 0.80).

4.2 Paper 2 (miR-21)

In a pilot study, our group screened tumor tissues from 10 worst and 10 best prognosis NSCLC cases as well as 10 normal lungs for the expression of 281 miRNAs, among these several angiogenesis-related miRNAs (52). Quantified by microarray hybridization and validated by qRT-PCR, miR-21 had a four-fold change in tumor when compared to normal NSCLC tissues. Previous results on miR-21 had been conflicting, and we wanted to explore the impact of miR-21 on DSS in our large NSCLC cohort. miR-21 was assessed in tumor and stromal cells.

4.2.1 Correlations

We did not observe any significant correlation between miR-21 and the angiogenesis-related protein markers protein kinase B (Akt), phosphatidylinositol-3-kinase (PI3K), hypoxia induced factor 1 (HIF1 α) or VEGF-A.

4.2.2 Univariate analyses

Tumor cell expression of miR-21 had no significant impact on DSS when assessed in the overall NSCLC cohort. In subgroup analyses of node positive patients, high tumor cell expression of miR-21 was associated with a better prognosis compared to low expression ($P = 0.024$). In stroma of all patients, high miR-21 expression was a negative prognostic indicator ($P = 0.022$). This was also the case in the node-negative patients ($P = 0.044$).

4.2.3 Multivariate analyses

miR-21 expression had no independent impact on survival in the whole cohort. In node positive patients, however, low tumor cell expression was independently associated with a worse prognosis (HR 2.03, CI 95% 1.09 – 3.78).

4.3 Paper 3 (miR-182)

In the same pilot study as referred to for paper 2, miR-182 was the only miRNA to be up-regulated in all three comparisons: worst prognosis versus normal lung, best prognosis versus normal lung and worst prognosis versus best prognosis (52). From previously published studies, miR-182 was often reported up-regulated in cancers. But the results were highly conflicting regarding the impact of miR-182 as an oncogene or as a tumor suppressor. This made it interesting for us to explore this miRNA in a large NSCLC cohort. miR-182 was expressed only in tumor cells.

4.3.1 Correlations

We found significant, although weak, correlations between miR-182 and fibroblast growth factor 2 (FGF2) ($r = -0.147$; $P = 0.010$), HIF2 α ($r = 0.115$; $P = 0.047$) and MMP-7 ($r = 0.172$; $P = 0.003$).

4.3.2 Univariate analyses

In the whole cohort there was a tendency towards a better prognosis for patients expressing high tumor cell miR-182 ($P = 0.062$). In subgroup analyses, we found a significantly improved survival for those expressing high miR-182 in stage II patients ($P = 0.003$) and in patients with SCC ($P = 0.042$).

4.3.3 Multivariate analyses

High tumor cell miR-182 tended to a positive prognostic impact for the whole cohort, but the multivariate analysis did not reach statistical significance (HR = 0.73, CI 95% 0.50 – 1.06). In subgroup analyses, however, we found high miR-182 expression in tumor cells to be

an independent positive prognostic factor in stage II patients (HR 0.50, CI 95 % 0.28 – 0.90) and in the histopathological SCC subgroup (HR 0.57, CI 95% 0.33 – 0.99).

5 DISCUSSION

5.1 A summary of strengths and weaknesses

We have a relatively large sample size with hardly any selection bias, as the cohort includes 90% of all operated stage I-III A NSCLC patients in our region during the specified period. The follow up time is significant, and we have performed a comprehensive collection of clinical data. We have also validated the data by contacting the local hospital or the patients' physician in cases of inadequate or missing data in the hospital journals. Staging and pathological diagnosis was revised by two experienced pathologists. For the TMAs, duplicate cores were taken from both tumor cell areas and stromal cell areas of the tissue blocks. The TMA production and the IHC and ISH procedures were performed by dedicated and experienced technicians. We assessed protein expression and microRNA expression both in stromal cells and neoplastic cells. The optimal cut-offs reduced chances of false negative results (type 2 errors).

A weakness may be that we did not perform any validation of the antibodies used in paper 1. However, in-house validation had been done by the manufacturer. Furthermore, using optimal cut-offs and not predefined or mean value cut-offs, the chance of false positive results (type 1 errors) increase. Another weakness is the unavailability of an external validation set.

5.2 Paper 1

In the first paper we explored the three matrix metalloproteinases MMP-2, MMP-7 and MMP-9. Our main conclusion was that high tumor cell MMP-7 and stromal MMP-9 expressions were independent positive prognostic factors in NSCLC. MMP-2 expression did not have any prognostic implications in our cohort.

We are the first researchers to find a positive prognostic impact of MMP-7 expression in NSCLC. From four small-sized previous studies on NSCLC and MMP-7 expression, two reported no impact on survival (76;77), while the other two found an independently worsened survival connected to high MMP-7 expression (78;79). A recently published paper failed to show any MMP-7-dependent impact on survival (80). However, consistent with our NSCLC data, high MMP-7 expression has been associated with a better survival in other tumor types. In salivary gland carcinoma and in papillary thyroid carcinoma, MMP-7 was related to an improved outcome (81;82)

We found no impact on DSS connected to MMP-9 expression in the tumor cells. High stromal expression of MMP-9, however, was independently associated with a good prognosis. Two papers reporting on stromal MMP-9 expression in NSCLC failed to show any impact on survival (76;83). In these studies, both patient cohorts were smaller than ours.

Since MMPs traditionally have been viewed as oncogenes, MMP inhibitors were invented as drugs in cancer treatment (37;84;85). Randomized trials with these MMP inhibitors failed, however, to show positive survival results. It is speculated whether the lack of effect is related to the complex pro- and antitumorigenic properties of MMPs.

The MMPs have complex functions. The MMPs of this paper have traditionally been connected to degradation of the extracellular matrix, but it is also well known that they contribute to signalling for cell growth, inflammation and angiogenesis (36;37) Functional studies have demonstrated that they can contribute to both tumor progression and tumor suppression (86-88). Among studies supporting the notion that MMP-7 may serve a protective role in lung cancer and thereby corroborating our results, is the one by Abdel-Ghany et al., which showed that MMP-7 inhibited lung cancer cell adhesion to lung endothelium (86). Besides, Acuff et al. found MMP-7 positive mice to form fewer lung tumors than MMP-7 null mice (87). Studies on MMP-9 have shown divergent impacts on tumor development. Skin

carcinomas in MMP-9-null mice were found to increase in number, but acted less aggressive than in mice with normal MMP-9 levels (89). In another study, higher MMP-9 levels were associated with more lung metastasis, but the subsequent growth of metastases was not affected by MMP-9 levels (87).

MMPs are associated with angiogenesis, and in a stimulatory fashion through their role in the angiogenic switch (38). But also here, their dual roles are evident, as they contribute to the release of several antiangiogenic factors. Both MMP-7 and -9 are known generators of angiostatin, a cleavage product of plasminogen. In mice, Pozzi et al. found a link between lower levels of MMP-9 and angiostatin, and a subsequent increase in tumor growth (90). The same MMPs produce endostatin, another angiogenesis inhibitor, from the basement membrane type XVIII collagen. Endostatin is associated with angiogenesis inhibition and reduced tumor growth in animal models (91;92). Finally, MMP-9 cleavage of basement membrane collagen IV α 3 generates tumstatin. Lower levels of this angiogenesis inhibitor are found to increase pathological angiogenesis and tumor growth (93).

We found a protective impact of MMP-7 and MMP-9, a result somewhat conflicting earlier studies and the established understanding of how these MMPs work. However, we have pointed out some possible explanations for our results. Our cohort is large, which makes our numbers more reliable. Further, studies on other tumor types are consistent with our results. We also discuss several studies which can explain why these MMPs may have tumor protecting features, and our results contribute to the understanding why the MMP inhibitors failed in clinical studies. Anyway, it is necessary to explore this field further. Validation in other cohorts would be warranted. It will also be of interest to see further functional studies.

5.3 Paper 2

In this paper, we investigated the impact of miR-21 in NSCLC. We found high tumor cell miR-21 expression in patients with lymph node metastasis to be an independent positive prognostic factor. For stromal cell expression of miR-21, we observed an opposite trend with high expression associated with a negative prognosis in the univariate analysis.

Our study is the first using ISH-technique for high throughput exploration of miR-21 expression, assessing its prognostic impact in NSCLC. There are a few previous publications in this field, most using qRT-PCR to assess the prognostic impact of miR-21 in NSCLC. Some of these publications find, inconsistent with our results, miR-21 to be a negative prognostic factor (94-96), while others find no prognostic impact of miR-21 (97;98).

One plausible reason for our divergent results when compared to some of the other studies, may be the fact that we used ISH, enabling us to assess tumor cells and stromal cells individually. In contrast, PCR can only give one overall assessment for the tissue investigated. So, in the previous studies it could not be discriminated between tumor and stromal cells, as the authors did not microdissect tumor cells versus stromal cells. With respect to our data, the positive contribution from miR-21 in tumor cells may have overridden possible negative contribution from the stroma. In line with this, Gregg and colleagues observed in prostate cancer a large difference regarding gene expression between cells of the tumor respective stromal compartment (99).

As miR-21 regulates a number of various genes, it may contribute to both tumor progression and suppression. Consistently, functional studies have revealed that miR-21 can act both pro- and anti-angiogenic. The proangiogenic features were shown in human prostate cancer cells, where miR-21 through up-regulation of HIF-1 α and VEGF and activation of the Akt and extracellular regulated kinases (ERK) pathways induced angiogenesis (100). Sabatel and colleagues used human umbilical vein endothelial cells (HUVECs) to demonstrate

possible angiogenesis inhibiting properties from miR-21 (101). Via inhibition of RhoB (ras homolog gene family, member B), endothelial proliferation and migration was reduced, leading to reduced vessel formation. It is possible that these pro- and anti-angiogenic properties dominate in different stages of the disease, and this may explain the difference we find for miR-21 impact between node negative and node positive patients.

5.4 Paper 3

In the last paper, we investigated the possible impact of miR-182 on the NSCLC prognosis. We found high tumor cell miR-182 expression in stage II patients and in SCC to be an independent favorable prognostic factor. In the whole cohort, high miR-182 expression tended to a favorable outcome without reaching statistical significance. miR-182 was not detected in stromal cells.

We have identified one smaller NSCLC study with 70 cases, in which the prognostic impact of miR-182 was explored (102). The authors found miR-182 to be a negative prognostic factor. They used PCR, not ISH, to detect miR-182 expression. In one study of 253 glioma patients using ISH, high miR-182 expression was a negative prognosticator (103). In another study on 148 colorectal patients, a similar conclusion was reached (104). Thus, these studies conclude opposite from our study.

In the literature, miR-182 has mainly been regarded as an oncogene. It is interesting to see, though, that many functional studies and studies on cell cultures find tumor suppressing properties connected to miR-182, supporting the conclusion in our study. In one study by Poell and coworkers, miR-182 was found to be a strong inhibitor of melanoma cell line proliferation (105). Supporting these findings, Yan et al. following transfection of miR-182 into cultured uveal melanoma cells, found a significant decrease in cell growth, migration and invasiveness (106). Microphthalmia-associated transcription factor (MITF), B-cell lymphoma 2 (BCL2) and cyclin D2 are believed to be targets of miR-182 leading to these tumor suppressing properties. There are also two studies on lung tumors corroborating our conclusion on miR-182 as a possible tumor suppressor. miR-182 was found to suppress lung tumorigenesis through regulation of Regulator of G-protein signaling 17 (RGS17) (107). Also, Zhang and coworkers found that invasion and proliferation of human lung

adenocarcinoma cells were inhibited via miR-182's effect on human cortical actin-associated protein (CTTN) (108).

miR-182 is also found to be differently expressed between primary tumors and metastases in the same organ (109;110). Other studies have revealed different expression profiles of miRNAs in AC and SCC of the lung (111). These findings indicate that miRNAs, and also miR-182 in particular, can be stage- and tissue specific. It may explain why miR-182 can have a prognostic impact in subgroups, even when a significant impact can not be seen for the whole cohort.

6 CONCLUSIONS AND IMPLICATIONS FOR FURTHER RESEARCH

We have been studying two different classes of markers, which are angiogenesis-related. We started with the MMPs, which are known to be important players in tumor angiogenesis, although they also have many other important functions. We found high expression of two of the MMPs to be significantly associated with an improved survival. We believe that the conflicting results from studies on MMP's prognostic impact in NSCLC and other cancers may explain the lack of positive results from large clinical phase III studies with MMP inhibitors. MMPs have angiogenesis stimulating features, but they also contribute to angiogenesis inhibition through release of natural angiogenesis inhibitors.

Research on MicroRNAs is a fast growing field, but there still is a long way before we can begin to get a functional overview of the vast number of different miRNAs. Our group is among the first to use ISH to perform large scale expression studies on miRNAs, at least in NSCLC. We believe that the distinction between tumor cells and stromal cells which the ISH methodology allows us to make, can contribute to new knowledge from more precise expression patterns and functions of miRNAs. We believe that the dissimilar prognostic impact of miR-21 depend on whether the methods used were cell specific or based on RT-qPCR. Our main results are from subgroups, and may underscore the complex functions of miRNAs with discrepant impacts in different compartments. There are functional studies showing miR-21 and miR-182 to act both pro- and antiangiogenic, hence acting as oncogenes as well as tumor suppressors. These findings may predict that it might be challenging to develop drugs targeting miRNA, either in the form of inhibitors or stimulators.

Although we have a large NSCLC material, a weakness of our work is the lack of validation cohorts. A solution is to establish a validation cohort through the collaboration with

another research group. This development is in progress. We are also expanding our own cohort by collecting tissue and data on patients operated in “our” hospitals between 2005 and 2010. We will then have the possibility to split our cohort between patients operated in our two hospitals (Tromsø and Bodø), using one of the groups as a validation set.

A major strength of our lung cancer research is the establishment of a large data set over time, with far beyond 100 molecular markers examined, and the number still rising. This facilitates exploration on relations between markers with potentially biological interactions. Our research team will continue to explore new groups of molecular markers. In the near future, we will investigate the potential impacts by immune cells and chemocines and hormonal markers.

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Paper 1

Paper 2

Paper 3

