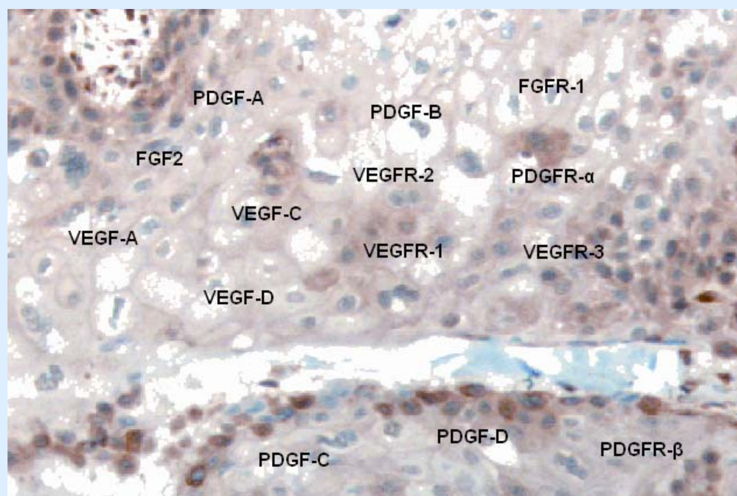
Molecular Analysis of Angiogenic Markers as Prognostic Factors for Non-Small Cell Lung Cancer (NSCLC)

A new Translational Research Strategy involving tissue microarray technology at the University Hospital North Norway and the University of Tromsø



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A dissertation for the degree of Philosophiae Doctor

UNIVERSITY OF TROMSØ
Institute of Clinical Medicine
Department of Oncology

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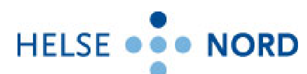

CONTENTS **Page**

Acknowledgements	3
List of papers	4
List of abbreviations	5
1. Introduction	
1.1 Lung cancer	6
1.1.1 Epidemiology and incidence	6
1.1.2 Histopathology	8
1.1.3 Staging, TNM and prognosis	8
1.1.4 Treatment of NSCLC stage I-IIIA	11
1.1.5 Potential of new molecular markers in NSCLC	11
1.2 Angiogenesis	12
1.2.1 Vascular endothelial growth factors and receptors	13
1.2.2 Platelet–derived growth factors and receptors	13
1.2.3 Fibroblast growth factors and receptors	14
1.3 Interaction tumor – stroma – vasculature	15
1.4 Angiogenic inhibitors and NSCLC	16
1.4.1 Tyrosine kinase inhibitors and monoclonal antibodies	16
1.4.2 Rationale and timing of anti-angiogenic treatment	17
1.4.3 Targeting different families of angiogenic markers	18
1.5 Tissue microarray (TMA)	19
2. Aims of thesis	20
3. Material and methods	21
3.1 Study population	21
3.2 Tissue microarray	22
3.2.1 TMA construction	22
3.2.2 TMA advantages/disadvantages	24
3.3 Immunohistochemistry (IHC)	26
3.3.1 IHC procedure	26
3.3.2 Scoring	29
3.3.3 Cut-off values	32
3.3.4 Controls and limitations	32
3.4 Statistical analysis	34
4. Main results	36
4.1 Paper I (VEGFs/VEGFRs)	36
4.2 Paper II (PDGFs/PDGFRs)	37
4.3 Paper III (FGF2/FGFR-1)	37
5. Discussion	39
5.1 Discussion of results	39
5.1.1 Paper I	39
5.1.2 Paper II	41
5.1.3 Paper III	42
6. Conclusions and implications for further research	44
Reference list	46
Papers I-III	
Appendix	

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LIST OF PAPERS

Paper I **Dønnem T, Al-Saad S, Al-Shibli K, Delghandi MP, Persson M, Nilsen MN, Busund R LT, Bremnes RM**. Inverse Prognostic Impact of Angiogenic Marker Expression in Tumor Cells versus Stromal Cells in Non Small Cell Lung Cancer. *Clin Cancer Res* 2007 Nov 15;13(22):6649-57.

Paper II **Dønnem T, Al-Saad S, Al-Shibli K, Andersen S, Busund R LT, Bremnes RM**. Prognostic Impact of Platelet-derived Growth Factors in NSCLC Tumor and Stromal Cells. *Journal of Thoracic Oncology*. Sept 2008;3(9):963-970.

Paper III **Dønnem T, Al-Shibli K, Al-Saad S, Busund R LT, Bremnes RM**. Prognostic Impact of Fibroblast Growth Factor 2 in NSCLC: Co-Expression with VEGFR-3 and PDGF-B Predicts Poor Survival. *Journal of Thoracic Oncology*. Accepted.

LIST OF ABBREVIATIONS

Ab	Antibody
b-FGF	Basic fibroblast growth factor (=FGF2)
DNA	Deoxyribonucleic acid
DSS	Disease-specific survival
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
IHC	Immunohistochemistry
Mab	Monoclonal antibody
MVD	Micro vessel density
NH	Nordland Central Hospital
NSCLC	Non-small cell lung cancer
OS	Overall survival
PFS	Progression-free survival
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
RNA	Ribonucleic acid
TKI	Tyrosine kinase inhibitor
TMA	Tissue microarray
UNN	University Hospital of North Norway
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1. INTRODUCTION

1.1 Lung cancer

1.1.1 Epidemiology and incidence

Lung cancer is the leading cause of cancer death in the western world, and in some countries, lung cancer accounts for more deaths than prostate cancer, breast cancer, and colorectal cancer combined (1). About 5 persons die from lung cancer every day in Norway. From being a rare disease at the beginning of the 20th century, about 1.35 million new lung cancer patients were registered in 2002 (2). In Norway, 1369 men and 953 women were diagnosed with lung cancer in 2006 (3).

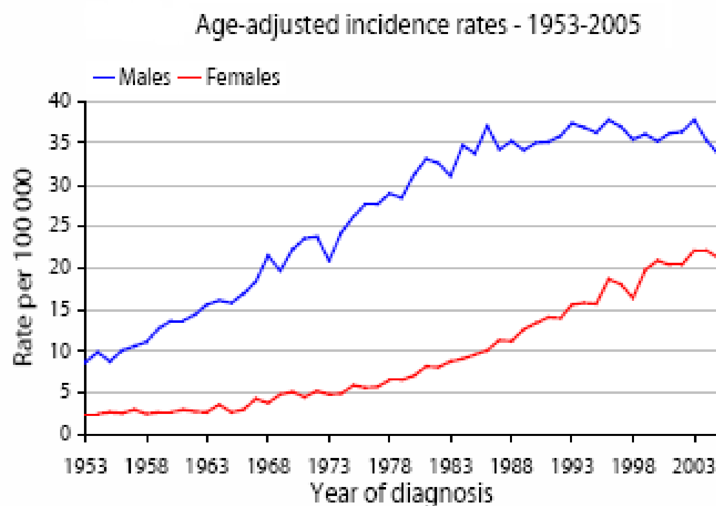


Figure 1. Age-adjusted lung cancer incidence rates among men and women (Adapted from www.kreftregisteret.no).

As shown in Figure 1, we see an increase in incidence in both genders, but while the ratio between men and woman in the early fifties was 1:4, it was 1:1.6 in 2003. When looking at age-specific incidence rates in the period 2001-2005 (Figure 2), more females than males are diagnosed with lung cancer below the age of 50 years.

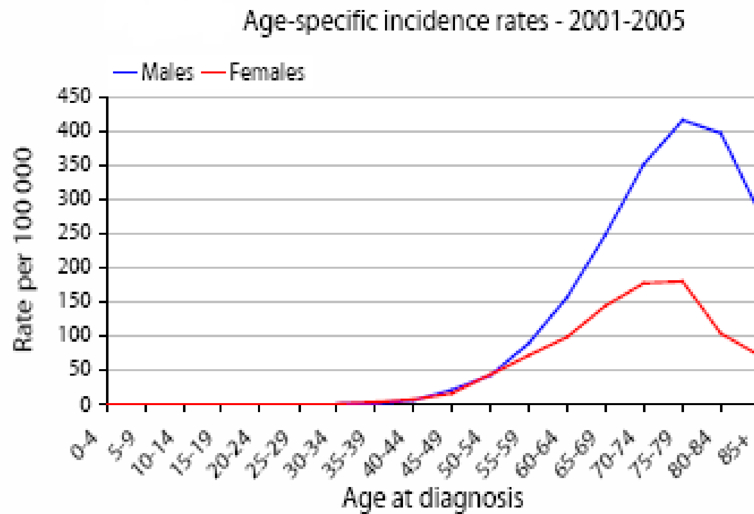


Figure 2. Age-specific incidence rates among men and women in Norway (Adapted from www.kreftregisteret.no)

Cigarette smoking is by far the most important etiologic factor for lung cancer, responsible for about 90% of the cases (4). Asbestos, radon, arsenic, nickel, chromates etc. are also related to lung cancer etiology. Lung cancer among never-smokers appears more frequent among females. But for lung cancer in general there seems to be a strong association between smoking history and the incidence of lung cancer 20-30 years later.

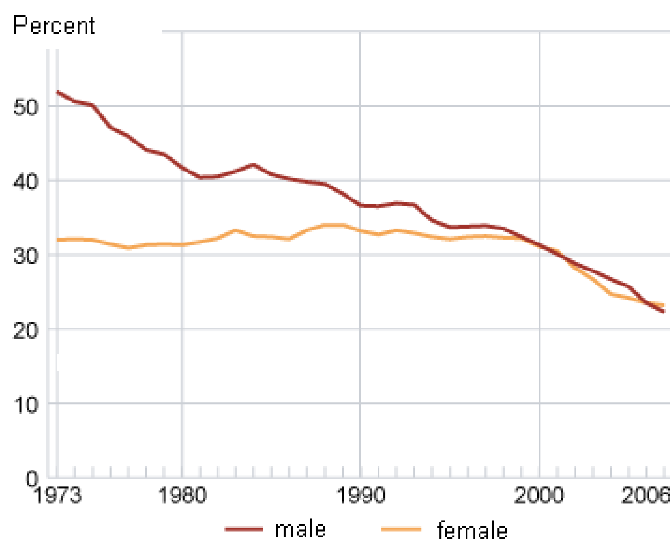


Figure 3. Smoking rates among males and females in Norway 1973-2006. (Adapted from www.ssb.no).

As shown in Figure 3, the fraction of daily smoker has decreased, but still about one out of five smoke at a daily basis in Norway. For heavy smokers, there is more than 20 fold increased risk of developing lung cancer compared with never smokers.

1.1.2 Histopathology

There are two main categories of lung cancer: Non-small cell lung cancer (NSCLC; 80%) and small cell lung cancer (SCLC; 20%). The major NSCLC histological subtypes are squamous cell carcinomas, adenocarcinomas and large cell carcinomas (5). Previously, squamous cell carcinomas were most common, but the percentage of adenocarcinomas is increasing probably due to present use of filter-cigarettes. Adenocarcinoma is also the most common subtype among never-smokers. For a more comprehensive table of the WHO histological classification, see appendix 1.

1.1.3 Staging and TNM

The majority of patients have symptoms at the time of diagnosis, and the most common symptoms are cough, dyspnoea and hemoptysis. As many of the lung cancer patients are asymptomatic for long periods, only 20% (4346/21968) of NSCLC patients in Norway, 1993-2005, were diagnosed at an early stage and got surgery with a curative goal (6). The diagnostic procedure should end up in a conclusive histology and clinical disease stage of each patient. All patients with suspected lung cancer undergo a chest x-ray and a CT of the chest including the upper abdomen with the adrenal glands. Tissue biopsies are usually obtained by bronchoscopy, but for peripheral tumors CT guided biopsy is often performed. In patients with enlarged mediastinal glands, mediastinoscopy or open surgery was regularly done to confirm or to rule out N2-status. Today, positron emission tomography (PET),

transoesophageal or endobronchial ultrasound have been added to the staging procedure. These procedures are vital to correct staging, treatment and prognosis. Clinical TNM (cTNM) are based on clinical examination of the patients while pathological TNM (pTNM) are based on examination of the surgical specimen. Table 1 shows the 1- and 5- year survival rates based on cTNM.

Table 1. Clinical staging and survival of Non-small cell lung cancer

Stage	Tumor	Node	Metastasis	Definition	Survival rate (%) 1 Yr	Survival rate (%) 5 Yr
cIA	T1	N0	M0	T1: Tumor ≤3cm, without bronchosopic evidence of invasion proximal to the lobar bronchus	91	61
cIB	T2	N0	M0	T2: Tumor > 3 cm, or tumor of any size with one or more of the following characteristics: - infiltration of the visceral pleura - invades the main bronchus but > 2 cm distal to the main carina - atelectasis or obstructive pneumonitis that extends to the hilus but does not involve the entire lung and without pleural effusion	72	38
cIIA	T1	N1	M0	N1: Metastasis to ipsilateral peribronchial and/or ipsilateral hilar lymph nodes, including direct invasion	79	34
cIIB	T2	N1	M0	T3: Tumor of any size with invasion of	61	24
	T3	N0	M0	the chest wall including adjacent rib(s), diaphragm, mediastinal pleura, parietal pericardium, or tumor in the main bronchus < 2 cm distal to the carina; or tumor associated with atelectasis or obstructive pneumonitis of the entire lung	55	22
cIIIA	T3	N1	M0	N2: Metastasis to ipsilateral mediastinal	56	9
	T1-T3	N2	M0	and/or subcarinal lymph nodes	50	13
cIIIB	T4	N0-N2	M0	N3: Metastasis to contralateral	37	7
	Any T	N3	M0	mediastinal, contralateral hilar, or ipsilateral and/or contralateral supraclavicular or scalene lymph nodes	32	3
cIV	Any T	Any N	M1	M1: Distant metastasis, including separate tumor nodules in a different lobe	20	1

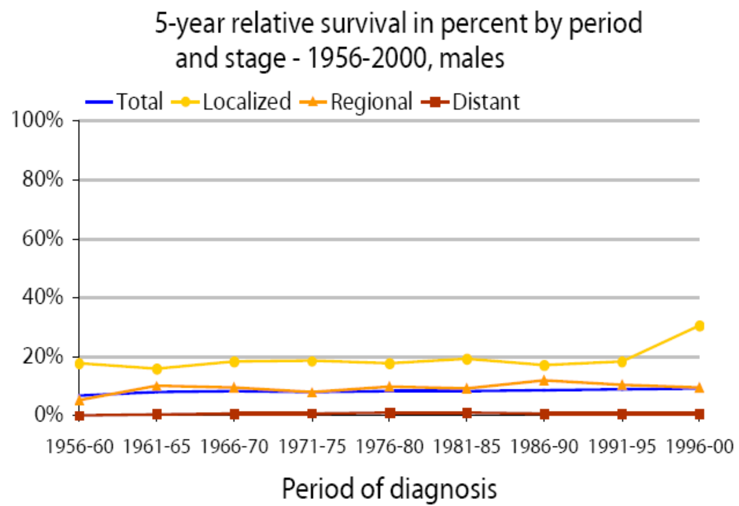
Adapted from CF Mountain. Revisions in the International System for Staging of Lung Cancer. Chest 111:1710, 1997.

There is a significant difference in survival between the cTNM and the pTNM status. This discrepancy is due to the fact that several patients are up-staged during surgery as they have more advanced disease than concluded with after the presurgical

staging (c-stage). For instance, pTNM five-year survival rates range from 67% for T1N0 (IA) disease to 23% for patients with T1-3N2 (IIIA) (7), as compared to 61% and 13% in cTNM, respectively.

Due to the fact that most lung cancer patients get diagnosed at a late stage the overall survival is poor, and relatively little improvement has been made in the 5-year survival rate during the last 50 years (Figure 4).

A



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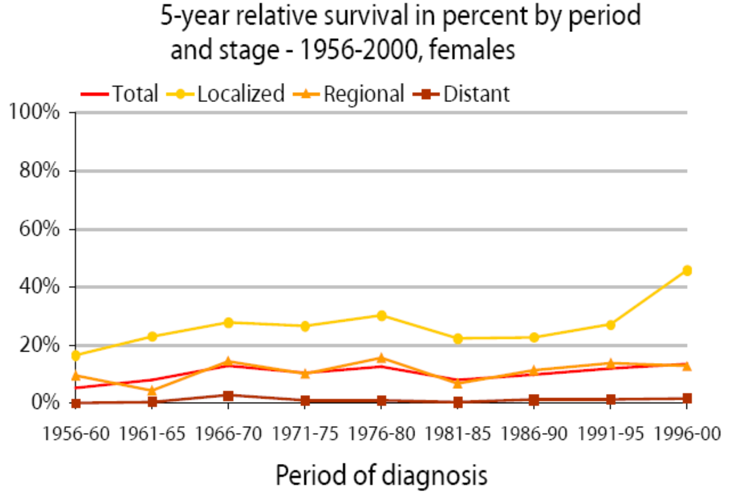

Figure 4. Five year survival rates; (A) males, (B) females (Adapted from www.kreftregisteret.no)

1.1.4 Treatment of NSCLC stage I - IIIA

For many years, surgery alone was the standard treatment for NSCLC patients with stage I-IIIA. To improve survival for patients with resectable NSCLC, clinicians have examined the use for chemotherapy and radiation therapy in both the preoperative (neoadjuvant) and postoperative (adjuvant) settings (8). Adjuvant chemotherapy is not recommended for stage IA, under debate for stage IB, while cisplatin-based chemotherapy is recommended for stage II-IIIA (8-12).

Radiotherapy in stage I-II is only indicated for patients not medically fit for surgery. For stage IIIA with clinical TNM status radical radiotherapy is administered, provided good prognostic factors (tumor size, performance status, weight loss). Patients with pathological N2 disease or incomplete resection margins are postoperatively given radiotherapy (8;10;13;14).

1.1.5 Potential of new molecular markers in NSCLC

An underlying hypothesis in the modern era of cancer research is that prediction of a patient's prognosis or response to therapy can be improved by combining standard clinical variables (i.e., tumor size, differentiation, or stage), with intrinsic genetic or biochemical characteristics of the tumors. These characteristics have been defined by evaluating the DNA, RNA or protein expression levels of selected candidate molecules. Given the possibility to prognosticate on basis of the molecular marker expressions, patients with resectable lung cancer found to be at high risk of recurrent disease, may theoretically be considered to benefit from postoperative chemotherapy or novel targeted therapies to reduce the risk of relapse and improve survival.

Consequently, there has been increased interest in the identification and validation of new biomarkers to help us better understand tumor biology, to guide us more precisely in clinical decision-making processes, and to aid in drug development strategies (15).

1.2 Angiogenesis

Angiogenesis is a physiological process involving the growth of new blood vessels from pre-existing vessels. In the late 1960s, the first preliminary evidence indicating that tumor angiogenesis was mediated by diffusible factors produced by tumor cells (16;17) Few years later Folkman stated that the malignant tumor requires angiogenesis to grow beyond 1 to 2 mm^3 (18). In the well-known review by Hanahan and Weinberg in 2000, angiogenesis is considered one of the hallmarks of cancer development together with self-sufficiency in growth signals, tissue invasion and metastasis, insensitivity to anti-growth signals and evasion of apoptosis (19).

The so-called “switch” to an angiogenic phenotype is considered important in the malignant process whereby proangiogenic mechanisms overwhelm or circumvent negative regulators of angiongenesis (19). Angiogenesis may be divided into four stages (20): (I) activation of the endothelial cells leads to the localized degradation of the basal membrane of the parent vessel and of the extra-cellular surrounding matrix; (II) oriented migration of endothelial cells in the extracellular matrix; (III) proliferation of endothelial cells; (IV) differentiation of these cells with organization into tubular structures with a new basal lamina. Through these stages the new capillaries form a new vascular network.

Various angiogenic mechanisms may be differentially important in different tumor types and/or stages of neoplastic progression (21). However, three of the major families of growth factors involved in angiogenesis are the vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) families of ligands and receptors.

1.2.1 Vascular endothelial growth factors and receptors.

Much attention has been focused on the VEGF family of growth factors and the receptor tyrosine kinases that mediate their proangiogenic effect (22). The VEGF family comprises six secreted glycoproteins of which VEGF-A, VEGF-C, and VEGF-D are of great significance (21;23). These VEGF ligands mediate their angiogenic effect via the receptor tyrosine kinases (RTKs) VEGFR-1 (flt-1), VEGFR-2 (KDR or Flk-1) and VEGFR-3 (Flt-4)(24-26). VEGF-A has been regarded as the major player for angiogenesis and usually referred to as VEGF. It binds to VEGFR-1 and VEGFR-2, of which VEGFR-2 is the major mediator of the mitogenic and angiogenic effects of VEGF-A. VEGF-C and VEGF-D activate VEGFR-3 and appear important for lymphatic endothelial cell growth, migration and survival (27-30) . However, proteolytically processed VEGF-C and VEGF-D can also induce blood-vessel growth by activating VEGFR-2 (31;32). VEGFR-3 deletion leads to defects in blood-vessel remodeling and embryonic death at mid-gestation and blocking of VEGFR-3 suppress angiogenic sprouting, indicating that activation of VEGFR-3 promote angiogenesis in addition to lymphangiogenesis (33-35).

1.2.2 Platelet-derived growth factors and receptors.

Signaling through PDGF ligands and receptors contributes to multiple tumor-associated processes and the angiogenic activity was first described in the early

nineties (36). The PDGF family consists of five isoforms of A-, B-, C- and –D polypeptide chains, which is the homodimers PDGF-AA, -BB, -CC, -DD, and one hetereodimer PDGF-AB (37;38). The PDGF isoforms exert their cellular effects by binding to structurally similar α- and β-tyrosine kinase PDGF receptors. The PDGF-AA, -AB, -BB and –CC dimers bind to the α-receptor with high affinity, whereas PDGF-BB binds preferentially to the β-receptor and PDGF-DD activates the β-receptor only (36;39). PDGF-B and PDGFR- β is essential for recruitment of pericytes (supportive cells to endothelium) and in maturation of the microvasculature (40). Recent studies have emphasized the significance of tumor-derived PDGF-A (and potentially PDGF-C) and PDGFR- α signaling in recruitment of the angiogenic stroma to produce VEGF-A and other angiogenic factors (41).

1.2.3 Fibroblast growth factors and receptors.

The FGF family represents a group of heparin-binding, multifunctional polypeptides and act as broad-spectrum mitogens also involved in angiogenesis (42;43). Fibroblast growth factor 2 (FGF2 = basic fibroblast growth factor, b-FGF) is considered a potent stimulator of angiogenesis and binds with high affinity mainly to fibroblast growth factor receptor-1 (FGFR-1), a tyrosine kinase receptor (44). FGF2 may contribute to cancer progression by acting directly on the tumor cells (44) . In angiogenesis, FGF2 may exert its effect on endothelial cells via a paracrine mode as a consequence to its release from tumor and stromal cells. It is also suggested that FGF2 plays an autocrine role in endothelial cells (44;45).

Although the activity of individual angiogenic factors is relatively well studied, less is known about the interplay between various tumor-produced angiogenic factors and their cooperative efforts in promoting tumor neovascularization. Interestingly, murine

studies have observed an intimate cross-talk between FGF2/FGFR-1 and different members of the VEGF and PDGF family during hemangiogenesis and lymphangiogenesis (44;46-48).

1.3 Interaction tumor - stroma - vasculature

VEGFs/VEGFRs, PDGFs/PDGFRs and FGFs/FGFRs are all important in the angiogenic cellular cross-talk. In tumor development, angiogenic switch is associated with the onset of expression and secretion of angiogenic factors by tumor cells. The tumor cell secretion of growth factors leads to a complex interplay with subsequent stromal secretion of growth factors and activation of endothelial cell receptors. A distinction is made between paracrine and autocrine signaling (Figure 5).

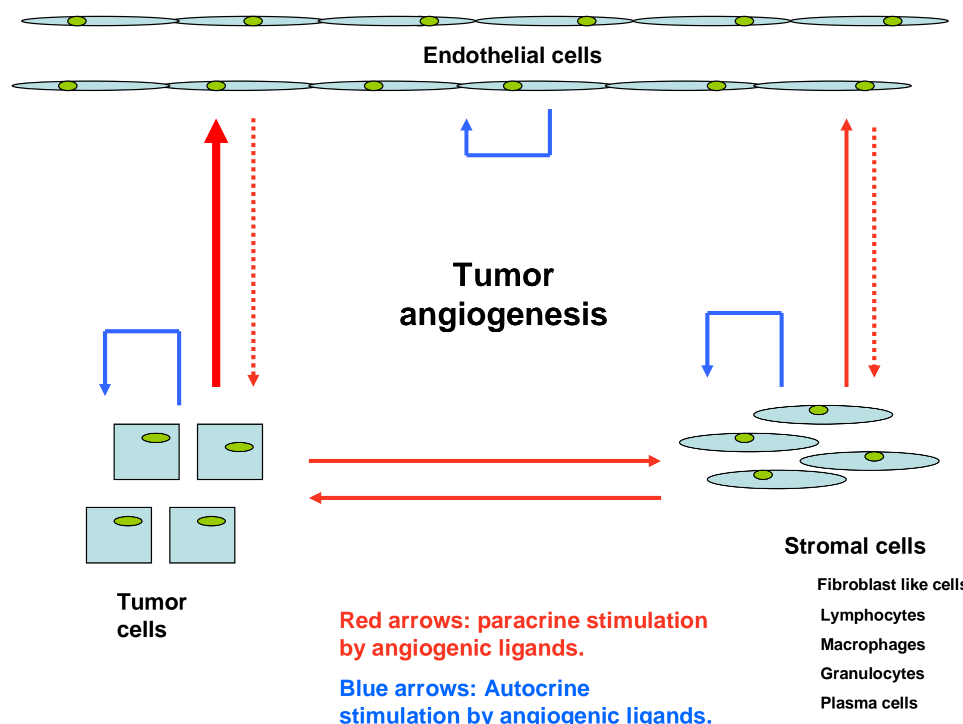

Figure 5. The three compartments, tumor cells (neoplastic cells), stromal cells and endothelial cells interconnected with possible autocrine and paracrine stimulation in tumor angiogenesis. Broad arrows indicating autocrine (blue) and paracrine (red) stimulation established in the literature, while dotted lines indicate paracrine stimulation far less described.

Both affect neighboring cells, but whereas *autocrine* signaling occurs among the same cells, *paracrine* signaling affects other cells.

It was conceived that the angiogenic growth factors act in a paracrine fashion by being produced in the tumor cells and activating endothelial cell receptors. However, finding VEGF-/PDGF-/FGF- receptors in the tumor cells indicates that tumor-produced ligands sometimes act as a direct (cell autonomous) autocrine growth factors for the tumor cells. Additionally, it is now clear that angiogenic growth factors, in amounts sufficient to drive tumor angiogenesis, include contributions from various host cells and tumor associated stromal cells.

Unlike normal tissue, the tumor stroma contains increased amounts of inflammatory infiltrates, an increased micro vessel density with dysfunctional lymphatics and blood vessels, and a denser extracellular matrix with reactive fibroblasts (49). Today, there is growing recognition that the tumor stroma plays a crucial role in tumorigenesis (50) but many questions regarding the cross-talk between stromal, endothelial and tumor cells, as a part of the angiogenic process, remain to be answered.

1.4 Angiogenic inhibitors and NSCLC

1.4.1 Tyrosine kinase inhibitors and monoclonal antibodies

Tumor blood vessels are distinct from normal resting blood vessels, and the discrepancy regarding these tumor vessels feature them as good targets for cancer therapies. In anti-angiogenic therapy there are two major groups of targeted therapies, the tyrosine kinase inhibitors (TKIs) and the monoclonal antibodies (mabs) (51). However, when administered as single agents, antiangiogenic drugs have

produced modest objective responses, and had limited effect on the overall survival (52). In contrast, when given in combination with chemotherapy, bevacizumab (mab against VEGF/VEGFR2) was first presented with an increased overall survival in metastatic colorectal cancer (53). In metastatic NSCLC patients the E4599 trial (54), using chemotherapy alone or combined with bevacizumab, demonstrated significant improvements in response rate, progression-free survival (PFS) and overall survival (OS) in the bevacizumab arm. Though the median survival benefit was modest, from 10.3 to 12.3 months, the drug has been approved by both EMEA and FDA (55). The European AVAiL trial found improvements in response rate and PFS, but as presented at ESMO September 2008 (56), it could not reproduce the overall survival benefit. As a consequence, the use of bevacizumab in advanced NSCLC in Norway is a matter of debate. Bevacizumab is now also being studied in earlier-stage disease as neoadjuvant or adjuvant therapy and in locally advanced NSCLC (55).

Within the expanding group of TKIs, VEGFRs, PDGFRs and FGFRs are well represented as targets. For instance, sunitinib (FDA approved in renal cell carcinoma) and sorafenib (FDA approved in hepatocellular carcinoma), both target VEGFRs and PDGFRs. Evaluation of these drugs in phase II NSCLC studies have shown promising efficacy (57).

1.4.2 Rationale and timing of antiangiogenic treatment

An important question in antiangiogenic treatment is the timing of administration when given together with chemotherapy or radiotherapy. Tumor vascular abnormalities lead to an abnormal tumor microenvironment characterized by interstitial hypertension (IFP), hypoxia and acidosis (52;58). Impaired blood supply

and high IFP interfere with the delivery of therapeutics to solid tumors. Hypoxia renders tumor cell resistant to both radiation and several cytotoxic drugs (58). One may expect that destroying the vasculature would compromise the delivery of oxygen and therapeutics to the solid tumor, producing hypoxia that would render many chemotherapeutics, as well as radiation, less effective. However, to resolve this paradox, it has been hypothesized that antiangiogenic agents may “normalize” the abnormal vasculature by making it less leaky, less dilated, with a more normal basement membrane and better coverage by pericytes, resulting in more efficient delivery of drugs and oxygen to targeted cancer cells (52;58). Optimal timing of antiangiogenic treatment with chemotherapy and/or radiation therapy requires knowledge of the time window during which the vessels become “normalized”, as well as knowledge regarding how long they remain in this state.

1.4.3 Targeting different families of angiogenic markers

Different molecular markers appear influential at different stages of tumor angiogenesis, and the VEGF pathway seems more important in the early stages of tumor development (52). Unfortunately, current anti-angiogenic therapies are unable to maintain vascular regression durably, as tumors relapse and return to abnormal vessels formation, most probably by activation of other angiogenic families as FGF2 and PDGF pathways rather than VEGFs/VEGFRs (52;58).

Although the activity of individual angiogenic factors is relatively well studied, less is known about the interplay between various tumor-produced angiogenic factors and their cooperative efforts in promoting tumor neovascularization. Fibroblast growth factors (FGFs), vascular endothelial growth factors (VEGFs) and platelet-derived

growth factors (PDGFs) are important in angiogenesis and several new agents try to achieve enhanced anti-angiogenic effect by combining VEGFs/PDGFs/FGFs-antagonists. Identifying patients with tumors having biological signatures which match these known targets may therefore be of interest.

1.5 Tissue microarray

Tissue microarrays (TMAs) may be used for large-scale investigation of the biologic and prognostic value of molecular marker families. TMA allows rapid visualization of molecular targets in hundreds of tissue specimens on a single slide, either at DNA, RNA or protein level (59). The technique may facilitate rapid translation of molecular discoveries to clinical applications.

The history of TMAs is relatively short and one of the first premature multicore blocks was used in 1986, when Battifora described a method of embedding 100 or more different tissue samples in a normal sized paraffin block (60). But only during the last decade has this high-throughput technique been commonly used. One of the first large scale TMA studies on NSCLC was published by Bremnes et al. in 2002 (61). To our knowledge, we are the first using TMA technique to study angiogenesis in NSCLC tumor cells and tumor related stroma.

2. AIMS OF THESIS

The present thesis was aimed at exploring potential prognostic angiogenic markers for NSCLC and to better understand the biology of tumor angiogenesis, by assessments of marker expression in both tumor cells and tumor related stroma.

More specifically the aims were to:

Elucidate the prognostic significance of VEGFs and VEGFRs in tumor cells as well as in the tumor stroma of resected NSCLC tumors.

Investigate the prognostic impact of PDGFs and PDGFRs and their interaction with VEGFs and VEGFRs in the complex interplay between tumor and stromal cells.

Assess the prognostic impact of FGF2 and FGFR-1 in tumor cells and tumor stroma of resected NSCLC and explore the relevance of their co-expression with VEGFR-3 and PDGF-B.

3. MATERIAL AND METHODS

3.1 Study population

The same study population was used in all three papers. Primary tumor tissues from anonymized patients diagnosed with NSCLC pathologic stage I to IIIA at the University Hospital of Northern Norway (UNN) and the Nordland Central Hospital (NH) from 1990 through 2004 were used in this retrospective study. As shown in Figure 6, 371 patients were registered from the hospital databases. 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). Thus, 335 patients with complete medical records and adequate paraffin-embedded tissue blocks were eligible.

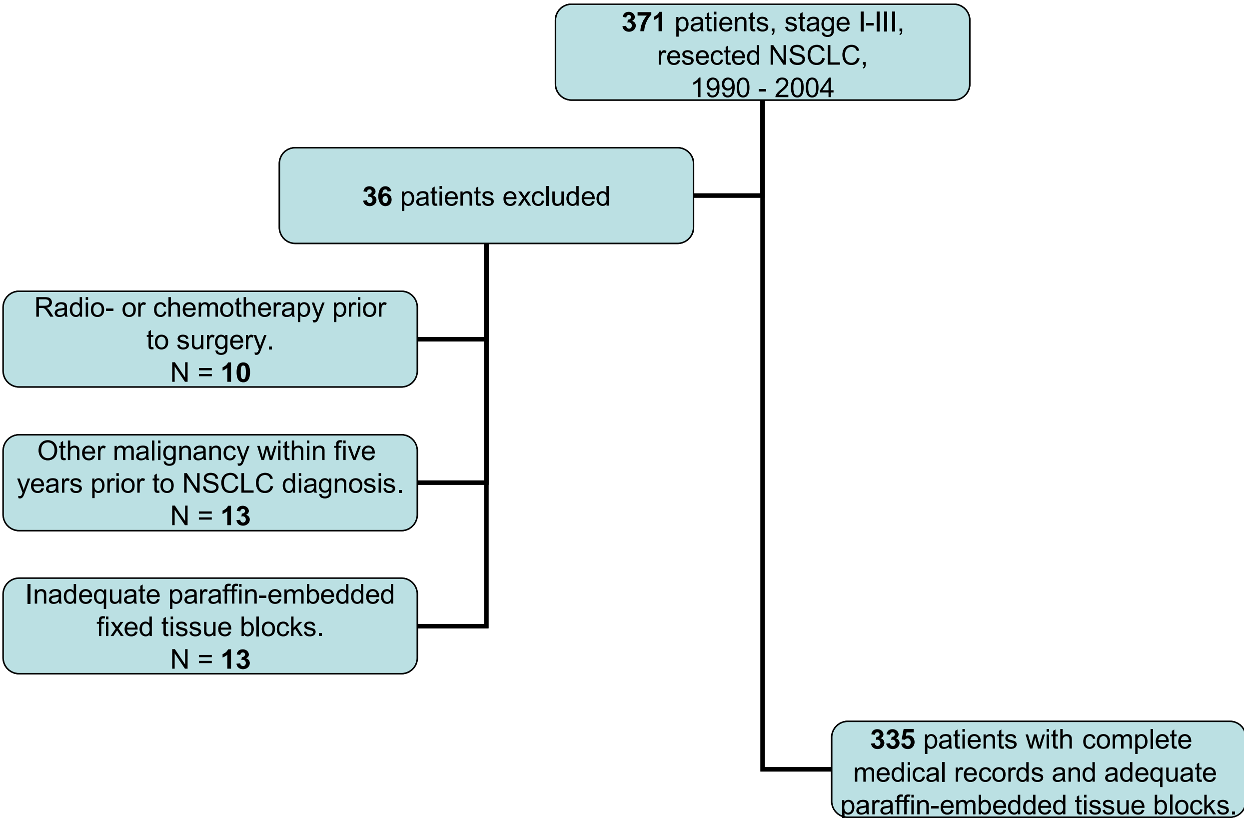

Figure 6. Study population.

This report includes follow-up data as of September 30, 2005. The median follow-up was 96 (range 10-179) months. Complete demographic and clinical data were collected retrospectively. Formalin-fixed and paraffin-embedded tumor specimens were obtained from the archives of the Departments of Pathology at UNN and NH. The tumors were staged according to the International Union Against Cancer's TNM classification (7) and histologically subtyped and graded according to the World Health Organization guidelines (5).The National Data Inspection Board and The Regional Committee for Research Ethics approved the study.

3.2 Tissue microarray (TMA)

3.2.1 TMA construction

All lung cancer cases were histologically reviewed by two pathologists (S. Al-Saad and K. Al-Shibli) and the most representative areas of tumor cells (neoplastic epithelial cells) and tumor stroma were carefully selected and marked on the hematoxylin and eosin (H/E) slide and sampled for the TMA blocks. The TMAs were assembled using a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD), consisting of thin-walled stainless steel biopsy needles and stylets used to biopsy the donor block, transfer the needle content and empty it into the recipient block. The recipient block was held in an X-Y position guide that was manually adjusted by micrometers. The instrument was used to create holes in the recipient paraffin block and to acquire tissue cores from the donor block by a thin-walled needle. The cylindrical samples was retrieved from the selected regions in the donor block and extruded directly into the recipient block at defined array coordinates. A solid stylet, closely fit in the needle, was used to transfer the tissue cores into the

recipient block. We used a 0.6 mm diameter stylet, and the study specimens were routinely sampled with two replicate core samples (different areas) of neoplastic tissue and two of tumor stroma.

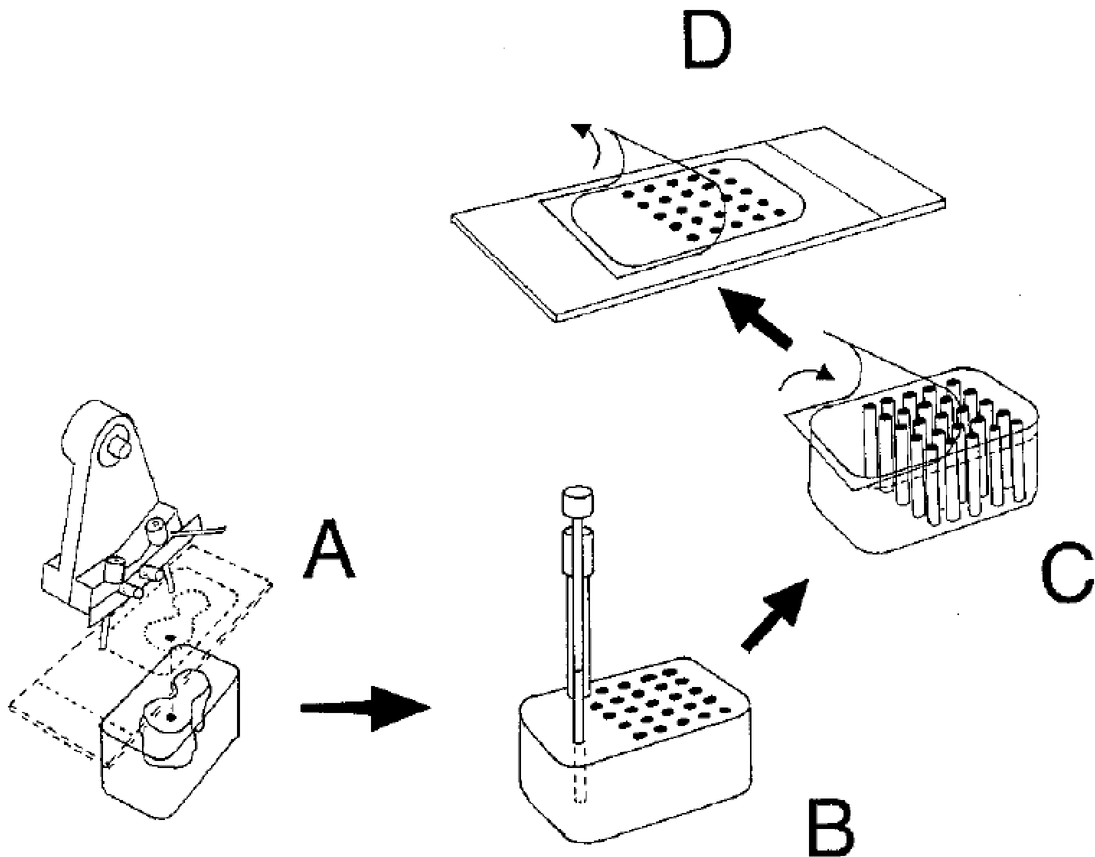

Figure 7. (A) A tissue core biopsy is punched from a preselected region of neoplastic cells or tumor related stroma (0.6 mm in diameter). (B) The cylindrical samples were extruded directly into the recipient block at defined array coordinates. (C) Multiple 5-μm sections were cut and (D) final TMA slides were ready for IHC staining. Figure adapted from Bubendorf et al. (62).

To include all core samples, eight tissue array blocks were constructed. Multiple 5-μm sections were cut with a Micron microtome (HM355S) and stained by specific antibodies for immunohistochemistry (IHC) analysis.

3.2.2 TMA - advantages and disadvantages

Table 2 summarizes some of the advantages and disadvantages with TMA technology.

Advantages	Disadvantages
- Time saving	- Possible low representativity in heterogeneous tissue
- Reduced costs	- Not suited for individual diagnosis
- Tissue saving	
- Study large samples	
- Standardization	
- Anonymization	
- Suited for educational purposes	
- Exchanging slides between laboratories	

Table 2. Advantages and disadvantages with TMA technology.

One of the main advantages with TMA technology is of course the high level of standardization and the ability to rapid visualization of many molecular targets of tissue specimens in one procedure. This is not only time saving, but has also an economical advantage using considerable less antibodies than with conventional section analyses. Using 0.6 mm core diameters can also theoretically give more than 300 punches from a tumor block containing a tumor area of 18 x 18 mm (62).

The literature on the protein expression in tumors is often conflicting (62). Variations in antibodies used, staining protocols, fixation of tissues, selection of patients, and criteria for interpretation of staining are routinely discussed as possible sources for discrepancy regarding results. Using TMAs there is the possibility of exchanging slides between different laboratories. Both unstained and stained slides may be of interest. Exchanging unstained slides (on site staining) will help reveal any discrepancies in IHC procedures, while exchanging stained sections may clarify possible differences in the interpretation of the IHC results (scoring, cut-off values

etc). TMA technique is also a good tool for tissue anonymization and is ideally suited for educational purposes. While the interpretation of large sections reflects an attempt to integrate the observations of multiple different regions of a tissue section, the reading of TMAs is theoretically easier and more reproducible.

It is essential to understand that the TMA approach has been designed to study tumor populations and not to examine individual tumors (62). It is obvious that some alterations are not detected if the analysis of potentially heterogeneous tumors is restricted to samples measuring 0.6 mm in diameter. However, it can be assumed that the probability of error will be similar in all tumor groups represented on one array. Associations between molecular alterations and clinical and morphological parameters are therefore likely to be representative from sufficiently large TMAs. However, even in these large scale analyses the absolute frequency of a given alteration may be underestimated of its true prevalence.

Due to tumor heterogeneity both the core diameter and the number of cores have been an important issue. Some investigators have used core samples that are larger in diameter (≥ 2- 4 mm) to improve the representativity (59). Since the chance of finding heterogeneity within a small area is often quite low, this does not necessarily increase the information content of TMA analyses. In contrast, punching multiple small cores from different regions captures the heterogeneity of the tumors more effectively. If a better representation of an individual tumor is requested, it will be much more advantageous to array two or more samples from different areas of each tumor (62). However, a larger diameter may be preferable on complex tissues that require simultaneous investigation of various regions of an organ. This was

experienced in liver research where at least one acinus were necessary to be included in each core, and 2 mm core diameter seem to fulfill this requirement (63).

3.3 Immunohistochemistry (IHC)

3.3.1 IHC procedyre

In short, IHC staining techniques allow 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 at the antigen site. As this is a multi-step process there are potential pitfalls. On the other hand, TMA technique gives the advantage of standardization compared to conventional tissue sections. Besides, trained pathologists can evaluate the final IHC staining with respect to both specificity and background staining.

The 5 µm sections were deparaffinized with xylene and rehydrated with ethanol. Regarding VEGFs, VEGFRs , PDGFs, FGF2 and FGFR-1 antigen retrieval was performed by placing the specimen in 0.01M citrate buffer at pH 6.0 and exposed to two repeated microwave heating of 10 min (except VEGFR-3, 2 x 5 min) at 450W. VEGF-D was heated for 45 minutes in a water bath in 0.01 M citrate buffer and FGFR-1 heated by pressure boiler in 2 min. The DAKO EnVision + System-HRP (DAB) kit was used as endogen peroxidase blocking. As negative staining controls, the primary antibodies were replaced with the primary antibody diluent. Additionally, for the VEGFs and VEGFRs isotype controls for each antibody were performed. Primary antibodies were incubated for 30 min in room temperature (except VEGFR-3 20 min, FGFR-1 60 min and VEGF-D and PDGF-D over night in 4 °C). The DAB-kit

was used to visualize the antigens. This was followed by application of liquid diaminobenzidine and substrate-chromogen, yielding a brown reaction product at the site of the target antigen. PDGF-D was visualized by adding a secondary antibody conjugated with Biotin, followed by an Avidin/Biotin/Peroxydase complex (Vectastein ABC Elite kit from Vector Laboratories). Finally, all slides were counterstained with hematoxylin to visualize the nuclei.

PDGFR-α and –β were stained using Ventana BenchMark XT (Ventana Medical Systems Inc.), procedure iView DAB®. Antigen retrieval was done in Tris/EDTA buffer at pH 8.4 for 30 min (PDGFR-α) or 60 min (PDGFR-β) at 37°C. The primary antibodies were incubated for 30 min in room temperature.

The applied antibodies had been subjected to in-house validation by the manufacturer for IHC analysis on paraffin-embedded material. The antibodies used in the study are shown in Table 3.

Antigen	Antibody	Catalog #	Source	Dilution
VEGFR-1	Rabbit polyclonal	RB-1527	NeoMarkers	1:10
VEGFR-2	Rabbit polyclonal	RB-9239	NeoMarkers	1:25
VEGFR-3	Rabbit polyclonal	Sc-321	Santa Cruz Biotechnology	1:10
VEGF-A	Rabbit polyclonal	RB-1678	NoeMarkers	1:10
VEGF-C	Rabbit polyclonal	18-2255	Zymed laboratories	1:25
VEGF-D	Mouse monoclonal	MAB286	R&D Systems	1:40
PDGF-AA	Goat polyclonal	AB-221-NA	R&D Systems	1:200
PDGF-AB/BB	Rabbit polyclonal	RB-9257	Neomarkers	1:15
PDGF-CC	Goat polyclonal	GT15151	Neuromics	1:80
PDGF-DD	Goat polyclonal	AF1159	R&D Systems	1:400
PDGFR-α	Rabbit polyclonal	RB-9027	Neomarkers	1:75
PDGFR-β	Rabbit polyclonal	RB-9032	Neomarkers	1:25
FGF2	Rabbit polyclonal	AB1458	Chemicon	1:200
FGFR-1	Rabbit polyclonal	Sc-121	Santa Cruz Biotechnology	1:50

Table 3. Antibodies

For each antibody, included negative controls, all TMA staining procedures were performed in one single experiment.

3.3.2 Scoring

The same scoring system was used in all three papers. By light microscopy, representative and viable tissue sections were scored semiquantitatively for cytoplasmic staining. The dominant staining intensity in both tumor cells and stromal cells was scored as: 0 = negative; 1 = weak; 2 = intermediate; 3 = strong.

The cell density of the stroma was scored as: 1 = low density; 2 = intermediate density; 3 = high density. All samples were anonymized and independently scored by two pathologists (S. Al-Saad and K. Al-Shibli). In case of disagreement, the slides were re-examined and a consensus was reached by the observers. In most tumor cores as well as in some stromal cores there is a mixture of stromal cells and tumor cells. However, by morphological criteria we have only scored staining intensity of tumor cells in tumor cores and intensity and density of tumor related stroma in stromal cores. When assessing a variable for a given core, the observers were blinded to the scores of the other variables and to outcome. In paper I, the interobserver scoring agreement was assessed for one ligand (VEGF-C) and one receptor (VEGFR-3).

Mean score for duplicate cores from each individual was calculated separately in tumor cells and stroma. High expression in tumor cells was defined as score > 1 (FGFR-1), ≥1.5 (PDGF-C), ≥ 2 (VEGF-C, VEGF-D, VEGFR-2, PDGF-A, PDGFR-α and PDGFR-β), > 2 (VEGF-A, VEGFR-1, VEGFR-3) or = 3 (PDGF-B, PDGF-D and FGF2). Examples of tumor cell scoring are shown in Figure 8.

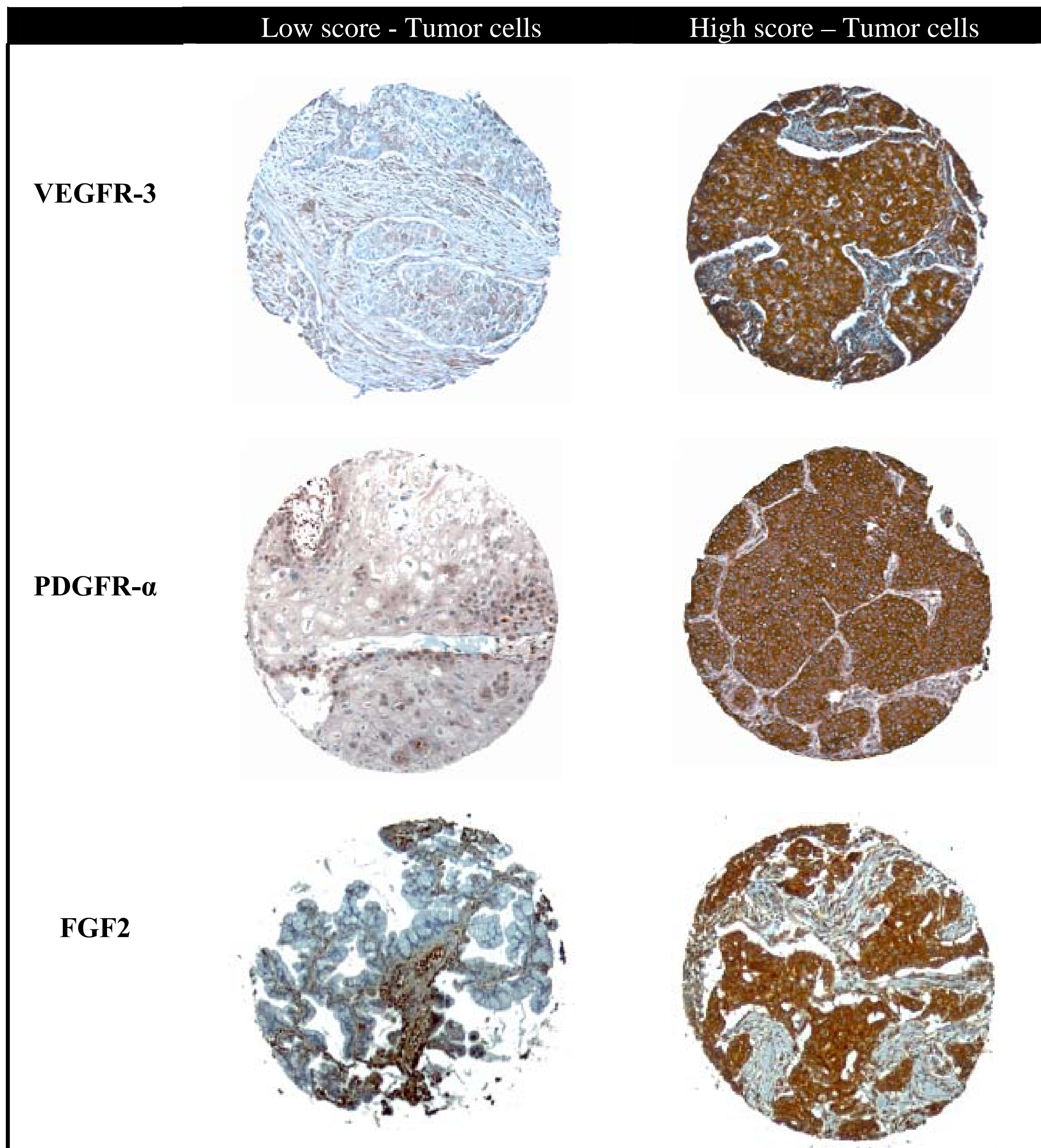

Figure 8. Examples of high and low tumor cell score within each angiogenic family.

Stromal expression was calculated by summarizing density score (1-3) and intensity score (0-3) prior to categorizing into low and high expression (Figure 9). High expression in stroma was defined as score ≥ 2.5 (PDGFR-β), ≥ 4 (VEGF-C, VEGF-D, VEGFR-1, VEGFR-2, VEGFR-3, PDGF-B and FGFR-1), ≥ 4.5 (VEGF-A, PDGF-A, PDGF-C, PDGFR-α and FGF2) or ≥ 5.5 (PDGF-D).

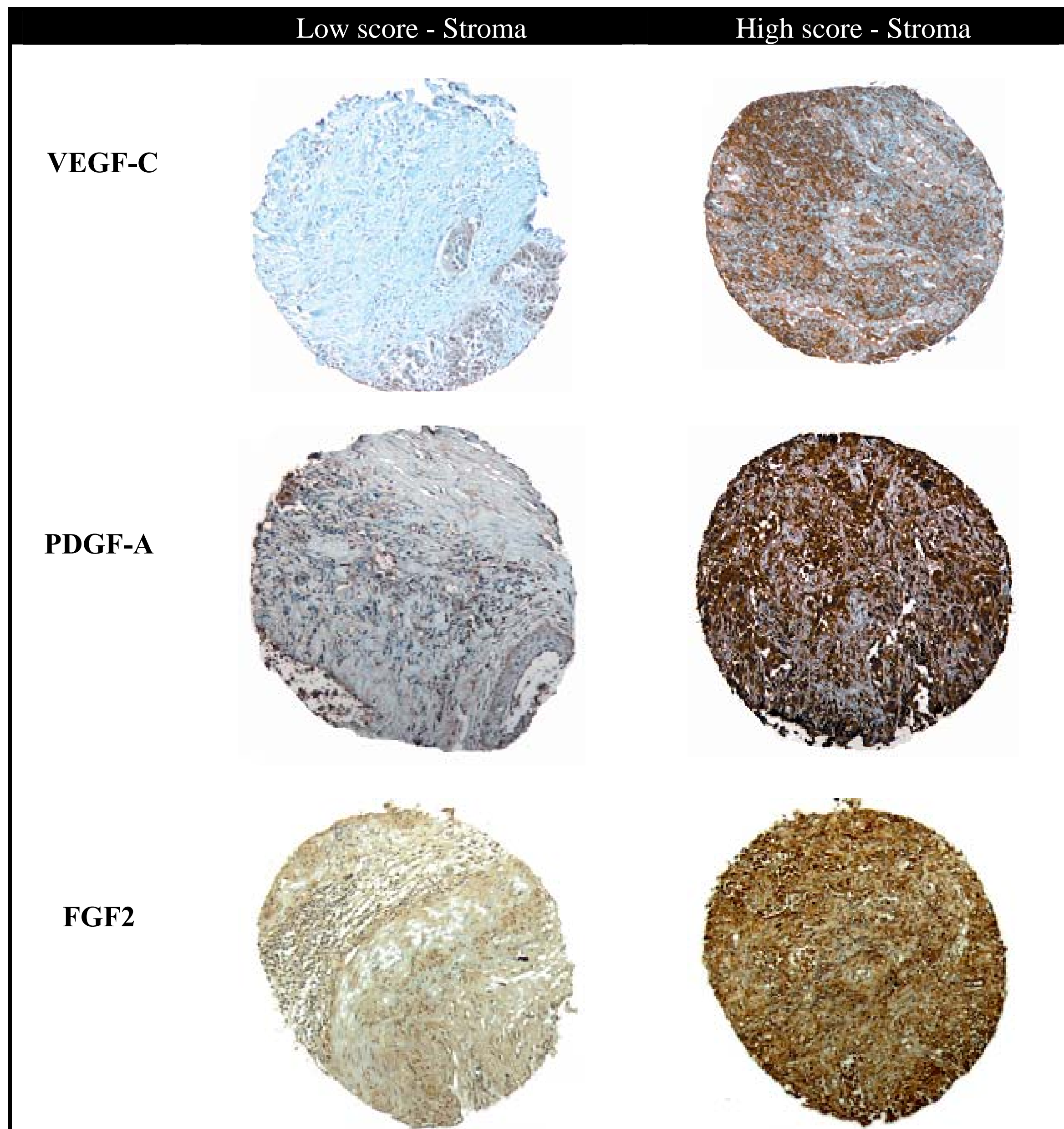

Figure 9. Examples of high and low stromal score within each angiogenic family.

In paper one, we assessed micro vessel density (MVD) by CD34 IHC staining and any stained endothelial cell or endothelial cell cluster separated from other stromal elements was considered as single countable microvessels. The MVD was defined as the number of micro vessels identified within one array core (0.6 mm diameter). Tumor or stromal MVD was scored as: 0 = negative; 1 = 1-10 vessels per core; 2 = 11-20 vessels per core; 3 ≥ 20 vessels per core. In tumor cores, only micro vessels

surrounded by viable tumor cells were counted, whereas in stromal cores, only micro vessels adjacent to other stromal cells were scored. As for the angiogenic ligands and receptors, a mean score for duplicate cores from each individual was calculated separately in tumor cells and stroma. High MVD in tumor cores was defined as a mean score = 2.5 or 3, whereas in stromal cores high MVD was defined as a mean score = 3.

3.3.3 Cut-off values

Variation in methods including differences in tissue preparation, antigen retrieval, and assessment of positive staining makes it difficult to standardize cut-off values. Many studies use the median as cut-off value, but the obvious disadvantage with this approach is missing biological interesting mechanisms. For instance, this may be the case where only the minority or the majority of the patients had a high expression level linked to a certain biological effect. In our binary cut-off points of biomarkers, the cut-off point was determined for each variable so that the two resulting subgroups were the most different according to DSS. The main drawback with this approach is the danger of false positive results, and especially borderline significant results in the analyses must be interpreted carefully.

3.3.4 Controls and limitations

Both reagent and tissue controls were used. Of all components used for IHC analyses, the primary antibody is the most critical. Though, occasionally other reagents may need to be replaced. As reagent control, diluent without primary antibody was used as well as isotype control for the VEGFs/VEGFRs. As tissue

controls, both tissue distant from the primary tumor and lung tissue from individuals without a malignant diagnosis were used.

Another concern is if improper tissue storage over years has affected the results. The oldest tissue blocks used were prepared in 1990, and archival blocks dating back 20-40 years are considered adequate provided initial fixation in 4% buffered formalin (59). When testing for tumor cell-VEGF-A expression, there was no significant difference between specimens with long (>10 years) or short (< 10 years) storage.

It is important to note that only the total expression of each protein was assessed. It means that receptors in both phosphorylated (active) and non-phosphorylated (inactive) state were scored. Most of the studied receptors have no proper antibodies, validated for paraffin-embedded tissue, to measure only the phosphorylated (active) receptors. We have, however, looked at important intracellular downstream molecules (Akts) in both phosphorylated and non-phosphorylated forms (64).

Whether our findings are the result of overexpression of the wild-type protein or whether there is a novel mutation causing overexpression will remain undetected in our analyses. This will be of importance when trying to understand the biology and efficacy of targeted drugs, but less important when evaluating prognostic markers.

3.4 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 hazards 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.

In all three papers, statistical analyses were done using the statistical package SPSS (Chicago, IL), version 14 or 15. In paper one, the IHC 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. 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 by using the Kaplan-Meier method, and statistical significance between survival curves was assessed by the log rank test. Disease-specific survival (DSS) was determined from the date of surgery to the time of lung cancer death. To assess the independent value of different pretreatment variables on survival, in the presence of other variables, 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. Probability for stepwise entry and removal was set at .05 and .10, respectively. The significance level was defined at $p < 0.05$.

4. MAIN RESULTS

4.1 Paper I (VEGFs/VEGFRs)

This study aimed to investigate the prognostic significance of the VEGFs -A, -C, -D and the VEGFRs -1, -2 and -3 in tumor cells as well as in the tumor stroma of resected NSCLC tumors. Interobserver scoring agreement was tested for one ligand (VEGF-C) and one receptor (VEGFR-3). For VEGF-C: tumor $r = 0.95$, $P < .001$; stroma intensity $r = 0.93$, $P < .001$; stroma density $r = 0.93$, $P < .001$. For VEGFR-3: tumor $r = 0.98$, $P < .001$; stroma intensity $r = 0.96$, $P < .001$; stroma density $r = 0.97$, $P < .001$.

In general, high tumor cell angiogenic marker expression appeared to indicate a poor prognosis, while high expression in the stromal compartment appeared associated with a favorable prognosis. In univariate analyses, a high tumor cell expression of VEGF-A ($p = 0.0005$), VEGFR-1 ($p = 0.013$), VEGFR-2 ($p = 0.006$) and VEGFR-3 ($p = 0.0003$) were negative prognostic indicators for disease-specific survival (DSS). In tumor stroma, however, high expression of VEGF-A ($p = 0.017$), VEGF-C ($p = 0.003$), VEGF-D ($p = 0.009$), VEGFR-1 ($p = 0.01$) and VEGFR-2 ($p = 0.019$), was associated with a good prognosis. There was no significant correlation between micro vessel density (MVD) and DSS. In multivariate analyses, high expression in tumor cells of VEGFR-3 was an independent negative prognostic factor for DSS (HR 1.7, CI95% 1.2 – 2.5) whereas in stromal cells high VEGF-C (HR 2.3, CI95% 1.3 – 4.0) expression had an independent positive survival impact.

4.2 Paper II (PDGFs/PDGFRs)

PDGFs and PDGFRs are pivotal in the complex interplay between endothelial, stromal and tumor cells (neoplastic epithelial cells in tumor angiogenesis). This study investigated the prognostic impact of PDGF-A,-B, -C and -D and PDGFR-α and -β in tumor cells and tumor stroma as well as any possible correlations with VEGF-A. In univariate analyses, high tumor cell expression of PDGF-B ($p = .001$), PDGF-C ($p = .01$) and PDGFR-α ($p= .026$) were negative prognostic indicators for DSS. In tumor stroma, high expression of PDGF-A ($p= .009$), PDGF-B ($p= .04$), PDGF-D ($p= .019$) and PDGFR-α ($p= .019$), correlated with a good prognosis. Tumor cell PDGF-A correlated positively with tumor cell VEGF-A expression and stromal PDGF-A were positively associated with stromal VEGF-A expression. In multivariate analyses, high tumor cell PDGF-B (HR 2.1, CI95% 1.4 – 3.3) and PDGFR-α (HR 1.5, CI95% 1.0 – 2.3) expression were independent negative prognostic factors for DSS, whereas in stromal cells high PDGF-A (HR 2.0, CI95% 1.3 – 3.0) expression had an independent positive survival impact.

4.3 Paper III (FGF2/FGFR-1)

This study assessed the prognostic impact of FGF2 and FGFR-1 in tumor cells and tumor stroma of resected non-small cell lung carcinomas (NSCLC) and, based on interesting preclinical trials, explores the importance of their co-expression with VEGFR-3 or PDGF-B.

In univariate analyses, high tumor cell FGF2 expression ($p= 0.015$) was a negative prognostic indicator for DSS. High tumor cell FGFR-1 expression was not significant

associated with DSS ($p = 0.15$). In tumor stroma, high FGF2 ($p = 0.024$) expression correlated with a good prognosis. In multivariate analyses, high expression of FGF2 in tumor cells (HR 1.8, CI95% 1.0 – 3.1) was an independent negative prognostic factor whereas increased FGF2 in stroma (HR 1.8, CI95% 1.1 – 2.8) was a positive prognosticator.

Tumor cell co-expressions of FGF2/VEGFR-3 ($P < 0.001$) and FGFR-1/PDGF-B ($P = 0.002$) were significant indicators of poor prognosis. Tumor cell high FGF2 / high VEGFR-3 expression had a 5-year survival of 10 months versus 64 months in the low FGF2 / low VEGFR-3 group, whereas tumor cell high FGFR-1 / high PDGF-B expression had a 5-year survival of 41 months versus 61 months in the low FGFR-1 / low PDGF-B group.

5. DISCUSSION

5.1 Discussion of results

The major strengths of these discussed studies are the large sample size, an unselective patient cohort and data supplied also from the lung cancer stromal compartment. The possibility to study correlations between angiogenic families and exploring co-expressions of different angiogenic markers, are other major advantages. In contrast, the question whether TMA is suited for evaluating MVD, and lacking data on endothelial expression of the different angiogenic ligands and receptors may be a major limitation when trying to understand the biology of tumor angiogenesis.

5.1.1 Paper I

In this first paper we identified a positive prognostic impact by highly expressed angiogenic markers in tumor stroma, with VEGF-C as a significant independent prognostic indicator.

Much mental capacity has been invested to find an explanation for this novel and somewhat surprising finding. One of our main hypotheses has been that these results are linked to the adaptive immune system's ability to protect against tumor development (65). The regulation of VEGF-C is complex, but it seems like VEGF-C, unlike VEGF-A, is not regulated primarily by hypoxia, but increased by proinflammatory cytokines indicating a possible role in inflammatory responses (66). As we concluded in this paper, knowing more about the individual contribution by the

different categories of stromal cells could be a step forward to better explain this finding. We have studied the prognostic impact of both the innate and adaptive immune system and found high densities of CD4+ and CD8+ lymphocytes (important in the adaptive immune system) in the stroma to be independent positive prognostic indicators (67). This suggests an antitumor immune response mediated by immune cells. Although there are some correlations between these lymphocytes and the VEGF-C expression, this is probably not the only explanation to the independent prognostic impact of VEGF-C.

We found tumor cell VEGFR-3 to be an independent negative prognostic indicator of DSS, consistent with a smaller previous NSCLC study by Arinaga et al. (68). Several preclinical and clinical studies have in different malignancies found VEGF-C, VEGF-D and VEGFR-3 to be important players in lymphangiogenesis (27-30). Clinical studies have, hitherto, not been able to answer whether VEGF-C, VEGF-D and VEGFR-3 have an impact on lymphangiogenesis in lung cancer (68-75). In our NSCLC cohort, we have observed tumor cell VEGFR-3 to be significantly associated with lymph node metastasis (76). Whether the prognostic impact of VEGFR-3 corresponds to hemangiogenesis remain debatable, but in a recent paper Tammela et al. (35), demonstrated VEGFR-3 to be important also in angiogenic sprouting.

To our knowledge, this paper was the first TMA study to evaluate the impact of MVD in NSCLC. Earlier studies have demonstrated a negative prognostic impact of high MVD in NSCLC (77-79). Though, most studies investigating angiogenesis in tumors have determined MVD by estimating the number of micro vessels in the most vascular areas (so-called “hot spots”), as described by Weidner (80), or applied the

Chalkley counting technique (81). The lack of prognostic significance of MVD in our study may be due to the TMA technique's unsuitability for evaluating MVD in NSCLC. However, even when using conventional tissue sections the prognostic impact of MVD in NSCLC is debated (82). In a meta-analysis in Lancet, the authors argue that MVD appears not to be a prognostic factor in surgically resected NSCLC when using all-vessel measurement, and only a week association was seen using the Chalkley method (82).

5.1.2 Paper II

Herein, we observed high tumor cell PDGF-B and PDGFR-α expression to be independent negative prognostic factors for DSS, whereas in stromal cells high PDGF-A expression had an independent positive survival impact. We also had the opportunity to correlate our PDGFs results with previous data on VEGFs/VEGFRs and found stromal PDGF-A to be associated with high stromal VEGF-A expression and tumor cell PDGF-A to correlate with tumor cell VEGF-A expression. The latter is consistent with a study by Shikada et al. (83), demonstrating PDGF-A to stimulate VEGF-expression in NSCLC.

We are the first to report tumor cell PDGFR-α expression as an independent prognostic factor in NSCLC, which is consistent with previous associations between PDGFR-α and a poor prognosis in other malignancies (84-86). This finding may possibly be explained by an autocrine loop in the tumor cells or by ligand-activation from neighboring cells.

Identifying tumor cell PDGF-B as an independent negative prognostic factor was consistent with a smaller lung cancer study by Kawai et al (87). The negative

prognostic effect may at least in part be explained by this ligand's contribution to increased IFP. Besides, the prognostic impact of PDGF-B may be caused by stimulation of the pericytes and subsequent maturation of the microvasculature. Of interest, there seems to be an enhanced antiangiogenic effect by combining VEGF- and PDGF- antagonists by simultaneously stimulating antiendothelial and antipericyte effects (88). In fact, clinical phase II NSCLC trials on drug inhibition of both the VEGFR and PDGFR - axis show promising efficacy (57;89).

5.1.3 Paper III

In this paper we found high tumor cell FGF2 expression to be independently associated with a poor prognosis, while high stromal FGF2 expression correlates with a good prognosis. Based on interesting preclinical data and our previously reported data on PDGF-B and VEGFR-3, we could demonstrate tumor cell co-expression of both FGF2/VEGFR-3 and of FGFR-1/PDGF-B to correlate with an extremely poor prognosis.

Previous data on FGF2's prognostic impact in NSCLC has been conflicting (90-95). In a recent large scale TMA-based NSCLC study, Behrens et al.(96) observed FGF2, FGFR-1 and -2 to be overexpressed in both squamous cell carcinomas and adenocarcinomas. In addition they differentiated the cellular IHC expression according to cytoplasmic and nuclear localization. Somewhat surprisingly, they found the cytoplasmic overexpression of FGF2 in squamous cell carcinomas to correlate with a better prognosis, while there were no significant associations in adenocarcinomas, neither in cytoplasma nor nuclei.

Several clinical NSCLC studies have explored the prognostic role of elevated serum-FGF2, but no consensus has been reached (90;97-101). One study reported high serum level of FGF2 to indicate a favorable prognosis (102). The latter may be consistent with our finding of high stromal FGF2 expression as a favorable prognostic indicator, as it can be argued that both stromal and tumor cell FGF2 may contribute to the serum level of FGF2.

In the first prospective randomized phase II/III study including chemotherapy and bevacizumab and the impact of FGF2 plasma levels (in addition to other markers), FGF2 failed as both a prognostic and predictive marker (103). However, bevacizumab target VEGF-A/VEGFR-2 axis and one may expect FGF2 more likely as a predictive marker for e.g. tyrosine kinase inhibitors targeting the FGF2/FGFR-1 axis. The severely detrimental survival mediated by the co-expression of FGF2/VEGFR-3 or FGFR-1/PDGF-B should be of interest for future choices of candidate predictive markers and combinations of therapy targets for upcoming NSCLC therapy.

6. CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH

As angiogenesis is a critical and universal process in tumor development and the role of novel agents directed at the angiogenic process continues to increase, the interest in biomarker identification and validation has increased. Angiogenic prognostic and predictive markers are important to help us better understand tumor biology, and will in the future guide us more precisely in the clinical decision-making processes and to help in drug development strategies.

By studying three important families of angiogenic markers, we have identified several independent prognostic factors and found prognostically highly important co-expression. As several novel target agents are designed to enhance the antiangiogenic efficacy by combining VEGFs/PDGFs/FGFs- antagonists, one may expect that co-expressions of these angiogenic markers may be of predictive value in the future. There are many promising prognostic factors, but so far no predictive factor has been established for anti-angiogenic treatment. Future prospective studies are expected to elucidate this topic and subsequently give us the tools to select patients most likely to benefit from anti-angiogenic treatment.

The TMA technology, facilitating a large-scale high-throughput study with unselected data, has been a good platform to study angiogenic molecular mechanisms in NSCLC tumors. Our research group will continue to explore the co-expressions of different angiogenic markers as there is a considerable interplay between the different marker families. We are presently introducing new research strategies to enhance the knowledge with basic angiogenesis mechanisms. Cell culture studies have been initialized to examine angiogenic marker associations over time in human

cancer cell lines, exposed to hypoxia or normoxia. Additionally, new technology has given us the opportunity to measure different short, non-coding, functional RNA molecules (microRNAs) in formalin-fixed paraffin-embedded tissue. Relating microRNA results to our previous angiogenic protein expression data will be a novel and interesting approach.

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Paper 1

Paper 2

Paper 3

Appendix

WHO histological classification of tumours of the lung

Malignant epithelial tumours

- Squamous cell carcinoma 8070/3
  - Papillary 8052/3
  - Clear cell 8084/3
  - Small cell 8073/3
  - Basaloid 8083/3

- Small cell carcinoma 8041/3
  - Combined small cell carcinoma 8045/3

- Adenocarcinoma 8140/3
  - Adenocarcinoma, mixed subtype 8255/3
  - Acinar adenocarcinoma 8550/3
  - Papillary adenocarcinoma 8260/3
  - Bronchioloalveolar carcinoma 8250/3
    - Nonmucinous 8252/3
    - Mucinous 8253/3
    - Mixed nonmucinous and mucinous or indeterminate 8254/3
  - Solid adenocarcinoma with mucin production 8230/3
    - Fetal adenocarcinoma 8333/3
    - Mucinous ("colloid") carcinoma 8480/3
    - Mucinous cystadenocarcinoma 8470/3
    - Signet ring adenocarcinoma 8490/3
    - Clear cell adenocarcinoma 8310/3

- Large cell carcinoma 8012/3
  - Large cell neuroendocrine carcinoma 8013/3
    - Combined large cell neuroendocrine carcinoma 8013/3
  - Basaloid carcinoma 8123/3
  - Lymphoepithelioma-like carcinoma 8082/3
  - Clear cell carcinoma 8310/3
  - Large cell carcinoma with rhabdoid phenotype 8014/3

- Adenosquamous carcinoma 8560/3

- Sarcomatoid carcinoma 8033/3
  - Pleomorphic carcinoma 8022/3
  - Spindle cell carcinoma 8032/3
  - Giant cell carcinoma 8031/3
  - Carcinosarcoma 8980/3
  - Pulmonary blastoma 8972/3

- Carcinoid tumour 8240/3
  - Typical carcinoid 8240/3
  - Atypical carcinoid 8249/3

- Salivary gland tumours
  - Mucoepidermoid carcinoma 8430/3
  - Adenoid cystic carcinoma 8200/3
  - Epithelial-myoepithelial carcinoma 8562/3

- Preinvasive lesions
  - Squamous carcinoma *in situ* 8070/2
  - Atypical adenomatous hyperplasia
  - Diffuse idiopathic pulmonary neuroendocrine cell hyperplasia

- Mesenchymal tumours
  - Epithelioid haemangioendothelioma 9133/1
  - Angiosarcoma 9120/3
  - Pleuropulmonary blastoma 8973/3
  - Chondroma 9220/0
  - Congenial peribronchial myofibroblastic tumour 8827/1
  - Diffuse pulmonary lymphangiomatosis
  - Inflammatory myofibroblastic tumour 8825/1
  - Lymphangioleiomyomatosis 9174/1
  - Synovial sarcoma 9040/3
    - Monophasic 9041/3
    - Biphasic 9043/3
  - Pulmonary artery sarcoma 8800/3
  - Pulmonary vein sarcoma 8800/3

Benign epithelial tumours

- Papillomas
  - Squamous cell papilloma 8052/0
    - Exophytic 8052/0
    - Inverted 8053/0
  - Glandular papilloma 8260/0
  - Mixed squamous cell and glandular papilloma 8560/0
- Adenomas
  - Alveolar adenoma 8251/0
  - Papillary adenoma 8260/0
  - Adenomas of the salivary gland type
    - Mucous gland adenoma 8140/0
    - Pleomorphic adenoma 8940/0
    - Others
  - Mucinous cystadenoma 8470/0

Lymphoproliferative tumours

- Marginal zone B-cell lymphoma of the MALT type 9699/3
- Diffuse large B-cell lymphoma 9680/3
- Lymphomatoid granulomatosis 9766/1
- Langerhans cell histiocytosis 9751/1

Miscellaneous tumours

- Harmatoma
- Sclerosing hemangioma 8832/0
- Clear cell tumour 8005/0
- Germ cell tumours
  - Teratoma, mature 9080/0
  - Immature 9080/3
  - Other germ cell tumours
- Intrapulmonary thymoma 8580/1
- Melanoma 8720/3

Metastatic tumours

[1] Morphology code of the International Classification of Diseases for Oncology (ICD-O) {6} and the Systematized Nomenclature of Medicine (http://snomed.org). Behaviour is coded /0 for benign tumours, /3 for malignant tumours, and /1 for borderline or uncertain behaviour.

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