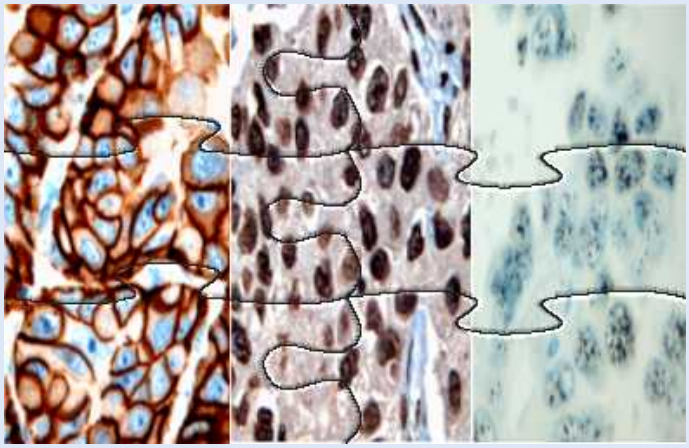
PROGNOSTIC IMPACT OF MOLECULAR MARKERS RELATED TO TUMOR PROLIFERATION AND DIFFERENTIATION IN NON-SMALL-CELL LUNG CANCER

A translational research strategy investigating the prevalence and the prognostic impact of cancer progression-related molecular markers



Samer Al-Saad

A dissertation for the degree of Philosophiae Doctor

University of Tromsø
Institute of Medical Biology
Department of Pathology

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PROGNOSTIC IMPACT OF MOLECULAR MARKERS RELATED TO TUMOR PROLIFERATION AND DIFFERENTIATION IN NON-SMALL-CELL LUNG CANCER (NSCLC)

A dissertation for the degree of Philosophiae Doctor

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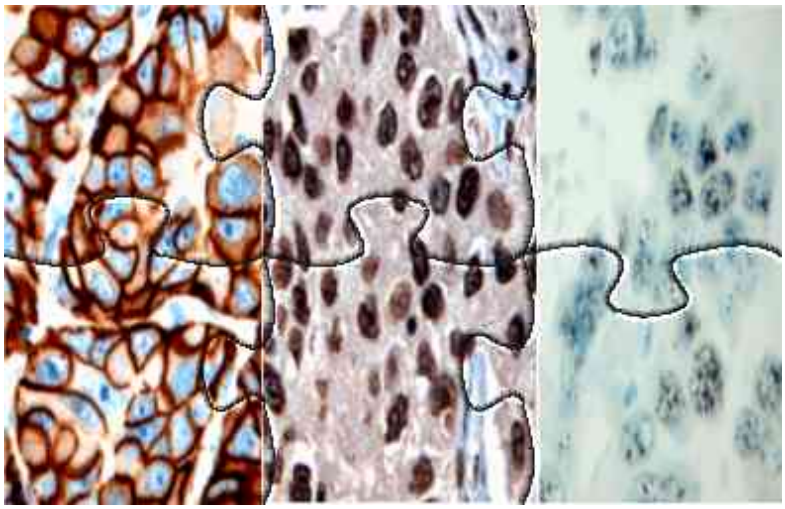
2010



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British Journal of Cancer (2008) 99, 1476 – 1483

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Anticancer Research 2009 Oct;29(10):4175-83.

PAPER III **Al-Saad S, Al-Shibli K, Donnem T, Andersen S, Bremnes RM and Busund L-T**

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Journal of Thoracic 2010 Oct;5(10):1536-43.

LIST OF ABBREVIATIONS

Ab	Antibody
Akt	Atypical protein kinase B
aPKC	Atypical protein kinase protein complexes C
DNA	Deoxyribonucleic acid
DSS	Disease-specific survival
EGFR	Epithelial growth factor receptor
EMT	Epithelial mesenchymal transition
ErbB	Erythroblastic leukemia oncogene
IHC	Immunohistochemistry
Mab	Monoclonal antibody
MYC	Myelocytomatosis oncogene
NF-κB	Nuclear factor kappa binding
NLSH	Nordland Central Hospital
NSCLC	Non-small-cell lung cancer
OS	Overall survival
Par6	Partitioning-defective protein-6
PFS	Progression-free survival
PTEN	Phosphatase and tensin homologue deleted on chromosome ten
RAS	Rat sarcoma oncogene
RNA	Ribonucleic acid
RR	Relative risk
TGF-ß	Transforming growth factor-beta
TP53	Tumor protein 53
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

1.1.3 Epidemiology and incidence

Lung cancer has now become one of the world's leading causes of death,[1] with an estimated annual incidence of more than 1.3 million cases and a mortality of more than 1.1 million cases worldwide. Epidemiological studies have shown a strong correlation between smoking and lung cancer. In fact, Lung cancer mortality can provide a useful measure of a population's exposure to smoking, especially in the population segment aged 35-54, as around 80-90% of lung cancer cases are caused by active tobacco consume.[2, 3] While lung cancer incidence and mortality in men has decreased in recent years (see Figure 1a), mortality in women is still increasing with the largest increase in Europe.[4, 5] In the EU as a whole, lung cancer mortality in men peaked at 55.4 per 100,000 men in 1988 and declined thereafter to 46.7 in 2000.[4] In women on the other hand, there was an increase in lung cancer mortality from 7.7 per 100,000 women in 1980 to 11.1 women in 2000.

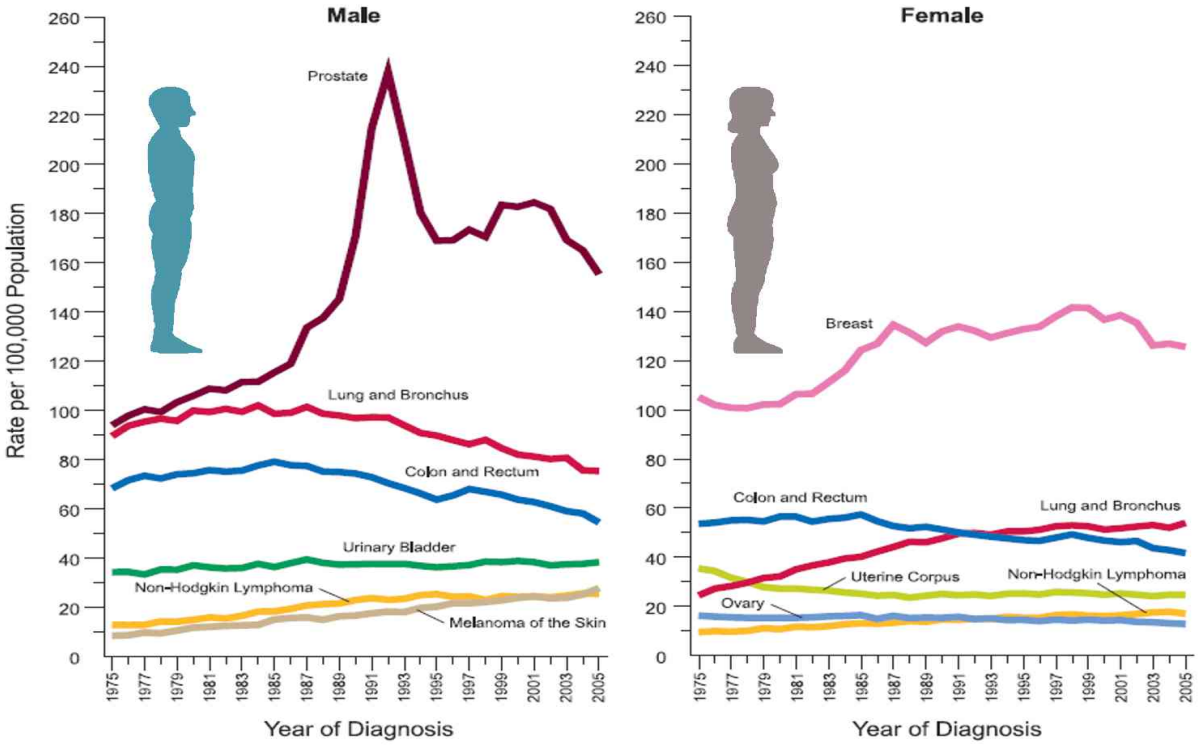

Figure 1a. Annual Age-adjusted Cancer Incidence Rates* for Selected Cancers by Sex, United States, 1975 to 2004. *From Jemal, A. et al. CA Cancer J Clin 2008:CA.2007.0010v1-20070010.* American Cancer Society 2008

An alarming feature is the 38% increase of lung cancer mortality in women aged under 55 years between 1990 and 2000 (from 2.16 per 100,000 women to 2.99 per 100,000 women), reflecting the worrisome spread of cigarette smoking among EU women over the last few decades.[5] With respect to lung cancer-related deaths before the age of 50 years in Norway, women surpassed men almost 13 years ago (Figure 1.b).

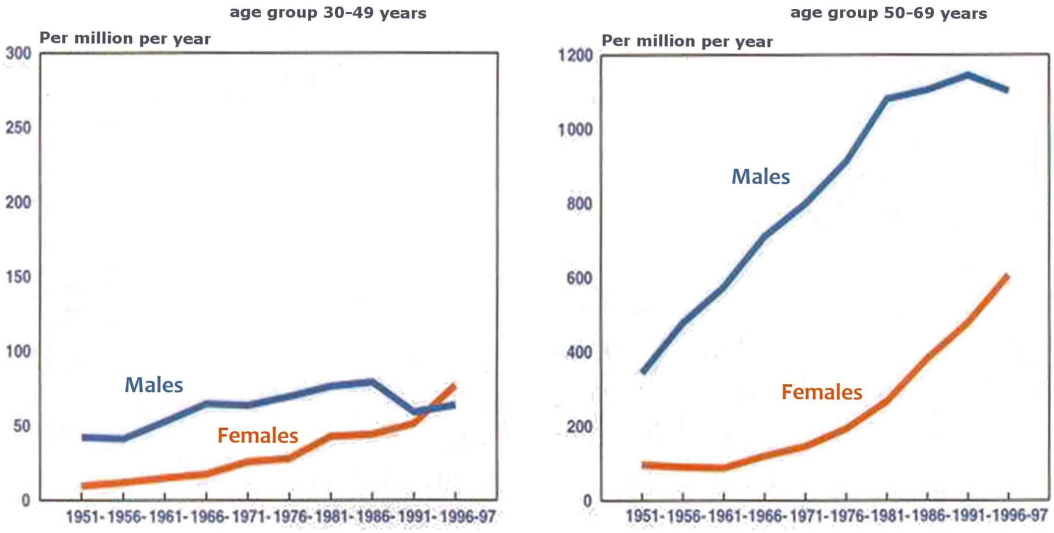

Figure 1b. Mortality rates of lung cancer among men and women in different age groups in Norway (1951-1997). With respect to lung cancer-related deaths before the age of 50, women surpassed men almost 13 years ago. *Source: Kreftregisteret / Cancer Registry of Norway*

In the the EU member countries there were in 2002 over 227.000 fatal outcomes of patients diagnosed with lung cancer.[6] In Norway, 1422 men and 1107 women were diagnosed with lung cancer in 2008.[7] For lung cancer mortality in both genders in Norway see Figure 2.

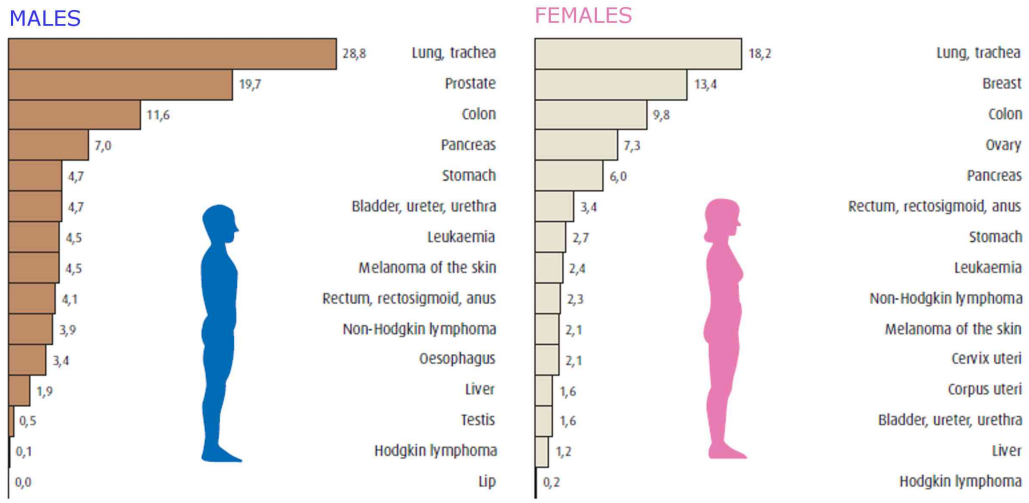

Figure 2. Age-standardised (world) mortality rates in Norway 2007 for selected cancers
Source: Kreftregisteret / Cancer Registry of Norway

Other causes of lung cancer are radiation exposure, uranium (with a relative risk RR of 4:1 for nonsmokers and 10:1 for smokers vs. general population); asbestos (RR with asbestos exposure is 5:1 for nonsmokers, 50-90:1 for smokers vs. general population), exposure to nickel, chromate, coal, mustard gas, arsenic, beryllium, iron, vinyl chloride and radon radiation.[8-11]

1.1.2 Histopathology

Lung cancers are classified according to non-small-cell carcinoma (NSCLC, 80%) and small-cell cancer (SCLC, 20%)**.** NSCLC is histologically a wide classification, when compared to SCLC. The major NSCLC histological subtypes are squamous cell carcinoma, adenocarcinoma with bronchioloalveolar carcinoma and large cell carcinoma (see Figure 3). Mixed histological subtypes in the same tumor can also occur.[12] Patients with bronchioloalveolar carcinoma have a significant better clinical outcome.

Large cell carcinoma is also known as undifferentiated large cell carcinoma or anaplastic carcinoma. Previously, squamous cell carcinomas were most common, but the percentage of adenocarcinomas is now increasing probably due to increased use of filter cigarettes. Adenocarcinoma is also the most common subtype among never smokers.

For a more comprehensive table of WHO classification of NSCLC see Appendix 1.

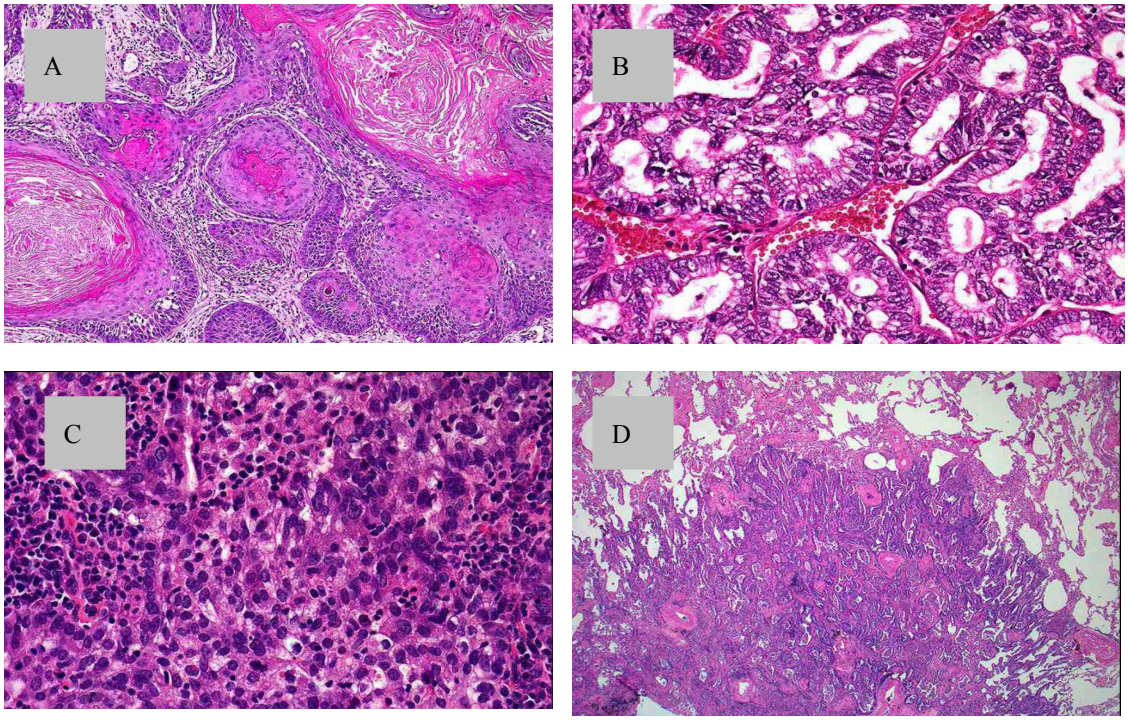

Figure 3. Squamous cell carcinoma A, Adenocarcinoma B, Large cell carcinoma C and bronchioloalveolar carcinoma D

1.1.3 Staging and TNM

The majority of lung cancer patients are already clinically symptomatic at the time of diagnosis. The most common symptoms are cough, dyspnea and hemoptysis. Due to diagnosis at a late stage, only (4346/21968; mean 20%) of NSCLC patients in Norway, 1993-2005, were clinically operable at the time of diagnoses.[7] Therapeutical strategies are based on both histological and clinical examinations. Clinical examinations include chest x-ray and a CT of the chest including the upper abdomen with the adrenal glands. Histological biopsies are usually obtained by bronchoscopy for central/hilar tumors or by a CT guided procedure for peripheral tumors. According to patients with enlarged mediastinal glands, a mediastinoscopy or an open thoracal surgery is regularly performed to rule out N2-status (see Table 1). Recently, additional staging tools like positron emission tomography (PET) and transesophageal or endobronchial ultrasound have been employed. Clinical TNM (cTNM) are based on clinical patient examinations, while pathological TNM (pTNM) is based on histological examinations. Prognostic significance of cTNM is demonstrated in Table 1. For pTNM and clinical stage IIIA see Figure 4 and Appendix 2.

Table 1. Prognostic significance of cTNM stages

Stage	Tumor	Node	Metastasis	Definition	Survival rate (%) 1 Yr	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.

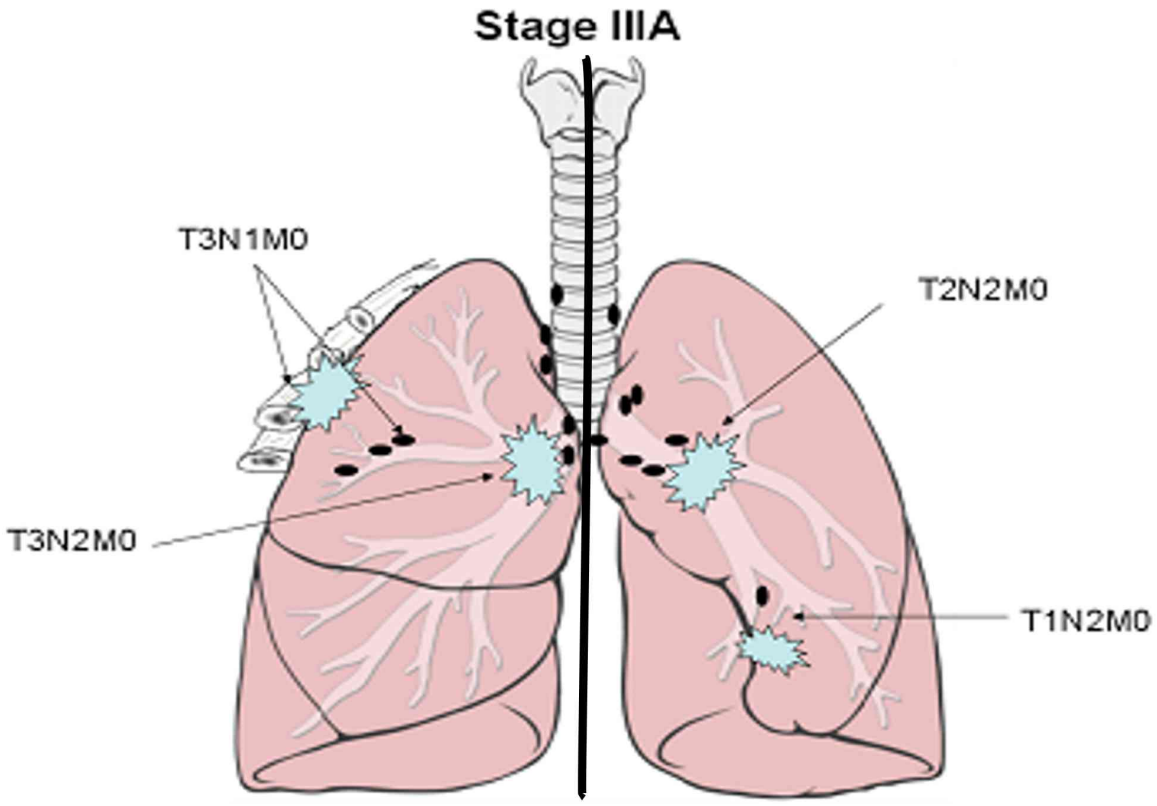

Figure 4. Adapted from C F Mountain. *A new international staging system for lung cancer. CHEST 2000*

1.1.4 Treatment of NSCLC stage I-IIIA

Complete surgical resection is the standard treatment for patients in early clinical stage (I-IIIA) NSCLC. Clinical trials have shown improved survival rates in subsets of resectable NSCLC patients administered preoperative (neoadjuvant) and postoperative (adjuvant) chemotherapy and/or radiotherapy. Adjuvant chemotherapy is, however, not recommended for stage IA and IB disease.[13] For patients with clinical stage II-IIIA, cisplatin-based adjuvant chemotherapy is recommended.[13-17] Radiotherapy, in stead for surgery, in stage I-II is only indicated for inoperable patients due to age, concurrent diseases or poor clinical performance status. For patients with pathological N2 disease stage (metastases to ipsilateral mediastinal and/or subcarinal lymphnodes) or incomplete tumor resection postoperative radiotherapy is indicated.[13-17] For inoperable patients with stage IIIA disease, radical radiotherapy is indicated only for those with good prognostic factors (regarding tumor size, performance status, degree of weight loss). Otherwise, chemotherapy or palliative radiotherapy is administered.

1.2 MOLECULAR AND GENETIC ABNORMALITIES

1.2.1 Molecular alterations in NSCLC

Several genetic abnormalities and biomolecular alterations have been demonstrated in NSCLC.[18] Mutations of the tumor suppressor gene TP53 due to loss of one allele at 17p13, a locus that harbors the TP53 has been identified in lung cancer.[19] There is evidence that such alterations in the p53 gene may be induced by tobacco inhalation.[20] Other promoter genes involved in lung cancer tumorigenesis include tyrosine kinase receptors such as EGFR, as well as the *myc* and *ras* oncogenes family. While alteration of the *ras* oncogene is a frequently observed event in adenocarcinomas, it is most frequently expressed in smokers.[21] Additionally, there are observations regarding increased DNA-methyltransferase (DNA-MTase) activity followed by a hypermethylation of normally unmethylated gene regions, representing an alternative method for inactivation of tumor suppressor genes, including p16 and E-cadherin.[22]

1.2.2 Therapeutic molecular targets in tumor cells

Preclinical investigations on various types of cancers, including NSCLC, observed a frequent overexpression of the tyrosine kinase receptor EGFR. EGFR activation elicits the activation of downstream pathways, resulting in cell proliferation, invasion, metastasis, and loss of apoptosis. Interruption of this signaling pathway with a variety of EGFR inhibitors has been shown to decrease tumor cell viability, prevent proliferation, or both, *in vitro* and *in vivo*.[23] Gefitinib and erlotinib are selective EGFR tyrosine kinase inhibitors (TKIs) and were the first EGFR-targeted agents to be studied in patients with NSCLC. In phase II trials of gefitinib in patients with pretreated metastatic NSCLC, objective tumor response rates of 9–19% were seen, and approximately 40% of patients showed improvement of symptoms.[24, 25] Meanwhile, somatic mutations in exons 19 and 21, corresponding to the tyrosine kinase domain of EGFR where revealed in most patients responding to EGFR TKI.[26, 27] However, these specific mutations most frequently occur in subsets of lung adenocarcinomas, females, Asians, and/or non-smokers.[28, 29]

Although most EGFR mutant NSCLCs initially respond to EGFR inhibitors, the vast majority of these tumors ultimately become resistant to the drug. It is proposed that activation of other Erb family members can play an essential role in development of this drug resistance. This has led to a search for combinations of drugs, simultaneously inhibiting different tyrosine kinase receptors. Clinical NSCLC trials have also reported improved survival in patients with

HER2 gene amplification after blockade of the HER2 receptor.[30] Investigations concerning HER3 activation are also ongoing. Recently, the MET proto-oncogene (a HER3 activator) has become an interesting inhibition target in NSCLC.[31] Angiogenesis i.e. formation of new vasculature for tumor blood supply has also emerged as an interesting target in cancer therapy, also in lung cancer. A phase III trial in NSCLC patients using bevacizumab (inhibitor of VEGF/VEGFR2) presented an improved response rate, progression-free survival and overall survival. Though the median survival benefit was modest, from 10.3 to 12.3 months, the drug has been approved by both EMEA and FDA.[32] However, a similar later study did not show any difference regarding overall survival.[33]

Research within molecular cancer biology found the protein kinase B (Akt) signaling pathway to be central in cellular proliferation and survival. Being disrupted in many human malignancies with wide-ranging biological consequences, the PI3K/Akt signaling pathway is considered an essential target of various antitumor drugs, including UCN-01 (7-hydroxystaurosporine) and geldanamycin analogues.[34]

1.2.3 Potential of new molecular targets in cancer

Molecular markers of neoplastic tissue are now gaining ground as prognosticators and possible therapeutical targets. Identification of molecular markers involved in critical cancer pathways may present interesting approaches for therapeutical regimes. Thus it is of huge interest to investigate the prognostic and/or the predictive significance of different biological markers in malignancies. Having mentioned this, we should not underestimate the prognostic significant power of various clinical and histological variables (such as tumor size, differentiation, tumor stage and performance status). Since new potential molecular targets are discovered almost on a daily basis, a serious concern may be the necessary selection of which biomarkers to investigate. Immunohistochemistry has played a critical role in assessing new biomarkers, but new, more comprehensive and effective methods seem to be needed in the near future to improve our understanding of tumor biology and progression.[35]

1.3 Tumor differentiation and proliferation

Cancer cells undergo complex genetic aberrations through the different stages of carcinogenesis. Genomic instability or selection leads to aberrations that can be grouped into six essential pathways as proposed by Hanahan and Weinberg[36] in 2000: Acquisition of (1) self-sufficient or autonomous growth signals; (2) insensitivity to anti-growth signals; (3) resistance to signals of apoptosis; (4) unlimited replicative potential; (5) sustained angiogenesis; and (6) invasion of tissue and metastasis. Each of these mechanisms is regulated of several pathways which further interact with a complex roadmap of subpathways. Despite this complexity, there is mounting evidence that certain molecular aberrations are more likely to influence the clinical behavior of a malignant tumor, including the risk of metastasis.[37-40] Investigating such aberrations in prognostic markers can not only potentially influence and predict the clinical outcome of a cancer patient, but also open possibilities for new interesting therapeutical targets.[41] There are several methods for determining alterations in molecular prognostic markers: Changes in gene copy number by means of silver/fluorescence *in situ* hybridization; messenger RNA (mRNA) expression, and protein expression levels using immunohistochemistry (IHC).

1.3.1 Tumor proliferation

A balance between stimulators and inhibitors of cell proliferation maintains growth homeostasis in normal cells. Resistance to growth-inhibitory factors is an essential step in carcinogenesis. The cell population of tumor tissues is determined by the rates of cell proliferation, differentiation, and death by apoptosis.[42] The proliferative behavior of a tumor is important for establishing therapeutic regimes and for predicting prognosis. Various techniques have been developed to quantify tumor proliferation rates. Mitotic count, using light microscope, are widely used as a simple measure of cellular proliferation and are often incorporated into tumor grading systems.[43] Other methods have been developed, such as the detection of cells undergoing DNA synthesis,[44] flow cytometry to estimate the percentage of cells in S phase of the cell cycle or the detection of antigens associated with proliferation. Evaluating the mitotic index can be performed on paraffin-embedded specimens using light microscopy with simple staining methods. However, there may be inter- and even intra-observer variability in counting. Some morphologic changes in cells (e.g. apoptosis, or nuclear pyknosis) can also be miscounted as mitosis. On the other hand, using specific antigens can be both expensive and time consuming but examinations can be performed on a

small amount of tissue. A known immunohistochemical marker is the nuclear antigen Ki67/MIB-1 which is widely used on routine basis for diagnostic purposes. Different marker expressions which were investigated in this study are correlated both to proliferation and differentiation of tissue.

1.3.2 Tumor differentiation and epithelial-mesenchymal transition

Differentiation refers to the extent to which neoplastic cells both morphologically and functionally resemble normal mature cells of the tissue of origin in a given organ. Malignant neoplasms range from well differentiated to undifferentiated. Poorly differentiated tumors consist of primitive-appearing, unspecialized cells. This lack of differentiation (also called anaplasia) is marked by a number of morphologic changes, such as variation in size and shape, i.e. pleomorphism, dark stained nuclei due to abundance of DNA (called hyperchromasia), loss of orientation (loss of polarity, i.e. poorly differentiated tumor cells grow in disorganized sheets). There is mounting evidence suggesting that this change in tumor tissue architecture takes place through a peculiar phenotype modulation known as epithelial-mesenchymal transition (EMT). EMT involves dedifferentiation of epithelial cells to fibroblastoid, migratory and more invasive cells, showing a profoundly altered mesenchymal gene expression profile.[45, 46] The essential features of EMT are the disruption of intercellular contacts and the enhancement of cell motility leading to metastases. Although the molecular bases of EMT have not been completely elucidated, several cellular transduction pathways and a number of signaling molecules potentially involved have been identified. EMT also occurs during embryonic development as epithelial cells lose their polarized phenotype, a process which is regulated by an interplay of different signaling pathways.[47-50] An example of these processes includes tubulogenesis and branching in the mammary gland, mesoderm formation during gastrulation, and palate fusion and emigration of neural-crest cells from the neural tube.

1.3.3 Factors and receptors of tumor proliferation and differentiation

1.3.3.1 Proteins involved in inflammatory processes and tumorigenesis

Epidemiological studies have revealed inflammation as a critical component of tumor progression. Many cancers arise from sites of infection and chronic irritation. There is also evidence that usage of non-steroidal anti-inflammatory agents is associated with protection against certain malignancies. Different cellular components are involved in the inflammatory process at the invading edge of cancer,[51-53] including white blood cells, macrophages and fibrocytes. Several proteins[54] and proliferation markers[55, 56] are found in areas with inflammation. The transcription factor Nuclear factor-kappa binding (NF-κB) is activated in a range of inflammatory processes and human cancers and is thought to promote tumorigenesis.[55, 57] A link between aberrant NF-κB activity and cancer was initially implied by the identification of v-Rel, a viral homolog of c-Rel, as the transforming oncogene of an avian retrovirus that causes aggressive tumors in chickens.[58] Moreover NF-κB is activated by oncogenic viruses, such as human T cell leukemia virus I or Epstein-Barr virus.[59, 60] Constitutive nuclear NF-κB activity has emerged as a hallmark of many other human leukemias, lymphomas, and solid tumors.[57, 61]

NF-κB is a group of proteins that control inflammation, cell survival, transformation, proliferation, angiogenesis and apoptosis.[62] It is normally retained in the cytoplasm in an inactive state through interaction with inhibitor kB (IkB) (Figure 5).[57] Degradation of the IkB proteins results in the liberation of NF-κB , allowing nuclear translocation and the activation of target genes, including Snail and Bcl-2.[63] Five mammalian NF-κB proteins have been identified: p65 (RelA), NF-κB 1 (p50 and its precursor p105), NF-κB 2 (p52 and its precursor p100), c-rel and RelB. These bind to DNA as homo- or heterodimers.[64] Unlike RelA, RelB, and c-Rel, the p50 and p52 NF-κB subunits do not contain transactivation domains in their C terminal domain. Nevertheless, the p50 and p52 NF-κB members play critical roles in modulating the specificity of NF-κB function. Although homodimers of p50 and p52 have been described as repressors of kB site transcription, both p50 and p52 participate in target gene transactivation by forming heterodimers with RelA, RelB, or c-Rel.[65] In addition, p50 and p52 homodimers also bind to the nuclear protein Bcl-3, and such complexes can function as transcriptional activators.[66-68]

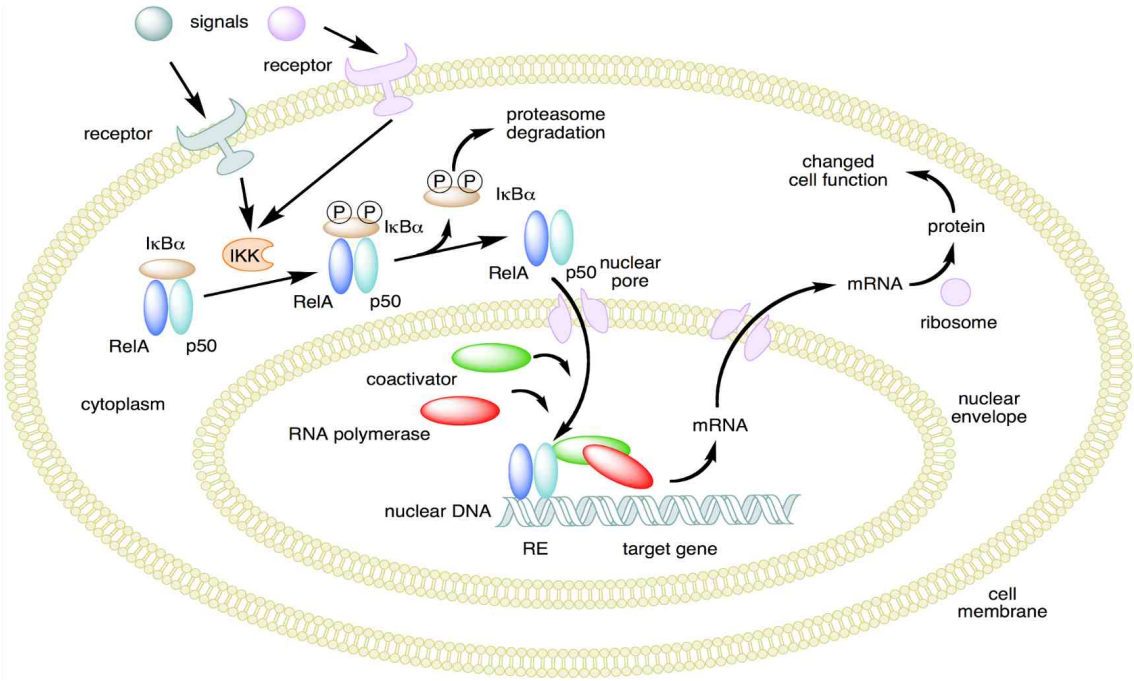

Figure 5. Mechanisms of NF-kB activation. Adapted from Glimore TD (2006). "introduction to NF-kB: players, pathways, perspectives". *Oncogene 25 (51): 6680-4.*

1.3.3.2 Regulators of cell polarity

Polarization of epithelial cells resting on the basal membrane is a feature of differentiated tissue. On the other hand, loss of polarization accompanied by other morphologic and functional changes such as fibroblastoid (mesenchymal) morphology and enhanced motility is a feature of aggressive tumors. While mechanisms of cell polarity are quite complex, the partitioning-defective protein-6 and atypical protein kinase protein complexes C (Par6-aPKC) localized to the apical membrane domain have emerged as central players in the regulation of cell polarity and the asymmetric division of cells.[69-72]

The intermediate filament protein vimentin is a structural protein from cells of mesenchymal origin[73] and was initially isolated from a mouse fibroblast culture.[74, 75] Its expression is higher in migratory epithelial cells and may contribute to the migratory and invasive phenotype of metastatic cells.[76] Significant correlations between high vimentin tumor cell expression and poor prognosis have previously been reported in various malignancies.[77, 78]

Fascin is an actin-bundling and crosslinking protein that binds to preformed filaments and regulates their organization and stability. It is presumed to regulate cortical cell membrane protrusions.[79] Its overexpression is induced by Epstein Bar virus infection in a subset of B-lymphocytes[80] and is proposed to increase the motility of epithelial cells.[81, 82]

1.3.3.3 Growth factors

Transforming growth factor-beta (TGF-ß) is a chemokine with multifunctional regulatory aspects of cellular functions including cellular proliferation, differentiation,[45] migration, apoptosis, adhesion, angiogenesis, immune surveillance, and survival.[83, 84] Although TGF-ß regulates proliferation of normal tissue and suppresses early tumorigenesis; it demonstrates however, a paradoxical action as a tumor promoter in later stages of tumor progression.[85] It can also facilitate malignant transformation through manipulating a more hospitable environment for tumor invasion and the development of metastases. Overexpression of TGF-ß is proposed to enhance tumorigenesis not only by stimulating angiogenesis and suppressing the immune system, but also by acting directly on neoplastic cells in some malignancies.[86-90] Elevated expression of TGF-ß has been correlated to poor clinical outcome in different malignancies,[91] hence various components of the TGF-ß signaling pathway offer potentially attractive therapeutic targets for cancer treatment.[92]

1.3.3.4 Regulators of cell adhesions

E-cadherin is a cell adhesion molecule expressed in epithelial cells[93, 94] and plays an essential role in epithelial tissue formation. In the cytoplasm, E-cadherin is anchored to the actin cytoskeleton via the E-cadherin–binding h-catenin and a-catenin,[95] which is crucial for the dynamic regulation of cellular differentiation, proliferation and migration.[95] There is evidence that instability of the E-cadherin complex in malignant cells contribute to a functional loss of cell-cell adhesion.[96, 97] Interaction of tumor stromal cells with epithelial cells has been shown to result in a hyperphosphorylation of E-cadherin complexes, inducing the disassembly of their adhesion complexes and contribution to tumor progression.[98]

1.3.3.5 Growth factor tyrosine kinase receptors

The human epidermal growth factor receptor (Erb) family consists of four membranous tyrosine kinases (EGFR, HER2, HER3 and HER4), which differ in ligand specificity.[99, 100] The genes for the HER family of receptors are designated erbB1 (EGFR), erbB2, erbB3, and erbB4.[101] The amino acid sequences of the HER receptors are highly homologous to the epidermal growth factor receptor (EGFR). The binding to ligands elicits their homo- or heterodimerization and kinase domain activation initiating cascades of cytoplasmic and

nuclear mitogenic pathways including the RAS-MAP and PI3K-Akt pathways, which lead to gene activation and cell proliferation (Figure 6).[102] HER1 (EGFR) binds to different ligands (e.g., TGF-α and amphiregulin).[103] While there are no known ligands for HER2,[104] evidence indicates that isomorphs of the new differentiation factor (NDF or neuregulins) function as ligands for both HER3 and HER4.[103] It has been proposed that HER3 lacks intrinsic kinase activity as a single protein but can transduce signals following heterodimerization.[105, 106] Amplification of HER2 gene and overexpression of its encoded protein appeared to negatively influence survival in about 30% of breast cancer patients.[107] Whereas specific inhibition of HER2 gene amplified breast cancers resulted in an improved patient survival.[108]

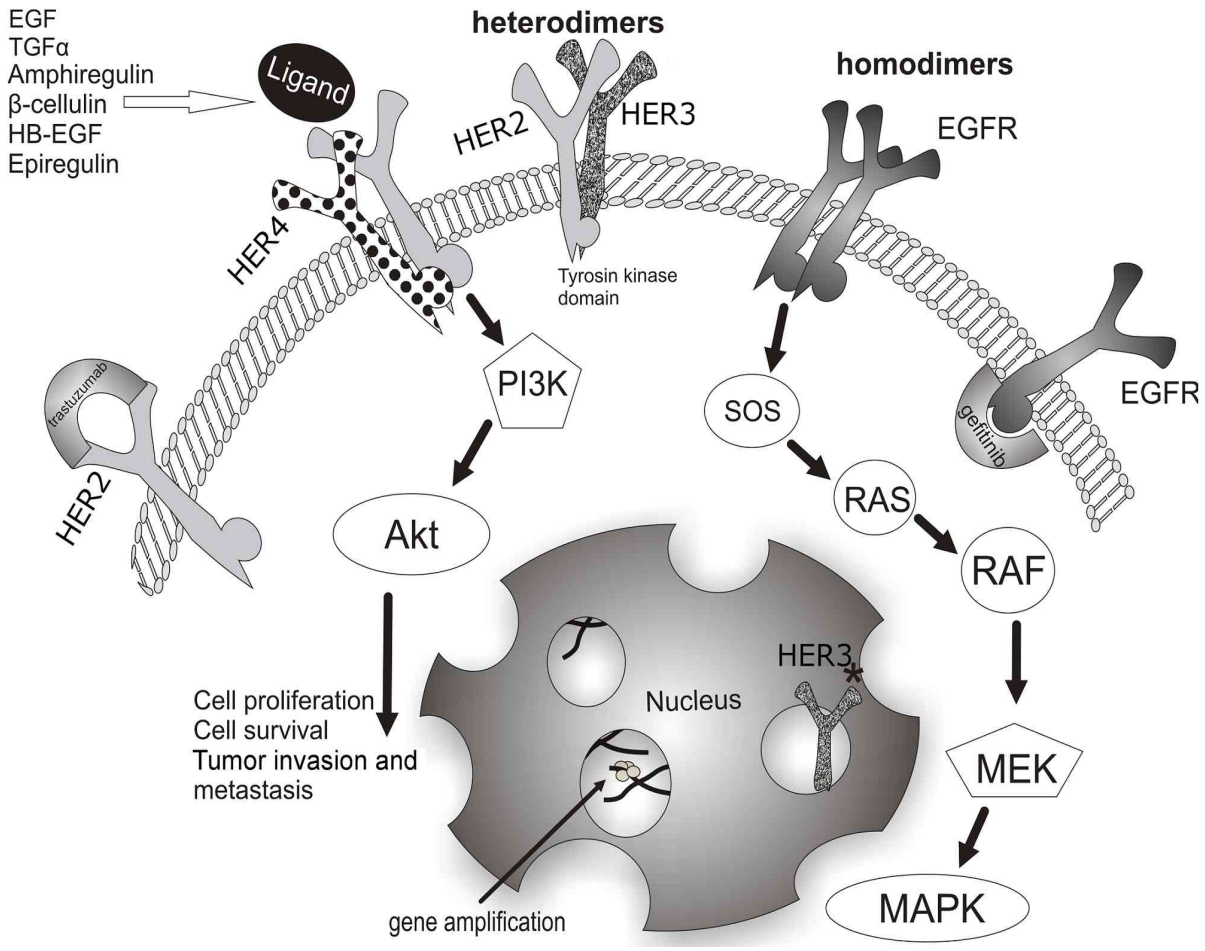

Figure 6. Simplified schematic illustration of homo- and heterodimers of erb family members transducing signals to different oncogenes. *Presumed nuclear translocation of membranous HER3.

1.3.3.6 Signaling Pathway linking EGFR activation

Ligands binding to human EGFR elicit kinase domain activation initiating cascades of cytoplasmic pathways. A cytoplasmic pathway of central role in cell activity is the PI3K-Akt pathway.[102] The atypical protein kinase B (PKB/Akt) family consists of three serine/threonine protein kinase isoforms Akt1/PKBα, Akt2/PKBß and Akt3/PKBγ, which function as key regulators for cell growth, survival and proliferation. Other processes regulated by Akt isoforms include cell size, cell response to nutrient availability, intermediary metabolism, angiogenesis and tissue invasion.[109] Deregulations of these kinases have been described in human malignancies.[110] In order to be activated, Akt1 is recruited to the cellular membrane by binding of its amino terminal pleckstrin homology (PH) domain to membrane-bound phosphatidylinositol 3,4,5 triphosphate (PIP3),[111] which is followed by the phosphorylation of two key amino acids: i) threonine 308 (Thr308) in the P-loop of the protein kinase domain and ii) serine 473 (Ser473) in the carboxy-tail region.[112]

This pathway is regulated by other kinases; PI3K activates Akt by catalyzing the production of its dependent kinases phosphoinositide-dependent kinase and integrin-linked kinase.[113-115] The tumor suppressor phosphatase and tensin homologue deleted on chromosome ten (PTEN) is a phospholipid phosphatase which negatively regulates phosphatidylinositol triphosphate levels thus antagonizing PI3K.[116] Frequent inactivation and loss of function mutations have been described for PTEN in different malignancies.[117, 118]

1.4 Epithelial mesenchymal transition and relation to tumor stroma

Stromal–epithelial interactions are considered critical for regulating tissue development and for the maintenance of tissue homoeostasis.[119] Consequently, it seems essential to study molecular marker alterations in stromal cells surrounding neoplasia to better understand the mechanisms of tumor growth and proliferation. Stromal cells surrounding the advancing tumor edge (also called tumor stroma) consists of 1) non-malignant cells related to the tumor; activated fibroblasts, specialized mesenchymal cell types distinctive to each tissue environment, innate and adaptive immune cells, and the vasculature with endothelial cells and pericytes, as well as 2) the extracellular matrix (ECM).[120] ECM consists of structural proteins (collagen, elastin), specialized proteins (fibrilin, fibronectin, elastin) and proteoglycans. It is now becoming clear that the tumor microenvironment, which is largely orchestrated by inflammatory cells, is a major participant in the neoplastic process, fostering proliferation, survival and migration. Thus a rather paradoxical role of stromal cells is to be appreciated. Stromal cells appear, in some instances, to support growth and motility of tumor cells,[121] and in other instances to be a part of the microenvironment preventing tumor cell invasion, in concert with the assumed function of the immune system. This complex interplay between tumor epithelial cells and stromal cells can be further complicated by morphological observations describing tumor epithelial cells to gain a mesenchymal or a stromal cell appearance.[122] Since 1989, this phenomenon of morphological alterations (later called epithelial mesenchymal transition; EMT) has been linked to cancer development, as rat bladder carcinoma cells were seen to have a changed morphology. These cells were missing desmosome junctions, and had a rearranged cytoskeleton.[123, 124] All the above mentioned prognostic markers (chapter 1.3.3) are described to play a role, either directly or indirectly, in the differentiation of tumor tissue and can, to a varying degree, be linked to the phenomenon of EMT in tumor tissue. However, even though EMT can be described as a set of changes where malignant epithelial cells lose their polarity, and acquire a spindle-shaped, highly motile fibroblastoid phenotype following loss or redistribution of tight- and adherens-junction proteins and alteration in gene-expression, there is still no consensus about a clear-cut definition of this morphological and functional phenomenon or an established number of altered molecular markers in these cells. As previously mentioned EMT also occurs as a physiological process during embryonic development, and is regulated by an increasingly complex pattern of signaling pathways.[47-50]

1.5 Tissue microarray

Tissue microarray (TMA) is a powerful technology tool designed to investigate the expression of proteins or genes efficiently and economically across large sets of tissue specimens, assembled on a single microscope slide.[125] This method implies the extraction of small tissue cylinders from a donor tissue block (usually formalin-fixed paraffin-embedded tissue) to be embedded in a "recipient" paraffin block (see Figure 7).

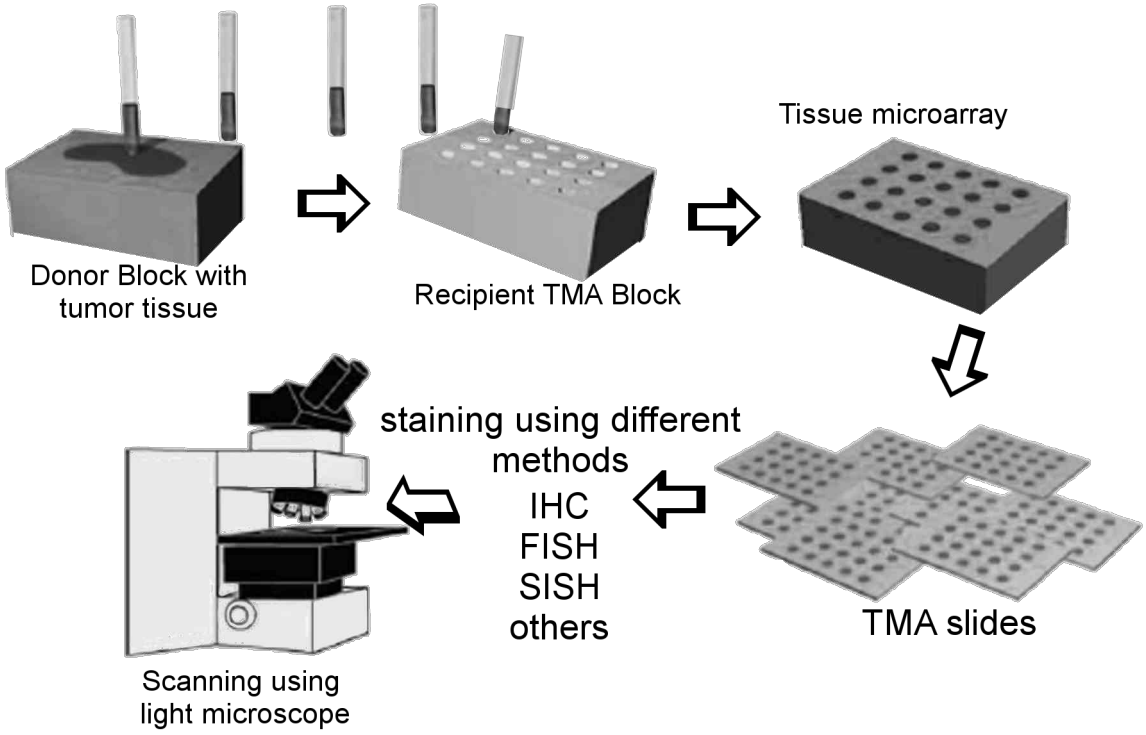

Figure 7. Tissue microarray method with donor block and recipient. Slices can be cut and used for various staining methods. Adpated from W. Chen, D.J Foran/ Analytica Chemica Acta 564 (2006) 74-81

About 200 consecutive sections of 4–8 micrometer thickness can be cut from each tissue microarray block. These sections can then be stained with different markers to establish a large-scale protein or gene expression profile of cancers. This method allows the assessment of; DNA using fluorescent/silver *in situ* hybridization (FISH/SISH), RNA using mRNA *in situ* hybridization (RNA-ISH), or protein expression using immunohistochemistry (IHC). The terminology should not lead to confusion with the DNA microarrays, in which each tiny spot on the grid represents a unique cloned complementary DNA (cDNA) or oligonucleotide. A major advantage using TMA is saving time and money assessing protein expression, since a

large number of small core biopsies can be assessed on just one slide representing numerous tumors, rather than staining individual slides for each tumor. And only few microliters of antibody are enough to stain multiple tumors on one slide. Furthermore, staining and assessing several tumors on one slide implies that the different specimens are treated in an identical manner. This is critical, when comparing the molecular expression of different tumors.

2. Aims of thesis

A major goal of this study was to elucidate the prognostic roles of tumor proliferation and differentiation markers in NSCLC, and to explore their prevalence in both stromal and epithelial malignant cells.

More specifically the aims were:

● To assess the prevalence and prognostic significance of NF-κB p105, vimentin, E-cadherin, atypical PKC, Par6, fascin and transforming growth factor-ß (TGF-ß) in tumor stromal and tumor epithelial cells of resected NSCLC tumors

● To determine the prevalence and prognostic significance of all three known Akt isoforms (Phosphorylated Akt1, non-phosphorylated Akt2 and total Akt3) as well as PTEN and PI3K, upstream members of the PI3K/Akt signaling pathway

● To examine the prevalence and prognostic role of Erb family members (EGFR, HER2, HER3 and HER4) in NSCLC as single proteins and as pairs
○ Compare the expression of HER2 as detected by IHC with its gene amplification by means of SISH stratified for clinical variables
○ Investigate the correlation between nuclear and cytoplasmic expression of HER3 and their prognostic significance
○ Determine the correlation between polysomy of chromosome 17, the chromosome on which the HER2 gene is located, and disease-specific survival in NSCLC patients

3. Patients and methods

3.1 Patients and clinical samples

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 North Norway (UNN) and the Nordland Central Hospital (NLSH) from 1990 through 2004 were used in this retrospective study. As shown in Figure 8, 371 patients were registered from both hospital databases. Of these, 36 patients were non-eligible for 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. 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 for all patients were collected retrospectively by an oncologist (T. Dønnem). The last patient update was performed in November 30, 2008. Formalin-fixed and paraffin-embedded NSCLC tumor specimens were obtained from the archives of the Departments of Pathology at UNN and NLSH. The tumors were staged according to the International Union Against Cancer's TNM classification[126] and histologically subtyped and graded according to the World Health Organization guidelines.[12] The National Data Inspection Board and The Regional Committee for Research Ethics approved the study.

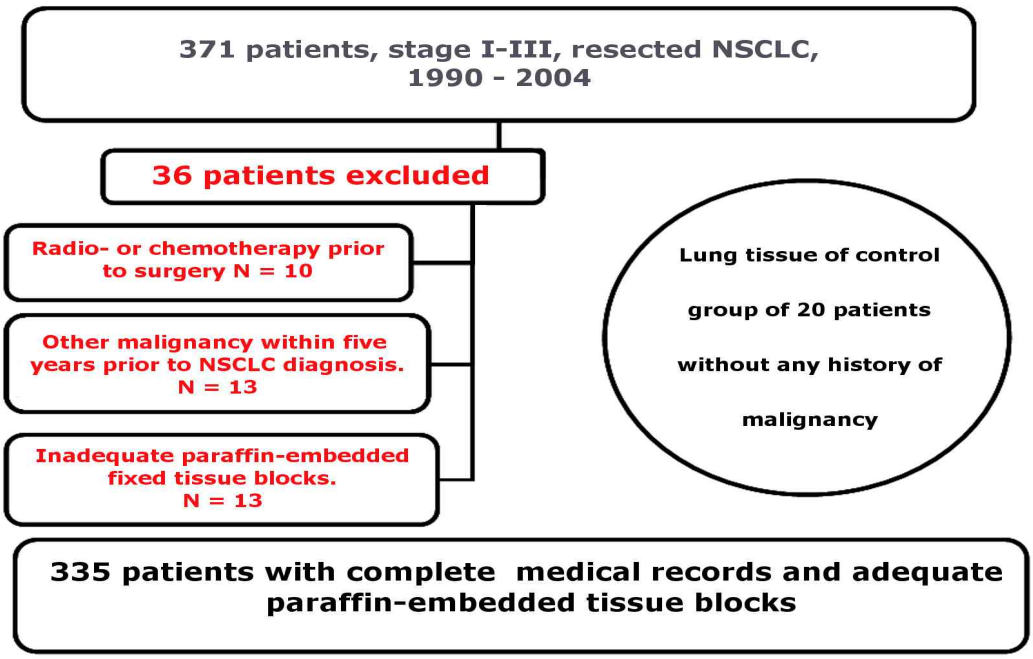

Figure 8. 335 patients with complete medical records were included

3.2.1 Tissue microarray 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, USA), consisting of thin-walled stainless steel biopsy needles and stylets used to extract tissue cylinders from the donor block, transfer the needle content and empty it into the recipient block. Tissue cylinders were retrieved from selected regions of the donor block. The recipient block was held in an X-Y position guide that was manually adjusted by micrometers, and the instrument was used to create holes in the recipient paraffin block. Thereafter, cores from the donor blocks where transferred to the pre-made holes in the recipient block by a solid stylet, closely fit in the needle. The tissue cores were transferred to the recipient block at defined array coordinates. We used a 0.6 mm diameter needle, and the study specimens were routinely sampled with two replicate core samples (different areas) of neoplastic tissue and two of tumor stroma. To include all core samples including controls, eight tissue array blocks were constructed. Multiple 4 μm sections were cut with a Micron microtome (HM355S) and stained by specific antibodies for immunohistochemistry (IHC) analyses. For paper III, sections were also stained by specific antibodies for silver *in situ* hybridization (SISH) analyses.

3.2.2 Advantages and disadvantages with TMA technology

As previously mentioned (see 1.5) TMA technology is both an efficient (time saving) and economical (antibody saving) method. Further it allows a more objective comparison of antibody expression in a large number of tissues (up to 300) obtained from different tumors on the same slide.[127] A common concern is whether the small core samples used in TMA analysis give meaningful information about the large tumor specimens. Instead of 0.6 mm cores, some investigators have used larger cores (2-4 mm or more) to increase representativity. Others suggest that punching multiple small cores from different regions captures the heterogeneity of the tumors better.[125] Hence, we chose to use duplicate cores of both tumor cells and stromal cells, and to sample from as representative sites as possible after reviewing all the original sections of the tumor and taking tumor heterogeneity in consideration. In addition, the total surface areas of all cores were counted (rather than using 1-2 high power fields). Up to 95% correlation has been demonstrated when comparing tumor

cell assessment in duplicate 0.6 mm cores versus the whole slide.[125] However, representativity of using TMA for other variables (including lymphocytes) may be slightly lower when compared to evaluations of bigger sections, but it is still highly representative.[128] Still, there are no published data on the TMA's representativity with respect to the assessment of stromal cells. This has been explained further in the methods. However, in complex tissues that require simultaneous investigation of various regions of an organ, a larger core diameter may be preferable. This was concluded in research performed on liver tissue, where at least one acinus was necessary to be included in each core, and 2 mm core diameter was the most adequate.[129]

3.3 Immunohistochemistry (IHC)

IHC as a method for detecting and visualizing antigens in paraffin-embedded tissue is both highly sensitive and specific[130] and is considered the most practical method of assessing protein expression changes in diagnostic histopathology. Using IHC does not only enable a semiquantitative assessment of protein abundance, but can also localize the protein expression. It may also detect functionally important post-translational protein modifications, such as phosphorylation. These considerations have led to the extensive use of IHC in studies on prognostic and predictive markers for different tumors.[41] Yet, the specificity of an immunohistochemical test would never exceed the specificity of the antibody provided by the manufacturer. Nevertheless, an additional possible source of error can still be the biological variation of protein expression in different areas of tumor tissue. Nonetheless, this source of bias can be reduced by increasing the number of examined tissue as in this study. IHC as a method is briefly based on applying a specific primary antibody which binds to the antigen of interest. This reaction is usually amplified by a biotinylated secondary antibody, an enzyme complex and a chromogenic substrate for visualizing the aimed antigen.

3.3.1 IHC procedure

Paper I: For staining with fascin and NF-κB p105, sections were deparaffinised with xylene and rehydrated with ethanol. Antigen retrieval was performed by placing the specimen in 0.01 mol citrate buffer at pH 6.0 and exposed to two repeated microwave heatings of 10 min at 450W. Primary antibodies for fascin and NF-κB p105 were incubated for 30 min at room temperature. For staining with vimentin, E-cadherin, atypical PKC, Par6, and transforming growth factor-b (TGF-ß) the slides were transferred to the Ventana Benchmark, XT

automated slide stainer (Ventana Medical System, Illkirch, France). The DAKO En- Vision+ System-HRP (DAB) kit was used to visualise the antigens for all stains. This yielded a brown reaction product at the site of the target antigen. Tissue sections were incubated with primary antibodies recognising vimentin, E-cadherin, Par6, aPKC and TGF-ß. Primary antibodies were incubated at 37˚C (vimentin 24 min, E-cadherin 32 min, aPKC 28 min, Par6 52 min and TGF-ß 28 min).

Paper II: The antibodies used in the study were as follows: Phospho-Akt (Ser473) (1:5; rabbit monoclonal, clone 736E11; #3787; Cell Signaling Technology, Danvers, USA), which detects Akt1 only when phosphorylated at serine 473, and Akt2 and Akt3 only when phosphorylated at equivalent sites; Phospho-Akt (Thr308) (1:50; rabbit monoclonal, clone 244F9; #4056; Cell Signaling Technology), which recognizes all three Akt isoforms when phosphorylated at threonine 308; Akt2 (1:18; rabbit monoclonal, clone 54G8; #4057; Cell Signaling Technology), which preferentially binds to non-phosphorylated endogenous levels of Akt2 and does not cross-react with recombinant Akt1 or Akt3; Akt3 (1:8; rabbit polyclonal, #4059; Cell Signaling Technology), which detects endogenous levels of total Akt3, but does not recognize the truncated form of rat Akt3 nor does it cross-react with recombinant Akt1 or Akt2; PTEN (1:10; rabbit monoclonal; #9559; Cell Signaling Technology), which detects endogenous levels of total PTEN protein; PI3-kinase (1:25; rabbit polyclonal; #4254; Cell Signaling Technology), which detects endogenous levels of total PI3K. Sections were deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval was performed by placing the specimen in 0.1 mol/l citrate buffer at pH 6.0 and exposing it to two repeated microwave heating of 10 minutes at 450 W. The DAKO EnVision+ System-HRP (DAB) kit (Glostrup, Denmark) was used as endogen peroxidise blocking. Primary antibodies were incubated overnight at 4˚C (except PI3K, for 32 minutes at room temperature).

Paper III: The antibodies used in the study were as follows: Phospho-EGF Receptor/EGFR (1:250; rabbit monoclonal, clone 53A5; #4407; Cell Signaling Technology, Danvers, MA, USA); HER2/neu (pre diluted by the manufacturer; rabbit monoclonal, clone 4B5; #790-100; Ventana Medical Systems, Illkirch, France); Nuclear non-phosphorylated HER3 receptor (HER3n) (1:250; mouse monoclonal, clone RTJ2; #NB100-2691; Novus Biologicals, Littleton, CO, USA); Phospho-HER3 (p-HER3) (1:250; rabbit monoclonal, clone 21D3;

#4791; Cell Signaling Technology, Danvers, MA, USA); HER4 (1:50; rabbit polyclonal, #RB-9045-R7; Thermo Fisher Scientific Anatomical Pathology, Fremont, CA, USA).

All the used antibodies in the three above mentioned studies had been subjected to in-house validation by the manufacturer for IHC analysis on paraffin-embedded material. The DAKO EnVision+ System-HRP (DAB) kit was used to visualize the antigens for all stains. This yielded a brown reaction product at the site of the target antigen. As negative staining controls, primary antibodies were replaced with the primary antibody diluent. All slides were counterstained with haematoxylin to visualize the nuclei. For each antibody, including negative controls, all TMA stainings were performed in a single experiment. The antibodies used in the three studies are shown in Table 2.

Table 2. The investigated antibodies using immunohistochemistry

Antigen	Antibody	Catalog #	Source	Dilution
NF-kB p105	Rabbit monoclonal	4808S	Cell signaling	1:50
Vimentin	Mouse monoclonal	790-2917	Ventana	a
E-cadherin	Mouse monoclonal	760-2830	Cell Marque	a
Par6	Rabbit polyclonal	Sc-25525	Santa Cruz	1:10
aPKC	Rabbit polyclonal	Sc-216	Santa Cruz	1:100
Fascin	Mouse monoclonal	MAB3582	Chemicon	1:25
TGF-ß	Rabbit polyclonal	Sc-146	Santa Cruz	1:50
p-Akt Ser^{473}	Rabbit monoclonal	3787	Cell Signaling	1:5
p-Akt Thr^{308}	Rabbit monoclonal	4056	Cell Signaling	1:50
Akt2	Rabbit monoclonal	4057	Cell Signaling	1:18
Akt3	Rabbit polyclonal	4059	Cell Signaling	1:8
PTEN	Rabbit monoclonal	9559	Cell Signaling	1:10
PI3K	Rabbit polyclonal	4254	Cell Signaling	1:25
p-EGFR	Rabbit monoclonal	4407	Cell Signaling	1:250
HER2/neu	Rabbit monoclonal	790-100	Ventana	a
Nuclear HER3	Mouse monoclonal	NB-100-2691	Novus Bio.	1:250
p-HER3	Rabbit monoclonal	4791	Cell Signaling	1:250
HER4	Rabbit polyclonal	RB-9045-R7	Thermo Fisher	1:50

a=Prediluted by the manufacturer

3.4 Silver *in situ* hybridization (SISH)

In recent years, more focus has been attributed to specific genetic changes in the DNA of neoplastic cells. Therefore, methods visualizing nucleic acids for investigating gene status were highly needed. In 1969, a method called fluorescence in situ hybridization (FISH) was introduced by Gall and Pardue, where they used radiolabeled probes for visualizing specific nucleic acids.[131] As recent as 1980, Bauman and Wiegant et al, used FISH to visualize specific DNA sequences in the insect *Crithidia luciliae*.[132] This method has been developed over time and has now become a specific and reliable method for investigating different gene status.[133] However, this technique is not easy to perform or to interpret and it requires expensive instruments such as highly sensitive immunofluorescence microscopy, making it a particular challenge for many laboratories. Recently, a new method, called silver *in situ* hybridization (SISH), has been introduced. This new method offers the accuracy of FISH with the use of silver staining, instead of fluorescent signals, thus allowing the use of ordinary light microscopy which is available for all histopathologists. The method is also fully automated, which ensures consistency with respect to methodology and results.[134]

In our study, automated SISH was performed on a Ventana Benchmark XT, according to the manufacturer's protocols for the INFORMHER2 DNA and chromosome 17 probes. The applied antibodies (see Table 3) have been subjected to in-house validation by the manufacturer for detection of the HER2 gene (HER2sish) and chromosome 17 (chrom17) in paraffin-embedded material. The antibodies used for SISH staining were as follows: HER2 DNA probe (anti-rabbit, ref: 780-4332; Ventana Medical Systems, Illkirch, France) and chromosome 17 probe (anti-rabbit, ref: 780-4331; Ventana Medical Systems, Illkirch, France). Sections were deparaffinized with xylene and rehydrated with ethanol. The probes were labelled with dinitrophenol (DNP) and visualized using the rabbit anti-DNP primary antibody and the Ultraview SISH Detection Kit. Both the HER2 DNA and the chromosome 17 probes were denatured at 95°C for 12 min, and hybridization was performed at 52°C for 8h. The final reaction product was black metallic silver, which was produced by the sequential addition of silver acetate, hydroquinone and hydrogen peroxide to the peroxidase-conjugated goat anti-rabbit antibody from the detection kit. The metallic silver was deposited in the nuclei, and a single copy of the HER2 gene or chromosome 17 was seen as a discrete black dot. The slides were then counterstained with hematoxylin for interpretation by light microscopy.

For each antibody, including negative controls, all TMA stains were performed in one single

experiment. HER2 gene status was detectable on one slide, and chromosome 17 probe was detectable on a matched slide, which allowed the HER2 gene status to be investigated in the context of its chromosomal state using standard light microscopy. Two discrete black dots were observed in the nuclei of cells with normal HER2 gene copy numbers (non-malignant cells served as internal positive controls for staining). Small or large clusters of black dots were seen in cells with multiple HER2 gene copies, representing HER2 gene-amplified cells. More than two discrete black dots were seen in the nuclei of cells with abnormal numbers of chromosome 17, indicating polysomy.

Table 3. The investigated antibodies using silver *in situ* hybridization

Antigen	Antibody	Catalog #	Source	Dilution
HER2 (SISH)	Anti-rabbit	05273439001	Ventana	a
Chromosome 17 (SISH)	Anti-rabbit	05273412001	Ventana	a

a=Prediluted by the manufacturer

3.5 Scoring

3.5.1 Scoring of immunohistochemistry:

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 a mixture of stromal cells and tumor cells was observed. However, by morphological criteria we have only scored staining intensity of tumor cells in tumor cores and intensity of expression 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 a study performed by our group,[135] the interobserver scoring agreement was assessed for one ligand (VEGF-C) and one receptor (VEGFR-3). The mean correlation coefficient (r) was 0.95 (range 0.93-0.98). 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 ≥ 2 (E-cadherin, p-Akt Thr^{308}, Akt2). For

both vimentin and NF-κB p105 an additional moderate expression was defined as >1 and < 3. Examples of different grades of expression in tumor cells are presented in Figure 9.

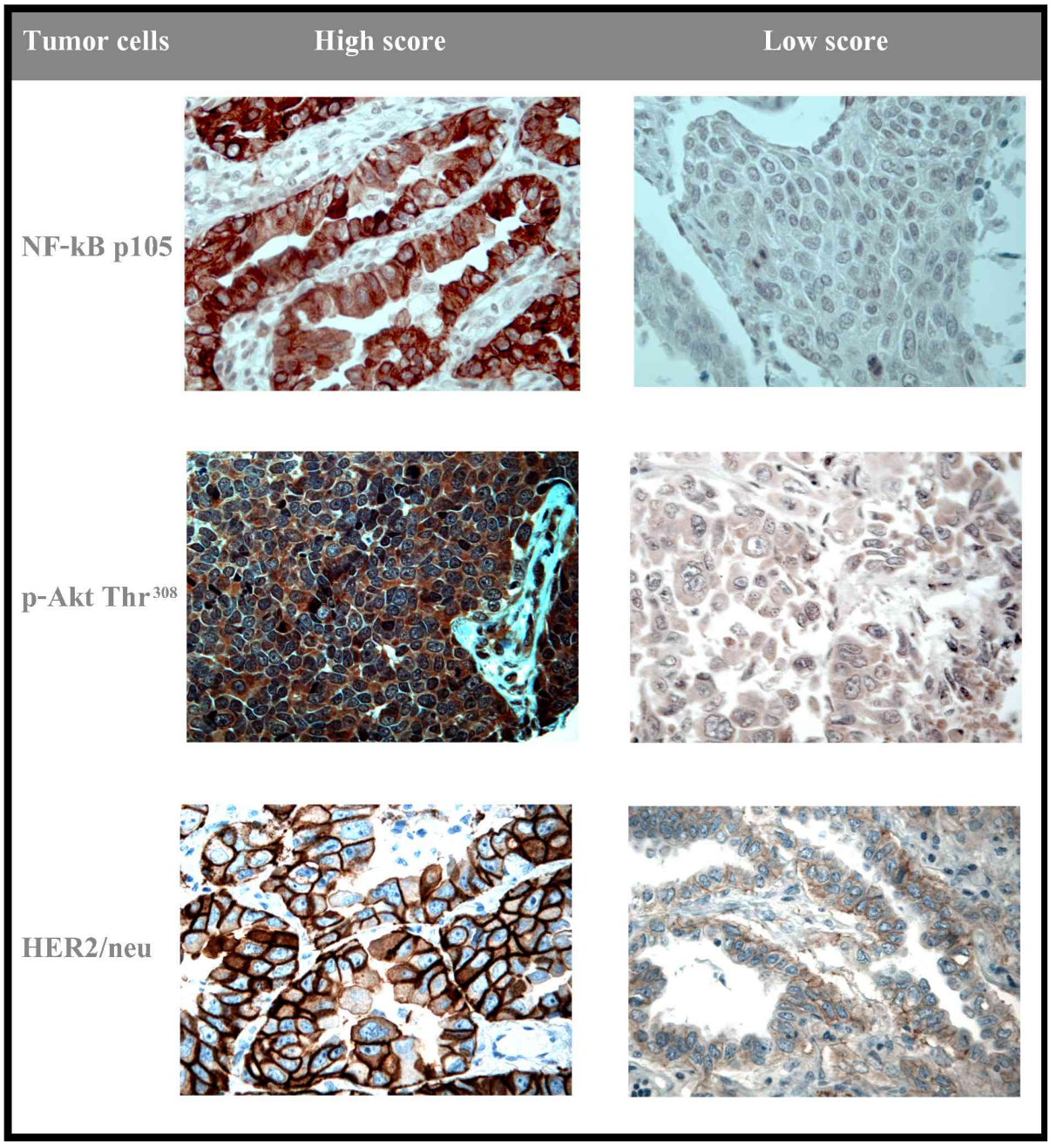

Figure 9. Examples of different grades of IHC expression in tumor cells

Stromal expression was calculated by summarizing density score (1-3) and intensity score (0-3) prior to categorizing into low and high expression (Figure 10). High expression in stroma was defined as score ≥ 2 (NFkB p105, Par6, Akt3 and PI3K).

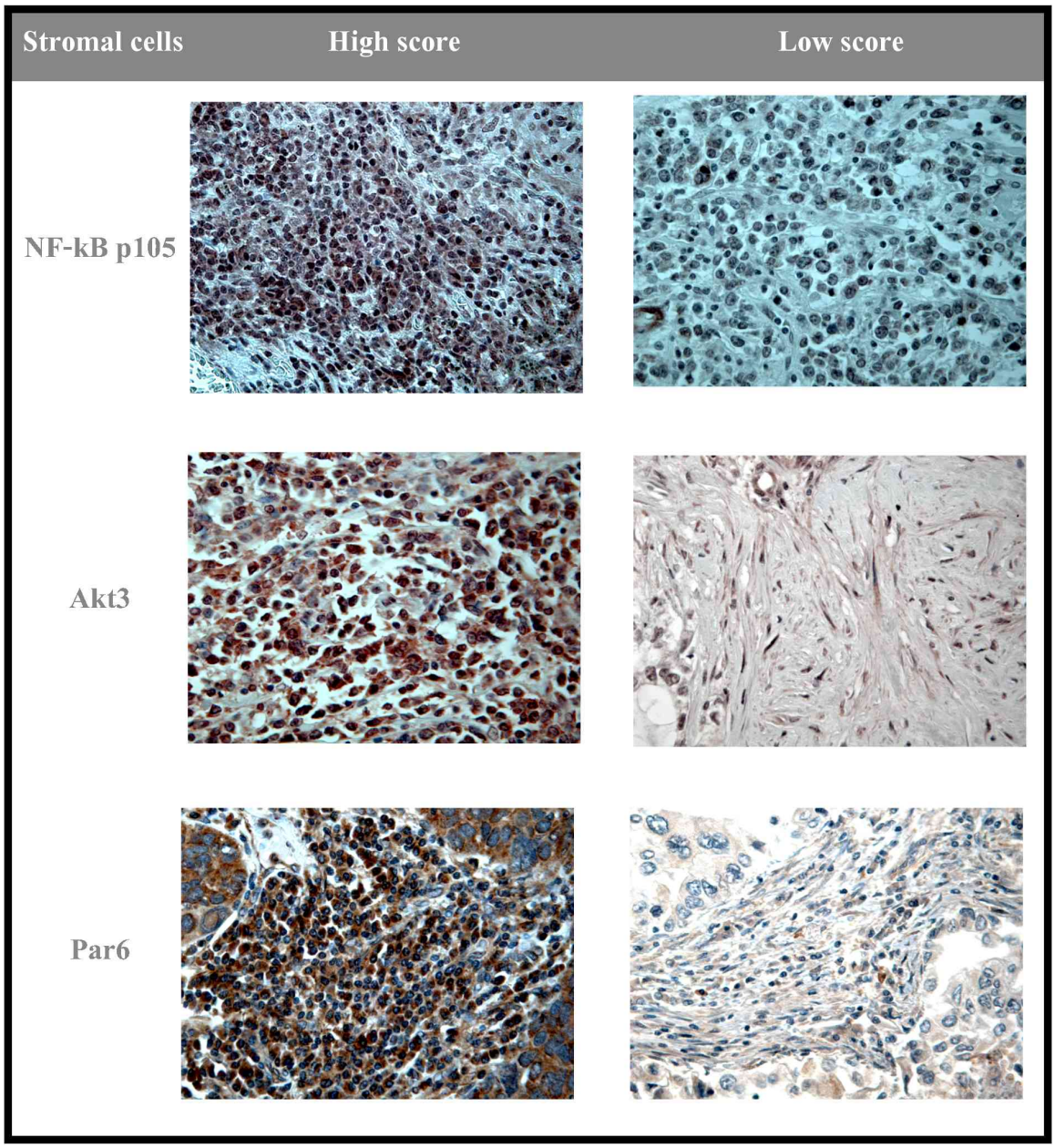

Figure 10. Examples of different grades of IHC expression in stromal cells

3.5.2 Scoring of silver *in situ* hybridization:

There are still no clear guidelines established for measuring HER2 gene amplification in NSCLC. In paper III we sought to determine whether the number of HER2 gene copies detected by SISH (i.e., the number of black dots observed in the nuclei of tumor cells in the HER2sish assay) would add prognostic significance beyond that established by the

HER2/chr17 ratio. The number of gene copies was counted according to the manufacturer's protocols for INFORMHER2 DNA. Briefly, a discrete dot was counted as a single copy of HER2sish or chromosome 17. Some nuclei showed multiple discrete copies. Clusters of dots representing many copies of the HER2 gene were also observed; a small cluster of multiple signals was counted as 6 copies and a large cluster was counted as 12 copies (Figure 11). According to HER2 gene copies, the mean value was used as cut-off point, defining patients having 2.7 gene > copies as patients with high gene copy number.

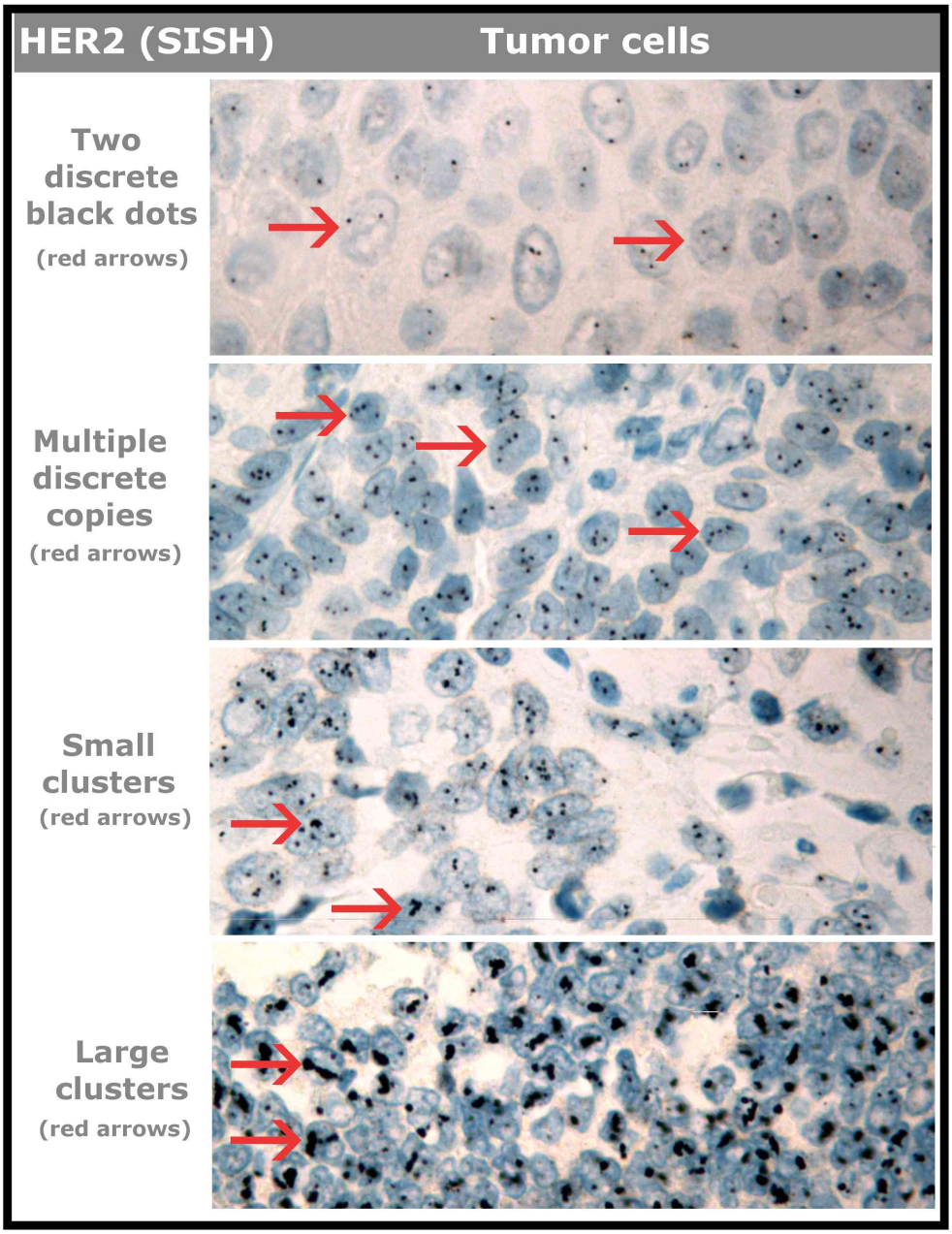

Figure 11. Examples of different grades of SISH expression in tumor cells

3.6 Cut-off values

An ideal situation for the evaluation of different markers expression would be defining standardized cut-offs points for each specific method. Unfortunately, such standardized cut-offs are extremely difficult- if not impossible- to imply in daily practice due to variations in tissue fixation and preparation, antigen retrieval, and antibody dilution. Additionally, inter- and intraobserver variations can play a major role in defining standardized cut-off values. Using the mean value or the median as a cut-off point would appear to be the least statically biased decision. However, using the mean value or median as cut-off value will at the same time ignore interesting alterations and prognostic expressions of markers in a small group of the study population. In our binary and ternary cut-off points of biomarker expressions, the cut-offs were determined for each variable so that the resulting subgroups were the most dissimilar 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.

3.7 Controls and limitations

Both reagent and tissue controls were used. Of all components used for IHC and SISH 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 negative control. As tissue controls, both normal lung tissue distant from the primary tumor and lung tissue from individuals without any history of malignancy were used. Another concern to be mentioned is whether variations in tissue storage period over years affected the results. The oldest paraffin imbedded tissue blocks used were obtained in 1990. In general, archival blocks dating back 20-40 years are considered adequate for evaluation, provided initial fixation in 4% buffered formalin.[125] While examining the slides, we did not observe differences in markers expressions related to variations of storage period. An important limitation to be mentioned when using immunohistochemistry, is the inability to completely exclude positive cross-reactions, i.e. other factors which would cause positive staining reaction using an antibody. Besides, other methods would help investigating the specifity of various antibodies. Nevertheless, the specifity of a defined antibody would never exceed the specifity of the antibody provided by the manufacturer.

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 hazards regression with a specific marker at a specific level as a dichotomous independent variable, 300 subjects were considered 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 a former study,[135] 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 survival 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, a multivariate analysis was carried out using the Cox proportional hazards model. Only statistically significant variables 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 (Markers related to EMT)

The aim of this study was to investigate the prognostic impact of different markers known to play an important role in cell differentiation. The expression of the seven markers, vimentin, NF-κB p105, fascin, E-cadherin, TGF-ß, Par6 and atypical PKC, was investigated in both tumor cells and stromal cells in 335 primary resected NSCLC tumors.

All the investigated markers except E-cadherin were expressed in the cytoplasm of tumor epithelial cells. E-cadherin showed a membranous staining. Expression of the investigated markers in tumor epithelial cells or stroma did not correlate with clinical performance status, vascular infiltration or histological subgroups. Expression of vimentin and Par6 in tumor epithelial cells correlated significantly (r=0.2, *P*=0.001), as did vimentin and tumor differentiation (r=-0.1, *P*=0.01). High expression of vimentin was seen in 61% of poorly differentiated tumors, 39% of moderately differentiated tumors and no expression was seen in well differentiated tumors. Even among the patient group with poorly differentiated tumors, high tumor epithelial cell vimentin expression tended to correlate with poor survival (*P*=0.08). Further, a significant correlation was seen between tumor epithelial expression of aPKC and Par6 (r=0.2, *P*=0.001).

In univariate analyses, high tumor epithelial cell expressions of NF-κB p105 (*P*=0.02) and E-cadherin (*P*=0.03) were positive prognostic indicators for disease-specific survival (DSS), whereas high tumor epithelial cell expression of vimentin (*P*=0.001) was a negative prognostic indicator. In the stromal compartment, high expression of NF-κB p105 (*P*=0.001) and Par6 (*P*=0.0001) correlated with a good prognosis.

In multivariate analyses, low tumor epithelial cell expression of NF-κB p105 (HR 7.1, CI 95% 2.18-23.2, *P*=0.0001) and high vimentin expression (HR 2.70, CI 95% 1.44-5.04, *P*=0.005) were unfavorable independent prognostic factors. Furthermore, low stromal cell expression of NF-κB p105 (HR 2.16, CI 95% 1.23-3.80, *P*=0.007) and low Par6 expression (HR 2.46, CI 95% 1.66-3.64, *P*=0.0001) were unfavorable independent prognostic factors for DSS.

4.2 Paper II (PI3K/Akt pathway)

In this study we investigated the prevalence and prognostic significance of the expression of altered Akt isoforms, PTEN and PI3K in primary resected NSCLC tissue. When compared to 20 lung tissue cores from patients without any history of malignancy PTEN expression was significantly higher in control tissue when compared to tumor tissue (*P*=0.001). There was a significantly negative correlation between PI3K expression in control versus tumor tissue (*P*=0.001, r=–0.2).

All the investigated Akt markers were expressed in the cytoplasm of the tumor epithelial cells. A weak positive nuclear staining was observed in some cores. Nuclear staining was seen to be related to cytoplasmic staining, with more pronounced nuclear staining in cores with high cytoplasmic expression. Expression of the investigated markers in tumor epithelial cells and stroma did not correlate with age, gender, smoking, clinical performance status, vascular infiltration, tumor differentiation or histological type.

In univariate analyses, high tumor epithelial cell expression of non-phosphorylated Akt2 (*P*=0.014) was a positive prognostic indicator for disease-specific survival (DSS), while high tumor epithelial cell expression of p-Akt Thr^{308} (*P*=0.045) was a negative prognosticator. High stromal expression of total Akt3 (*P*=0.0008) and total PI3K (*P*=0.0003) correlated with a good prognosis.

Prognostic relevance of the concomitant phosphorylation of both sites of activated Aktl (p-Akt $Ser^{473}$and p-Akt Thr^{308}) was evaluated. Ninty-eight percent of the tumors were phosphorylated at both sites. There was no significant association between DSS and the concomitant expression of phosphorylated Akt (Ser^{473} and Thr^{308}) in tumor epithelial (*P*=0.2) or stromal cells (*P* =0.09).

In the multivariate analysis, high tumor epithelial cell expression of p-Akt Thr^{308} (HR 1.98, CI 95% 1.36-2.88, *P*=0.0009) and low tumor epithelial cell expression of Akt2 (HR 2.91, CI 95% 1.39-6.08, *P*=0.004) were unfavorable independent prognostic factors. Furthermore, low stromal cell expression of Akt3 (HR 3.82, CI 95% 1.96-7.45, *P*=0.0008) and PI3K (HR 1.84, CI 95% 1.15-2.96, *P*=0.012) were unfavorable independent prognostic factors for DSS.

4.3 Paper III (Erb family members)

Herein, we compared the efficacy of silver *in situ* hybridization (SISH) and immunohistochemistry (IHC) in detection of HER2 alterations and investigated the prevalence and prognostic significance of Erb family members in NSCLC.

We found overexpression of EGFR, HER2, HER3, HER3n (nuclear), HER4 and HER2sish in 41%, 38%, 34%, 38%, 39% and 38% of tumor samples, respectively. Polysomy of chromosome 17 was seen in 14% of valid cases. Synchronous high expression of all Erb family members was seen in 5% of tumor samples, while 19% of samples showed synchronous low expression.

Epidermal growth factor receptors did not correlate with gender, histological subtypes, WHO performance status or vascular infiltration. Overexpression of EGFR correlated positively with the expression of HER3 (P=0.0001), HER3n (P=0.0001) and HER4 (P=0.0001). A significantly higher expression of EGFR was noted in patients who were smokers or former smokers (P=0.028) when compared to non-smokers. Membranous HER2 overexpression correlated significantly with higher HER2 gene copy number (P=0.003). Overexpression of HER3n correlated significantly with the expression of cytoplasmic HER3 (P=0.0001) and HER4 (P=0.0001) and with higher HER2 copy numbers (P=0.015). Additionally, a higher nuclear HER3 expression was seen in patients with polysomy of chromosome 17 (P=0.003).

HER4 expression correlated positively with tumor status (T-stage, P=0.028). A high HER2 gene copy number correlated positively with nodal status (P=0.046). In 73% (77/105) of all patients with a high HER2 gene copy number, simultaneous polysomy of chromosome 17 was observed. There was a significantly negative correlation between polysomy of chromosome 17 and tumor differentiation (P=0.023). There was a significant correlation between the HER2 gene copy number and HER2 protein expression (P=0.003).

In univariate analyses, high HER2 gene copy number was a highly significant negative prognostic indicator for disease-specific survival (DSS) in female patients (P=0.005), while it did not show prognostic influence in men (P=0.9). Neither polysomy of chromosome 17 nor the IHC expression of Erb family member proteins as singles or pairs correlated significantly with survival. In the multivariate analysis, high HER2 gene copy number in tumor epithelial cells (HR 2.50, CI 95% 1.09-5.75, P=0.03) was an unfavorable independent prognostic factor for DSS in female patients.

5. Discussion

5.1 Discussion of results

Our study population is relatively large compared with other studies investigating NSCLC. Expressions of the investigated biological markers were performed by two pathologists adding more objectivity to the scoring procedure. An experienced oncologist collected comprehensive clinical data for each individual with a relatively long follow up (median 86 months, minimum 48 months) allowing sufficient statistic for correlative and also multivariate analyses. All patients had primary resected NSCLC without any other known malignancy. The histological diagnosis and pathological stage was revised by the two experienced pathologists for all patients. Due to the large number of commercial antibodies available for immunohistochemistry, we had the possibility to investigate the prevalence and prognostic impact not only of biological markers related to cell proliferation and differentiation, but also to investigate additional highly interesting markers related to angiogenesis[135-137] and *in-situ* immunity.[138, 139] Using an additional method, SISH, we were able to investigate the HER2 gene status and to correlate it to its encoded protein expression. On the other hand, a general concern using commercial antibodies would be the specifity of the antibodies provided by the manufacturer. However, local experiences at UNN and NLSH with different antibody providers were taken critically into consideration, while selecting our antibody providers.

5.1.1 Paper I

In this study we identified a positive independent prognostic impact by highly expressed NF-κB p105 both in epithelial cells and in stromal cells. Being activated in a range of inflammatory processes and human cancers, the transcription factor NF-κB is thought to play an important role in tumorigenesis. The functional link between inflammation and cancer is not new. As early as 1863, Virchow hypothesized a correlation between inflammatory processes and the development of tumors.[52] Among the best documented are the causal relationship between *Helicobacter pylori* and mucosa-associated lymphoid tissue lymphoma of the stomach, and between the helminth worm *Schistosoma hematobium* and bladder cancer. Furthermore, risk factors such as asbestos and tobacco inhalation cause a chronic inflammation state that can promote tumorigenesis.[53] NF-κB p105 is the cytoplasmic precursor protein for NF-κB 1 (p50). High cytoplasmic expression of NF-κB -as observed in our study- have been interpreted as a higher amount of inactivated cytoplasmic NF-κB

protein, since degradation of the IkB protein results in the liberation of NF-κB, allowing its nuclear translocation and the activation of target genes (Figure 5). The same positive prognostic impact for the precursor protein NF-κB was also observed in stromal cells. This correlation between NF-κB inactivation in stromal cells and better prognosis gives a special emphasis on the role of stromal cells in oncogenesis. There is mounting evidence, proposing the formation of tumor-specific stromal cells with paradoxical influence on tumor progression. In fact, Giatromanolaki and co-workers described a process termed "stromatogenesis" indicating a role for tumor-specific stromal cells promoting cancer cell progression and invasion.[140]

These observations both in tumor epithelial and stromal cells underscore the importance of considering the regulating of NF-κB activity as part of future anticancer therapy. Already, there are dithiocarbamate compounds which can inhibit the NF-κB cascade, but preclinical laboratory investigations are still ongoing to succeed making such substances applicable as cancer drug therapies.[141]

The finding that high tumor epithelial cell expression of vimentin is associated with a worse prognosis seems at first to be of minor interest taking into account the large number of papers reporting a similar association.[142-148] However, we decided to investigate the prognostic impact of vimentin in NSCLC not only because it is considered one of the most known tumor differentiation markers, but also because such a finding will give us more confidence in our method of staining and scoring and can be regarded as a control marker for our study construction.

We found high Par6 stromal cell expression to be an independent favorable prognostic factor, indicating an anti-tumor effect of motile and proliferative stromal cells. This effect is in concert with the assumed function of the immune system. Such observations with apparently different impact on tumorigenesis of the same marker in different tumor compartments will open new and unique horizons for investigating tumor biology and progression and seem to be critical in considering new targets for cancer therapies.

5.1.2 Paper II

In tumor epithelial cells, we observed high expression of p-Akt Thr^{308} to be an independent negative prognostic factor for DSS and high non-phosphorylated Akt2 expression to be a positive prognosticator. In stromal cells, high expression of total Akt3 and PI3K were both independent positive prognostic factors for DSS.

While several studies confirmed a negative prognostic role for phosphorylated Akt and its isoforms,[110, 149-151] only few studies[151] investigated its prognostic impact when phosphorylated at both its activation sites; the serine 473 and the threonine 308. Our investigations did not confirm an additional prognostic role for Akt, when phosphorylated at its serine 473 amino acid, as proposed by Tsurutani et al.,[151] but we were the first to report an independent positive prognostic role for the non-phosphorylated Akt2 isoform in NSCLC. Whether the observed overexpression of non-phosphorylated Akt2 indicates a lower presence of phosphorylated (active) Akt2 or whether these findings represent opposing prognostic roles of phosphorylated (active) and non-phosphorylated Akt isoforms deserves further investigation. An apparent limitation for such an investigation is the problem finding a sufficiently specific antibody for phosphorylated Akt2 on paraffin-embedded material. While performing our investigations, we were unable to find a commercially available antibody with reliable specificity. Additional studies will be of interest as soon as more specific antibodies are available. Identifying high stromal Akt3 and PI3K expressions as independent positive prognostic factors, demonstrates a complicated epithelial-stromal cell interaction, which deserves further attention. It is likely to assume that expression of a molecular marker or its isoforms can have opposing prognostic effects in different tumor compartments. Such an observation would encourage further investigations regarding the specifity of potential cancer drug therapies inhibiting the PI3K/Akt signaling pathway, and whether a specific targeting of only epithelial or stromal cells to inhibit the Akt cascade is possible.

5.1.3 Paper III

In this paper we report about a significant correlation between HER2 gene copy numbers assessed by means of silver *in situ* hybridization and gender specific survival in NSCLC.
While various studies[23, 27, 29] indicated EGFR as a possible prognostic or predictive target in NSCLC, other studies including our could not immunohistochemically confirm such an association. In stead we investigated the gene and were able to identify high HER2 gene copy number as a highly specific and independent negative prognosticator in female NSCLC patients, while its alteration in males did not have influence on prognosis. Our observations deserve further attention if taking into account specific mutations in the tyrosine kinase region of the EGFR gene, which were more frequently observed also in females in addition to lung adenocarcinomas, non-smokers and Asians.[28, 29] In a recent study using mRNA expression values, Vallbohmer et al,[152] found HER2 to be a substantial gender specific negative prognostic factor for females, but not for males. These gender differences in cancer tumorigenesis and prognosis are most likely related to different sex hormone effects on various genes, and seem to be of huge interest for planning anti-cancer drug therapy regimes.
The recent identifying of specific alterations of HER2 protein and gene expression in breast cancer has dramatically improved survival in about 30% of female breast cancer patients,[153-155] after introducing the anti-HER2 drug trastuzumab. In our study, about 38% of female patients showed a high HER2 gene copy number. These findings deserve further future translational prospective studies to confirm the role of HER2 as a prognostic and possibly as a predictive marker for female NSCLC patients.

6. Conclusions and implications for future research

Our research group has, in NSCLC patients, studied the prevalence and prognostic impact of multiple markers expected to influence tumor proliferation and differentiation.

Using the TMA technology gave us the possibility to study a large-scale of biological markers regarding tumor differentiation markers, angiogenic markers and markers related to the innate immune system. Several markers were found to be independent prognostic factors in NSCLC. We learned, however, a new aspect of epithelial-stromal cell interactions and were able to recognize -in some instances- opposing roles of protein isoforms in different tumoral compartments. Employing new methodology, such as SISH gave us the possibility to reveal an interesting correlation between gene status and survival in female NSCLC patients.

Our group has the aim to continue the translational work bridging basic and clinical research to identify new important markers in NSCLC. We are presently introducing new research strategies such as micro RNA expression to enhance the knowledge and understanding of tumor biology. Additionally, we are establishing short-term cell-lines from freshly resected NSCLC tumor tissue, which will give us additional possibilities to understand the somehow shadowy, but very challenging process of tumor progression. Enhancing our knowledge in this field is a prerequisite to develop new and more specific strategies to reduce the fatal outcomes of this aggressive malignancy.

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Paper I

The prognostic impact of NF-kB p105, vimentin, E-cadherin and Par6 expression in epithelial and stromal compartment in non-small-cell lung cancer

Paper II

Diverse prognostic roles of Akt isoforms,
PTEN and PI3K in tumor epithelial cells and
stromal compartment in non-small cell Lung Cancer

Paper III

Clinical Significance of Epidermal Growth Factor Receptors in Non-small Cell Lung Cancer and a Prognostic Role for HER2 Gene Copy Number in Female Patients

Appendix 1

Appendix 1

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 intermediate	8254/3
Solid adenocarcinoma with mucin production	8230/3
Fetal adenocarcinoma	8333/3
Mucinous ("colloid") carcinoma	8480/3
Mucinous cystadenocarcinoma	8473/3
Singet ring adenocarcinoma	8490/3
Clear cell adenocarcinoma	8310/3
Large cell carcinoma	8012/3
Large cell neuroendocrine carcinoma	8013/3
Combinded 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
Epithelia-myoepithelial carcinoma	8562/3
Perinvasive lesions	
Squamous carcinoma *in situ*	8070/2
Atypical adenomatous hyperplasia	
Diffus idiopathic pulmonary neuroendocrine cell hyperplasia	

Mesenchymal tumours	
Epitheloid haemangioendothelioma	9133/1
Angiosarcoma	9120/3
Pleuropulomnary blastoma	8973/3
Chondroma	9220/0
Congenial perboronchial myofibroblastic tumour	8827/1
Diffus pulmonary lymphangiomatosis	
Inflammatroy myofibroblastic tumour	8825/1
Lymphangioleiomyomatosis	9174/1
Synovial sarcoma	9040/3
Monophasic	9041/3
Bipahsic	9043/3
Pulmonary artery sarcoma	8800/3
Pulomnary 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
Diffus large B-cell lymphoma	9680/0
Lymphomatoid granulmatosis	9766/1
Langershans cell histiocytosis	9751/1
Miscellanous tumours	
Hamartoma	
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	

WHO classification of Tumours of the Lung, PLeura, Thymus and Heart. Travis WD, Brambilla E, Mueller-Hermelink HK, & Harris CC. Lyon 2004. International Agnecy for Research on Cancer (IARC)

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[1] Morphology code of the international Classification of Disease 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 unceratain behaviour

Appendix 2

Appendix 2

TNM classification of the lung

TNM classification of carcinomas of the lung (738,2045)

T - Primary Tumour

TX Primary tumour cannot be assessed, or tumour proven by the presence of malignant cells in sputum or bronchial washing but not visualized by imaging or bronchoscopy

T0 No evidence of primary tumour

Tis Carcinoma in situ

T1 Tumour 3 cm or less in greatest dimension, surrounded by lung or visceral pleura, without bronchoscopic evidence of invasion more proximal than the lobar bronchus, i.e., not in the main bronchus (1)

T2 Tumor with any of the following features of size or extent:
- More than 3 cm in greatest dimension
- Involves main bronchus, 2 cm or more distal to the carina
- Invades visceral pleura
- Associated with atelectasis or obstructive pneumonitis that extends to the hilar region but does not involve the entire lung

T3 Tumour of any size that directly invades any of the following: chest wall (including superior sulcus tumours), diaphragm, mediastinal pleura, parieatal pericardium, or tumour in the main bronchus less than 2 cm distal to the carinal but without involvement of the carina; or associated atelectsis or obstructive pneumonitis of the entire lung

T4 Tumour of any size that invades any of the following: mediastinum, heart, great vessels, trachea, oesophagus, vertebral body, carina; seperate tumour nodule(s) in the same lobe; tumour with malignant pleural effusion (2)

Notes: 1.The uncommon superficial spreading tumour of any size with its invasive component limited to the bronchial wall, which may extend proximal to the main bronchus, is also classified as T1.

2. Most pleural effusions with lung cancer are due to tumour. In a few patients, however, multiple cytopathological examinations of pleural fluid are negatvie for tumour, and the fluid is non-bloddy and is not an exudate. Where these elements and clinical judgment dictate that the effusion is not related to the tumour, the effusion should be excluded as a staging element and the patients should be classified as T1, T2, or T3.

N - Regional Lymph Nodes"

NX Regioanl lymph nodes cannot be assessed

N0 No regional lymph node metastasis

N1 Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension

N2 Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)

N3 Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s)

M - Distant Metastasis

MX Distant metastasis cannot be assessed

M0 No distant metastasis

M1 Distant metastasis, includes separate tumour nodule(s) in a different lobe (ipsilateral or contralateral)

Stage Grouping

Occult carcinoma	TX	N0	M0
Stage 0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IB	T2	N0	M0
Stage IIA	T1	N1	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T1,T2	N2	M0
	T3	N1, N2	M0
Stage IIIB	Any T	N3	M0
	T4	Any N	M0
Stage IV	Any T	Any N	M1

A help desk for specific questions about the TNM classification is available at http://www.uicc.org/tnm/

''The regional lymphnodes are the intrathoracic, scalene, and supraclavicular nodes.